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Oligodendrocytes and Myelination: The Role of Iron

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ABSTRACT
Iron is an essential trophic factor that is required for oxygen consumption and ATP production. Thus it plays a key role in vital cell functions. Although the brain has a relatively high rate of oxygen consumption 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 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 acquisitions are important prerequisites to developing effective therapies for dysmyelinating disorders. It is the purpose of this review to give a comprehensive overview of the existing literature on role of iron in oligodendrocytes and the mechanisms of iron acquisition and intracellular handling. © 2008 Wiley-Liss, Inc.

Oligodendrocytes 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. The focus of this review is the role of iron in oligodendrocyte development and myelin production. Oligodendrocytes stain for iron more robustly that any other cell in the normal adult brain (Benkovic and Connor, 1993; Connor et al., 1995; Connor and Menzies, 1996). Iron-enriched oligodendrocytes appear in rows in the white matter (see Fig. 1). The cell body of oligodendrocytes is clearly visible following iron staining, but processes can also be detected (Figs. 1, 2 and 3). Iron-enriched oligodendrocytes are found in lower animals forms such as chickens and flys (Erb et al., 1996). Thus, even in 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 (see Fig. 2) (Dwork et al., 1988; Hill, 1989; Benkovic and Connor, 1993). These data suggest that iron accumulation by oligodendrocytes contributes more to the function of these cells than only metabolic support for myelination, but data in areas other than myelination are not available. Therefore this review 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 deficiency of tuberculosis (Connor, 1994; de Benoist, 2001). The most common neurological signs of iron deficiency in children include poor school performance, decreased cognitive abilities, and behavior problems (Oski and Honig, 1978; Oski et al., 1983; Grantham-McGregor and Ani, 2001). Several studies have shown that these clinical outcomes in iron-deficient children can be traced to hypomyelination (Oski et al., 1983; Lozoff et al., 2006). Furthermore, these neurological sequelae persist even after iron supplementation (Lozoff et al., 2006; Lozoff and Georgieff, 2006; Beard, 2007). Thus, understanding the role of iron in myelination and iron acquisition mechanisms by oligodendrocytes, *including the timing of iron acquisition*, are the key to developing effective intervention strategies. It is therefore the purpose of this paper to review the current literature on physiological role of iron in the myelination and mechanisms of iron import into the oligodendrocytes.

IRON IN THE NORMAL BRAIN

In the brain, white matter stains more strongly for iron than gray matter (LeVine and Macklin, 1990). The iron distribution in the white matter, although unique to oligodendrocytes, is not homogeneous, but occurs rather in patches (Connor and Menzies, 1996). The functional significance of the patchy iron staining (see Fig. 4) is not clear, but indicates an epigenetic influence. Previous studies have shown that in the developing brain, iron is first detected in oligodendrocytes proximal to blood

