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The Challenge of Understanding Cerebral White Matter Injury in the Premature Infant

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Abstract

White matter injury in the premature infant leads to motor and more commonly behavioral and cognitive problems that are a tremendous burden to society. While there has been much progress in understanding unique vulnerabilities of developing oligodendrocytes over the past 30 years, there remain no proven therapies for the premature infant beyond supportive care. The lack of translational progress may be partially explained by the challenge of developing relevant animal models when the etiology remains unclear, as is the case in this disorder. There has been an emphasis on hypoxia-ischemia and infection/inflammation as upstream etiologies, but less consideration of other contributory factors. This review highlights the evolution of white matter pathology in the premature infant, discusses the prevailing proposed etiologies, critically analyzes a sampling of common animal models and provides detailed support for our hypothesis that nutritional and hormonal deprivation may be additional factors playing critical and overlooked roles in white matter pathology in the premature infant.

Introduction

White matter injury in the premature infant leads to substantial long-term motor and cognitive disabilities and remains one of the most challenging clinical problems in neonatal neurology. Very low birth weight infants (<1500 grams) are particularly at risk, as 10–15% are diagnosed with permanent motor deficits (cerebral palsy) and 25–50% have significant cognitive, attentional, behavioral or socialization problems (Hack et al., 2005, Volpe, 2009, Mercier et al., 2010, Anderson et al., 2011, Anderson, 2013). The emotional and economic burdens of these deficits are large, with cerebral palsy costs estimated at nearly a million dollars per patient (Honeycutt et al., 2004). Since preterm birth rates are increasing in nearly all countries (Goldenberg et al., 2008, Blencowe et al., 2012), particularly in the very low birth weight population at highest risk for white matter injury (Batton et al., 2011), the

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vulnerable cohort of infants will only grow. Somewhat surprisingly, the rate of preterm births in the United States more closely resembles that of many third world countries [see Figure 3 in (Blencowe et al., 2012)], likely reflecting a variety of socio-economic, lifestyle and healthcare delivery challenges, but critically highlighting the epidemic of preterm birth with resultant neurological disabilities in the United States. While much has been learned about potential etiologies and proposed molecular mechanisms for white matter injury in the last 20–30 years, the absence of any definitive therapeutic interventions highlights the importance of further studies for this costly and debilitating disorder.

As our knowledge of the neuropathology of brain injury in the premature infant has progressed, the patterns of injuries have evolved to include neurons and axons as well as white matter. The term “encephalopathy of prematurity” has been coined to highlight that the injury involves more than just the myelin (Volpe, 2005, 2009). Several recent reviews (Volpe, 2009, Volpe et al., 2011, Kinney and Volpe, 2012) highlight the importance of areas beyond white matter. Given the breadth of this topic, we will limit our discussion to white matter pathology, although it is critical to recognize the interplay between neuronal/axonal injury and the pathogenesis of white matter injury (Volpe, 2009). This review will trace the history of white matter pathology in the preterm infant, briefly discuss proposed etiologies, present an overview of relevant animal models, and then discuss our hypothesis that nutritional and hormonal deprivation may be playing an important and overlooked role in white matter pathology.

History of Periventricular Leukomalacia (PVL)

The origins of neonatal white matter research can be traced to Banker and Larroche in the 1960's who described a large number of primarily premature infants with unique lesions of the white matter consisting of coagulative necrosis, astrocytic proliferation and microglia activation which they termed “periventricular leukomalacia” (Banker and Larroche, 1962). Careful review of the clinical history of these infants showed that all had suffered an anoxic episode and they noted that the areas of injured white matter were found at arterial border zones, supporting their hypothesis that blood with either a lack of oxygen or an excess of oxygen contributed to the pathogenesis of the observed injury. (Of interesting historical note, they also mention that no experimental animal model had reproduced the findings observed in periventricular leukomalacia.) An alternative hypothesis implicating infection as an etiology of white matter injury initially developed from epidemiological work of Leviton and Gilles showing that gram-negative bacteremia at autopsy was highly associated with perinatal telencephalic leukoencephalopathy (Leviton and Gilles, 1973). Subsequent epidemiological studies and prospective cohort studies have confirmed an association of white matter injury with infection/inflammation (Nelson et al., 1998, Wu et al., 2003, Stoll et al., 2004, Shah et al., 2008, O'Shea et al., 2013).

The emergence of cranial ultrasonography in the late 1970's provided a “window to the neonatal brain” (Volpe, 1982) and while first used to identify hemorrhage and brain anomalies (Babcock and Han, 1981) was recognized to be sensitive at detecting cystic white matter injury as well (Dubowitz et al., 1985). As this pattern of brain injury became more recognized, neonatal intensive care units began screening more systematically with routine

cranial ultrasounds and cystic PVL lesions were identified. At the same time, neonatal intensive care improved, surfactant for lung development was introduced (~1990) and survival of the very low birth weight infants (<1500 grams) improved (Engle et al., 2008, Halliday, 2008, Volpe, 2008). A reappraisal of the major underlying lesion of PVL demonstrated that a more diffuse lesion with microcystic areas was now the predominant lesion (Miller et al., 2005, Woodward et al., 2006) and often detectable with brain MRI (Maalouf et al., 2001, Inder et al., 2003a, Inder et al., 2003b). Most very low birth weight infants now survive, making autopsy studies challenging and highlighting the importance of neuroimaging in evaluating these infants with the diffuse white matter lesion illustrated here on brain MRI (Figure 1).

Etiology: Pre-OL Targeted

Despite the shift in the predominance of the lesion type, the main hypothesized etiologies, especially hypoxia-ischemia, remain largely unchanged from the studies described above in the 1960's and 1970's, although the likelihood that an important subset of infants experiences a combined insult, infection/inflammation plus hypoxia-ischemia, is apparent. The target of these upstream injury mechanisms is the oligodendrocyte, the myelin-producing cell of the central nervous system. Oligodendrocytes progress through a well-characterized developmental lineage that can be identified through cell surface antigens recognized by monoclonal antibodies A2B5, O4, and O1 as well as antibodies against myelin basic protein (MBP). Oligodendrocyte progenitors express the A2B5 antigen, pre-oligodendrocytes express the O4 antigen, immature, post-mitotic oligodendrocytes express the O1 antigen in addition to the O4 antigen, and mature oligodendrocytes express MBP (Figure 2). Extensive investigation using oligodendrocyte cultures, pre-clinical animal models and human autopsy material have identified the O4-positive premyelinating oligodendrocyte (pre-OL) as the predominant form of the oligodendrocyte lineage in the human premature brain and the vulnerable cell population leading to white matter injury [reviewed in (Volpe et al., 2011)]. A variety of downstream mechanisms including excitotoxicity, oxidative/nitrative injury from free radicals, and microglial activation have all been implicated in pre-OL injury and death [see comprehensive reviews in (Back and Rosenberg, 2014, Rosenberg, 2014)]. More recent evidence suggests that following pre-OL injury there is replenishment of the pre-OL pool, but critically, a subsequent failure of proper oligodendrocyte maturation (Billiards et al., 2008, Buser et al., 2012). There has been some suggestion that hyaluronan digestion product accumulation (Preston et al., 2013) may contribute to this maturational block that ultimately leads to hypomyelination.

Modeling White Matter Injury in the Laboratory (Pre-Clinical Models)

Based on the hypotheses that hypoxia-ischemia and inflammation play critical upstream roles in the pathogenesis of white matter injury, a diversity of pre-clinical models have been developed in numerous species. These generally involve hypoxia-ischemia, chronic hypoxia, hyperoxia, or lipopolysaccharide-induced inflammation. Lissencephalic rodents are commonly used due to low cost, widespread availability of critical mouse/rat reagents, and the potential to use genetic approaches to investigate molecular pathways. Larger animal models (pigs, sheep, baboons) are less commonly used but offer benefits of physiologic

monitoring, longer gestations, and larger brains for regional studies. We will review a few examples to illustrate the diversity of the models.

