



# Dysmaturation of Premature Brain: Importance, Cellular Mechanisms, and Potential Interventions

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**Dysmaturation of Premature Brain: Importance, Cellular Mechanisms  
and Potential Interventions**

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**ABSTRACT**

Prematurity, especially very preterm birth (<32 weeks' gestation), is common and associated with high rates of both survival and neurodevelopmental disability, especially apparent in cognitive spheres. The neuropathological substrate of this disability is now recognized to be related to a variety of dysmaturational disturbances of brain. These disturbances follow initial brain injury, particularly cerebral white matter injury, and involve many of the extraordinary array of developmental events active in cerebral white and gray matter structures during the premature period. This review delineates these developmental events and the dysmaturational disturbances that occur in premature infants. The cellular mechanisms involved in the genesis of the dysmaturation are emphasized, with particular focus on the pre-oligodendrocyte (pre-OL). A central role for the diffusely distributed activated microglia and reactive astrocytes in the dysmaturation is now apparent. Because these dysmaturational cellular mechanisms appear to occur over a relatively long time-window, interventions to prevent or ameliorate the dysmaturation, i.e., neurorestorative interventions, seem possible. Such interventions include pharmacological agents, especially erythropoietin, but also particular attention to such nutritional factors as quality and source of milk, breastfeeding, polyunsaturated fatty acids, iron and zinc. Recent studies also suggest a potent role in enhancing brain development for interventions directed at various experiential factors in the neonatal period and infancy, i.e., provision of optimal auditory and visual exposures, minimization of pain and stress, and a variety of other means of environmental behavioral enrichment.

## INTRODUCTION

Preterm birth (<37 weeks' gestation) is an enormous public health problem worldwide. According to the WHO, approximately 15 million premature infants are born yearly and account for approximately 1 million deaths.<sup>1</sup> The USA ranks sixth among countries with the greatest number of preterm births. According to the CDC, from 2014 to 2017 the preterm birth rate rose in the US to approximately 10%. Of the approximately 4 million births in the USA, 1.4%, or about 56,000, are of very low birth weight (<1500 gm).<sup>2</sup> Survival rates vary markedly as a function of gestational age, but are at least 95% at 32 weeks' gestation, 90% at 28 weeks, and 60 – 65% at 24 weeks.<sup>3-5</sup> The substantial survival rates, unfortunately, are accompanied by relatively high incidences of neurological disability, e.g., cerebral palsy in 5 – 10%, other motor disturbances in 25 – 40%, and cognitive, attentional, behavioral and socialization disturbances in 25 – 50%.<sup>6-16</sup>

The neuropathological substrate of this disability in preterm infants, especially those very preterm (<32 weeks' gestation) and extremely preterm (<28 weeks' gestation), consists of a combination of cerebral white matter injury (WMI) and especially, subsequent dysmaturational events in both white matter and neuro-axonal structures (see later). This combination of WMI and disturbances of gray matter structures has been termed the “encephalopathy of prematurity”.<sup>17</sup> In the initial review describing this encephalopathy, a particular emphasis was placed on the initial injury.<sup>17</sup> Subsequent work now suggests that although WMI is an important and likely initiating event, multiple subsequent *dysmaturational events* are most critical in determining outcomes (see later). Moreover, because these dysmaturational events evolve over a very prolonged period (many months), a relatively long-time window exists for interventions to

prevent, counteract or ameliorate the dysmaturation, i.e., neurorestorative interventions (see later).

In the following, I will review the multiple maturational events occurring in infant brain during the premature period; the dysmaturational events observed in premature infants, including the importance of the initiating cerebral WMI; the dysmaturational events that may occur without WMI; and potential neuroprotective and neurorestorative interventions.

### **BRAIN MATURATION DURING THE PREMATURE PERIOD**

The brain dysmaturation that occurs in premature infants (see later) involves the multiple active developmental events occurring in human cerebrum during the period of 20 - 40 weeks of gestation and beyond. The rapidity and complexity of these cellular events underlie, to a considerable degree, their vulnerability to perturbations. The principal components involved include the oligodendroglial (OL) lineage, especially the pre-oligodendrocyte (pre-OL), cerebral white matter axons, subplate neurons, cerebral cortex, thalamus and basal ganglia (Fig. 1). Additionally, microglia and astrocytes, especially in white matter, are involved importantly in both normal development and dysmaturation of these principal components. The major developmental events during this period have been summarized in detail elsewhere.<sup>18, 19</sup> A brief review of the temporal aspects of these events is appropriate here (Table 1).

#### **Pre-OL as a Principal Cellular Target**

Pre-OLs are the principal cellular target in WMI of premature infants.<sup>20-22</sup> These cells are generated from OL progenitors and are the principal phase of the OL lineage during the premature period (Table 1, Fig. 2). Pre-OLs account for 90% of the lineage during the peak period of WMI in premature infants. Even at term, pre-OLs account for 50% of the lineage in

cerebral white matter, while approximately 50% of the lineage are the more differentiated “immature” OLs.<sup>23</sup> Mature, myelin-producing OLs do not develop in human cerebral white matter to an appreciable degree until post-term. The pre-OL begins ensheathment of white matter axons at approximately 30 weeks’ gestation (Fig. 3).<sup>24</sup> This process is critical for axonal differentiation<sup>25-35</sup> and, as a consequence, axonal function. The latter is the critical driving force for cerebral cortical development (see later), which evolves rapidly as an activity-dependent process during the third trimester of gestation.

The pre-OL is a highly vulnerable cell, with particular susceptibility to such insults as hypoxia, ischemia, inflammation, which lead to death via excitotoxic and free radical mediated mechanisms.<sup>20</sup> The particular molecular characteristics that underlie this pre-OL vulnerability have been reviewed elsewhere.<sup>22</sup> Suffice it to say here, many experimental studies of acute pre-OL death produced by hypoxia, ischemia and inflammation have shown protective benefit for such agents as anti-excitotoxic, anti-inflammatory and anti-oxidant compounds (see later). Notably, however, as will be discussed later, in the premature infant with WMI, pre-OLs are *replenished* in the subacute period but *fail to differentiate* over the ensuing weeks/months to later phases of the OL lineage. As a result, hypomyelination is a hallmark of the disease.

### **Axons**

Axonal development is remarkably active in the cerebrum during the premature period (and the early postnatal period) (Table 1).<sup>36</sup> Utilizing immunostaining with GAP-43, a protein expressed on growing axons, Haynes et al.,<sup>36</sup> showed marked expression in cerebral white matter to at least 37 weeks’ gestation. Growing white matter axons reach approximately the subplate region at 20 weeks, the deep layers of the cortical plate at 27 weeks, and the entire cortex by 37 weeks. (Fig 4). Axonal growth occurs primarily within the cortex after 37 weeks and into the

first year of life. Based on work by Kostovic and coworkers,<sup>37-40</sup> the likely anatomic correlates of this progression in cerebral white matter during the premature period are at 20 weeks, growth of axons from thalamus to subplate neurons, and at 27 weeks, from subplate neurons to cerebral cortex (Table 1). Also, at 27 weeks commissural and cortico-cortical cerebral white matter axons are actively growing, especially in the posterior periventricular regions, a so-called “crossroads” area. The increase in cerebral *cortical* expression of GAP-43 at 37 weeks may reflect a sum of continued cortical penetration from the subplate of thalamic ascending fibers and of commissural and cortico-cortical fibers (Fig. 4). Thus, it is apparent that the premature period is one of extraordinarily rapid axonal development, especially in cerebral white matter. Axons during this rapid growth period are exquisitely vulnerable to multiple insults (see later).

#### **Cerebral Cortex-Dendritic Development, Synaptogenesis**

The cerebral cortex undergoes dramatic changes during the premature period. These events include attainment of proper alignment, orientation and layering of cortical neurons (six layers apparent by 30 gestational weeks), arrival of late migrating GABAergic neurons (principally to upper cortical layers), elaboration of dendritic and axonal ramifications (neurite outgrowth), onset of synaptogenesis, and a marked increase in cortical surface area with gyral development (Table 1).<sup>18</sup>

*Neurite outgrowth, and particularly dendritic development, is most relevant in this context.*<sup>41-44</sup> Dendritic development is especially rapid in the third trimester (Fig 5) and is correlated with the development of cortical activity (Table 1).<sup>18</sup> Importantly, in this context the progress of dendritic development depends on the *establishment of afferent input from cerebral white matter and presumably then synaptic activity.*<sup>42, 45-56</sup> Thus, axonal input from subplate neurons and then from thalamic, commissural and cortico-cortical fibers are the principal driving

forces underlying cortical dendritic development.<sup>56</sup> *The importance of synaptogenesis in mediation of these effects of axonal input on cortical development has been emphasized in several studies.<sup>57</sup> (See ref 57 for review.) In a seminal study, Sarnat and coworkers studied synaptic development in human cerebral cortex from 6 to 41 weeks' gestational age with the immunomarker synaptophysin, which identified maturation of synaptic vesicles in axonal terminals. Thalamocortical axons exhibited intense staining in frontal cortex at approximately 26 weeks' gestation, and diffuse and uniformly strong staining was apparent throughout the cortex from 34 weeks onward.<sup>58</sup> The findings integrate closely with measures of axonal development in the last trimester of gestation and with previous delineations of EEG maturation in premature infants. Functional synaptic activity via the axonal input to cortex is mediated principally through excitatory amino acid receptors, both the excitatory Ca<sup>++</sup>-permeable NMDA and GluR2-deficient AMPA receptors, which exhibit exuberant expression in developing human cortex during this period.<sup>59,60</sup> *This role of functional activity has implications for the effects of a variety of environmental stimuli on cortical development in the premature infant, and for potential neurorestorative roles for such stimuli in the context of brain injury and dysmaturation (see later).**