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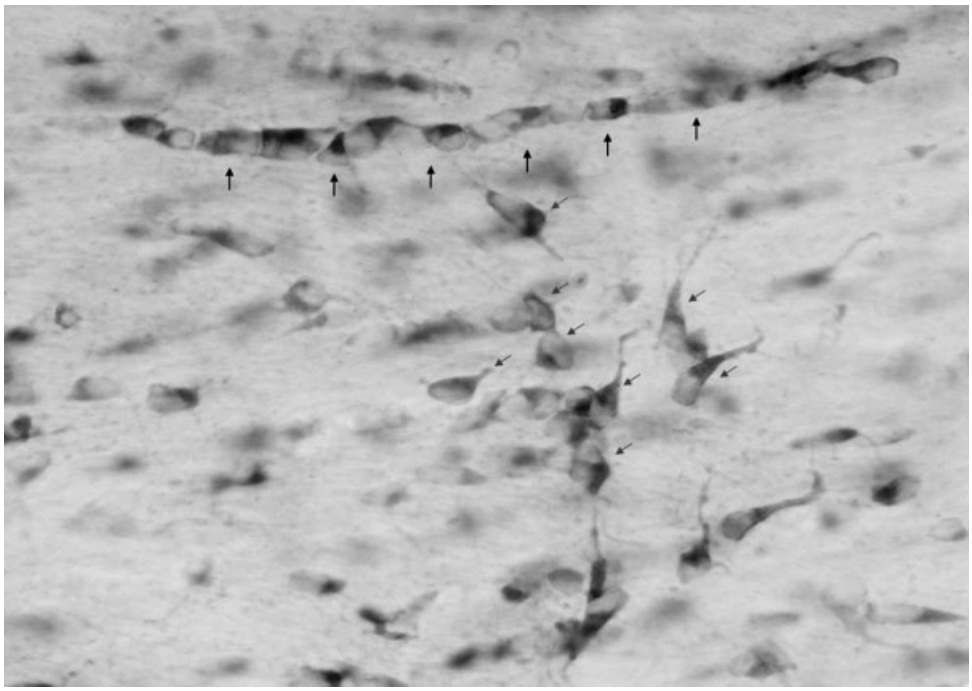

Fig. 1. Perl's staining of normal adult mouse brain is confined mainly to oligodendrocytes in white matter tracts. This representative micrograph of iron staining in corpus callosum from a normal mouse brain demonstrates labeling of small cells aligned in rows (vertical arrows), which is characteristic of oligodendrocytes. Additional oligodendrocytes are visible that are not aligned in rows (slanted 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.

vessels and subsequently oligodendrocytes farther from the blood vessels with the blood vessel remaining near the center of the patch (Burdo et al.,1999). During the second post-natal 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 (Taylor and Morgan, 1990; Connor and Menzies, 1996). At this time immature, iron-positive oligodendrocytes are clearly visible (see Fig. 5). 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 pentose-phosphate shunt, which provides reducing equivalents (NADPH) for synthesis of myelin fatty acids and also requires 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 (Cammer, 1984), but not decreased iron staining or apparently iron utilization. For example, oligodendrocytes synthesize transferrin, the iron mobilization protein, and the brain is the only organ in which transferrin mRNA increases post-natally (Bloch et al., 1985). This increase, as discussed later, 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 leads to hypomyelination, which has been documented both in human and animal studies. In human studies, increased latency of auditory brain stem potentials and visual evoked potentials (indirect markers of myelination) have been reported in iron-deficient children compared to normal controls (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 compared to controls (Yu et al., 1986; Ortiz et al., 2004). Interestingly, placing rats on iron-restricted diet after the post-weaning period (PNDs

Abbreviations

apoTf	apo-transferrin
ATP	adenosine tri-phosphate
CNPase	2′,3′-cyclic nucleotide 3′-phosphodiesterase
FGF	fibroblast growth factor
IGF-1	insulin-like growth factor 1
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
OLGcs	oligodendrocytes
OPCs	oligodendrocyte progenitor cells
PDGF	platelet derived growth factor
PLP	proteolipid protein
PND	post-natal day.

