Published in final edited form as:

Int J Dev Neurosci. 2011 June ; 29(4): 423–440. doi:10.1016/j.ijdevneu.2011.02.012.

The developing oligodendrocyte: key cellular target in brain injury in the premature infant

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Abstract

Brain injury in the premature infant, a problem of enormous importance, is associated with a high risk of neurodevelopmental disability. The major type of injury involves cerebral white matter and the principal cellular target is the developing oligodendrocyte. The specific phase of the oligodendroglial lineage affected has been defined from study of both human brain and experimental models. This premyelinating cell (pre-OL) is vulnerable because of a series of maturation-dependent events. The pathogenesis of pre-OL injury relates to operation of two upstream mechanisms, hypoxia-ischemia and systemic infection/inflammation, both of which are common occurrences in premature infants. The focus of this review and of our research over the past 15-20 years has been the cellular and molecular bases for the maturation-dependent vulnerability of the pre-OL to the action of the two upstream mechanisms. Three downstream mechanisms have been identified, i.e., microglial activation, excitotoxicity and free radical attack. The work in both experimental models and human brain has identified a remarkable confluence of maturation-dependent factors that render the pre-OL so exquisitely vulnerable to these downstream mechanisms. Most importantly, elucidation of these factors has led to delineation of a series of potential therapeutic interventions, which in experimental models show marked protective properties. The critical next step, i.e., clinical trials in the living infant, is now on the horizon.

Keywords

Developing oligodendrocyte; Excitotoxicity; Free radicals; Hypoxia-ischemia; Microglial activation; Premature brain injury

1. Introduction

Brain injury in the premature infant, particularly the infant of very low birth weight (VLBW), is a problem of major importance. Thus, currently in the U.S. approximately 1.5% of the 4,300 000 live births yearly, or nearly 65,000, are VLBW infants (Heron et al., 2010). Worldwide this number is likely in excess of several million such infants (Muglia and Katz, 2010). In the U.S., 90% of the 65,000 VLBW infants survive the neonatal period because of major advances in neonatal intensive care (Volpe, 2008b). Unfortunately 5-10% of these survivors later exhibit the spastic motor deficits categorized as cerebral palsy (Doyle et al.,

2010; Groenendaal et al., 2010; Munck et al., 2010; Beaino et al., 2010; Volpe, 2008b), and importantly fully 25-50% later exhibit cognitive/attentional/behavioral/socialization defects that significantly impair quality of life (Aarnoudse-Moens et al., 2009; Johnson et al., 2009; Roberts et al., 2009; Johnson et al., 2010; Msall, 2010; Limperopoulos, 2009; Volpe, 2008b). Because rates of prematurity have not declined in the past decade, at least in the U.S., this enormous burden of neurological disability can be expected to continue. Prevention of the brain injury underlying this disability is needed urgently. To accomplish prevention, insights into the underlying pathology and pathogenesis are needed first. This review will discuss research largely over the past 15 years concerning pathology, pathogenesis and prevention. The insights provided by the research give promise that preventative interventions are on the horizon.

2. Neuropathology

Multiple neuropathologies occur in premature infants (Volpe, 2008b). However, the dominant lesion is a form of cerebral white matter injury termed periventricular leukomalacia (PVL). PVL is the focus of this review, but it should be recognized that neuronal/axonal abnormalities often accompany PVL (Volpe, 2008b; Volpe, 2009). Indeed, we have used the term encephalopathy of prematurity for this combination of PVL and neuronal/axonal deficits. These deficits, studied in considerable detail in the past several years, involve abnormalities of cerebral cortex, thalamus, basal ganglia and white matter neurons (Pierson et al., 2007; Ligam et al., 2009; Haynes et al., 2008; Andiman et al., 2010; Kinney et al., 2011) but are not discussed here because of space limitations.

2.1 Two components in PVL – focal and diffuse

PVL consists of two basic components: focal necrosis with loss of all cellular elements, and a more diffuse and cell-specific lesion, consisting of acute loss of early differentiating oligodendrocytes (premyelinating oligodendrocytes – pre-OLs) with accompanying astrogliosis and microgliosis (Fig. 1). Pre-OLs, in this presentation, refer to both the 04-positive, 01-negative pre-oligodendrocyte and the 01-positive immature oligodendrocyte (Fig. 2). The 04-positive, 01-negative pre-oligodendrocyte predominates in the cerebral white matter of the human premature infant (Back et al., 2001) and is most vulnerable to the injurious mechanisms discussed later. Indeed the 04-positive cell comprises approximately 90% of the total oligodendroglial population until about 28 weeks of gestation but even at term accounts for 50%. The 01-positive cell accounts for only about 10% of the oligodendroglial population of the premature infant and is also vulnerable to these mechanisms (albeit somewhat less so than the 04-positive cell). The 01-positive cell does not reach 50% of the total oligodendroglial population until term in the human brain (Back et al., 2001).