### **Subplate Neurons**

This important transient population of neurons is well-established in subcortical white matter by 20 weeks' gestation.<sup>37-40, 61</sup> During the important period of 24 – 32 weeks (the peak period for the occurrence of cerebral WMI) the subplate reaches maximum size (several times thicker than the cortical plate at 27 – 30 weeks) (Fig. 1, Table 1). These neurons elaborate a dendritic arbor with spines, receive synaptic inputs from ascending afferents from thalamus and distant cortical sites,<sup>18</sup> and extend axon collaterals to the overlying cerebral cortex and to other



cortical and subcortical sites (thalamus, other cortical regions, corpus callosum). The crucial organizational functions of these neurons include provision of a transient synaptic site for ascending afferents from thalamus and other cortical sites, i.e., these “waiting” afferents cannot synapse yet in cortex because their neuronal targets have not yet differentiated. These afferents would undergo degeneration if they did not have the subplate neurons as transient targets. Moreover, the subplate neurons extend axons to cortex to promote cortical differentiation and to guide the afferent axons to cortex when sufficient cortical differentiation has occurred. Subplate axon collaterals also descend to pioneer or guide the initial axonal projections from cerebral cortex toward subcortical sites (e.g., thalamus, corpus callosum, other cortical sites). The subplate neuronal layer gradually decreases after 36 - 40 weeks’ gestation.

#### **Late Migrating GABAergic Neurons**

Particularly characteristic of human cerebral cortical development is the relatively late generation of GABAergic neurons from the dorsal telencephalic subventricular zone and from the ventral ganglionic eminence (Fig. 6).<sup>61-65</sup> The origin of these late generated neurons is approximately 65% from the dorsal subventricular zone and 35% from the ventral ganglionic eminence. A substantial proportion of the ultimate population of GABAergic cortical neurons migrate through the cerebral white matter to cortex in the third trimester. This migration peaks around term and then declines within the first 6 postnatal months.<sup>66</sup>

#### **Microglia, Astrocytes**

Microglia and astrocytes are key players in the development of the white and gray matter structures just described. These glial elements also play a major causal role in the dysmaturational events that occur with cerebral WMI. Emphasis in this section is on the roles of

microglia and astrocytes in normal development. Their role in dysmaturation is discussed later in the section on neuropathology.

### *Microglia*

Microglia play important roles in such aspects of brain development as axonal development, OL differentiation-myelination, vascularization, synaptogenesis, synaptic pruning, and neural circuit formation.<sup>67-76</sup> The roles in OL development involve microglial proteins that stimulate pre-OL proliferation, enhance pre-OL survival and provide iron for OL differentiation, and secreted cytokines that enhance differentiation.<sup>75</sup> These cells also are the principal neuroimmune cells involved in neuroinflammatory responses. As part of the neuroinflammatory responses microglia can be destructive to cellular elements, such as pre-OLs, principally by generating free radicals, secreting injurious cytokines and enhancing excitotoxicity (see later).<sup>73, 77-85</sup> Microglia have been characterized generally as pro-inflammatory (activated) (M1) or anti-inflammatory (M2). However, this bimodal characterization appears now to be too simplistic. Thus, a recent landmark study in the developing mouse, utilizing molecular characterization methods, identified at least nine distinct microglial subpopulations with unique molecular signatures that changed over the course of development and exhibited marked spatial differences.<sup>76</sup> One distinct population was highly concentrated in axon tracts of the pre-myelinated brain. The molecular signatures of the microglial subpopulations in early development identified pathways associated with cell metabolism, growth, motility and proliferation, among others. Studies in developing human brain will be of great interest.

Microglia become prominent in the human forebrain at 16-22 weeks of gestation, migrate progressively through the white matter from 20-35 weeks, and then to cerebral cortex.<sup>69, 80, 86, 87</sup> The critical point is that the cerebral white matter of the human premature infant is heavily

populated with microglia during a period when various maturational events are occurring and when a variety of pro-inflammatory insults can lead to “activation” to destructive microglial phenotypes and WMI (see later). Moreover, because of the important role of microglial subpopulations in such important developmental events as OL development, axonal guidance, synaptogenesis, sculpting of neural networks, cerebral connectivity, diversion of these normal cells to microglial phenotypes with primarily pro-inflammatory functions could contribute to disturbances in these maturational events observed in the premature brain.<sup>72, 73</sup>

### *Astrocytes*

The last half of human gestation also is a crucial time in astrocyte formation in the cerebrum.<sup>18</sup> Fibrous astrocytes (generated from radial glial fibers) increasingly populate cerebral white matter. During development astrocytes are important in axonal guidance, angiogenesis, formation of the blood-brain barrier, synaptogenesis, neuronal survival and axonal and synaptic pruning.<sup>72</sup> The molecular characteristics of astrocytes involved in facilitation of these events underlie such functions as expression of extracellular matrix proteins and axonal guidance molecules, secretion of angiogenic factors, secretion of synaptogenesis molecules, clearance of extracellular glutamate, and secretion of various neurotrophic molecules. As will be discussed later, in the context of various brain insults (e.g., inflammation, hypoxia-ischemia), astrocytes can become “reactive” and exhibit a variety of metabolic changes that are deleterious to other white matter components, including pre-OLs.

## **DYSMATURATION IN PREMATURE BRAIN**

### **Overview**

The principal manifestations of dysmaturation in premature brain have been elucidated by advanced MRI techniques in living infants (Table 2). Briefly, the abnormalities have been: by volumetric MRI, diminished regional volumes, especially of cerebral cortex, white matter, thalamus and basal ganglia; by diffusion-based imaging, in cerebral white matter, decreased fractional anisotropy (FA) with relatively greater involvement of radial diffusivity (consistent with impairment of pre-OL ensheathment of axons), and in cerebral cortex, blunting of the normal decline in FA (consistent with impaired dendritic development); by surface-based MRI measures, decreased cerebral cortical surface area and cortical folding/gyrification; and by functional MRI, impaired development of measures of connectivity, including especially thalamocortical connectivity. The abnormalities have been elucidated most commonly at term equivalent age, but generally persist, or may increase later in infancy, childhood, adolescence or adulthood.<sup>88-93</sup> The most common accompaniment by MRI has been cerebral WMI (see later). The dysmaturational events, in general, appear to be *secondary* to WMI (see later discussion of mechanisms). The constellation of WMI and the accompanying disturbances of neuronal/axonal structures is generally referred to as the encephalopathy of prematurity,<sup>17</sup> However, recent work suggests that some of the dysmaturational events documented in premature infants are not clearly related to WMI and perhaps are *primary* disturbances (see later). The emphasis in the following section is on the relation of cerebral WMI and dysmaturational events. Brief consideration of potentially primary dysmaturational events, perhaps independent of WMI, will then be presented.

## **Cerebral White Matter Injury — A Spectrum**

### ***Neuropathology***

Cerebral WMI encompasses a spectrum of neuropathology that ranges from overt periventricular leukomalacia (PVL) to diffuse white matter gliosis (DWMG) (without focal

necroses) (Fig.7A-C). The two fundamental characteristics of PVL are focal necroses with loss of all cellular elements in periventricular white matter and a more diffuse lesion in cerebral white matter, consisting initially of death of early differentiating pre-OLs, accompanied by vigorous and persistent astrogliosis and microgliosis (Fig 8).<sup>94-102</sup> The focal necroses are essentially infarcts. Temporally, in the more diffuse lesion the pre-OL disturbance consists acutely of cell death, followed subacutely and chronically initially by proliferation of pre-OLs but then critically, *a failure of maturation*.<sup>103-108</sup> As noted earlier, this pre-OL dysmaturation underlies the subsequent hypomyelination, a unifying feature of PVL. The mildest form of WMI, i.e., diffuse white matter gliosis (DWMG) *without* focal necroses, now is the most common form of WMI in premature infants and is also accompanied by the pre-OL dysmaturation.<sup>100-102</sup>

The relative distribution of the spectrum of cerebral WMI in the modern era has been delineated best by neuropathological studies. The two largest, most recent series demonstrate that compared with earlier studies, the areas of focal necrosis are smaller and, indeed, most cases have few or none.<sup>100, 101</sup> In the series of Pierson et al. (n=41), true PVL, i.e., with focal necroses, occurred in 17 (42%). Importantly, nearly all of these lesions were less than 1mm in size. In an additional 17 (42%) only DWMG without focal necroses was observed. (Only 7 of the 41 brains were free of white matter abnormality.) Critically, Busser and coworkers observed in association with DWMG the sequelae of pre-OL death, i.e., the excess of pre-OLs and failure of pre-OL maturation.<sup>101</sup> Thus, the full spectrum of cerebral WMI can be illustrated as shown in Fig. 7A-C.

### ***Pathogenesis***

The pathogenesis of the focal necroses characteristic of PVL relates primarily to decreases in cerebral blood flow, related to a variety of perinatal/neonatal events, and the presence in the periventricular area of vascular border zones and end zones.<sup>20, 22</sup> The diffuse

abnormality, DWMG, relates in considerable part to similar, albeit less severe clinical events (see later).