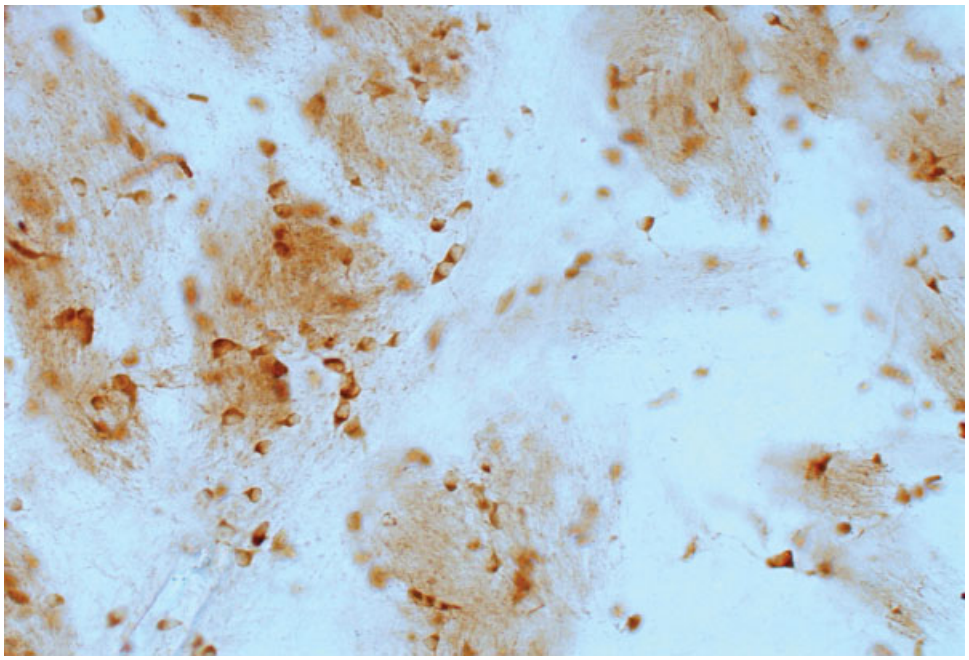

Fig. 2. Iron staining in adult mouse striatum. Another brain region that stains heavily for iron with Perl's is the striatum. In this brain region, the cells are also small and round with relatively scant cytoplasm and processes and an eccentric nucleus. The morphology is again typical of oligodendrocytes 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). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

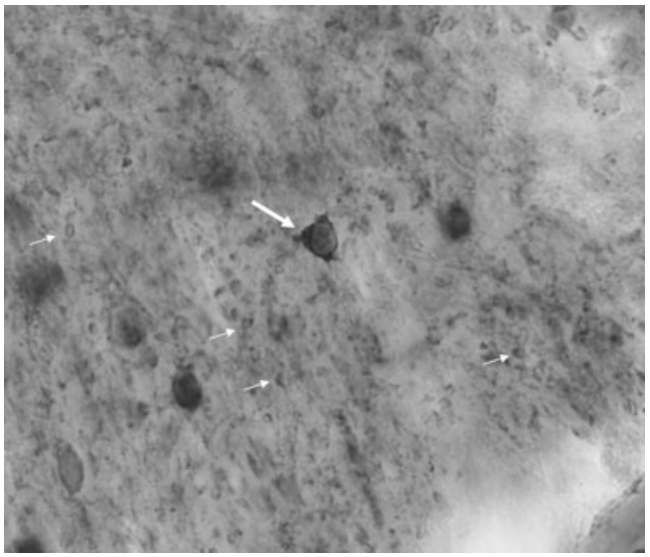

Fig. 3. Perl's staining in normal human white matter. Higher power (captured at 40×) micrograph demonstrating iron staining (Perl's reaction) in oligodendrocytes (e.g., large arrow) of normal human white matter. Staining is confined predominately in the cytoplasm, which surrounds larger eccentrically positioned nucleus. Diffuse background staining is observed with small rings (e.g., small arrows) or beads which are oligodendrocyte processes.

21–63) 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 require consistent iron delivery. The concept of continued myelination in adult, and thus continued iron requirements for myelin production and maintenance, is also true in humans (Bartzokis, 2002). Continued 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, Huntingdon's, and Parkinson's disease (Connor, 2002, 2004; Bartzokis et al., 2007a,b; Bartzokis et al., 2004; Todorich and Connor, 2004). Thus, the delivery mechanisms and regulation of those mechanisms is critical for normal brain function at all ages.