When the focal necrotic component of PVL is large, subsequent cyst formation occurs and the term cystic PVL is used (Fig. 3A). The cystic lesions are readily seen in premature newborns by ultrasonography (Volpe, 2008b). More commonly, the focal necrotic lesions are microscopic in size (1-2 mm or less) and evolve to glial scars rather than cysts, termed noncystic PVL (Fig. 3B). This latter form requires MRI for visualization in the living newborn (Volpe, 2008b). Over the past 10-15 years, cystic PVL has declined in incidence and currently occurs in less than 5% of living VLBW infants (Volpe, 2008b). Noncystic PVL has become the dominant lesion, accounting for more than 90% of PVL, and occurs in about 50% of VLBW infants (Volpe, 2008b). With either cystic or noncystic PVL the diffuse injury to pre-OLs occurs and is followed by a deficit in mature, myelin-producing OLs and as a consequence, cerebral hypomyelination.

2.2 Pre-OL “injury”

The nature of the pre-OL “injury” in PVL has been elucidated in recent years (Table 1). Thus, acutely pre-OL death occurs (Haynes et al., 2003; Bell et al., 2005; Back et al., 2005a; Robinson et al., 2006). Additionally pre-OLs may survive but with loss of cell processes (Billiards et al., 2008) (Fig. 4). These cells do not appear to differentiate subsequently. A third aspect of the pre-OL abnormality in PVL is an apparent replenishment of pre-OL progenitors, by proliferation and/or migration, but again with subsequent failure of differentiation (Billiards et al., 2008). Some cells may synthesize myelin basic protein (MBP), but an impairment in localization of MBP from perikaryon to peripheral sheaths prevents proper myelination (Billiards et al., 2008). The result in toto of these three pre-OL disturbances is the hallmark of PVL, subsequent *cerebral hypomyelination* (Volpe, 2008b) (Fig. 5). The particular emphasis of our research has been the pathogenesis of pre-OL injury in PVL, particularly the initial pre-OL death and the loss of cell processes despite apparent cell survival. The work also is relevant to the subsequent failure of pre-OL differentiation in PVL (see later). The remainder of this review will focus on pre-OL injury, in terms of its pathogenesis and potential prevention.

3. Pathogenesis

The pathogenesis of pre-OL injury in PVL relates to three major interacting factors, cerebral ischemia, systemic infection/inflammation and intrinsic vulnerability of cerebral white matter pre-OLs during the peak period of occurrence of PVL, i.e., especially 24-32 weeks of human gestation (Table 2). Cerebral ischemia and systemic infection/inflammation, the two major upstream pathogenetic mechanisms, have been discussed in detail elsewhere (Volpe, 2008b) and will be reviewed only briefly next. The intrinsic vulnerability of pre-OLs and the action of the three major downstream pathogenetic mechanisms, i.e., microglial activation, excitotoxicity and free radical attack, will be discussed in more detail (Fig. 6).

3.1 Cerebral ischemia

3.1.1 Pressure-passive cerebral circulation—Premature infants have a particular propensity for developing cerebral ischemia, especially in white matter (Table 2). This propensity relates to intrinsic vascular and physiological factors, as discussed elsewhere (Volpe, 2008b). The essential point is that the distal arterial fields in cerebral white matter are not fully developed, and in part, this relative underdevelopment is reflected in very low basal values for blood flow to cerebral white matter in the premature infant (Volpe, 2008b). Thus, there is a minimal margin of safety for blood flow. When this minimal margin is coupled with an underdeveloped cerebrovascular autoregulatory system and thereby a pressure-passive cerebral circulation, the infant is vulnerable to declines in blood pressure (Khawaja and Volpe, 2008; Volpe, 2008b). Such declines in blood pressure (and therefore blood flow to white matter) are very common in sick premature infants with severe respiratory disease, the latter a nearly constant feature because of lung immaturity (Soul et al., 2007).

3.1.2 Hypocarbia—Premature infants also are likely to develop cerebral white matter ischemia because hypocarbia occurs as a consequence of the ventilator management of their respiratory disease (Volpe, 2008b). Hypocarbia is a potent cerebral vasoconstrictor, and thus it is perhaps not surprising that cumulative hypocarbia has been shown to be highly associated with the subsequent development of PVL in premature infants (Shankaran et al., 2006).