The most well established rodent model of neonatal brain injury is the Rice-Vannucci model. This model is classically performed in the P7 rat and is produced by unilateral common carotid ligation followed by a variable period of systemic hypoxia leading to a unilateral injury to white matter, cerebral cortex and deep nuclei (Vannucci and Vannucci, 2005). It has been adapted to younger ages (P3) (Segovia et al., 2008), to mice (Graham et al., 2004, Sheldon et al., 2007) and has been used to investigate molecular mechanisms of emerging neuroprotective therapies such as hypothermia (Liu et al., 2013) and erythropoietin (Fang et al., 2013) which are primarily targeted at term infants. Others have modified the P7 model to produce relative selectivity for white matter [but see (Selip et al., 2012)] and demonstrated a potential role for excitotoxicity in white matter injury (Follett et al., 2000, Follett et al., 2004, Manning et al., 2008). However, the classical model often leads to substantial neuronal necrosis and/or infarction which are not typical features of diffuse white matter injury in the premature infant (see example in Figure 3).

More recently, a mouse model using unilateral carotid ligation alone (without hypoxia) at P5 has been developed that produces a bilateral white matter injury with some features of PVL (Fatemi et al., 2011). The authors argue that the relative selectivity for white matter is because the insult is milder and inflicted at an earlier age, although the mortality rate from the injury was 25% and, of the animals that survived, almost 10% suffered large hemispheric strokes and had to be excluded from analysis. In addition, appropriate placebo treated animals to control for anesthesia effects were not included which is critical as anesthesia alone has toxic effects on developing oligodendrocytes (Brambrink et al., 2012, Creeley et al., 2013). The relevance of this new model remains to be determined.

Other rodent models use chronic hypoxia from P3–P13 (Ment et al., 1998) or P3–P33 (Weiss et al., 2004) to induce white matter injury. The opposite condition, neonatal hyperoxia, can produce oligodendrocyte death, decrease myelination and produce abnormal structural changes in the myelin sheath (Gerstner et al., 2008, Ritter et al., 2013). While infants in neonatal intensive care units are frequently exposed to hypoxia from apneic spells due to prematurity or chronic lung disease and may be exposed to hyperoxia at delivery or during correction of desaturation events, the prolonged duration of the insults required to produce white matter lesions in these models is of concern regarding relevance. In addition, these models suffer from the inability to measure blood oxygen content and the chronic hypoxia models develop compensatory mechanisms such as robust corticosteroneogenesis (Fagel et al., 2006) with unclear relevance to human white matter injury.

A rabbit model of in utero placental insufficiency leading to hypoxia-ischemia in preterm (70% gestation) rabbit fetuses has been used to study white matter injury as well (Derrick et al., 2004). This model offers advantages as the rabbits develop hypertonic motor deficits that can be easily quantified using neurobehavioral tests. Placental insufficiency may explain some of the injuries seen in infants born small for gestational age, but these are a relative minority of premature infants.

Preterm sheep exposed to ischemia (Elitt et al., 2003, Bennet et al., 2007, Fraser et al., 2008) or LPS-induced inflammation (Duncan et al., 2002, Mallard et al., 2003, Dean et al., 2011b) develop white matter lesions. The advantages and disadvantages of the sheep models were reviewed recently by Back and colleagues (Back et al., 2012). The benefits include the ability to perform *in utero* physiologic monitoring and develop controlled brain insults, the relative abundance of white matter in the preterm sheep, and ability to produce a graded lesion with relative selectivity for white matter. Drawbacks include the reliance on histological and radiological endpoints, a lack of neurobehavioral testing and the *in utero* nature of the model. The inability to model an *ex utero* environment precludes investigation of postnatal respiratory complications and the effects of fetal deprivation from maternal hormones and nutritional factors.

Newborn piglets have also been used to study perinatal brain injury. Advantages of piglets include a brain growth pattern similar to humans (Figure 4) and the ability to readily perform physiologic monitoring and brain imaging. The majority of studies have modeled term hypoxic-ischemic encephalopathy (with primarily deep gray matter injury) and examined therapeutic interventions such as hypothermia, melatonin and xenon treatment (Faulkner et al., 2011, Faulkner et al., 2012, Kerenyi et al., 2012, Robertson et al., 2013). To our knowledge, there has not been detailed analysis of oligodendrocyte development or production of a diffuse white matter lesion relevant to the premature infant in the piglet. A preterm piglet model at 90% gestation has been used in gastroenterology research (Rasch et al., 2010, Sangild et al., 2013) but the need for a sow surgical facility, piglet intensive care unit, and significant technical expertise are limitations to this model.

White matter injury has also been modeled in non-human primates. A baboon model born at approximately 70% gestation and cared for in a neonatal intensive care unit under conditions attempting to mimic those of the human infant (Inder et al., 2005, Griffith et al., 2012) leads to white matter injury and intraventricular hemorrhages in some animals. Notably, this model does not involve any inflicted injury *per se*, but rather routine neonatal care with ventilatory support. However, the degree and mode of ventilation appears to impact brain growth complicating interpretation of the results in these experiments (Verney et al., 2010). The cost and practical aspects of primate studies also limit their utility.

Since white matter injury is frequently studied in rodent models, the issue of timing of experimental insults to best mimic the human disorder is important. Development in the rodent is much more rapid than in the human fetus (for example, days versus years for myelination, Figures 4 & 5) (Dobbing and Sands, 1979, Kinney et al., 1988) and attempts have been made to compare key developmental events across species (www.translatingtime.org) (Clancy et al., 2007). The prevailing thought is that the P7 rat is near term or term equivalent and earlier times represent preterm human equivalents [reviewed in (Semple et al., 2013)]. However, the central question is when the developing oligodendrocytes of particular interest, the pre-OLs, are present in the human and the rodent. Between 18–27 weeks gestation in human autopsy specimens, more than 90% of the oligodendrocytes are at the pre-OL (O4+, O1-) stage (Back et al., 2001). Beginning at about 28 weeks gestation, the immature-OL (O4+, O1+) population slowly increases, comprising approximately 30% of oligodendrocytes between 28–41 weeks gestation. Thus, in the

human the major cell population present during the peak incidence of white matter injury in the premature infant is the pre-OL. Both in vitro and in vivo studies in rodents also demonstrate that the vulnerable pre-OL population is present considerably earlier than P7, the age used for many of the rodent models discussed earlier. Immediate dissociation of rat forebrains have shown that O4-positive oligodendrocytes peak around P4 (Gard and Pfeiffer, 1989, 1993). Careful in vivo studies in mice and several strains of rats have shown that pre-OLs peak around P2 and by P5–P7 are replaced by immature oligodendrocytes (O4+, O1+) (Craig et al., 2003, Dean et al., 2011a). Thus, based on population dynamics of the pre-OL population, the relevant age to model human PVL in the rodent would be P2–P4.

Since excitotoxicity is thought to be a major component of white matter injury (Volpe et al., 2011), the timing of glutamate receptor expression on oligodendrocytes is important and available data is somewhat in conflict with the data discussed above regarding the presence of the pre-OLs in the white matter. The developmental expression of α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPA) have been studied in both rats and humans across development (Talos et al., 2006a, Talos et al., 2006b), specifically examining AMPAR subunit expression, as AMPARs lacking the GluR2 subunit are highly calcium permeant and thereby important for the production of excitotoxicity. In these studies, GluR2-lacking AMPAR seemed to be present on OLs between P7–P9 in the rat and were notably absent from P1–P5. Similar findings were demonstrated in human pathology specimens from 25–37 weeks gestation infants having a relative abundance of GluR2-lacking AMPARs on OLs. Based on these data, the time window for maximal white matter injury targeting OLs involving excitotoxic mechanisms would seem to be from P7–P9. Indeed, animal models show that white matter injury can be blocked using AMPAR antagonists at these ages (Follett et al., 2000). However, on close review of the localization studies of AMPAR subunits on oligodendrocytes, the cell stage that was actually stained is unclear. Double-labeling immunocytochemistry for the AMPAR subunits was performed using O4 as a marker for pre-OLs, but immature-OLs also express O4. Triple labeling with O4, O1 antibodies and antibodies against GluR subunits, or some other approach to discriminate between the pre-OL and immature OL populations, would be necessary to show that it is pre-OLs specifically that express GluR2-lacking AMPARs. The developmental expression of AMPARs noted may reflect the transition to immature OLs (O4+, O1+), as the timing of expression correlates with the transition from pre-OL to immature-OL in the rodent. The above mentioned absence of GluR2-lacking AMPAR on oligodendrocytes at P1–P5 in vivo suggests that, in contrast to what has been seen in vitro (Fern and Moller, 2000, Itoh et al., 2002, Deng et al., 2003, Rosenberg et al., 2003, Sanchez-Gomez et al., 2011), this type of AMPAR might be absent on pre-OLs in vivo. These observations raise the possibility that other glutamate receptors (NMDA, kainate receptors) or non-excitotoxic injury pathways may be more important for pre-OL injury/death in vivo. NMDA receptors are present on developing oligodendrocytes in both rodents and humans (Karadottir et al., 2005, Salter and Fern, 2005, Jantzie et al., 2013), but a detailed analysis of NMDA receptor expression on pre-OLs has not been completed. Thus, at present, it is uncertain whether excitotoxic mechanisms are important in pre-OL injury in the developing brain because, remarkably, glutamate receptors have not been clearly demonstrated on pre-OLs in vivo. This gap in our knowledge further complicates the development of relevant animal models.