In reference to the various studies on myelin and iron deficiency, Larkin and Rao (1990) suggested that the common thread among animal studies shows that even though globally decreased, the relative ratio of myelin components is in fact normal. 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, 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

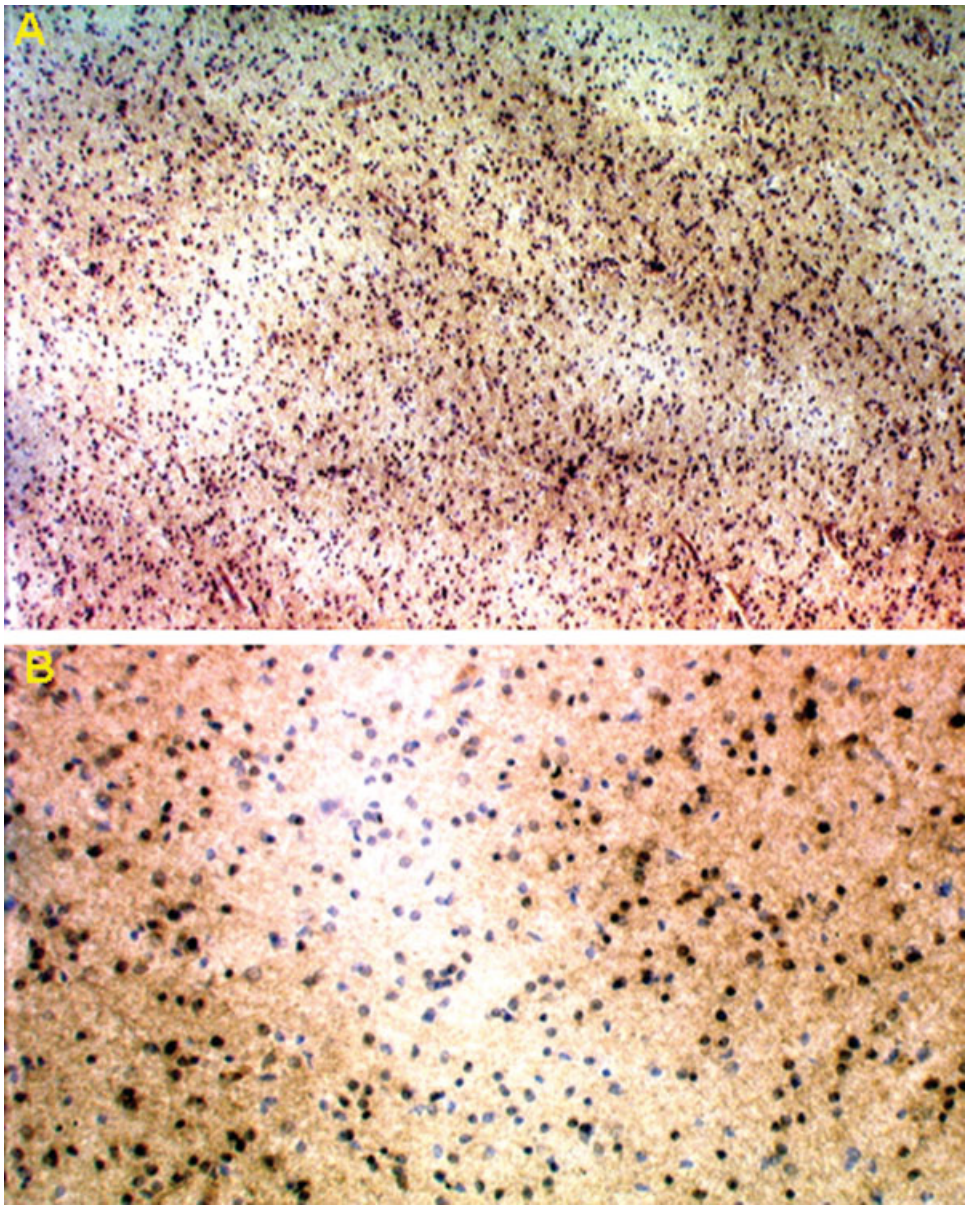

Fig. 4. Perl staining of normal human white matter is distributed in patches. Low power (**A**) and higher power (**B**) micrographs of 30 micron sliding microtome frozen sections of normal human white matter stained with the Perl's reaction for iron and counter-stained with cresyl violet to see the 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 that 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 h, (2) the thicker the section the better. The optimal sections are 30–50 μm from a freezing microtome. We have almost never observed the patchy staining on paraffin sections or thin cryostat sections. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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 (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.