3.1.3 Pre-OL as the principal cellular target of ischemia—The primacy of intrinsic cellular factors in determining the pre-OL as the principal cellular target of white matter

ischemia has been shown in studies of fetal sheep (Riddle et al., 2006; McClure et al., 2008). Thus, the topography of white matter injury with ischemia was determined particularly by the topographic distribution of pre-OLs. Variations in degree of white matter ischemia were less important than variations in distribution of pre-OLs.

3.2 Infection / inflammation

3.2.1 Maternal intrauterine and neonatal systemic infection—The second major upstream mechanism in the pathogenesis of pre-OL injury is infection and systemic inflammation (Volpe, 2008b) (Fig. 6). A number of epidemiological studies have shown an association between PVL and maternal intrauterine infection with fetal *systemic* inflammation or neonatal systemic infection with neonatal systemic inflammation (Volpe, 2008b; Volpe, 2008c; Wu and Colford, 2000; Shah et al., 2008; Chau et al., 2009; Leviton et al., 2010). Maternal intrauterine infection or neonatal systemic infection or both occur in as many as 65% of VLBW infants (Volpe, 2008b; Stoll et al., 2004; Stoll et al., 2005). As will be discussed later, activation of brain microglia is the principal initiating event in causation or accentuation of pre-OL injury in the context of systemic infection and inflammation (Kadhim et al., 2001; Haynes et al., 2003; Volpe, 2008b).

3.2.2 Potentiation of hypoxia-ischemia—A *potentiating interaction* between the two upstream mechanisms, hypoxia-ischemia and systemic infection /inflammation is likely important clinically and occurs on several levels. Thus, human neuropathological studies show markedly greater microgliosis when hypoxia-ischemia occurs in the context of systemic infection/inflammation than in its absence (Kadhim et al., 2001). Moreover, fetal and neonatal infection often is associated with persistent systemic hypotension and impaired cerebrovascular autoregulation (Yanowitz et al., 2006). Thus, systemic infection/inflammation may accentuate hypoxic-ischemic insults. Importantly, potentiation of ischemic cerebral injury by prior or concomitant systemic infection/inflammation has been documented in over a dozen excellent experimental studies (see later). The potentiation has caused *sub-threshold* ischemic insults to be overtly injurious (Volpe, 2008a; Eklind et al., 2001; Lehnardt et al., 2003; Ikeda et al., 2004; Larouche et al., 2005; Wang et al., 2007b; Wang et al., 2009; Wang et al., 2010; Degos et al., 2010). It is likely that this potentiation occurs largely at the level of cerebral white matter microglial activation.

The potentiation between systemic infection/inflammation and hypoxia-ischemia depends on the relative timing of the insults. In most potentiating paradigms LPS is administered 4 hours before the subthreshold hypoxic-ischemic insult. A similar potentiation has been observed when LPS was administered 72 hours earlier (Eklind et al., 2005). However, when LPS was administered 24 hours before hypoxia-ischemia, injury was reduced, i.e., tolerance rather than potentiation occurred. Of potential high clinical relevance is the additional observation by Hagberg and coworkers that mice injected with a single dose of LPS in utero at gestational day 15 and later subjected to hypoxia-ischemia at postnatal days 5 or 9 resulted in enhanced injury and hypomyelination at day 14 (Wang et al., 2007b). (Antenatal administration of LPS also was shown to sensitize neonatal rat brain in an excitotoxic model of white matter injury) (Rousset et al., 2008). Thus, if a similar sensitization occurs in human infants, prior fetal exposure to systemic infection/inflammation could render the premature infant sensitized to apparently modest postnatal hypoxic-ischemic insults.

3.3 Maturation-dependent vulnerability of cerebral white matter pre-OLs

3.3.1 Three maturation-dependent downstream mechanisms—The critical role of a series of *maturation-dependent factors* that underlie the particular vulnerability of cerebral white matter pre-OLs to injury in the human premature infant is at the core of the downstream mechanisms operative in this injury (Table 3). These three mechanisms,

microglial activation, excitotoxicity, and free radical attack, have been the focus of our National Institutes of Health-funded program project research grant over the past 15 years. Free radical attack appears to be the principal final common pathway to injury (Fig. 6). Excitotoxicity likely leads to pre-OL injury by promoting Ca^{2+} influx, and as a result, generation of reactive oxygen and nitrogen species (ROS/RNS). Microglial activation can lead to pre-OL injury by multiple mechanisms, but free radical generation likely is most important. Because free radical attack appears to be the principal final mediator of pre-OL injury, we will discuss this mechanism first.