The large diversity of animal models using different stimuli at various gestational ages all leading to white matter injury in itself suggests that the exact etiology of premature brain injury is unknown and likely multifactorial. So without knowing the precise etiology or etiologies of white matter injury, can we really develop an appropriate model in the laboratory?

Even when the primary cause of a disease is well established, the elucidation of pathogenetic mechanisms can be challenging. This point is well illustrated by a consideration of studies related to Wernicke's encephalopathy, a human disorder known to be produced by a dietary deficiency in thiamine (vitamin B1) and most common in alcoholics. Initial studies in animal models of thiamine deficiency suggested that the lesions showed similarities with anoxic injury, and excitotoxicity was proposed as the cause of cell death in the thalamus, mammillary bodies and periventricular regions (Langlais and Mair, 1990, Langlais and Zhang, 1993, Zhang et al., 1995). This hypothesis was consistent with the understanding that thiamine is necessary for metabolism of carbohydrates, and its absence produces energy failure in cells, similar to ischemia. However, subsequent studies showed that early events in the animal models were not blocked by glutamate antagonists, strong evidence against the hypothesis that excitotoxicity underlies the early pathogenesis of thiamine deficiency. Currently, the pathogenesis is thought to be a combination of oxidative stress, inflammatory processes involving microglial activation, and excitotoxicity (Hazell and Butterworth, 2009, Jhala and Hazell, 2011). There are (at least) two lessons to be learned from this example: 1) Even in such a case as Wernicke's encephalopathy in which a disorder is known to be produced by a single cause, the manifestations are complex and caution should be exercised in focusing on one particular mechanism of injury (for example excitotoxicity in the case of Wernicke's); 2) It is extremely difficult to determine primary etiologies from manifestations of tissue injury.

The diversity of pathogenetic mechanisms implicated in thiamine deficiency is strikingly similar to that proposed for PVL. By example, then, the different processes that seem to be involved in the pathogenesis of white matter injury in the premature infant could, conceivably, be manifestations of a single etiology. As we have discussed, the etiology of PVL remains to be established, although there is evidence supporting important roles for excitotoxicity, oxidative stress, and cytokine mediated injury. Since Wernicke's encephalopathy is known to be produced by a nutritional deficiency that leads to manifestations of these same pathogenetic mechanisms, we have a precedent for the idea that the pathology of PVL could be produced by a nutritional deficiency. Therefore, we propose that it might be fruitful to consider the possibility that PVL could arise, at least in part, from a nutritional deficit.

Potential Role of Nutrition

The developing human undergoes a tremendous *perinatal* "brain growth spurt" which is also observed *postnatally* in rodents (Figure 4) (Dobbing and Sands, 1979). Premature birth occurs during the upswing of this growth phase, and the extrauterine environment has a multitude of challenges, including the sudden early necessity to breathe, deliver oxygenated blood, adjust to hormonal changes and obtain adequate nutrition. The potential

complications from mechanical ventilation, apnea, bradycardia and hypotension highlight the significance of the respiratory and cardiac systems. However, the potential importance of perinatal nutrition, particularly the acquired nutrient deficits from premature birth and our incomplete understanding of appropriate dietary intake for the premature infant, should not be overlooked.

Composition of Myelin and Accumulation of Myelin Precursors

When considering nutritional influences on myelination, it is first important to understand the biochemical building blocks for developing myelin which have been extensively profiled in both animals and humans (O'Brien and Sampson, 1965, Kinney et al., 1994). At a basic level, myelin consists of wrappings of plasma membrane around axons and functions principally to enhance conduction of action potentials [reviewed in (Aggarwal et al., 2011)], and in humans myelination occurs primarily postnatally (Figure 5) (Kinney et al., 1988). However, the lipid and protein components of myelin are vastly different than plasma membranes of other cells. Myelin has very high lipid content (70–80%) and contains several myelin-specific proteins such as myelin basic protein and proteolipid protein (Figure 6) (Deber and Reynolds, 1991). Cholesterol in the brain is concentrated in myelin (~70%) and there is a high concentration of galactolipids, plasmalogens, and fatty acids. Many of these chemical components required for myelin synthesis accumulate rapidly just prior to and during the active myelination phase which is thought to be one driver for the brain growth spurt (Figure 4). These accumulations precede and parallel the rapid accumulation of myelin observed in human postnatal MRI studies of preterm and full term infants (Figure 7) (Huppi et al., 1998). For example, cerebroside, sulfatide and sphingomyelin concentrations dramatically increase beginning around 32 weeks gestation (Conde et al., 1974), notably also the period of transition from pre-OL population to the immature-OL population (Back et al., 2001, Craig et al., 2003)). A similar chemical spurt also occurs for phosphoglycerides (Martinez et al., 1974), plasmalogens (Martinez and Ballabriga, 1978), and cholesterol (Martinez and Ballabriga, 1978) in human infants (Figure 8, one example). Animals at the respective periods of peak brain growth also rapidly accumulate cholesterol (Dobbing, 1963), sulfatides (Davison and Gregson, 1962) and various lipids (Cuzner et al., 1965) (Figure 9), critical building blocks for the synthesis of myelin.

Undernutrition and Myelination

The concept that dietary intake could influence myelination was initially suggested by Sugita in the 1900's who reduced milk intake to rat pups using two methods: 1) maternal undernutrition leading to decreased milk production or 2) artificially increasing litter number leading to competition with overfed and underfed pups (Sugita, 1918). Rats with reduced milk intake or slow growth had relative hypomyelination. Dobbing and colleagues studied this effect more closely in the 1960's by transferring pups on the day of birth to produce small and large litters which yielded "fast-growing" and "slow-growing" rat pups, respectively. Under these conditions, brain cholesterol accumulation, used as a surrogate for myelin formation, was reduced in the underfed, "slow growing" rats (Dobbing, 1964). Similar reductions in brain sulfatides, lipids, cerebroside, proteolipids and histological myelin have been observed when rodents are specifically undernourished in the first three weeks of life, during the onset (P8–P10) myelination (Benton et al., 1966, Chase et al.,

1967, Bass et al., 1970a). Human autopsies of infants and children who died from undernutrition also show reduced white matter lipids, specifically proteolipid proteins, cerebroside and plasmalogens (Fishman et al., 1969, Chase, 1973, Martinez, 1982).

The Vulnerable Period: Timing, Degree, and Duration of Undernutrition

Hypomyelination is not observed if undernutrition occurs later in life, for example in the rat from P30–P51 (Donaldson, 1911), suggesting that there is a “vulnerable period” (Davison and Dobbing, 1966) for inadequate intake with potential long-term consequences on myelination. Several studies have attempted to define this critical period in the rodent. Undernutrition from P21–P77 produces a reduction in brain weight and brain cholesterol, but this is entirely reversed by a refeeding period with adequate nutrition (Dobbing and Widdowson, 1965). Wiggins et al showed that early undernourishment (P0–P20) significantly reduces the production of myelin protein (Figure 10) and a period of refeeding increases the accumulation of myelin but does not restore the synthetic capacity to control levels (Wiggins et al., 1976), suggesting that the deficits may be permanent. Substantially more modest reductions in intake during this critical period still lead to permanent myelin deficits (Fuller et al., 1984, Royland et al., 1992) and morphometric studies of axons from undernourished animals demonstrated decreased myelinated axons, thinner myelin sheaths and more “promyelinating fibers” (axons with one or two non-compacted myelin lamellae) (Krigman and Hogan, 1976).