Fig. 5. Iron staining of 15-day-old rat subcortical white matter. This representative micrograph illustrates iron accumulation by developing oligodendendrocyte progenitors (arrows) with multiple processes.

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 its post-translational effects on the iron-requiring proteins involved in energy metabolism and myelin synthesis (Beard and Pinero, 1996). Iron is needed as cofactor for cytochromes a, b, and c, and 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 100× the weight of an average oligodendrocyte (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 include HMG-CoA reductase, succinic dehydrogenase, NADH dehydrogenase, dioxygenase and glucose-6-phosphate dehydrogenase, and most of these are enriched in oligodendrocytes compared to other cells in CNS (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 cofactor (Lange and Que, 1998). Iron deficiency leads to impaired function and degradation of these proteins, which then impairs energy production leading to a decrease in myelin production (Iron, 2001). Several studies, albeit indirectly, reinforce the molecular connection between iron and myelin. In humans, tellurium causes peripheral neuropathy by inhibiting squalene epoxidase, a key enzyme in 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 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* was associated with 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 their function, development and myelination.

IRON TRANSPORT PROTEINS IN THE BRAIN

Clearly iron is essential for myelination, and understanding mechanisms of iron import by oligodendrocytes is a necessary prerequisite for developing therapies for demyelinating disorders 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 most important for oligodendrocytes.

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 and offered significant challenges to this long held belief that are beyond the scope of this review (Connor, 2002). In addition to the BBB transport, choroid plexus secretes transferrin, which could be another 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, 1998). 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.

Most of CNS transferrin in the brain is produced by the oligodendroglial cells, 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 oligodendrocytes is compelling. Tf gene expression in the brain (Bloch et al., 1985; Espinosa de los Monteros et al., 1994) is predominantly present in OLGcs and the appearance of Tf in OLGcs is key in the formation of myelin (Espinosa

de los Monteros et al., 1989, 1999; Connor et al., 1993). The brain is the only organ in which Tf mRNA expression increases after birth, and this increase is directly related to the maturation of oligodendrocytes (Bartlett et al., 1991). Transgenic mice overexpressing Tf, have increased levels of myelin components, including galactolipids, phospholipids, and proteins (CNPase, MBP, MAG) 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 (Saleh et al., 2003). Using the same mouse model, Sow et al. (2006) showed that OPCs cultured from brains of these mice show accelerated differentiation [expression of O4 and PLP markers (O4 and O1, markers of pro-oligodendrocytes)] 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 is important for mobilization of iron within the oligodendrocytes, although mice deficient in Tf still myelinate normally and their oligodendrocytes acquire iron (Dickinson and Connor, 1995; Ortiz et al., 2004; Beard et al., 2005).

In addition to the intracellular effects, the role of 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 diminishes as the animal ages and is not detected in white matter in adults in either rodents (Hulet et al., 1999, 2002), or humans (Hulet et al. 1999). Emerging evidence suggests there is an alternative Fe import to oligodendrocytes involving H-ferritin that becomes dominant as the animal ages and this system is also in humans (Hulet et al., 1999, 2000, 2002; Espinosa-Jeffrey et al., 2002). This topic will be discussed later.