3.3.2 Vulnerability to free radical attack

3.3.2.1 Evidence for free radical attack in PVL: The possibility that PVL is related to attack by ROS/RNS initially seemed plausible because experimental models have shown that both ischemia and inflammation lead to cell death principally by free radical mechanisms (Volpe, 2008b). The most compelling direct evidence that these mechanisms are operative in PVL comes from study of the human lesion (Haynes et al., 2003; Back et al., 2005a). In our study of 17 cases, Kinney and coworkers used immunocytochemical markers for oxidative (hydroxynonenal) and nitrative (nitrotyrosine) attack and showed abundant positive staining in pre-OLs in the diffuse component of PVL (Haynes et al., 2003) (Fig. 7). Similarly, Back and coworkers later showed evidence for oxidative attack by detection of F2-isoprostanes in the oligodendroglial lineage in PVL (Back et al., 2005a). These findings are consistent with our experimental observations (1) that pre-OLs, but not mature OLs, are exquisitely vulnerable to ROS attack (Back et al., 1998; Volpe, 2008b; Oka et al., 1993; Yonezawa et al., 1996; Li et al., 2003; Deng et al., 2003), (2) that pre-OLs are the predominant form of the oligodendroglial lineage in human cerebral white matter during the premature period, and (3) that the distribution of pre-OLs determines the distribution of pre-OL injury with ischemia (Riddle et al., 2006).

Studies of living VLBW infants also support the role of oxidative toxicity in PVL. Thus, in a longitudinal study of premature infants, cerebrospinal fluid levels of oxidative products (protein carbonyls), measured early in the neonatal period, were sharply increased in those infants with later MRI evidence for PVL at term, compared with levels in infants without later MRI evidence for PVL (Inder et al., 2002). Notably also, a recent study detected evidence for high serum levels of oxidative stress markers in the first hours of life in infants who later manifested PVL in the neonatal period (Kakita et al., 2009). These infants also exhibited signs of intrauterine systemic inflammation.

3.3.2.2 Mechanisms underlying vulnerability of pre-OLs to ROS attack: The mechanisms underlying the maturation-dependent vulnerability of pre-OLs to ROS attack have been addressed in both human brain and experimental models (Table 3). The findings in human brain indicate a delay in development of both copper – zinc and manganese superoxide dismutases, and to a lesser extent, catalase (Folkerth et al., 2004b) (Fig. 8). Additionally, our findings in cultured pre-OLs by Rosenberg and coworkers show a deficit in scavenging of hydrogen peroxide by glutathione peroxidase and catalase (Baud et al., 2004b; Baud et al., 2004c). Because of this defect at glutathione peroxidase or catalase or both, hydrogen peroxide would be expected to accumulate and in the presence of ferrous ion be converted to the hydroxyl radical by the Fenton reaction, a notion supported by the observations of others of the early appearance of iron in developing human white matter (Iida et al., 1995; Ozawa et al., 1994), as well as by the demonstration of acquisition of iron by developing OLs for differentiation (Connor and Menzies, 1996). In addition, non-protein-bound iron increases in cerebral white matter after hypoxia-ischemia (Savman et al., 2005). Further supportive of a relationship between iron and PVL are findings in a mouse model that iron pretreatment increases the amount of PVL (Dommergues et al., 1998). Moreover,

studies of human VLBW premature infants show that the presence of intraventricular hemorrhage (IVH) increases the likelihood of PVL by 5-9-fold (Kuban et al., 1999) and that after IVH, large amounts of non-heme iron are detectable in CSF for weeks thereafter (Savman et al., 2001). (IVH is present in as many as 30-40% of VLBW infants) (Volpe, 2008b). Taken together, the findings indicate a maturation-dependent window of vulnerability to oxidative attack during pre-OL development, related principally to delayed development of antioxidant enzymes, accentuated by acquisition of iron for differentiation and as a consequence of IVH and hypoxia-ischemia (Fig. 6).