In the diffuse lesion the pathogenesis of the acute pre-OL injury/death likely relates in part to the acute insults, noted above, as well as accompanying disturbances that may predispose the pre-OL to injury (e.g., intrauterine growth retardation, systemic infection, impaired nutrition, etc.) (see later). The stimulus for the subsequent proliferative response of OL progenitors to produce abundant pre-OLs remains unclear. The pathogenesis of the subacute and chronic failure of maturation of these newly generated pre-OLs appears to relate to deleterious effects of the abundant activated microglia and reactive astrocytes characteristic of the diffuse lesion. These components likely are involved in other dysmaturational events relating to axonal and neuronal structures, as described next.

**Deleterious Roles of Microglia and Reactive Astrocytes.** The *pro-inflammatory microglia* may impair pre-OL maturation by release of reactive oxygen/nitrogen species or cytokines (e.g., TNF- $\alpha$ , ILI- $\beta$ ) that then act on pre-OLs.<sup>20</sup> Recent studies of multiple sclerosis lesions have identified an inflammatory subpopulation of microglia that specifically targets myelin.<sup>76</sup> Whether such subpopulations are involved in pre-OL dysmaturation is unknown, but it is noteworthy that a large population of potentially activatable microglia are present in normal developing white matter during the premature period (see earlier). Additionally, *pro-inflammatory microglia* have been shown recently to induce formation of neurotoxic reactive astrocytes.<sup>109, 110</sup> As discussed next, such astrocytes are important in the pre-OL maturational failure. Finally, the shift in microglial phenotype from an anti-inflammatory to a pro-inflammatory, activated phenotype diverts the critical roles of “normal” microglia in OL development described earlier.

The abundant “reactive” astrocytes (A1) in DWMG also likely play critical roles in the failure of pre-OL maturation.<sup>108-111</sup> The best established mechanism in the context of WMI is based on seminal work by Back and coworkers.<sup>101, 108, 112</sup> The likely sequence involves the generation by reactive astrocytes of high molecular weight forms of hyaluronic acid. Astrocyte-associated extracellular matrix (ECM) is also involved in this generation. ECM also is a key source of hyaluronidases, which convert the high molecular weight forms of hyaluronic acid to lower molecular weight forms. The latter lead to failure of pre-OL maturation, probably by activating TLR-2 receptors on pre-OLs.<sup>113</sup> The particular role of hyaluronan is supported by the observation that pharmacological inhibition of hyaluronidases promotes pre-OL maturation and myelination (see later). Other products of reactive astrocytes may also be involved in the pre-OL dysmaturation. Thus, in human WMI reactive astrocytes express large amounts of interferon- $\gamma$ , and pre-OLs express the interferon- $\gamma$  receptor,<sup>114</sup> activation of which inhibits pre-OL differentiation.<sup>20</sup> Other products of astrocytes may contribute to the inhibition of pre-OL differentiation, e.g., bone morphogenetic proteins, Notch ligand Jagged 1, but data on human preterm WMI are not yet available.<sup>108</sup> Finally, as noted for activated microglia, the shift in astrocyte phenotype from normal fibrous astrocytes to the toxic reactive phenotype also diverts the critical roles of astrocytes in development of OLs (see earlier).

In view of the apparent critical roles of activated microglia and reactive astrocytes in disturbing pre-OL development (and likely also, aspects of axonal development), the question of the *duration* of DWMG in cerebral WMI of the premature infant becomes critical. Thus, available evidence by MRI in vivo suggests that dysmaturation continues for many months and likely longer. Not unexpectedly, neuropathological data in human infants concerning duration of DWMG are somewhat scanty. However, available information suggests that DWMG is present

for at least many months after the premature period and likely longer.<sup>98, 100, 103, 115</sup> There is precedent for microglia to be chronically activated in human neuropathology, e.g., after traumatic brain injury.<sup>116</sup> In the latter setting, these cells are considered important in subsequent degeneration of axons and neurons years later and to play a role in the enhanced incidence of degenerative disorders, such as Alzheimer's and Parkinson's disease.

### ***Identification In Vivo***

Neuroradiological identification of the cerebral WMI spectrum *in vivo* is made best by MRI but is not entirely satisfactory. Thus, the most severe end of the WMI spectrum, i.e., *severe WMI*, with large areas of necrosis and apparent cystic change, are readily identified as such (Fig 7D). However, such lesions are observed on MRI (and by neuropathology) in less than 5% of infants in modern neonatal intensive care facilities.<sup>117, 118</sup> More common are small areas of necrosis (>1mm) in periventricular and central cerebral white matter, seen at term equivalent age as (noncystic) punctate white matter lesions (PWMLs) in 15-25%, i.e., *moderate WMI* (Fig 7E).<sup>119-122</sup> Notably, this incidence of noncystic PVL (PWMLs) is appreciably higher if scans are performed early in the neonatal period — presumably the gliotic scars contract sufficiently to become invisible to MRI by term equivalent age. The least severe end of the WMI spectrum, i.e., *mild WMI*, is likely a heterogeneous group. Thus, the large majority of focal necroses observed postmortem are less than 1mm in size.<sup>100, 101</sup> and likely below the resolution of most conventional MRI scanners. Additionally, the MRI correlate of the very common DWMG, *without focal necroses*, also is unknown. Importantly, as with the diffuse gliotic component of overt PVL, DWMG alone appears to lead to pre-OL death and subsequent dysmaturational,<sup>101</sup> and thus may be very important clinically. The frequent isolated finding of diffuse signal abnormality in cerebral white matter (Fig. 7F) may be the MRI correlate of mild WMI, although both the



reproducibility of this imaging finding and the relation to outcome remain unclear.<sup>102</sup> The few excellent studies that identify WMI without detectable focal necroses by the presence of diminished FA on diffusion-based MRI (see later) may be the best in vivo correlate of the admixture of the WMI spectrum that includes the two large groups of (1) focal necroses too small for identification (with DWMG), and (2) DWMG without any focal necroses, the two forms that we refer to as *mild WMI*.

### ***Clinical Importance***

The clinical importance of the cerebral WMI spectrum relates to the motor and cognitive deficits associated with the lesion and the subsequent dysmaturation. The clinical phenomena associated with moderate and severe WMI have been described in detail elsewhere.<sup>118</sup> Identification of the neurodevelopmental sequelae of mild WMI is hindered by the difficulty in identifying the lesion by conventional MRI, as used in most large-scale studies. Large-scale MRI studies of premature infants show, as expected, worsening clinical outcomes as a function of severity of WMI. *However, it is noteworthy that infants with either no or "mild" abnormality in cerebral white matter by conventional MRI still exhibit neurological disability subsequently.* Although cognitive scales utilized among studies vary, cognitive scores for infants (<28 - 30 weeks' gestation) with no or "mild" WMI are approximately 85 – 93.<sup>121, 123-125</sup> In a particularly well-characterized study of 480 extremely preterm infants (<28 weeks' gestation), 20% of infants with no apparent WMI by conventional MRI had cognitive scores <85.<sup>124</sup> The possibility that neuro-axonal dysmaturation with mild WMI (see Mechanisms later) is important in determination of these outcomes is suggested by follow-up studies that included assessment of gray matter abnormalities (as well as WMI).<sup>126</sup> As will be discussed later, studies that assess

WMI by highly sensitive diffusion MRI measures show a clear association between mild WMI, dysmaturation and subsequent cognitive disturbances.

### **Mechanisms of Dysmaturation with Cerebral White Matter Injury**

The mechanisms of the dysmaturational features identified by MRI in premature brains (Table 2), especially in the context of WMI, are likely multiple. The prevailing theme is a sequence whereby *the initial insult (hypoxia, ischemia, inflammation, infection, etc.) leads to primary cellular injury/death, which in turn results in subsequent replenishment of pre-OLs but secondary dysmaturation.* The cellular elements injured likely depend on the severity of the WMI. Thus, in moderate-severe WMI (Fig 7A,B), all the rapidly developing cellular elements, as outlined next, appear to be injured, whereas in mild WMI (Fig 7C), the pre-OL may be the principal or only cellular element undergoing primary injury.

#### ***Dysmaturation with Moderate-Severe White Matter Injury***

**Pre-OL Injury.** Primary injury/death to the pre-OL, which is exquisitely vulnerable to hypoxic-ischemic, inflammatory or related insults, is a consistent early feature of all forms of WMI.<sup>21, 108, 127</sup> Cell death (or irreversible process loss) or both have been documented acutely.<sup>21, 103, 105, 108, 127</sup> *Subsequently, over the ensuing weeks replenishment of the pre-OL pool occurs but subsequent maturation to mature, myelin-producing OLs fails.* The important role of reactive astrocytes and activated microglia in this dysmaturation was described earlier. The result of this pre-OL dysmaturation is hypomyelination (Fig. 9A). Additionally, however, pre-OL dysmaturation likely leads to failure of pre-OL ensheathment of axons, and as a consequence, impaired development, i.e., dysmaturation, of axons. The important trophic role of pre-OLs for axonal development, survival and function was noted earlier. Indeed, this process is likely crucial

for the exuberant axonal growth in cerebral white matter illustrated earlier (Fig 4) and the activity-dependent development of cerebral cortex (Fig. 5). The consequences of the axonal disturbance would be diminished volumes of cerebral cortex and thalamus/basal ganglia, secondary to retrograde and anterograde (trans-synaptic) effects, i.e., involving projection fibers to and from cortex, thalamus and basal ganglia, i.e., thalamocortical, corticospinal, corticostriatal, and commissural and association fibers to and from cortex, i.e., cortico-cortical). (Fig. 9A).