Since 1994 numerous studies designed to determine if transferrin has a trophic effect on myelin production independent of iron have been performed by the Pasquini group. These investigators have found that the intracranial injection of a single dose of apoTf (iron-free 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, 1997). Subsequently, it was demonstrated that in the brain of rats receiving ICI with apoTf 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 apoTf acts on pro-oligodendrocytes inducing their rapid differentiation. However these effects occurred only when the animals were injected at days 2–7 of age, suggesting that there was a "developmental temporal window" during which apoTf was effective (Marta et al., 2000) and is likely related to the loss of Tf receptor expression on the oligodendrocytes (Han et al., 2003). In another study run off transcription experiments showed that the MBP mRNA was significantly increased at the nuclear levels, but that the PLP mRNA was unaffected. These results seemed to indicate that apoTf 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 apoTf would result in upregulation of only MBP mRNA is not known and is presumably indirect.

In congruence with the biochemical studies, morphologic evaluation of apoTf injected rats showed increased deposition of myelin in the optic nerves and the corpus callosum. Specifically the ultrastructural analysis showed that the intracranial treatment with apoTf 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 apoTf-treated rats is directly related to the abnormal myelin composition that have been previously described in theses 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 apoTf-treated rats.

The effects of apoTf on OLGcs differentiation in *in vitro* experiments were also studied and yielded similar results to those obtained *in vivo*. In the presence of apoTf, OLGcs in culture developed a multipolar morphology and showed an increased expression of MBP and MAG as well as enhanced reactivity to O4 and O1 compared to controls. Migration studies using the agarose drop assay showed that addition of apoTf strongly inhibited oligodendrocyte precursor cell migration. This effect was abolished with an antibody against the transferrin receptor. These results suggest that apoTf 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 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 in newborn rats (Marta et al., 2002; Garcia et al., 2003).

As mentioned before apoTf has been shown to increase the expression of different components of the myelin cytoskeleton (Baracskay et al., 2002). When OLGcs in culture are treated with apoTf, this glycoprotein has a punctate distribution pattern along the OLGcs processes. Treatment with colchicine, cytochalasin, or taxol

induced a displacement of the immunoreactivity of apoTf towards the OLGcs soma. Analysis of the effects of apoTf on the cell distribution of tyrosinated and detyrosinated tubulin and STOP (stable tubule only polypeptide), showed it 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 apoTf, suggesting that these motors participate in the transport of the added glycoprotein. Moreover, after treatment with apoTf, clathrin immunoreactivity was displaced from the OLGcs body toward the cell processes. The results indicated that apoTf 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 (Ortiz et al., 2005).

The mechanisms involved in the effect of Tf on OLGcs are complex and not understood. In studies in which the progression of the cell cycle was analyzed in primary cultures of OPCs treated with apoTf and/or with different combinations of mitogenic factors, apoTf decreased the number of BrdU+ cells and increased the cell cycle time, while the number of cells in 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. Apotransferrin 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. These results indicated that inhibition of the progression of the cell cycle of OPCs by apoTf, even in the presence of PDGF, leads to an early beginning of the differentiation program, evaluated by different maturation markers (O4, GC and MBP) and by morphological criteria. The modulation by apoTf 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., 2006).

The studies on apoTf suggest that apoTf should be considered as a possible useful factor which 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 the animals showed histological and biochemical evidence of marked demyelination. Treatment of these animals with a single intracranial injection of apoTf (350 ng) at the time of cuprizone withdrawal was associated with a marked increase in myelin deposition and significant improvement in remyelination 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 apoTf-treated animals, these phenomena occurred much faster, and remyelination was much more efficient than in the untreated controls.

A critical question in the apoTf studies is whether or not the apoTf has acquired iron once it is injected *in vivo* or from the cell culture media. The presence of iron is ubiquitous and compounded with the high affinity of apoTf for iron is (association constant of 10^{22} M^{-1}) suggest that even small concentrations of iron would rapidly bind to injected apoTf (Aisen and Listowsky, 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. The elevated production of myelin is consistent with elevated myelination associated with over-expression of Tf by oligodendrocytes (Saleh et al., 2003, Sow et al., 2006).