Wiggins and Fuller further delineated the timing of the vulnerable period. Rats underfed from P0–P14 or P0–P20 had significant reductions in myelination in multiple brain regions including the cerebral cortex that persisted at P60. However, briefer periods of undernutrition from P0–P8 or at later periods P14–30 did not lead to hypomyelination (Wiggins and Fuller, 1978). This finding suggests that the vulnerable period for undernutrition in the rodent is just before the onset of active myelination between P8 and P14. Taken as a whole, the data suggest that an early period of even mild undernutrition during the brain growth spurt leads to long lasting hypomyelination. Unfortunately, similar studies have not been performed in larger species.

The vulnerable period in the human infant is less clearly defined as studies of human undernutrition are much more limited. The active phase of myelination in the human infant proceeds from just before birth to approximately age 2, with robust myelination occurring during the first 6–8 months and maturation of myelination occurring from 7–15 months (Kinney et al., 1988). Presumably this entire period and the preceding fetal period of oligodendrocyte development may be sensitive to undernutrition. Limited studies of undernourished infants and young children suggest that malnourishment can impair myelination (Fox et al., 1972) and longitudinal studies of Barbadian children malnourished for the first year of life suggest that they have slower performance on motor tasks (Galler et al., 1984) and reduced intelligence (Galler et al., 1990) which may reflect reductions in white matter. Alternatively, neuronal injury from malnourishment may also account for these deficits as diminished cortical thickness has been demonstrated in undernourished rodents (Bass et al., 1970b) and recently in premature infants with poor postnatal growth (Vinall et al., 2013). These cortical deficits are attributed to reduced axodendritic processes

and fewer mature oligodendrocytes, rather than neuronal loss. However, without more detailed studies in undernourished populations, ideally using state-of-the-art MRI measurements, it is difficult to more clearly define the vulnerable period for nutritional insults in the human.

Specific Nutritional Deficiencies Implicated in Myelination

The lack of numerous dietary components, many of which are enriched in breast milk and carefully supplemented in premature infants, can lead to hypomyelination. A postnatal diet deficient in protein reduces myelin accumulation at the time of weaning (Nakhasi et al., 1975) and longer durations of protein deficiency up to 4 months decreases myelin staining in the anterior/posterior commissures of rats (Pacagnella et al., 2013). A prenatal diet deficient in essential fatty acids and continued through the twelfth postnatal month reduces brain lipids and alters fatty acid composition such as decreasing linoleic acid and docosahexaenoic acid amounts by more than 50% (Galli et al., 1970). A postnatal diet deficient in essential fatty acids results in hypomyelination and myelin that has abnormal composition (McKenna and Campagnoni, 1979, Berkow and Campagnoni, 1983). The opposite, a diet enriched in lipids (odd-chain fatty acids) accelerates myelinogenesis in the rat producing oligodendrocytes with proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) mRNA as early as postnatal day 5 (Salvati et al., 1996). Artificially inhibiting the synthesis of sphingomyelin (a key precursor to galactosylceramide) reduces brain myelin content and decreases myelin thickness in the optic nerve of postnatal rats preceding and during active myelination. These findings are reversed by dietary supplementation with sphingomyelin, which is enriched in human milk (Oshida et al., 2003). Taken together, these studies suggest that a multitude of dietary components can influence myelination.

Cholesterol

The importance of cholesterol biosynthesis in myelination has been shown using mice that lack the ability to synthesize cholesterol in oligodendrocytes (Saher et al., 2005). These mice have severely disrupted myelination and develop ataxia and tremors. However, over several months in adulthood their symptoms improved. This improvement was attributed to horizontal transfer of cholesterol from neighboring healthy cells as cholesterol is reportedly not transferred from the bloodstream to the brain (Jurevics and Morell, 1995, Edmond, 2001), at least with an intact blood-brain barrier.

However, the same group demonstrated that *dietary* cholesterol plays a critical role in myelin synthesis. In a mouse model of Pelizaeus-Merzbacher Disease, a fatal leukodystrophy characterized by dysmyelination followed by demyelination related to overexpression of the proteolipid protein gene 1 (PLP1), a diet-enriched in cholesterol improved myelination and reduced motor deficits (Saher et al., 2012). They also demonstrated that circulating cholesterol enters the brain and appears to be incorporated into the myelin sheath, again highlighting the potent effects of diet on myelination, in this case under conditions of injury or inflammation. Early studies using hypocholesteremic agents from P15–P20 also reduced myelination (Fumagalli et al., 1969), supporting a role for dietary cholesterol during active myelination.

Long Chain Polyunsaturated Fatty Acids

Long chain polyunsaturated fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been studied extensively in preterm infants with mixed results (Lapillonne et al., 2013), although current recommendations are for lactating and pregnant mothers to supplement with DHA. Preterm infants are a unique population because they develop a severe DHA deficit over the first few weeks of life secondary to parenteral nutrition (Lapillonne et al., 2010). A variety of clinical trials in full term infants have shown improvements in visual acuity with increased DHA supplementation (Birch et al., 2010, Jacques et al., 2011). Studies in preterm infants have not shown a benefit on visual acuity but have had scattered positive benefits on mental development and attention scores (Fewtrell et al., 2004, Smithers et al., 2010, Westerberg et al., 2011). However, a recent meta-analysis of long chain polyunsaturated fatty acid supplementation in preterm infants found no conclusive benefits on visual acuity or neurodevelopmental outcome to recommend supplementation (Schulzke et al., 2011). The prior studies in preterm infants did not aim to replace the initial DHA deficits and the optimal dose of supplementation remains unclear, so it is not surprising that the studies have been somewhat inconclusive.

In contrast, in animal models of white matter injury, DHA has generally been protective. DHA pretreatment in a P7 hypoxia-ischemia rat model improved forepaw placing response and attenuated tissue damage (Berman et al., 2009). DHA combined with hypothermia attenuates brain injury and promotes sensorimotor recovery in the P7 rat exposed to hypoxia-ischemia (Berman et al., 2013). A maternal diet enriched in DHA and EPA was protective against LPS-induced white matter lesions in rats (Tuzun et al., 2012). Thus, it is possible that DHA may be an important protective factor for developing white matter that may need to be replaced.

Vitamin K

Under conditions of inflammation or injury, dietary nutrients might also modulate injury to developing oligodendrocytes. Vitamin K is a fat-soluble vitamin given to both term and preterm infants to prevent bleeding due to known deficiency at birth, although the dose provided in preterm infants may vary up to 20-fold (Clarke, 2010). We have previously shown that vitamin K can prevent oxidative injury to undifferentiated oligodendrocytes (Li et al., 2003). Other studies have shown that dietary vitamin K intake correlates with accumulation of glycolipids, specifically sulfatides, in brain myelin (Crivello et al., 2010) and can modulate the activity of galactocerebroside sulfotransferase (GST) (Sundaram et al., 1996), which is required for synthesis of sulfatides unique to the myelin membrane. No studies have evaluated the potential protective role of vitamin K on white matter in in vivo models of inflammatory or ischemic injury, although it appears that most premature infants are being appropriately supplemented even to supratherapeutic levels (Kumar et al., 2001). Nonetheless, this again suggests a role for dietary intake in modulating myelination.

Breastmilk

Many infants are exclusively formula fed and less than 50% are exclusively breastfed at 6 months of age (CDC, 2013). Breastfeeding is the recommended method for feeding premature infants and NICUs are now using banked donor milk when necessary (Parker et

al., 2013). Numerous observational studies have shown the benefits of breastfeeding on later cognitive measures (Lucas et al., 1992, Mortensen et al., 2002, Bernard et al., 2013). A study in premature infants showed a dose response in which every 10 mL/kg per day of breast milk contributed 0.53 points to the Bayley Mental Development Index (Vohr et al., 2006). While confounding variables such as social environment and maternal intelligence can bias these types of studies, with controls for these factors the benefit is still present (Belfort et al., 2013). Looking specifically at the role of breast milk on white matter development, others have correlated increased white matter development on MRI scans with increased breastfeeding (Isaacs et al., 2010, Deoni et al., 2013). Numerous components of breast milk such as long-chain polyunsaturated fatty acids, hormones or growth factors have been suggested as possible mechanisms for this effect on myelin, but all remain unproven.