**Axonal Injury.** Primary injury to the rapidly developing, vulnerable, premyelinating axons in cerebral white matter could be a primary event with WMI (Fig. 9B). Although axonal injury is shown readily in the areas of focal necrosis, a more widespread degeneration of axons detected by the apoptotic marker, fractin, also has been identified.<sup>128</sup> This finding is consistent with related experimental observations concerning the vulnerability of developing axons.<sup>129-133</sup> The dysmaturational events subsequent to axonal injury (Fig 9B) by anterograde and retrograde effects would result in the impairments of cortical and thalamic development and related abnormalities detected by MRI (see Table 2). An impairment of pre-OL maturation would result from the loss of trophic axonal signals, with the ultimate consequence, hypomyelination. A contributory role for deleterious effects of activated microglia and reactive astrocytes (see earlier) also seems likely. Moreover, because of the role of both these glial types in normal axonal development, diversion to activated/reactive phenotypes may further impair axonal development.

**Thalamic Injury.** Primary injury to thalamus is suggested by a neuropathological study of human infants with moderate to severe WMI and thalamic abnormalities (neuronal loss, gliosis, axonal degeneration) detected in approximately 60%.<sup>100, 134</sup> A particular vulnerability of

thalamus has also been shown in an experimental model.<sup>135, 136</sup> Primary injury to thalamus could lead to degeneration of axons originating in and terminating in thalamus and, as a consequence, to pre-OL dysmaturation and hypomyelination (Fig. 9C).

**Subplate Neuronal Injury.** Primary injury to subplate neurons would be expected to have major secondary dysmaturational effects on thalamus by retrograde degenerative effects on ascending thalamic axons (“waiting afferents”), as well as on cerebral cortex by anterograde effects via loss of subplate neuronal axons to cortex and on descending cortical axonal projections by loss of guidance from subplate axonal collaterals (Fig. 9D). Considerable experimental data support these contentions.<sup>37, 137-144</sup> With axonal degeneration, subsequent disturbances in pre-OL development would be expected (Fig. 9D). Although data are not entirely consistent, experimental studies suggest that subplate neurons are particularly vulnerable to hypoxia-ischemia.<sup>145</sup> Two reports suggest a loss of subplate neurons in premature infants with moderate-severe white matter injury.<sup>146, 147</sup>

**Late Migrating GABAergic Neurons.** Primary injury to late migrating GABAergic neurons seems possible because the migratory path of these late generated cells is from the dorsal subventricular zone through cerebral white matter to the cerebral cortex (Fig. 6). Two neuropathological studies of moderate-severe WMI show a deficit in central white matter neurons consistent with late migrating GABAergic neurons.<sup>146, 147</sup> The result of a disturbance in these neurons would be a deficit in cerebral cortical neurons, especially the upper cortical layers (Fig 9E). The MRI result would be diminished cerebral cortical volume, surface area, gyrification and connectivity, as noted in advanced MRI studies (Table 2).

**Conclusions.** Thus, in moderate to severe WMI, i.e., identified by neonatal MRI by PWMLs (relatively common) or by cystic lesions (rare), several potential sequences of primary

injury leading to dysmaturation and the developmental impairments detected by advanced MRI techniques seem likely. Although pre-OL death and subsequent replenishment of pre-OLs which then fail to mature appear most consistent (Fig. 9A), the other sequences depicted in Fig. 9 may also occur to varying degrees, dependent in part on such factors as the gestational age of the infant, the nature, severity and timing of the initiating insult(s), and the presence of other potentiating factors, e.g., intrauterine adversity, postnatal infection, undernutrition, etc.

### *Dysmaturation with Mild White Matter Injury*

Mild WMI, as discussed earlier is characterized by focal necrotic lesions less than approximately 1mm in size, and thus undetectable by conventional MRI, or DWMG without focal necroses. (Fig.7C) The dysmaturational features apparent subsequently in vivo by advanced MRI are similar in many respects to those described earlier for moderate and severe WMI (Table 2) but are less pronounced.<sup>118, 148-155</sup> Thus, a series of careful studies of premature infants without major WMI and utilizing diffusion-based MRI determinations of FA and related measures in cerebral white matter as a means to detect mild WMI, not readily apparent on conventional MRI, show at term-equivalent age disturbances in volumetric development of cerebral cortex, cerebral white matter, thalamus, basal ganglia, cortical folding, cortical and white matter microstructure, and thalamocortical connectivity.<sup>148-153, 155-157</sup> In a particularly large, recent series (n = 491), Barnett et al. identified lower FA in cerebral white matter with particularly high radial (vs. axial) diffusion (RD).<sup>155</sup> The high RD is consistent with an impairment of pre-OL ensheathment.<sup>118, 158</sup> *The findings suggest that impaired pre-OL maturation is the critical finding in mild cerebral WMI.* The lower FA values were independently associated with increased number of days on ventilation, perhaps consistent with chronic hypoxia or related insults and with fetal growth restriction. The latter has been shown to

be associated with a degree of hypoxia and in experimental studies to lead to delayed OL maturation<sup>155, 159</sup> – recall that the pre-OL is exquisitely vulnerable to hypoxic and related insults (see earlier). The white matter findings also related to prolonged parenteral nutrition and suggest that impaired nutrition may lead to impaired pre-OL development (see later). Importantly, the abnormal FA values in the large study of Barnett et al. were associated with impaired neurodevelopmental performance at 20 months of age.<sup>155</sup>

As noted earlier, two major neuropathological series indicate that mild WMI, as defined here, is the dominant form of cerebral WMI currently.<sup>100, 101</sup> As noted earlier, detection of this milder but prevalent form of WMI cannot be made consistently by conventional MRI. The recent work just described with diffusion-based MRI indicates promise for detection in vivo.

Although the mechanisms for dysmaturation with mild WMI may overlap with those just described for moderate to severe WMI, major differences are likely. Thus, with mild WMI clear evidence for primary injury to components other than the pre-OL is lacking. *It is most likely that with mild WMI the deleterious effects of the abundant activated microglia and reactive astrocytes are the dominant mediators of dysmaturation, especially to the pre-OL, and perhaps also to axons.*

**Pre-OL Injury.** Primary injury/death to the pre-OL with subsequent replenishment of pre-OLs but failure of maturation, as described for moderate-severe WMI (see earlier), may be the major mechanism for the widespread dysmaturation just described. The important role of activated microglia and reactive astrocytes was discussed earlier concerning moderate to severe WMI. The scenario to widespread dysmaturation, thus, would be similar to that described for moderate-severe WMI (Fig. 9A).

**Axonal Injury.** Although evidence for primary *injury* to axons in mild WMI is lacking, the deleterious effects of the abundant activated microglia and reactive astrocytes may disturb axonal *development*, separate from any effects on pre-OLs. Additionally, as noted earlier, during normal development these glia are critical for axonal guidance and growth, and phenotypic diversion to activated/reactive cells could lead to dysmaturation. Thus, a scenario similar to that depicted in Fig. 9B seems possible.

**Thalamic, Subplate, Late Migrating GABAergic Neuron Injury.** The scenarios described earlier for primary injury to these neural structures leading to dysmaturational gray matter disturbances in the setting of moderate-severe WMI (Fig 9C-E) cannot be ruled out in mild WMI but do not seem highly likely. For example, in the careful neuropathological study of Pierson et al.,<sup>100</sup> in the 17 infants with DWMG (and no focal necroses), neuronal loss in cortex, thalamus, and basal ganglia was observed in none.

**Conclusions.** The dysmaturational disturbances of white matter and gray matter structures apparent by advanced MRI methods in infants with mild WMI do not appear to be related to widespread injury. Pre-OL injury and dysmaturation do seem apparent, and thus the possibility of the multiple secondary developmental disturbances of gray and white matter structures described earlier (Fig. 9A) is real. The abundant reactive astrocytes and activated microglia in cerebral white matter, i.e., DWMG, also are likely important in the pre-OL injury/dysmaturation. Additionally, axonal injury and dysmaturation also are a potential consequence of the deleterious actions of these two glial types (Fig. 9B).

### **Primary Dysmaturation of Gray Matter Structures**

The possibility that the gray matter structures shown to exhibit secondary impaired development with the encephalopathy of prematurity, as outlined in the preceding, may exhibit *primary* dysmaturation is suggested by recent clinical and experimental studies. If primary dysmaturation does occur, the approaches to neuroprotection and neurorestoration (see later) could be quite different than those directed at secondary dysmaturation in the context of cerebral WMI.