To determine if the apoTf injections were effective in an iron-deficient environment in the brain, apoTf was injected intracranially to study the possibility of reversing the hypomyelination processes produced by iron deprivation. At PND 24, all the myelin components were markedly diminished in the ID rats and the ICI of apoTf 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 apoTf 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 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 apoTf, 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 apoTf. The *in vitro* treatment with apoTf produced a correction in the differentiation of OLGcs isolated from ID rats consistent with *in vivo* experiments (Ortiz et al., 2005).

Despite of the apparent importance of transferrin for oligodendrocytes, mature oligodendrocytes in the adult white matter express much lower levels of transferrin receptor than other parts of the brain including cortex. Several studies have independently failed to detect Tf receptor in white matter tracts in adult animals with different technical approaches. Immunohistochemical studies assessing distribution of transferrin receptor in the brain have shown strong staining of cortex and the neurons, but found none or at most faint staining of Tf receptors in the oligodendrocytes (Giometto et al., 1990; Connor and Menzies, 1995). *In situ* hybridization

detected abundant transcripts of Tf receptor mRNA in adult mouse cortex, but low to none in the white matter tracts (Han et al., 2003). The levels of transferrin receptor mRNA in white matter did not increase even in conditions of severe iron deficiency (Han et al., 2003). Radiolabeled transferrin binds gray matter, but not white matter tracts in adult mouse, rat and human brains (Mash et al., 1990) (Hill et al., 1985; Hulet et al., 1999). *In vitro* oligodendrocyte progenitors express transferrin receptors, but if they are allowed to differentiate into mature oligodendrocytes, transferrin receptor protein expression becomes undetectable by Western blot (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 PND 3, but not at PND 20 (Escobar Cabrera et al., 1994; Marta et al., 2000). It appears that there is a "critical temporal window" in the mouse brain development in which oligodendrocytes are responsive to apotransferrin injections can be explained by presence of transferrin receptors on early OPCs. When oligodendrocytes reach maturation at PND 20 their complete 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 stains for iron. Presumably there is a continued need for iron uptake given that iron deficiency in the adult can lead to loss of myelin (Beard et al., 2003). In support of this idea, despite having transferrin levels of less than 1% of normal controls in the brain, hypotransferrinemic mice are not hypomyelinated (Dickinson and Connor, 1995, 1998). These mice even have higher than normal levels of iron in white matter. 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 brain and in oligodendrocytes (Dickinson and Connor, 1995). Nonetheless, uptake of iron into the brain continued in the absence of transferrin (Takeda et al., 1998), while in oligodendrocytes cultured in media lacking transferrin, both normal and oligodendrocytes isolated from hypotransferrinemic mice actually *increased* internalization of Fe-59 from culture media (Takeda et al. 1998). These data prompted us to consider alternatives to transferrin for delivery of iron into oligodendrocytes.

Ferritin: Role in Iron Storage, Transport, and Oligodendrocyte Function

Ferritin is a high capacity iron storage protein; theoretically capable of binding over 4,500 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). 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 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). Distribution of ferritin subunits in these cells is consistent with their function and pattern of iron utilization: L-ferritin is the predominant type in microglia, and associated with iron storage, while neurons and oligodendrocytes, which are high in H-ferritin have a high metabolic rate and also high iron turnover (Connor and Menzies, 1996). Blissman et al. (1996) showed that in the developing pig brain accumulation of iron coincides with the appearance of immunostainable H-ferritin in CNPase positive cells, a standard marker of mature oligodendrocytes (Blissman et al., 1996). 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 pre-requisite 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 PNDs 3.5 and 10.5 post-fertilization (Ferreira et al., 2000, 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. Initially, we interpreted the decreased myelin profile in the H-ferritin deficient mice as resulting from a compromised ability to handle the intracellular iron and these data support the notion that H-ferritin accumulation by oligodendrocytes is important for myelination. However, now that H-ferritin has been identified as a potential iron delivery protein for oligodendrocytes (Hulet et al., 2000) an alternative interpretation that the hypomyelination in these mice is due to decreased iron delivery by 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 (Unger and Hershko, 1974)