Rodent milk is presumably influenced by diet as well and it is important to note that rodents are exclusively breastfed in the preclinical models in which white matter injury is studied. If breastfeeding has a benefit on myelination, then preclinical models may be confounded by the exclusive use and relatively unlimited quantity of breast milk. Maternal milk content is also dependent on laboratory chow which has its own set of variations across institutions and time as ingredients can vary with manufacturing batch or seasonal variable in raw starting materials (Barnard et al., 2009). Free access to unlimited food creates “metabolically morbid” maternal rodents (Martin et al., 2010) which likely influences milk content and may further complicate translation to the preterm infant.

Possible Mechanisms for Hypomyelination as a Consequence of Undernutrition

The mechanism by which diet influences myelination is unclear. One possibility is that there are simply inadequate precursors to maintain robust myelin synthesis and this is plausible given the extrauterine growth restriction observed in premature infants (see below). There may also be direct effects on oligodendrocyte maturation producing cell death or a maturation block. Total brain DNA is reduced with undernutrition, which is primarily attributed to reduced glia, possibly oligodendrocytes (Krigman and Hogan, 1976). One report however did not show any substantial reduction in oligodendrocyte number to account for a nearly 50% reduction in brain myelination in the rat (Sikes et al., 1981), although these investigators did not examine specific subpopulations of developing oligodendrocytes shown in Figure 2. Sikes and colleagues suggest that instead of oligodendrocyte death, there may be aberrant oligodendrocyte differentiation or decreased myelin synthesis on an individual cell basis. There is some evidence that undernutrition slows or blocks oligodendrocyte maturation, thereby resulting in hypomyelination (Robain and Ponsot, 1978) and others have observed a block in oligodendrocyte maturation in premature infants with white matter injury (Billiards et al., 2008, Buser et al., 2012). A role for polyunsaturated fatty acids has been suggested as supplementation with omega-3 or omega-6 fatty acids stimulates differentiation of oligodendrocytes in culture (van Meeteren et al., 2006). However, the direct or indirect effects of undernutrition on oligodendrocytes and myelination require more detailed investigation.

Malnutrition in Neonatal Intensive Care Units?

While a small percentage of premature infants are born growth restricted presumably from intrauterine factors, a much larger percentage develop *extrauterine* growth restriction which often persists into childhood. Postnatal daily growth goals have been set by the American Academy of Pediatrics that in essence mimic in-utero growth rates (15–20grams/kg/day) (AAP, 1985) and extrauterine growth restriction is typically defined as growth values 10th percentile of intrauterine growth expectation based on estimated gestational age (Clark et al., 2003). The majority of premature infants do not meet these recommendations and develop extrauterine growth failure. In one study looking at multiple intensive care units, 98% of moderately premature infants did not meet these recommendations for growth (Figure 11) (Blackwell et al., 2005). Extrauterine growth restriction is particularly a problem for very low birth weight infants (Lemons et al., 2001, Clark et al., 2003), the subpopulation most at risk for white matter injury. Inadequate nutrient intake beginning immediately after birth leads to a cumulative nutrient deficit (Figure 12) accounting for at least a portion of the extrauterine growth restriction (Embleton et al., 2001, Cooke, 2005). While there have been efforts to standardize dietary regimens for premature infants, there remains remarkable heterogeneity in intake and the recommendations continue to evolve as our knowledge about optimal nutrition improves (Blackwell et al., 2005, Lafeber et al., 2013). The heterogeneity in the intensive care units can be attributed to a variety of factors outlined in Table 1. At least a few recent studies (Senterre and Rigo, 2011) have shown that the extrauterine growth deficit can be reversed by early “aggressive nutrition” which some authors now equate with “adequate nutrition” (Lapillonne and Griffin, 2013).

Extrauterine growth restriction is obviously of great concern, particularly given the animal studies discussed above demonstrating hypomyelination with malnutrition and numerous clinical studies correlating poor growth with worse developmental outcome and increased incidence of cerebral palsy (Figure 13) (Latal-Hajnal et al., 2003, Ehrenkranz et al., 2006, Stephens et al., 2009). Growth is a multifactorial process and nutrition intake may be a reflection of illness or injury. Medical complications such as bronchopulmonary dysplasia, intraventricular hemorrhage, and sepsis are known to delay the time to full oral feeding (Hwang et al., 2013) for example. However, premature infants who are well also develop the growth restriction (Blackwell et al., 2005), indicating that inadequate dietary intake or absorption may be additional variables in white matter injury in the premature infant. A synergy between inadequate nutritional support and postnatal injuries (Ehrenkranz et al., 2011) could further accentuate white matter damage in the sickest of infants. Post-discharge feeding difficulties have also been associated with impaired language development (Adams-Chapman et al., 2013), highlighting the potential importance of post-hospitalization nutrition.

Hormonal Factors

While nutritional substrate is important for growth and myelination, premature birth also produces a variety of hormonal deprivations. These under-supplied metabolic and endocrine factors may influence the ability of premature infants to respond to stressors such as infection and hypoxia-ischemia. IGF-1, estrogens and thyroid hormones are three examples

among many that likely influence white matter development and have been studied in both humans and animals.

IGF-1

Insulin-like growth factor-I (IGF-1) plays a critical role in fetal growth particularly during the third trimester. Concentrations increase throughout pregnancy (Figure 14) (Lassarre et al., 1991, Langford et al., 1998) and then decrease immediately following birth (Hansen-Pupp et al., 2007). As a consequence of early birth, premature infants have decreased concentrations of IGF-1 compared to full term infants (Bennett et al., 1983), which may partially explain their extrauterine growth restriction (Hansen-Pupp et al., 2011b). In infants born at term, IGF-1 concentrations slowly increase over the subsequent postnatal weeks, but in premature infants concentrations remain low, with no postnatal surge observed in infants born at 26–33 weeks gestation (Lineham et al., 1986).

Decreased concentrations of IGF-1 raise several concerns based on animal studies. Mice deficient in the IGF-1 gene have reduced brain size and hypomyelination (Beck et al., 1995). Conditional knockout of the IGF-1 receptor in mouse oligodendrocytes leads to decreased oligodendrocyte differentiation and reduced myelin formation (Zeger et al., 2007). In culture, IGF-1 protects developing oligodendrocytes from glutamate-mediated apoptosis (Ness and Wood, 2002, Ness et al., 2004). In vivo, IGF-1 also protects against hypoxic-ischemic and inflammatory injury to developing white matter. For example, intraventricularly administered IGF-1 reduces white matter injury in the P7 rat hypoxia-ischemia model and in near-term sheep fetuses exposed to in utero ischemia and reperfusion (Guan et al., 2001). Delayed administration of intraventricular IGF-1 following hypoxia-ischemia in rats rescues oligodendrocyte precursors and promotes myelination (Wood et al., 2007). Intranasal administration of IGF-1 attenuates lipopolysaccharide-induced myelin injury and reverses some behavioral deficits in rats (Cai et al., 2011). IGF-1 is also protective against nutritional insults as overexpression of IGF-1 in mouse brain partially protects against nutritional insults (Ye et al., 2000). The evidence is quite clear that IGF-1 may be an endogenous protective factor for oligodendrocytes during states of injury and has important roles in normal white matter development. It is conceivable that IGF-1 deprivation in the premature infant could have detrimental effects on developing white matter.

Emerging clinical studies support the hypothesis that IGF-1 plays an important role in brain development. In very low birth weight infants, there are correlations with low IGF-1 concentration and subsequent decreased brain volume and impaired cognitive development (Hansen-Pupp et al., 2011a, Hansen-Pupp et al., 2013). A recent phase II pharmacokinetics and safety study using continuous infusions of IGF-1 with IGF-binding protein in premature infants at 26–28 weeks gestation age was shown to be safe and able to maintain IGF-1 concentrations at in utero concentrations (Ley et al., 2013). An expanded study with prolonged administration of IGF-1 is planned to assess the effects on white matter volume, motor function and cognitive abilities. It will be quite interesting to see if IGF-1 *replacement* therapy influences white matter development and myelination in the premature infant.