3.3.2.3 Mechanisms underlying vulnerability of pre-OLs to RNS attack: The mechanisms underlying the maturation-dependent vulnerability of pre-OLs to RNS attack have been investigated in both experimental models and human brain and overlap with the mechanisms just discussed concerning ROS (Table 3). Our studies of the oligodendroglial lineage in cell culture show that NO toxicity to OLs is maturation-dependent, with pre-OLs much more vulnerable than mature, MBP-expressing OLs (Baud et al., 2004a). Although NO may lead to pre-OL injury via combination with superoxide anion to produce peroxynitrite (Li et al., 2005), NO also may act directly on pre-OLs as a mitochondrial poison with subsequent translocation of apoptosis-inducing factor from mitochondria to nuclei and resulting caspase-independent cell death (Baud et al., 2004a). Several lines of evidence indicate relevance of these studies to human PVL. Thus, strong nitrotyrosine staining indicative of attack by peroxynitrite, in white matter pre-OLs in the diffuse component of PVL was shown in our studies of the human lesion (Haynes et al., 2003). The sources of the superoxide and NO required for generation of peroxynitrite likely are reactive astrocytes and activated microglia. The diffuse component of PVL includes abundant reactive astrocytes and activated microglia. We have shown in PVL intense iNOS expression both in the reactive astrocytes and activated microglia (Haynes et al., 2009). Superoxide anion appears to be generated both by activated microglia and by pre-OLs themselves (see earlier).

3.3.3 Vulnerability to excitotoxicity—An intrinsic maturation-dependent vulnerability of pre-OLs to excitotoxicity is supported both by experimental studies and our recent work with human brain (Table 3). Thus, excitotoxicity is the second major downstream mechanism activated by cerebral ischemia (Fig. 6). Both receptor and nonreceptor-mediated mechanisms of excitotoxicity are recognized (see later).

3.3.3.1 Elevations of glutamate in vivo: Elevations of extracellular glutamate have been documented in a sheep model of PVL (Loeliger et al., 2003). The extent of the increase in glutamate correlated directly with the ultimate extent of the white matter injury. Notably, the major increase in glutamate occurred over the *hours* after the insult, a delayed increase that suggests that a perturbation of glutamate transport is responsible.

3.3.3.2 Sources of glutamate in vivo: The principal sources of elevated glutamate in cerebral white matter and ischemia are glutamate transporters (Matute et al., 2006; Matute et al., 2007; Tekkok et al., 2007; Benarroch, 2010). Under conditions of ischemia and a failure of the ATP-dependent Na^+/K^+ pump and the consequent loss of the Na^+ gradient across the plasma membrane, the high affinity Na^+ -dependent glutamate transporters fail and operate in reverse. The principal cellular sources of the glutamate in cerebral white matter appear to be principally pre-OLs but probably also axons (Back et al., 2007). The maturation dependence of the transporter source in human premature white matter is supported by our finding that the principal transporter (EAAT2) is transiently exuberantly expressed in human pre-OLs during the peak period of occurrence of PVL (DeSilva et al., 2007). Notably, EAAT2 is upregulated in reactive astrocytes in human PVL (DeSilva et al., 2008). This

upregulation may be an adaptive response to prevent accumulation of extracellular glutamate, but possibly under conditions conducive to reversal of transport, EAAT2 could become a source of glutamate. Additionally, with inflammation, activated microglia likely contribute to extracellular glutamate via several mechanisms -- reversal of a Na⁺-dependent glutamate transporter, operation of the cystine-glutamate antiporter, and inhibition of glutamate transport with accelerated glutamate release in multiple other cell types (Matute et al., 2006; Pitt et al., 2003; Takahashi et al., 2003; Barger et al., 2007; Brown and Bal-Price, 2003). The latter effects are caused by cytokines or NO or both, released by microglia.

3.3.3.3 Receptor-mediated excitotoxicity: The principal mode of glutamate toxicity to pre-OLs is receptor-mediated. Only in approximately the past decade has it become clear that pre-OLs exuberantly express glutamate receptors which, when excessively activated, lead to cell injury. The best studied glutamate receptor in pre-OLs, the AMPA/kainate (AMPA/KA) type, is concentrated in cell somata and when excessively activated, leads to cell death. The more recently discovered ionotropic receptor in pre-OLs, the NMDA receptor, is concentrated in oligodendroglial processes and leads to process loss (but perhaps a viable cell) with excessive activation.