first hinted at the possibility of existence of plasma membrane receptor for ferritin in liver. Since then, ferritin receptors have been reported in binding studies in variety of cell types including rat (Mack et al., 1985), pig (Adams et al., 1988) and human hepatocytes (Adams et al., 1988), 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. 1999, 2000, 2002). Developmentally, H-ferritin binding followed the spatial and temporal sequence associated with myelinogenesis (Hulet et al. 2002). The binding constant (K_d) for H-ferritin in the brain and on oligodendrocytes was 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., 1999). These results suggest that two alternative iron delivery mechanisms exist in the adult brain: transferrin in grey matter and neurons, and ferritin in white matter and oligodendrocytes. The advantage of a separate iron delivery for oligodendrocytes in form of H-ferritin may be avoiding competition for transferrin-bound iron with the neurons and other brain cells that primarily use transferrin system while still preserving iron supply to oligodendrocytes in form of 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 transferrin mediated. A critical question that required immediate attention was to find the source of H-ferritin. Currently, two potential sources have been identified (see Fig. 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 (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 rat, 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, but it is 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 post-natal period, microglia first accumulate iron, serving as a sort of a iron capacitor, and releasing it to developing OPCs during myelination when the oligodendrocyte requirement for iron is the highest (see Fig. 6). This concept was further explored *in vitro* showing that media from iron-loaded primary rat microglia contained factors that promoted 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 they may contribute iron for the myelination process, mainly in form of H-ferritin.

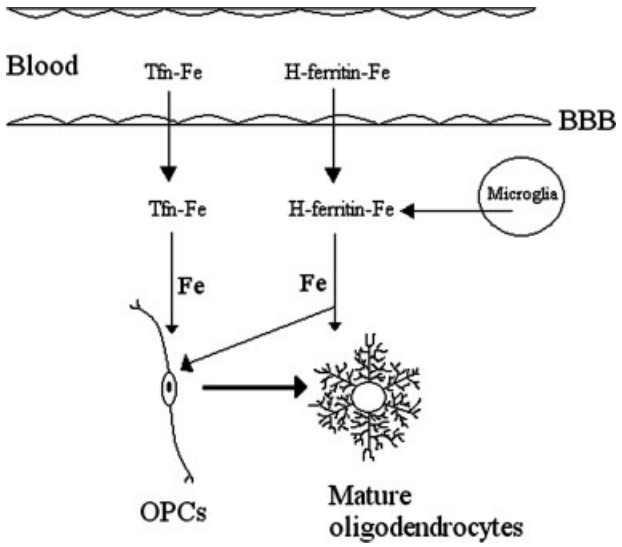

Fig. 6. Schematic of current understanding of iron sources for developing oligodendrocytes in CNS. Transferrin delivers iron to oligodendrocyte progenitor cells (OPCs). The source of transferrin for these cells is not known but is thought to come across the blood–brain barrier (BBB) from serum. The transferrin 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 OPCs and mature oligodendrocytes (Hulet et al., 2000).

The other major question if H-ferritin was an iron source for oligodendrocytes was the identity of the H-ferritin receptor. 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., 2008).

In conclusion, there is compelling evidence that iron is essential for normal myelin production and maintenance and iron accumulation by oligodendrocytes is an early event in the development of oligodendrocytes. Iron delivery to oligodendrocytes is mediated by transferrin ini-

tially and subsequently by H-ferritin (see Fig. 6). The role of H-ferritin and transferrin in both iron delivery and intracellur iron handling further emphasizes the importance of these proteins and iron in oligodendrocyte function. Knowledge of the timing of H-ferritin and transferrin expression will be essential for developing therapeutic strategies for treating iron deficiency related hypomyelination and supporting reparative mechanisms for treating dysmyelinating disorders.

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