### *Clinical Data*

Primary dysmaturation of cerebral cortex, in the absence of evidence for WMI, is suggested by a study of 95 premature infants studied by MRI at two time points in the neonatal period (32 and 40 weeks post-conception).<sup>160</sup> The principal finding was evidence for delayed microstructural development of cerebral cortical gray matter at multiple sites. Diffusion-based measurements showed delayed microstructural development in cerebral cortex, but *not cerebral white matter*, in association with *impaired somatic growth*. The expected normal developmental decline in FA in cortex was blunted, whereas the expected increase in FA in white matter was not. Thus, no evidence for WMI or impaired white matter development could be identified. As described earlier,<sup>18, 161</sup> FA decreases in cortex principally with dendritic development. In the study of Vinall et al.<sup>160</sup> radial diffusion and not axial diffusion in cortex was particularly affected, again most consistent with impaired dendritic development. The association with impaired somatic growth raises *the possibility that undernutrition is particularly involved*, although detailed data re: nutrition, caloric intake, feeding were not available. However, it is noteworthy that several studies of premature newborns with intrauterine growth retardation also show a particular involvement of cerebral cortical development, including reduced cortical volume, reduced cortical surface area and impaired gyification.<sup>162-166</sup> However, other studies of



such infants have shown abnormalities in microstructural development of white matter.<sup>155, 167</sup> Nonetheless, on balance, it does appear that disturbances in growth, perhaps secondary to undernutrition, either in the premature infant postnatally or in utero may have a primary dysmaturational effect on cerebral cortex. More data clearly are needed.

### ***Experimental Data***

Three recent studies in a well-characterized preterm large animal (fetal sheep) model of cerebral ischemia raise the possibility of primary dysmaturation of cerebral cortex, subplate neurons and caudate neurons.<sup>168-170</sup> Thus, utilizing elegant neurobiological methods, Back and coworkers have shown disturbances in cortex, in dendritic development and synapse formation; in subplate neurons, in dendritic arborization and synaptic activity; and in caudate, in dendritic arborization, synaptogenesis and synaptic activity.<sup>168-170</sup> These findings were apparent four weeks after the hypoxic-ischemic insult, but not after two weeks.<sup>168</sup> Because the basic experimental paradigm was designed originally to replicate cerebral WMI of the premature infant, these examples of neuronal dysmaturation were accompanied by pre-OL degeneration and dysmaturation and diffuse gliosis with reactive astrocytes and reactive microglia. A reasonable question is whether the 4-week period required for the evolution of the cortical, subplate or caudate neuronal dysmaturation is necessary because the dysmaturation is secondary to the pre-OL degeneration and dysmaturation as described earlier (Fig 9A). In the absence of a definitive answer to this question, the possibility that the hypoxic-ischemic insult leads primarily and directly to the neuronal dysmaturation is real. Coupled with the clinical study described earlier, the latter possibility demands further research.

### ***Conclusions***

The clinical studies of premature infants with impaired somatic growth and of those with intrauterine growth retardation raise the possibility that cerebral cortical development may be affected directly, i.e., *primarily*, perhaps by nutritional factors. In view of the rapid development of cortex during the premature period and therefore its likely vulnerability to neonatal insults, such a possibility seems reasonable. Experimental data also raise the possibility of a primary dysmaturational effect for hypoxia-ischemia on cortical, subplate and caudate neurons. However, as discussed, the available data do not rule out a primary effect on pre-OLs with secondary neuronal dysmaturation.

## NEUROPROTECTIVE AND NEURORESTORATIVE INTERVENTIONS

Because the pervasive theme in this review is that pre-OL *death* leads to subsequent *dysmaturation* of both white and gray matter structures, interventions are best considered (1) as preventative of the initial death (i.e., *neuroprotection*) or (2) as amelioration or prevention of the subsequent dysmaturation (i.e., *neurorestorative*). Although there is overlap in this categorization, the distinction best facilitates the discussion that follows.

### Neuroprotective Interventions

Neuroprotective interventions have focused on prevention of pre-OL injury or death. Many excellent recent reviews have addressed this issue and will not be discussed further.<sup>20, 22, 108, 171</sup> The principal neuroprotective interventions and the likely mechanism(s) affected in the cascade to pre-OL death are shown in Table 3. Most of the mechanisms also are relevant to those examples of WMI that are accompanied by direct injury to axons and neurons as well as to

pre-OLs. Of the interventions shown in Table 3 only erythropoietin (EPO) has been studied in detail in human premature infants and will be discussed here.

### *Erythropoietin*

Because EPO has anti-excitotoxic, anti-oxidant, anti-inflammatory and anti-apoptotic effects,<sup>172</sup> the agent is a prime candidate for prevention of pre-OL injury or death, the critical initial event in genesis of preterm WMI. EPO has been shown to prevent/mitigate WMI in a variety of experimental models.<sup>172-174</sup> Although numerous studies of EPO in premature infants have been carried out, a recent meta-analysis of four randomized, controlled trials (RCT) comprising 1133 infants is especially useful.<sup>175</sup> Prophylactic EPO administration reduced the incidence of Mental Developmental Index (MDI) scores of <70 (odds ratio 0.51 [0.31 – 0.81], P<.005) at 18 – 24 months. Because total numbers of infants <28 weeks' gestational age were not large enough to assess adequately the outcome in this critical group, more data are needed. A large multicenter RCT in the United States (PENUT Trial, NCT 01378273) is focused on this critical group, and results should be available this year.

A closer assessment of the key EPO trials suggests that *the timing of EPO administration* may be critical in the likelihood of benefit. Thus, in one series of studies utilizing early, relatively brief administrations of EPO (at <3 hrs, at 12-18 hours and at 36-42 hours after birth), no significant differences in outcome at 2 years could be discerned<sup>176</sup> (although MRI at term equivalent age showed decreased WMI and better white matter maturation in the EPO-treated infants).<sup>177, 178</sup> However, in a study utilizing EPO administration (as EPO or its higher glycosylated derivative darbepoietin) 3 x /week through 35 weeks' postconceptual age, the treated infants had better cognitive outcomes and less neurodevelopmental impairment at 3.5 to 4 years of age, when compared to placebo-treated infants.<sup>179</sup> Thus, the two different protocols re:

timing of EPO administration suggest that with the early, relatively brief approach, EPO was functioning only as a neuroprotective agent, whereas with the more prolonged approach the agent may have functioned *both* as a neuroprotective *and* a neurorestorative intervention. Perhaps consistent with this notion, the largest study to date randomized 800 infants of <32 weeks' gestation to placebo or EPO administered intravenously within 72 hours of birth and then once every other day for 2 weeks.<sup>180</sup> The rate of moderate/severe neurological disability at 18 months' corrected age was significantly lower in the EPO group (7.1%) versus the placebo group (18.8%) (OR = 0.22, CI 0.19 – 0.55, P<.001). Dosing in the aforementioned PENUT trial will be still more prolonged, i.e., initially, single doses intravenously, every other day, from day 1 to day 11, and subsequently, doses subcutaneously every other day until 32 weeks. The potential mechanisms for EPO's benefit concerning brain maturation, i.e., neurorestorative effects, will be discussed in the next section.

### **Neurorestorative Interventions**

The emphasis of this review has been the evolution of the widespread dysmaturational events that follow the initial insult(s) and injury/death, especially to pre-OLs. These events develop over many weeks to months, and perhaps longer. This relatively protracted period raises the possibility of a long time-window for interventions potentially capable of ameliorating or preventing the dysmaturation. I will term these interventions *neurorestorative*. The principal such interventions, shown in Tables 4 and 5, are classified based on their study in experimental settings only (Table 4) or in clinical settings with human infants, principally preterm infants (Table 5).

### **Experimental Studies**

**EGF, IGF-1.** Both epidermal growth factor (EGF) and insulin-like growth factor (IGF-1) have beneficial effects in experimental models of preterm WMI (Table 4). The agents appear to exhibit both neuroprotective and neurorestorative properties. In a mouse model of preterm WMI, Scafidi et al. showed that either selective overexpression of human EGF receptor in OL lineage cells or the intranasal administration of EGF immediately after injury led to decreased OL death, enhanced generation of new OLs from progenitor cells, and promoted functional recovery.<sup>181</sup> The benign mode of administration of the EGF suggests potential clinical applicability.

IGF-1 has shown protective effects versus WMI both in neonatal animal models (hypoxia-ischemia, lipopolysaccharide-induced inflammation) and in cultured pre-OLs.<sup>182-186</sup> The agent also showed restorative effects, i.e., rescue of pre-OLs and promotion of myelination. Two issues limit enthusiasm for IGF-1: first, the peptide must be administered intraventricularly, and second, its effects are dose-related, with lower doses effective but higher doses toxic.

**Hyaluronidase Inhibitors.** Pre-OL dysmaturation in chronic WMI appears related at least in considerable part to the astrocytic component of the diffuse gliosis characteristic of the lesion. Thus, Back and coworkers have shown that reactive astrocytes synthesize high molecular weight forms of hyaluronic acid, which are readily detectable in the human lesion.<sup>101, 108</sup> As described earlier, hyaluronic acid digestion products, generated from hyaluronidases in the disrupted extracellular matrix of WMI, lead to a block in pre-OL maturation. This block could be prevented by pharmacologic inhibition of hyaluronidase in vitro and in an animal model.<sup>187</sup> Whether use of a hyaluronidase inhibitor has value in preventing pre-OL dysmaturation in the human infant requires further study.