Pre-OLs *express AMPA/KA receptors*, the activation of which results in cell death (McDonald et al., 1998; Follett et al., 2000; Tekkok and Goldberg, 2001; Yoshioka et al., 2000; Matute et al., 2006; Itoh et al., 2002; Rosenberg et al., 2003; Sanchez-Gomez et al., 2003; Follett et al., 2004; Karadottir and Attwell, 2007). Our studies of the major phases of the oligodendroglial lineage in cell culture showed that the toxicity is maturation-dependent and that both functional activity and subunit expression of AMPA/KA receptors are upregulated in pre-OLs but not in mature OLs (Follett et al., 2000; Rosenberg et al., 2003; Jensen, 2005; Talos et al., 2006a; Talos et al., 2006b). Relevance of these findings to hypoxia-ischemia was suggested by the demonstration in culture by others and by us that receptor-mediated excitotoxicity is the principal mechanisms for pre-OL death with oxygen-glucose deprivation, an *in vitro* model of ischemia (Deng et al., 2003; Fern and Moller, 2000; Deng et al., 2004; Deng et al., 2006). Relevance to hypoxia-ischemia *in vivo* was shown by the development by Jensen and coworkers of a rodent model of hypoxia-ischemia induced PVL. Thus, the P7 rat subjected to unilateral carotid ligation and hypoxemia incurred death of pre-OLs and subsequent failure of myelination, as in human PVL (Follett et al., 2000). This white matter injury, i.e., both the pre-OL death and subsequent hypomyelination, could be prevented by systemic administration *after* termination of the insult of NBQX, an AMPA/KA antagonist (Follett et al., 2000). Because NBQX may not be clinically safe, in later work, topiramate, a clinically safe anticonvulsant drug with AMPA receptor blocking properties, was studied and shown to have a similar protective effect (Follett et al., 2004) (Fig. 9). Relevance of this experimental work to the human premature brain is supported by the observations that as in human brain, pre-OLs are the major OL type at P7, that AMPA receptors are overexpressed at this age, and that at this age hypoxia-ischemia preferentially affects white matter rather than cortex (Talos et al., 2006a).

The *mechanism of the AMPA receptor-mediated toxicity* appears to involve Ca²⁺ influx and subsequently generation of ROS/RNS (Deng et al., 2004; Itoh et al., 2002; Deng et al., 2003; Fern and Moller, 2000; Alberdi et al., 2002; Liu et al., 2002) (Fig. 6). Indeed, this pre-OL excitotoxicity can be blocked by SOD/catalase mimetics, Euks 8 and 134, nonpeptidyl molecules with *in vivo* neuroprotective properties. The basis for the Ca²⁺ influx relates to the expression in pre-OLs (but not mature OLs) of AMPA receptors that lack the GluR2 subunit, the subunit that renders the receptor Ca²⁺-impermeable (Deng et al., 2003; Itoh et al., 2002; Follett et al., 2004). Direct relevance of these observations to ischemic white matter injury is suggested by the demonstration in cultured cells that pre-OL killing induced by oxygen-glucose deprivation occurs by Joro spider toxin-sensitive Ca²⁺-permeable AMPA

receptors (Deng et al., 2003). Moreover, of potential clinical relevance is the additional finding that *sublethal* oxygen-glucose deprivation resulted in enhanced toxicity to pre-OLs on subsequent exposure to this insult because of down-regulation of the GluR2 subunit and an increase in Ca^{2+} influx (Deng et al., 2003). This observation is relevant to the important role of *recurrent* hypoxic-ischemic insults in genesis of PVL (see later). Finally, and perhaps most importantly, Jensen and coworkers showed that in the *human brain* (as in the developing rodent), not only are AMPA receptors *overexpressed* during the peak period of vulnerability to PVL, but also these receptors are relatively deficient in the GluR2 subunit and are thereby Ca^{2+} -permeable (Talós et al., 2006a; Talós et al., 2006b).

Pre-OLs also express *NMDA receptors*, which, unlike the AMPA receptors, are concentrated in the *processes* of pre-OLs (Karadottir and Attwell, 2007; Salter and Fern, 2005; Karadottir et al., 2005; Micu et al., 2006; Matute, 2006; Manning et al., 2008; Bakiri et al., 2009). When activated by ischemic conditions, loss of processes occurs. Moreover, because NMDA receptors are Ca^{2+} permeable, it is probable that the downstream mechanisms related to Ca^{2+} influx and generation of ROS/RNS, account for the deleterious effects. Because axons can release glutamate, one likely physiological role for the NMDA receptors on pre-OL processes may involve axonal-pre-OL signaling related to myelination (Bakiri et al., 2009). However, with excessive glutamate, as occurs with ischemia, this physiological mechanism becomes pathological, process loss occurs, and hypomyelination, the hallmark of PVL, could result. Supporting this notion is our recent demonstration in the P7 rat model of PVL of a potent protective effect of memantine, a NMDA receptor blockers (Manning et al., 2008) (Fig. 10). Notably a safety study in the P7 rat suggests that, unlike other NMDA receptor antagonists, memantine is relatively safe (Manning et al., 2010). Relevance to human PVL is suggested by the observation that analogous to the findings with AMPA receptors, NMDA receptors are exuberantly expressed on human cerebral white matter pre-OLs during the peak period of occurrence of PVL (Talós et al., in preparation). The findings of NMDA receptors on pre-OLs also may help explain the white matter injury observed in developing animals by intracerebral injection of ibotenate, an agonist of the NMDA receptor (Hennebert et al., 2004; Tahraoui et al., 2001; Sfaello et al., 2005).