Estrogens

While concentrations of IGF-1 decrease several-fold following premature birth, estrogen concentrations decrease much more significantly. During pregnancy, the placenta synthesizes estrogens, including estradiol (E2), estrone (E1) and estriol (E3), from precursors of fetal and maternal origin ultimately producing levels that are 100 fold higher than in non-pregnant women (Tulchinsky et al., 1972). Within the first 24hrs after birth, the concentrations of these hormones decrease nearly 1000 fold to non-gestational levels (Figure 15) (Tulchinsky et al., 1972, Ishida et al., 1985, Trotter et al., 2001). The idea that estrogen supplementation might be beneficial to the premature infant dates back over 80 years (Aschheim, 1927), but studies in the 1930's focused on weight gain with postnatal estrogen supplementation and judged the small effects to be insignificant (Moncrieff, 1936). However, there is renewed interest in using estrogen supplementation to improve pulmonary development (Trotter et al., 1999) and neurodevelopmental outcome (Trotter et al., 2001, Trotter et al., 2012).

Estrogen receptors are expressed on the oligodendrocyte plasma membrane and within the myelin sheath (Arvanitis et al., 2004) and estradiol induces morphological changes and increases myelin basic protein expression (Jung-Testas et al., 1992). Supplementation of rat pups with estradiol accelerates white matter maturation of the anterior optic pathways and hemisphere commissures (Prayer et al., 1997). Both in vivo and in vitro, estradiol is protective against hyperoxia induced oligodendrocyte injury in P6 rats (Gerstner et al., 2007). Similar studies using oxygen-glucose deprivation in developing oligodendrocytes and hypoxic-ischemia injury P6 rats again showed a protective role for estradiol in the developing brain (Gerstner et al., 2009).

There has been hesitation to translate estrogen replacement therapy to premature infants because estradiol induces masculinization of the brain and behavior in female rodents (Feder et al., 1974, McEwen et al., 1977). However, in the primate brain, estrogens have no role in masculinization, as this function is performed by prenatal androgens, social context and rearing conditions (Wallen, 2005, McCarthy, 2008). The lack of a role for estrogen in masculinization of the primate brain would also explain the observation that diethylstilbestrol, administered to millions of mothers from 1940 until 1971 to prevent miscarriage, did not produce masculinized females (McCarthy, 2008). More importantly, the very high in utero concentration of estrogen does not masculinize the female infants. Thus, the concerns about replacement therapy appear unfounded.

In a randomized controlled trial of approximately 100 premature infants weighing less than 1000 grams, Trotter et al examined the effects of estrogen and progesterone replacement, which maintained postnatal concentrations at the high in utero concentrations, on the incidence of bronchopulmonary dysplasia (Trotter et al., 2012). Neurological status was assessed at five years of age as a secondary measure and showed a linear relationship between duration of treatment and cognitive outcomes. Each day of treatment was also associated with a reduced risk for cerebral palsy and spasticity, suggesting that hormone supplementation may be protective for in vivo white matter. Additional studies with larger numbers of infants are necessary, but the results using supplementation of naturally occurring hormones are encouraging.

Taken together, these clinical and animal studies suggest that the deprivation of gonadal steroids postnatally may be harmful to developing white matter. Endogenous high estrogen is lost at precisely the time when it is most needed, namely the postnatal period when premature infants are prone to infections, hypoxia-ischemic episodes and other stressors. Exogenous replacement therapy to maintain blood levels present in utero, similar to the approach being taken for IGF-1, could potentially substantially reduce the incidence and severity of white matter injury in premature infants.

Thyroid Hormones

Thyroid hormones play an important role in regulating growth and development in numerous organs including the brain. Thyroid hormones consist of thyroxine (T4) and the biologically active form triiodothyronine (T3) that act through nuclear receptors encoded by two genes, thyroid receptor alpha (TR α) and thyroid receptor beta (TR β) which bind T3 with high affinity (reviewed in (Williams, 2008)). A potential role for thyroid hormones in myelination was initially suggested by clinical studies of infants with congenital hypothyroidism, where a major feature of the neuropathology was a paucity of myelin (Rosman, 1972). It is now well recognized that early treatment for congenital hypothyroidism is essential to minimize subsequent cognitive and motor deficits that have been attributed to both deficiencies in white matter and gray matter (Klein et al., 1972, Rosman, 1972, Klein et al., 1974, Collaborative, 1981, Annunziata et al., 1983).

However, as a serendipitous result of routine screening for congenital hypothyroidism, it was subsequently recognized that many premature infants develop transient hypothyroxinemia, a condition characterized by low concentrations of serum T3 and T4 but normal concentrations of thyroid stimulating hormone. It is a particularly common condition in early gestation infants where more than 50% of infants are affected (Uhrmann et al., 1981, Adams et al., 1995, Den Ouden et al., 1996, Reuss et al., 1997). Longitudinal assessments suggest that free T4 concentrations in premature infants do not reach concentrations observed in term infants until 6–8 weeks after birth (Fuse et al., 1990), a prolonged period of deprivation (potentially from 26–34 weeks, for example) coinciding with the rapid period of oligodendrocyte maturation and peak incidence of white matter injury. The decreased concentration of thyroid hormone likely reflects a combination of factors unique to the premature infant including an immature hypothalamic-pituitary-thyroid axis, loss of maternal/placental transfer, reduced iodine availability and other adverse perinatal events (reviewed in (La Gamma and Paneth, 2012)).

Transiently decreased concentrations of thyroid hormones are concerning given animal studies showing potent effects of thyroid hormone on myelination. For example, rats pups radiothyroidectomized shortly after birth have decreased myelination in the corpus callosum and decreased deposition of myelin lipids (Figure 16), a pattern quite similar to the malnourished animals discussed earlier (Rosman et al., 1972). Other groups have shown that hypothyroidism attenuates myelination and hyperthyroidism produces accelerated myelination that is then terminated too early (Walters and Morell, 1981). In both cases, alterations from a euthyroid state ultimately led to reduced myelination that persisted at least through 40 days of age in the rat, suggesting that having an optimal concentration of thyroid

hormone during the active phase of myelination is important. Reduced myelination has also been observed in mice with congenital hypothyroidism from a hypoplastic thyroid gland and interestingly these deficits were partially reversed by parenteral or breast milk administration of T4 (Noguchi et al., 1985). T4 supplementation was only beneficial if administered within the first 20 days of age, again suggesting a critical window during the active phase of rodent myelination.

The trophic effects of thyroid hormones on cultured oligodendrocytes and in vivo myelination have been investigated extensively. Both developing and mature oligodendrocytes express thyroid hormone receptors (Yusta et al., 1988, Fierro-Renoy et al., 1995, Baas et al., 2002). There appears to be sequential expression of the TR α and TR β gene products such that all oligodendrocytes express the alpha receptors but only mature oligodendrocytes express the beta receptors (Baas et al., 1994, Carre et al., 1998). Genetic disruption of TR α in the optic nerve results in reduced numbers of mature oligodendrocytes (Billon et al., 2002). Mice devoid of all thyroid hormone receptors have persistent oligodendrocyte precursor proliferation and abnormal myelin formation, suggesting that thyroid hormone receptor binding stops self-renewal and triggers differentiation to the mature oligodendrocyte lineage (Baas et al., 2002). Numerous groups have also demonstrated that T3 is sufficient to inhibit proliferation and promote oligodendrocyte differentiation in culture (Barres et al., 1994, Baas et al., 1997). In fact, adding T3 to oligodendrocyte progenitor cells is a widely used method for obtaining mature oligodendrocytes in culture (Dugas and Emery, 2013), further highlighting the importance of thyroid hormones on oligodendrocyte development.

Severe transient hypothyroxinemia is associated with adverse motor and cognitive outcomes in a variety of studies (Lucas et al., 1988, Den Ouden et al., 1996, Reuss et al., 1996). More specifically, Reuss et al found that severe hypothyroxinemia (>2.6 standard deviations below the newborn mean) was associated with a four fold increased risk of developing disabling cerebral palsy, even after adjustment for multiple confounding variables (Reuss et al., 1996). Others have correlated decreased thyroid hormones with risk for developing white matter lesions on ultrasound. Infants weighing 500–1500 grams with hypothyroxinemia of prematurity (defined as <5.3 $\mu\text{g/dL}$) had twice the risk of cerebral white matter echolucency compared to peers with normal thyroid concentrations (Leviton et al., 1999). It is unclear if a similar association is present with the more diffuse white matter injury observed on MRI in current practice. While not causal, the association of decreased concentrations of thyroid hormones and poor neurodevelopmental outcome merit further investigation.