**Microglial or Astrocytic Phenotypic Manipulation.** Abundant microglia are important components of the diffuse gliotic component of WMI (see earlier). These cells are principally in *an activated, pro-inflammatory state (M1 phenotype)*. Their role in *acute* pre-OL injury/death likely relates to the generation of reactive oxygen and nitrogen species and secretion of injurious cytokines.<sup>20</sup> However, a variety of studies, performed in *in vitro* and *in vivo* models, including adult human lesions with failure of OL differentiation and myelin development (e.g., multiple sclerosis), suggest involvement of activated microglia in the subsequent pre-OL dysmaturation in human preterm WMI.<sup>188-190</sup> The data raise the possibility that interventions capable of converting microglia from a pro-inflammatory phenotype (M1) to an anti-inflammatory phenotype (M2) would have major potential as a neurorestorative therapy. Such *immunomodulatory* agents that cross the blood-brain barrier have been identified (e.g., minocycline, melatonin, minozac, etanercept) and are under study in human adult demyelinating diseases.<sup>116, 189, 191</sup> Their safety and efficacy in the premature infant for long-term use are not established.<sup>192</sup>

A recent relevant area of interest in regulation of microglial phenotype involves *microRNAs*<sup>191, 193</sup> These components are short non-coding RNAs (18-22 nucleotides) which are transcriptional regulators of gene expression. Several microRNAs have been shown to promote or inhibit inflammatory responses in microglia. One prominent microRNA of activated microglia is mir-155, which is elevated in microglia in multiple sclerosis lesions. When silenced *in vivo* by intracerebral injection of IL-17 in early stages of experimental allergic encephalomyelitis, the pathological and clinical effects of the demyelinating disease are blunted.<sup>193</sup> Thus, the possibility of such systemic therapy seems real. Indeed, recent research shows that intravenous delivery of another microRNA (miR-124) that promotes polarization of microglia from an inflammatory (M1) to an anti-inflammatory (M2) phenotype via miR-124-enriched exosomes improves

hippocampal neurogenesis and neurological function over four weeks after traumatic brain injury.<sup>194</sup> (See also later discussion of exosomes.) Notably, because the anti-inflammatory phenotype of microglia (M2) is important in the facilitation of many brain developmental events as described earlier, these avenues of research suggest a major neurorestorative possibility for in vivo manipulation of microglia phenotype.

Similar considerations concerning glial manipulation from a “harmful” to developmentally “helpful” phenotype apply to the *reactive astrocytes* in the diffuse gliotic component. Their involvement in pre-OL injury and dysmaturation and the potential value of hyaluronidase inhibitors were discussed earlier. Prevention of the microglial induction of harmful, “neurotoxic” astrocytes (A1 phenotype) is an area of active current research.<sup>109, 110</sup> The valuable results would be inhibition of pre-OL death and preservation of the maturational effects of the developmentally beneficial astrocytic phenotype (A2). A variety of drugs and neutralizing antibodies (e.g., to TNF $\alpha$  and IL $\alpha$  from microglia) to prevent induction of harmful reactive astrocytes are under study in animal models and in adult human neurodegenerative disorders.<sup>109</sup>

**Stem Cells.** Experimental studies of stroke and related ischemic brain injuries in neonatal animals suggest that stem cell therapies may be effective for restoration, particularly of OLs.<sup>195-200</sup> The major types of cells used thus far include neural, embryonic, mesenchymal, umbilical cord and induced pluripotent cells. In vitro manipulation of neural precursor cells prior to transplantation can enhance their capacity to undergo OL differentiation and axonal remyelination.<sup>201</sup> A variety of routes of cell administration have been utilized, and *intranasal* administration may be the most efficient. Stem cells administered by this route appear to target the injury site after entering the brain via olfactory neural processes traversing the cribriform plate.<sup>202</sup> Studies of rodent models of preterm brain injury have shown that the intranasal route of

administration can be effective not only for mitigating injury to myelin but also for improving behavioral outcome.<sup>196, 203</sup>

Of particular relevance to cerebral WMI and pre-OL dysmaturation/myelination failure in the premature infant is a recent study of such injury produced by hypoxia-ischemia in the 3-day-old rat.<sup>204</sup> OL progenitor cells produced from embryonic stem cells were transplanted into the injured cerebrum. The transplanted cells survived, underwent differentiation, formed myelin sheaths and stimulated proliferation of endogenous neural stem cells. Supporting a neurorestorative effect was the demonstration of functional benefit after 6 weeks. It will be of particular interest if the results can be replicated after intranasal administration.

A relevant human study in this context involves the transplantation of human neural stem cells into the brains of four infants with congenital Pelizaeus-Merzbacher disease.<sup>205</sup> After 1 year, evidence of myelin ensheathment of axons was obtained by diffusion tensor imaging. Direct extrapolation to the human preterm infant with WMI is difficult because of the mode of administration used. Nevertheless, the findings suggest promise for stem cell therapy as a neurorestorative therapy in such infants.

**Exosomes.** The precise neuroprotective factors released by stem cells are not known with certainty and may vary as a function of the injury. Notably, however, effects on pre-OL and myelin development and on behavioral outcome in a rodent model of preterm WMI was achieved with *intravenous* administration of extracellular vesicles, i.e., *exosomes*, derived from stem cells, in lieu of stem cells per se.<sup>206</sup> (Exosomes are a type of extracellular vesicle and can carry membrane and cytosolic proteins, various types of RNA and lipids, and perhaps DNA).<sup>207</sup> Similar benefit from the use of exosomes has been demonstrated in other animal models of brain injury.<sup>208-210</sup> The great therapeutic potential of exosomes, isolated from blood, has been



recognized only recently and the capacity to induce OL differentiation and myelination could serve a crucial neurorestorative function in the premature infant. Human studies will be of great interest.

**Dendrimers.** Dendrimers are unique nanoparticles synthesized for a variety of functions, including targeted delivery of therapeutic agents to brain.<sup>211,212</sup> Their small size and tailorable surface functional groups make them valuable for this role. *Drugs, and perhaps ultimately, microRNA's or silencing RNA's can be attached to the dendrimer.* Several recent models of ischemia- or inflammation-induced neonatal or fetal brain injury have shown marked beneficial effects of dendrimer – N-acetylcysteine conjugates.<sup>213-217</sup> N-Acetylcysteine is an anti-oxidant, and after intravenous administration of the conjugate, uptake into activated microglia, reactive astrocytes and differentiating OLs could be demonstrated. Sustained prevention of OL injury and improved myelination were shown.<sup>215</sup> The principal cellular target appeared to be inflammatory microglia. Further studies will be of great interest.

### ***Clinical Studies***

A burgeoning clinical literature suggests the possibility that the dysmaturation of both pre-OLs and gray matter structures after premature brain injury can be counteracted to a considerable extent. These neurorestorative interventions include pharmacologic agents, i.e., EPO, and modifications of nutrition and other environmental factors (Table 5). Implementation of these interventions during the vulnerable neonatal period, when the remarkable array of developmental events described earlier are proceeding most rapidly, is of critical importance. However, the beneficial effects of these interventions likely continue beyond the neonatal period (see later). The mechanisms of the benefits and the specific maturational events affected are not yet entirely understood. Our current understanding of these interventions is discussed next.

**Erythropoietin.** EPO was discussed earlier in relation to its neuroprotective effects. Notably as discussed earlier, current data suggest that more prolonged exposure to EPO is more beneficial than only early and brief exposure, thus suggesting that EPO has neurorestorative as well as neuroprotective properties.

Experimental data suggest that neurorestorative effects of EPO involve particularly OL development, although promotion of angiogenesis and neurogenesis may also occur.<sup>118, 172, 218</sup> In view of the likely importance of the failure of differentiation of pre-OLs in the genesis of axonal and neuronal dysmaturation, the decisive role of EPO in promoting pre-OL development after hypoxic-ischemic insults in experimental models is particularly relevant here.<sup>219-221</sup> In vivo, EPO appears to be generated primarily from astrocytes, abundantly present in the diffuse gliotic component of WMI. However, the EPO receptor is upregulated in pre-OLs after hypoxic-ischemic insults, and if sufficient endogenous EPO is not present, the unoccupied receptor leads to a failure of differentiation. The provision of abundant *exogenous* EPO may explain the benefit of EPO therapy vis à vis pre-OL differentiation. An indirect effect of EPO on pre-OL and neuronal/axonal maturation may also relate to its action of decreasing microglial recruitment, the other key glial element in diffuse white matter gliosis, and thereby the deleterious effects of inflammation.<sup>222</sup> Additionally, in an animal model,<sup>223</sup> EPO also promotes *cerebral cortical development* after hypoxia-ischemia and associated subplate neuronal loss, again consistent with its multifaceted effects on cellular development in brain. Recall that in moderate to severe WMI in premature infants, subplate neuronal loss and impaired cortical development are important features (see earlier).

A major question re: EPO as a restorative therapy relates to the prolonged duration likely required. Reactive astrocytes and activated microglia are likely present for many months after the

initial injury. More data are needed re: the safety of such prolonged treatment of an agent with multifaceted developmental effects.