The findings concerning AMPA and NMDA receptors on pre-OL cell bodies and processes respectively suggest that pre-OL excitotoxicity could proceed in a step-wise manner, progression of which is dependent on the severity and/or persistence of the insult (Fig. 11). Thus, initially activation of the NMDA receptor likely occurs because the NMDA receptor has a higher affinity for glutamate than does the AMPA receptor. The result would be loss of cell processes but not cell death. Recall that viable cell somata devoid of cell processes have been observed in human PVL (see earlier). More pronounced or prolonged exposure to glutamate may be required for activation of the AMPA receptors in the cell body, with cell death the consequence. The result of both scenarios would be deficient myelin formation and also axonal degeneration due to loss of the trophic support of OL processes and myelin (Fig. 11). Axonal degeneration has been documented in human PVL in the diffuse component of the lesion (Haynes et al., 2008).

3.3.3.4. Non-receptor mediated glutamate toxicity: Glutamate is capable of inducing maturation-dependent death of pre-OLs by a nonreceptor mediated mechanism as well as the receptor mediated mechanisms just discussed. The nonreceptor-mediated mechanism, which has been called oxidative glutamate toxicity or oxytosis (Albrecht et al., 2010), has been shown in neurons to be caused by competition of glutamate with cystine for the glutamate/cystine antiporter [xCT or solute carrier family 7, member 11 (Slc7a11)]. Cystine is reduced within cells to cysteine, which is a necessary precursor of glutathione, and the consequence of blockade of cystine transport by glutamate is intracellular glutathione depletion, oxidative stress induced injury and death (Murphy et al., 1989). Oxytosis has been

demonstrated in OLs, both by exposure to glutamate and by deprivation of extracellular cystine, and is a maturation-dependent phenomenon to which pre-OLs are much more vulnerable than mature OLs (Oka et al., 1993; Yonezawa et al., 1996; Back et al., 1998). This maturational dependence has been shown to be due to low levels of expression of glutathione peroxidase and MnSOD in the pre-OLs (Baud et al., 2004b; Baud et al., 2004c). Oxytosis depends upon the activation of an enzyme of arachidonic acid metabolism, 12-lipoxygenase, as well as MAP kinases (Li et al., 1997; Wang et al., 2004a; Ho et al., 2008; Zhang et al., 2007; Zhang et al., 2006; Zhang et al., 2004; Stanciu et al., 2000) and involves translocation of apoptosis inducing factor to the nucleus (Li et al., 1997; Wang et al., 2004a; Seiler et al., 2008). Oxytosis appears to be one of several pathways of programmed non-apoptotic cell death now known, generally, as necroptosis (Hitomi et al., 2008; Degterev and Yuan, 2008).

The relative importance of oxytosis in the brain injury induced by ischemia is not completely understood but may be considerable. The levels of glutamate required to activate oxytosis depend upon the extracellular cystine concentration (Yonezawa et al., 1996). In tissue culture, where the cystine concentration is typically 100-200 μM (Ham and McKeehan, 1979), millimolar concentrations of glutamate are required to induce oxytosis. Significantly lower concentrations may be effective in vivo where low levels of cystine are found; for example, 0.13 μM in the striatum (Baker et al., 2003).

Additionally, oxytosis may be one of several pathways of oxidative injury provoked by excitotoxicity. In fact, Deng et al. (Deng et al., 2004) found that both kainate and oxygen-glucose deprivation induced glutathione depletion in pre-OLs. A metabotropic agonist blocked glutathione depletion induced by both insults, and also was protective against their toxicity (Deng et al., 2004). Therefore, oxytosis may occur independent of excitotoxicity, but it may also contribute to the toxicity of glutamate receptor activation, as has been suggested previously (Schubert and Piasecki, 2001). Recent evidence documenting the protective effects of 12-lipoxygenase inhibitors (see later) and translocation of AIF into nuclei in adult stroke models (Pallast et al., 2010) strengthens the potential clinical relevance of oxytosis.