Almost no basic science studies have investigated the role of thyroid hormones in white matter injury models using genetic manipulation of thyroid hormone/receptors or exogenous thyroid replacement therapy. Interestingly, a recent study using P7 rat pups exposed to hypoxia-ischemia showed that T4 concentrations were decreased compared to placebo treated animals by P11, suggesting that neonatal brain injury may itself lead to transient hypothyroxinemia, although TSH concentrations were not measured (Hung et al., 2013). In the same study, intraperitoneally injected T4 was able to increase pre-oligodendrocyte survival, increase myelination and improve neuromotor behavioral performance after

hypoxic-ischemic insults at P7 (Hung et al., 2013) supporting the hypothesis that thyroid hormones may attenuate white matter injury.

Several limited studies using thyroid hormone replacement therapy for premature infants with hypothyroxinemia have been undertaken (Chowdhry et al., 1984, Vanhole et al., 1997, van Wassenae et al., 2005), but the neurodevelopmental results remain inconclusive (Osborn and Hunt, 2007) and the appropriate target goals for thyroid supplementation have not been fully delineated. The largest study of 156 infants demonstrated improved school and motor outcomes in infants <27 weeks gestation but worsened outcomes in 29 weeks gestation infants (van Wassenae et al., 2005). In addition to the inadequate power, one central problem with the early studies was suppression of TSH suggesting the infants were being overtreated. A recent phase 1 trial of thyroid supplementation in extremely premature neonates demonstrated that it is possible to increase total thyroxine (T4) concentration without completely suppressing thyrotropin (TSH) when administered as a continuous infusion for 42 days (La Gamma et al., 2009). A properly powered clinical trial with neurodevelopmental outcomes is planned but given the animal and in vitro studies discussed above, it may also be important to evaluate the trajectory of white matter development using serial MRI measurements in these studies of thyroid supplementation therapy.

Concluding Remarks

While much progress has been made in understanding how ischemia or infection/inflammation might promote developing oligodendrocyte injury, there still are no proven targeted therapies to ameliorate white matter injury in the human premature infant, a growing and costly problem to society. The etiology of white matter injury is likely multifactorial. It is challenging to develop an appropriate animal model when many of these factors remain unclear and potentially synergize to produce injury. Among these factors might be nutritional and hormonal deprivation as highlighted with examples here, suggesting the need for postnatal replacement therapies. These areas have received less attention than ischemia and inflammation, partially due to limitations of our current animal models of injury to developing white matter, as it is difficult to model extrauterine growth restriction in the in utero sheep model, or in rodent models in which a substantial portion of brain development normally occurs postnatally. New models should incorporate the types of deprivation that a human infant born prematurely must overcome, beyond the now obvious hurdles of infection, inflammation and hypoxia-ischemia. Recreating the in utero hormonal and nutritional state may provide new insights into brain development and therapeutic approaches to this longstanding problem. It is critical to continue and to intensify the dialogue between providers in neonatal intensive care units and neurobiologists to identify and critically test extrauterine factors that may be playing an important role in the pathogenesis of white matter injury in the premature infant.

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Highlights

White matter injury (WMI) of prematurity is prevalent and costly.

Multiple etiologies for WMI have been proposed. None are definitive.

Development of accurate animal models has been challenging.

Premature infants are at risk for injury from ischemia and inflammation.

Deprivation of factors normally provided in utero may contribute to WMI.

Figure 1. MRI showing diffuse white matter injury

Magnetic resonance images of diffuse white matter injury in a former 24 weeks gestational age infant studied at 12 months of age (A&C) and an age-matched control (B&D). Sagittal T1 images in the injured infant (A) compared to the control infant (B) demonstrate diffuse thinning of the corpus callosum (arrow). Axial T2 fluid-attenuated inversion-recovery (FLAIR) images (C&D) demonstrate diffusely increased signal in the periventricular white matter (arrows) and ventriculomegaly in the injured infant (C) compared to the control infant (D). This injured child developed mild spastic diparesis and later cognitive problems in school. [Images courtesy of Dr. Janet Soul]

Figure 2. Oligodendrocyte Lineage

Progression of the oligodendroglial lineage (OL) through the four major developmental stages. A2B5 identifies OL progenitors, O4 identifies Pre-OLs, O1 identifies Immature OLs and MBP identifies Mature OLs. The predominant form in cerebral white matter of the premature infant is the O4-positive pre-oligodendrocyte. [from: Volpe JJ, Kinney HC, Jensen FE, Rosenberg PA (2011) The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *International journal of developmental neuroscience* : the official journal of the International Society for Developmental Neuroscience 29:423–440. Figure 2]

Figure 3. Spectrum of Injury in Rice-Vannucci Model

A–D, Nissl stains of coronal brain sections from rat sacrificed on P14 after left carotid artery ligation and subsequent two-hour hypoxia on P7. Panel A, B, C and D represents two coronal brain sections illustrating the brain injury score of 0, 1, 2, and 3, respectively. [from: Lai PC, Huang YT, Wu CC, Lai CJ, Wang PJ, Chiu TH (2011) Ceftriaxone attenuates hypoxic-ischemic brain injury in neonatal rats. *Journal of biomedical science* 18:69. Figure 1]

Figure 4. Brain Growth Spurt

The brain growth spurts of 7 mammalian species expressed as first-order velocity curves of the increase in weight with age. The units of time for each species are as follows: guinea pig: days; rhesus monkey: 4 days; sheep: 5 days; pig: weeks; man: months; rabbit: 2 days; rat: days. Rates are expressed as weight gain as a percentage of adult weight for each unit of time. [from: Dobbing J, Sands J (1979) Comparative aspects of the brain growth spurt. *Early human development* 3:79–83. Figure 1]

	I. Posterior fronto-parieto-occipital sites	II. Anterior fronto-temporal sites
Internal capsules	Posterior limb: 44 weeks* (6 weeks)†	Anterior limb: 87 weeks (37 weeks)
Sensory radiation	Optic radiation mid-distal: 52 weeks (9 weeks)	Heschl's gyrus: 88 weeks (44 weeks)
Commissures	Body corpus callosum: 60 weeks (10 weeks) Splenum: 65 weeks (11 weeks)	Rostrum corpus callosum: 87 weeks (30 weeks) Anterior commissure inner: 139 weeks (75 weeks)
Central white matter	Distal radiation precentral gyrus: 70 weeks (29 weeks) Posterior frontal: 80 weeks (33 weeks) Posterior parietal: 99 weeks (46 weeks) Occipital pole: 87 weeks (40 weeks)	Temporal lobe at LGN‡: 119 weeks (65 weeks) Temporal pole: 122 weeks (75 weeks) Frontal pole: 119 weeks (69 weeks)
Crus pedunculi of midbrain	Lateral: 107 weeks (57 weeks)	Medial: 139 weeks (75 weeks)

Figure 5. Human Myelination is Mostly Postnatal

Median age when mature myelin (degree 3) is reached. *age in postconceptional weeks at which 50% of infants attain mature myelin. †myelin maturity interval, i.e. the time interval in weeks from the age at which 50% of infants reach degree 1 myelin to the age at which 50% reach degree 3 myelin at an individual white matter site. [from: Kinney HC, Brody BA, Kloman AS, Gilles FH (1988) Sequence of central nervous system myelination in human infancy. II. Patterns of myelination in autopsied infants. *Journal of neuropathology and experimental neurology* 47:217–234. Table 3]

DEBER AND REYNOLDS

TABLE 2
Lipid and Protein Composition of Central Nervous System Myelin^c

	<u>% Total Dry Weight</u>
Lipid	75.0
Protein	25.0
<hr/>	
<u>Protein</u>	<u>% Total Protein Weight</u>
Myelin basic protein (MBP)	22.5
Proteolipid proteins ^a	
PLP/lipophilin	30.0
Other LH-20 components	17.5
Thioethanol soluble	2.5
Others (Wolfgang, glycoproteins, etc.)	27.5
<hr/>	
<u>Lipid Weight</u>	<u>% Total Lipid</u>
Cholesterol	27.7
Cerebroside	22.7
Cerebroside sulfate	3.8
Ethanolamine phospholipids	15.6
Phosphatidylcholine	11.2
Plasmalogens ^b	12.3
Sphingomyelin	7.9
Phosphatidylserine	4.8
Phosphatidylinositol	0.6
Gangliosides (primarily GM ₁ and GM ₄)	<1.0