**Nutritional Factors.** The importance of appropriate nutrition during the premature period for neurodevelopmental outcome and the deleterious effects of postnatal undernutrition are supported by a large corpus of clinical, epidemiological and experimental studies.<sup>224-238</sup> In the context of this section the data raise the possibility that optimal nutrition, *both* in the neonatal period *and* the subsequent posthospitalization period, could be a restorative intervention in premature infants with WMI and the encephalopathy of prematurity. The high prevalence of impaired nutrition in premature infants is illustrated by observations that 50% of VLBW preterm infants had a discharge weight less than the 10th percentile for postmenstrual age, and 27% had a discharge weight less than the 3rd percentile.<sup>239, 240</sup>

The *maturational events apparently vulnerable to nutritional disturbance* in infancy have been elucidated at least preliminarily, by several MRI studies.<sup>155, 160, 241, 242</sup> Although results vary somewhat, impairments of cerebral cortical volumetric growth and microstructural differentiation, cerebral white matter microstructural maturation, and basal ganglia volumetric growth have been identified. Similar disturbances of brain maturation have been identified in infants born small for gestational age.<sup>164, 166, 167</sup> Impaired nutrition in the postnatal period in premature infants has been determined either directly (e.g., energy and lipid intake) or indirectly (e.g., deficient enteral nutrition, prolonged parenteral nutrition). Whether some of the dysmaturational events in these settings are primary or secondary to pre-OL dysmaturation is unclear. Nevertheless, the implication is that optimal nutrition could be restorative in this context.

The *quality of the milk provided to the infant* is another nutritional factor that appears important for neurological outcome and suggests a means for neurorestoration. Available data indicate particular value concerning neurodevelopmental outcome for fortified preterm versus term formula, for human milk versus formula, and for fortified human milk versus no fortification.<sup>236, 238, 243-249</sup>

*Breast-feeding* is of particular value, and several volumetric and diffusion tensor MRI studies have shown better white maturation in such infants.<sup>235, 236, 244, 247, 248</sup> Perhaps even more importantly, breast-feeding beyond the neonatal period appears to have long-lasting beneficial effects on cerebral white matter. Thus, a study of 133 (healthy) term-born infants either exclusively breast-fed for a mean of 413 days or exclusively formula fed or fed a mixture of formula and breast milk utilized advanced MRI measures at 10 months to 4 years of age to assess white matter development.<sup>247</sup> The data show that early exclusive breast-feeding was associated with better development in relatively late maturing white matter regions, including frontal and temporal white matter, corticospinal tracts, and superior longitudinal and occipitofrontal fasciculi. Notably, several of these regions are important for specific higher-order cognitive domains, in which breast-fed infants have been shown to have improved performance.<sup>247, 250</sup> Thus, current data suggest that breast-feeding could have neurorestorative potential and that the principal initial effect may involve OL development and myelination.

*Polyunsaturated fatty acids (PUFAs)* are crucial for brain development and are particularly concentrated in phospholipids of neural membranes, especially in cerebral cortex and other gray matter structures.<sup>251-253</sup> Most brain PUFAs are acquired in the last trimester of gestation and first two years of life, during rapid brain growth.<sup>254</sup> Thus, the preterm infant does not receive this critical transplacental transfer of PUFAs. Notably, however, breast milk is an

excellent source of PUFAs. Two recent studies utilizing advanced MRI techniques and determinations of PUFA levels in red blood cells noted positive correlations between PUFA levels and microstructural maturation of cerebral white matter and macrostructural (volumetric) development of cerebral cortex and basal ganglia/thalamus near-term equivalent age.<sup>254, 255</sup> Concerning the potential value of supplementation with PUFAs, although not all findings are consistent, favorable effects on visual function and cognitive function have been reported.<sup>238, 256, 257</sup> Supplementation to the lactating mother who is breast-feeding has been most effective. Thus, the possibility of PUFA supplementation in this manner to the preterm infant, especially with the dysmaturational abnormalities of white and gray matter, is worthy of study as a neurorestorative intervention.

*Iron is a critical nutrient for OL maturation.* Iron deficiency can be an important contributing factor in causation of brain dysmaturation in premature infants, with particular involvement of OL maturation and subsequent myelination. Thus, iron supplementation as needed can be considered a potential neurorestorative intervention. Although the data are not entirely consistent, most studies show impairments of motor, cognitive and behavioral development in iron-deficient infants.<sup>258-262</sup> Iron deficiency in the neonatal period is usually related to dietary deficiency, particularly in the context of breast-feeding and prematurity.<sup>263-265</sup> Indeed, as many as 10% of infants in the first two years of life in the United States and 15% of breast-fed Canadian infants exhibit iron deficiency. Because premature birth deprives the infant of the primary period of fetal iron deposition, i.e., the third trimester of gestation, the risks are still higher in such infants. Supportive of an effect on myelin development in iron-deficient infants is the finding on studies of auditory and visual evoked potentials of prolonged latencies, without impairment of amplitudes.<sup>266-269</sup> (The normal maturational decline in latencies relates to

acquisition of myelin, whereas changes in amplitude relate more to neuronal development<sup>161</sup>). A recent study of delayed umbilical cord clamping (DCC) in full-term infants suggests a beneficial effect of iron on MRI-quantitated myelin at four months of age.<sup>270</sup> Thus, infants followed after DCC had 48% higher serum ferritin levels and myelin content at 4 months of age, when compared to infants who had immediate cord clamping. Moreover, because infants with higher iron stores at 4 months of age have been shown to persist with higher stores later, the findings suggest that the positive effect on myelination may persist.<sup>271</sup> More data are needed, but higher iron stores could represent an important neurorestorative goal in premature infants. The clinical findings re: iron and myelination are consistent with experimental studies showing the crucial role of iron in the processes of OL differentiation and myelination.<sup>263, 265, 272, 273</sup> Because iron also plays an important role in neurotransmitter metabolism, the neural effects of iron deficiency may extend beyond an impairment of OL development/myelination.<sup>274</sup> *Quantitative* studies of myelination in living iron-deficient infants by advanced MRI measures would be of great interest.

*Zinc is another critical nutrient for OL maturation.* Zinc is critical for a variety of aspects of brain development, including OL development.<sup>275-279</sup> The effect is mediated particularly by an OL-specific zinc finger protein (Zfp 488) that functions as a transcriptional co-regulator important for OL differentiation.<sup>280</sup> Recent work has shown that zinc concentrations in developing OLs are relatively high during differentiation and decline after maturation is achieved.<sup>281</sup> Moreover, altered zinc balance is involved in experimental models of ischemic and excitotoxic OL death.<sup>282-284</sup> The clinical relevance of this work remains to be clarified fully, but it is notable that preterm infants are vulnerable to zinc deficiency because of high zinc requirements, diminished zinc stores (most zinc stores are acquired in the last trimester of

gestation), and suboptimal zinc absorption.<sup>285-287</sup> Moreover, zinc concentrations in human milk are highly variable, and current dietary guidelines for zinc intake for preterm infants are based on limited data.<sup>286, 288</sup> More data are needed in human preterm infants on OL maturation, myelination and neurological development, in relation to zinc status, and on ideal amounts of dietary zinc intake.

**Experiential Factors.** Experiential factors (Table 5) can play a major role in regulation of OL differentiation/myelination and neuronal/axonal development in human infants.<sup>18, 19</sup> Although emphasis is often placed on the *deleterious* effects of altered experience, especially in the neonatal period and particularly in the premature infant, the potential benefit of experiential factors as *restorative therapies* is important to consider. In this section, emphasis is placed on factors related to care in the neonatal period and later in infancy (Table 5).

A potential beneficial effect of modification of the *neonatal auditory environment* on cortical development and language outcome is suggested by recent clinical studies. The particular relevance of this work relates in part to the lay-out of neonatal intensive care units and associated attempts to minimize ambient noise. In part, because sound levels in many neonatal intensive care units, incubators and ventilators can exceed current recommendations of the American Academy of Pediatrics,<sup>289-293</sup> many units have been designed to minimize such noise, often by maintaining infants in single rooms. Two recent studies suggest that this approach may have an adverse effect on language development and cerebral cortical development in premature infants.<sup>294, 295</sup> In a study of 136 preterm infants assigned to either open ward or single room bedspaces, infants cared for in single patient rooms had lower language scores at age 2 years, accompanied by abnormalities of cortical folding in the superior temporal area (after controlling for potential confounders) (Fig. 10).<sup>294</sup> The difference in outcomes was attributed to differences

in exposure to language in open areas vs. single rooms. In a subsequent study of the role of maternal involvement with the infant, outcomes were assessed for infants from both single patient rooms and open ward bedspaces while taking into account maternal involvement in the NICU care.<sup>295</sup> Infants with *high maternal involvement* from *both* single patient and open ward bedspaces had higher cognitive and language scores at age 18 months than did infants with *low* maternal involvement. Notably, the effect size was greater for children from single rooms. This second study suggests that the level of language exposure depends on a variety of factors, including room type, but also other maternal characteristics that allow maternal verbal contact with the infant (e.g., availability of maternal leave, socio-economic status, time spent with the infant, a NICU culture that encourages verbal contact from parents). These latter issues also are important subsequent to the infant's discharge (see later).

The underlying pathophysiology of these effects of auditory experience on language development relates in part to the striking development of the auditory system during the premature period. Thus, connections between the cochlea and the brain stem are established by 24 to 25 weeks and connections to temporal lobe and auditory cortex by 30 to 31 weeks.<sup>296, 297</sup> Subsequently, cortical development occurs rapidly. Thus, it is biologically plausible that an important factor in the subsequent language impairment relates to a disruption of development of this key activity-dependent system. Consistent with this notion, functional connectivity MRI data show that disruption of brain networks detected at term equivalent age persists into childhood.<sup>298</sup>

The importance of the *nature of the auditory input* is shown by a recent study of the effects of different varieties of *music exposure* on cortical connectivity. A careful initial study utilizing fMRI showed that music exposure in preterm infants had at term equivalent age lasting learning effects on music processing — with an increased connectivity between primary auditory



cortex and brain regions involved in several aspects of music processing (e.g., temporal and cingulate cortex, basal ganglia).<sup>299</sup> Subsequent work will focus on the duration of such effects and effects on language processing. The initial findings suggest that details of the neonatal (and subsequent) auditory experience can have lasting beneficial effects on important areas of brain development.