Figure 6. Components of Myelin

Lipid and protein composition of central nervous system myelin. [from: Deber CM, Reynolds SJ (1991) Central nervous system myelin: structure, function, and pathology. Clinical biochemistry 24:113–134. Table 2]

Figure 7. White Matter Volume in Premature and Mature Newborns

(A) The scatterplot shows the relative volume of myelinated WM as a percentage of intracranial volume in single gestation preterm and full-term infants ($n = 35$). The polynomial function fits the data. (Myelinated WM = $0.002 \times \text{postconceptional age [weeks]} - 0.22 \times \text{postconceptional age [weeks]} + 6.3 \times \text{postconceptional age [weeks]} - 60.2$.) A logarithmic transform of myelinated WM was used for the calculation of the linear regression model with $r^2 = 0.6$ and $p < 0.001$. (B) The scatterplot shows the absolute volume of myelinated WM in single-gestation preterm and full-term infants ($n = 35$). The polynomial function fits the data. (Myelinated WM = $0.006 \times \text{postconceptional age [weeks]} - 0.5 \times \text{postconceptional age [weeks]} + 13.5 \times \text{postconceptional age [weeks]} - 111.3$.) Log (myelinated WM) was used for the calculation of the linear regression model with $r^2 = 0.8$ and $p < 0.001$. [from: Huppi PS, Warfield S, Kikinis R, Barnes PD, Zientara GP, Jolesz FA, Tsuji MK, Volpe JJ (1998) Quantitative magnetic resonance imaging of brain development in premature and mature newborns. *Annals of neurology* 43:224–235. Figure 8]

Figure 8. Accretion of Plasmalogens and Other Lipids in the Human Brain

A) Concentration profile of total plasmalogens in the human forebrain and cerebellum, in $\mu\text{mole/g}$ wet tissue plotted with gestational age (weeks) and postnatal age (months). In the cerebrum the increase before 32 weeks is not significant, while it is very rapid and significant between this point and the limit of the prenatal period ($r=0.94$; $P<0.001$). In the cerebellum, the plasmalogen concentration profile during the prenatal period is parabolic and highly significant ($r=0.89$, $P<0.001$). [from: Martinez M, Ballabriga A (1978) A chemical study on the development of the human forebrain and cerebellum during the brain 'growth spurt' period. I. Gangliosides and plasmalogens. *Brain research* 159:351–362. Figures 2 and 3]

Figure 9. Accumulation of Brain Lipids and Myelination

Lecithin (unfilled circles) and plasmalogen (filled circles) content of rat brain during development with intensity of myelin staining shown by luxol fast blue staining. [from: Cuzner ML, Davison AN, Gregson NA (1965) Chemical and Metabolic Studies of Rat Myelin of the Central Nervous System. *Annals of the New York Academy of Sciences* 122:86-94. Figure 3]

Figure 10. Myelin Protein in Well Nourished and Undernourished Rats

Recovery of myelin protein from brains of well-nourished (open circles) and undernourished (closed circles) rats. Each point represents a single preparation of myelin from two or more animals. [from: Wiggins RC, Miller SL, Benjamins JA, Krigman MR, Morell P (1976) Myelin synthesis during postnatal nutritional deprivation and subsequent rehabilitation. Brain research 107:257–273. Figure 1]

Figure 11. Below Goal Growth Velocity in 30–35 Week Gestation Infants

Growth velocity (g/kg/d) from 15 NICUs from birth to discharge. * $p < 0.01$ vs “best net growth” NICU #1. Differences between NICUs remain significant after controlling for BW, BW Z-score, GA and gender and LOS. Gray box indicates goal growth 15–20g/kg/day. [from: Blackwell MT, Eichenwald EC, McAlmon K, Petit K, Linton PT, McCormick MC, Richardson DK (2005) Interneonatal intensive care unit variation in growth rates and feeding practices in healthy moderately premature infants. *Journal of perinatology : official journal of the California Perinatal Association* 25:478–485. Figure 2D]

Figure 12. Cumulative Energy Deficit Leads to Extrauterine Growth Restriction

Energy intake, cumulative energy deficit and change in Z-score for body weight during the first 7 weeks of life. It takes 1 to 2 weeks to establish adequate dietary intakes with the result that all infants accrue a significant energy deficit, which is paralleled by significant postnatal growth retardation. [from: Cooke R (2005) Postnatal growth in preterm infants: have we got it right? *Journal of perinatology* : official journal of the California Perinatal Association 25 Suppl 2:S12–14. Figure 1]

Figure 13. Neurodevelopmental Outcome and In-Hospital Growth

Relation between in-hospital growth velocity and neurodevelopment in a cohort of North American neonatal intensive care unit infants with a birth weight between 501 and 1000 g. Weight-gain quartiles were divided into 12 (n = 124), 15.6 (n = 122), 17.8 (n = 123), and 21.2 (n = 121) g·kg⁻¹·d⁻¹. Weight-gain quartiles were compared by using Mantel-Haenszel chi-square test. MDI, motor developmental index; PDI, physical developmental index. [from: Lafeber HN, van de Lagemaat M, Rotteveel J, van Weissenbruch M (2013) Timing of nutritional interventions in very-low-birth-weight infants: optimal neurodevelopment compared with the onset of the metabolic syndrome. The American journal of clinical nutrition. Figure 1, adapted from Ehrenkranz et al., 2006]

Figure 14. IGF-1 Concentrations Increase with Gestational Age

Individual values of fetal insulin-like growth factor-I (IGF-I) ($\mu\text{g/l}$) and the range for gestational age (mean, fifth and 95th confidence intervals). [from: Langford K, Nicolaides K, Miell JP (1998) Maternal and fetal insulin-like growth factors and their binding proteins in the second and third trimesters of human pregnancy. *Human reproduction* 13:1389–1393. Figure 1]

Estrogen level drops approximately 1000 fold following birth

Figure 15. Sudden Drop of Estrogen After Birth

During pregnancy, maternal 17 β -estradiol (E2) plasma levels increase up to 100-fold (15,000 pg/mL, approximately 55 nM), largely because of placental aromatization of C-19 steroids produced by fetal adrenal glands, compared with plasma estrogen concentrations in nonpregnant females. After birth, E2 decreases to low levels (5–35 pg/mL, or 20–130 pM) within 24 hr. [Courtesy B. Gerstner, adapted from: Tulchinsky D, Hobel CJ, Yeager E, Marshall JR (1972) Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. *American journal of obstetrics and gynecology* 112:1095–1100 and Ishida T, Seo F, Hirato K, Fukuda T, Yanaihara T, Araki H, Nakayama T (1985) Changes in placental enzymatic activities in relation to estrogen production during pregnancy. *Nihon Sanka Fujinka Gakkai zasshi* 37:547–554].

Figure 16. Hypothyroidism Decreases Myelin Lipids

Proteolipid content in brains of hypothyroid and control rats during the critical period for myelination. [from: Rosman NP, Malone MJ, Helfenstein M, Kraft E (1972) The effect of thyroid deficiency on myelination of brain. A morphological and biochemical study. *Neurology* 22: 99–106. Figure 9]

Table 1

Reasons for Heterogenous Nutritional Intake in Premature Infants

Infant Factors	Caregiver Factors	Diet Factors
1) Impaired Absorption	1) Time to Initiate Feeds	1) Breastmilk Only
2) Prenatal and Postnatal Aggregation Deficits	2) Availability of Close Nutrition Support	2) Formula Only
3) Increased Nutritional Needs from Systemic Stressor	3) Feeding Regimen: Traditional vs "Aggressive"	3) Combination Formula/Breastmilk
4) Genetic Factors such as metabolic rate		4) Duration of Total Parental Nutrition
		5) Supplementation (DHA, other lipids)
		6) Post-Hospitalization Feeding Regimen