*Neonatal visual experience* also appears to play an important role in the infant's brain development. Because WMI in the premature infant tends to be most severe in the parieto-occipital region,<sup>127</sup> central visual impairment is a common neurological sequelae.<sup>118</sup> Notably, development of the axons of the geniculocalcarine tract, ensheathment of these axons by pre-OLs, early myelin formation of the tract and development of visual cortex are very active during the premature period and into infancy.<sup>18</sup> Whether optimal visual experience in premature infants could capitalize on this actively developing system to counteract the deleterious central visual effects of WMI, i.e., have *restorative* properties, is important to consider. The data just reviewed re: auditory experiences are encouraging in this regard. Experimental studies in normal and preterm monkeys showed that premature visual stimulation resulted in increases in size and proportions of synapses in visual cortex, presumably by activity-dependent alterations in synaptogenesis, synaptic modification or synapse elimination.<sup>300</sup> Visual deprivation had opposite and unfavorable effects. The somewhat limited data available in human infants suggest that, as in monkeys, visual experiential effects are important mediators of cortical development. Thus, visual experience of premature infants is associated with accentuation of the development of the visual evoked potential, a finding consistent with enhancement of axonal and dendritic development and synaptogenesis.<sup>301, 302</sup> More data are needed to address how visual experience

could enhance development of the cerebral visual system and perhaps counteract the effects of dysmaturation described earlier.

*Pain and stress in the neonatal period*, common experiences for the preterm infant, have been shown to have adverse effects on neurodevelopmental, behavioral and cognitive outcomes.<sup>303-308</sup> Abnormalities of brain development accompany these effects (see next). The findings suggest that reduction of pain and stress in these infants could serve as a neurorestorative intervention. (Table 5).

Several studies that quantitated pain and stress (either number of stressful events<sup>309</sup> or number of painful (skinbreaks) procedures<sup>307, 308, 310, 311</sup>) showed correlative disturbances in brain development involving cerebral cortex, thalamus, cerebral white matter and functional connectivity. Functional connectivity disturbance has been demonstrated as early as term equivalent age (Fig. 11).<sup>309</sup> Although most abnormalities were detected at term equivalent age, thinning of cerebral cortex, especially in parietal and frontal areas, was identified at a mean age of 7.9 years.<sup>311</sup> Additional evidence concerning a relation of stress to cerebral white matter development emanates from a later randomized controlled clinical trial that evaluated the effectiveness of training parents in reducing stressful experiences in premature infants in the neonatal intensive care unit.<sup>312</sup> At term equivalent age the infants of the mothers in the intervention group showed by advanced MRI significantly enhanced maturation and connectivity of cerebral white matter. These and related data caused the American Academy of Pediatrics to emphasize the need in neonatal intensive care facilities for “a pain-prevention program that includes strategies for minimizing the number of painful procedures performed”.<sup>313</sup> *This evidence for a deleterious effect of pain and stress in the neonatal period suggests that interventions to reduce the responsible events could be neurorestorative.* Moreover, the

potential importance of stress as a deleterious factor *beyond the neonatal period* and prevention/amelioration of such stress as a neurorestorative intervention will be discussed later.

One approach to diminish neonatal stress has been the individualized developmental care approach pioneered by Als.<sup>314</sup> The possible neurorestorative role of this approach was suggested by a randomized clinical trial of the Als care program, which showed at 9 months' corrected age, improved neurobehavioral function, quantitative EEG evidence of enhanced maturation, and diffusion-tensor MRI evidence of more advanced cerebral white matter fiber tract development.<sup>315</sup> Benefits were still present at 8 years of age.<sup>316</sup> Many elements of developmental care were identified, including patient positioning, light, sound, handling, approaches to feeding and inclusion of parents in care.<sup>317-319</sup> The initial findings from this work regarding beneficial effects on brain maturation suggest the possibility that at least some of these approaches could lead to mitigation and recovery of the dysmaturational features of the encephalopathy of prematurity.

The *mechanisms for the deleterious effects of stress on brain maturation*, although not entirely understood in the human newborn and young infant, may involve brain microglia.<sup>320</sup> Such stress mediators as glucocorticoids and catecholamines are known to lead to pro-inflammatory activation of microglia in experimental studies. In view of the key role of microglia in various aspects of brain maturation in early life and the substantial number of microglia in cerebral white matter in the human infant, especially in the diffuse gliosis of WMI (see earlier), such modulation of microglia toward a pro-inflammatory state could lead to dysmaturational events. Moreover, this modulation of microglia has been related to a potentiated response to *subsequent* stress, e.g., in infancy and beyond, with the exacerbation of

dysmaturation and the promotion of cognitive and emotional disorders. Thus, in this context, elimination of stress would be a major neurorestorative intervention.

*Parenting/educational/social factors* are critical in the infant's experience, especially beyond the neonatal period, and suggest opportunities for neurorestorative intervention (Table 5). Many studies in recent years have shown that factors relating to parenting, parental education and socio-economic context beyond the neonatal period are critical determinants of ultimate neurodevelopmental outcome, especially relating to cognition, language and behavior.<sup>10, 321-329</sup> Initial research with MRI suggests that the anatomical substrate for impaired neurodevelopment in this setting involves changes in volumetric growth of cortex, deep nuclear structures and cerebral white matter.<sup>328, 330</sup> and in functional network maturation.<sup>328, 331</sup> *The particular importance of early parenting behavior, particularly maternal affective involvement, parent-child synchrony, positive and responsive parenting, has been delineated and could represent a neurorestorative intervention.*<sup>327-329</sup>

Although the deleterious experiential effects just described begin in the neonatal period, their persistence into infancy and early childhood raises the possibility that *myelin development, a post-term event in human brain, could be especially affected.* This possibility is supported by recent elegant studies of infants institutionally reared in orphanages under circumstances associated with lack of parenting and neglect, including social, emotional and linguistic deficiencies. Although disturbances in subsequent behavioral and cognitive functions in such infants are well-known, impairments in macrostructural and microstructural white matter development have been shown only recently.<sup>332-337</sup> The potential to counteract this dysmaturation, i.e., a neurorestorative intervention, was shown by a large study of Romanian orphans randomly selected to remain in an institution or to be placed in supervised foster care at

2 years of age.<sup>338</sup> Studies by diffusion tensor MRI at 8 years of age showed significant associations between *neglect in early life and impaired microstructural integrity of multiple white matter structures*. Particular affection of radial diffusivity suggested impaired myelin ensheathment of axons. Early intervention (i.e., foster care) promoted more nearly normative white matter development among the previously neglected children. *The particular link between environmental enrichment and white matter development* is reminiscent of studies of specific skill development, e.g., musicians, which show a relation of enhanced skill to induced changes in white matter microstructure. Importantly, these changes are effected best during early sensitive periods, e.g., in childhood, and, moreover, enhance additional practiced-based changes in white matter microstructure and performance later in life.<sup>339</sup> Notably, a large experimental literature supports maturational value for environmental enrichment in many animal models.<sup>328</sup>

Randomized intervention studies of older infants and young children in lower SES conditions, albeit less severely neglected than the aforementioned orphans, also show benefit for development of language, emotional regulation and cognitive skills.<sup>328, 340, 341</sup> The anatomical substrate of these effects remain to be established. However, the demonstrated benefit raises important and complex societal issues.

## Conclusions

This major section focused on interventions directed at pre-OL death and subsequent dysmaturation of white and gray matter structures. Interventions directed at protection vs. pre-OL *death*, termed “neuroprotective”, discussed in detail elsewhere, were not emphasized. Of these, only EPO has been studied in human infants, and prolonged therapy seems preferable to brief courses. Interventions considered to be promising for amelioration or prevention of the subsequent dysmaturation, termed “neurorestorative”, were emphasized. Promising pre-clinical

data were reviewed. Clinical studies suggest value for EPO, optimal nutrition (concerning quality and source of milk, breastfeeding, polyunsaturated fatty acids, iron and zinc), and critical experiential interventions. The latter relate in the neonatal period to optimal auditory and visual experience and reduction of pain and stress. Importance for brain maturation also can be attributed to such interventions in infancy and early childhood as breastfeeding, carefully designed intervention programs, environmental enrichment and parental/educational/social factors.

### **SUMMARY/CONCLUSIONS**

The principal focus of this review has been the dysmaturation of brain in infants born prematurely, especially very preterm. The dysmaturational details have been identified primarily by advanced MRI studies. The review has delineated (a) the dysmaturational features involving both white and gray matter structures; (b) the context of their occurrence, i.e., the remarkable array of developmental events in the human brain during the premature period; (c) the spectrum of the cerebral white matter injury that appears to initiate the dysmaturational events; (d) the likely mechanisms by which the white matter injury, particularly the mild forms now most prevalent in neonatal intensive care facilities, lead to the dysmaturation; and (e) the potential neurorestorative interventions suggested by a burgeoning amount of recent work, both clinical and experimental.

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