3.3.4 Vulnerability to systemic infection/inflammation – central role of microglial activation—Clinical data support a relation of PVL, pre-OL injury and systemic infection / inflammation (see earlier). Activation of microglia is the principal cerebral white matter event in this scenario, and the effects of microglial activation represent a third major downstream mechanism leading to pre-OL injury (Fig. 6) (Table 3). How systemic inflammation leads to cerebral microglial activation is unclear, and the multiple potential mechanisms have been discussed elsewhere (Volpe, 2008b; Volpe, 2008c). The possibilities include transfer to brain of specific molecular patterns derived from bacterial pathogens [pathogen-associated molecular patterns (PAMPs)] found in blood, transfer of pathogen-activated immune cells into brain, stimulatory effects of systemic cytokines on brain endothelial cells, disruption of the blood-brain barrier by systemic cytokines and entry into brain of PAMPs, cytokines, or other compounds, etc., (Degos et al., 2010; D’Mello et al., 2009; Owens et al., 2008; Ransohoff, 2007; Volpe, 2008b). The central point in this context, however, is that systemic inflammation, induced, for example, by the Gram-negative bacterial product, lipopolysaccharide (LPS), does lead to striking responses in brain, including alteration of hundreds of genes (Hagberg and Mallard, 2005; Chakravarty and Herkenham, 2005; Eklind et al., 2006; Degos et al., 2010). *Notably many of the responses involve brain microglia and include up-regulation of toll-like receptors (TLRs) that mediate innate immunity in brain.* The latter may mediate the key deleterious effects of microglia in this context (see later).

The particular importance of microglia in the pathogenesis of PVL is supported by recent studies of a P7 rat model of selective white matter injury (Lechpammer et al., 2008). Thus, minocycline, an agent that suppresses microglial activation, when administered systemically post hypoxia-ischemia was shown to markedly ameliorate pre-OL injury and subsequently hypomyelination. In parallel, the number of activated microglia was reduced in the minocycline-treated animals.

3.3.4.1 Developmental abundance of cerebral white matter microglia: Although the abundance of activated microglia in the diffuse component of PVL is well-established (Haynes et al., 2003), the particular involvement of microglia in the pathogenesis of pre-OL injury is strengthened by recently discovered developmental features. Thus, it is noteworthy that microglial cells can be identified in human brain very early, become abundant in forebrain from 16 to 22 weeks of gestation and importantly are concentrated in a deep to superficial gradient (Rezaie et al., 2005; Rivest, 2003; Monier et al., 2006; Billiards et al., 2006). In a recent longitudinal study of human brain, density of microglia in white matter reached a peak during the period of greatest vulnerability to PVL (early third trimester of gestation) and then declined rapidly in white matter after 37 weeks of gestation (Billiards et al., 2006). As microglia declined in white matter, they increased in cortex. These observations suggest that a wave of possibly migrating microglia is present in cerebral white matter at the optimum time for activation by systemic infection or hypoxia-ischemia or both. *Thus, a maturation-dependent population of microglia may be concentrated in the cerebral white matter of the human premature infant at the right time and in the right place to be activated and produce injury to pre-OLs.*

3.3.4.2 Importance of innate immunity mediated by microglia: Innate immunity is mediated in brain by microglia, the resident-immune cell (Falsig et al., 2008; Buchanan et al., 2010; Lehnardt, 2010; Sloane et al., 2009). The key receptors involved in activation of microglia and mediation of innate immune responses are toll-like receptors (TLRs), most of which are present on microglia. TLRs are activated by specific PAMPs, the most important of which with bacterial sepsis are TLRs 2 (Gram-positive organisms) and 4 (Gram-negative organisms). Activation of microglia via TLRs cause release ultimately of ROS and RNS as well as cytokines and glutamate (Fig. 12). Thus, these products feed directly into the cascade leading to pre-OL injury (Fig. 6).

Cytokines released by activated microglia may also play an important role in pre-OL toxicity. Thus, in one autopsy series (n = 19), TNF α was shown to be abundant in PVL lesions, all of which were associated with “asphyxia”, and levels were still greater when evidence for systemic fetal or neonatal infection was also present (Kadhim et al., 2001). TNF α leads to pre-OL toxicity at least in part by potentiation of the toxicity of interferon γ , and the toxicity of both of these cytokines is maturation-dependent, i.e., greater to pre-OLs than to mature OLs (Agresti et al., 1996; Andrews et al., 1998; Buntinx et al., 2004; Pang et al., 2005; Baerwald and Popko, 1998; Popko and Baerwald, 1999; Vartanian et al., 1995). The major source of interferon γ may be astrocytes, which contain the cytokine in abundance in diffuse PVL, and the major target of its toxicity, the pre-OL, expresses the interferon γ receptor (Folkerth et al., 2004a). Induction of iNOS and thereby RNS appears to be the principal mode of cell death induced by interferon γ (Gendron et al., 2003). Thus, taken together, the cytokine data suggest that RNS toxicity, likely fueled by release of superoxide anion from microglia (see earlier) and NO from astrocytes and activated microglia, is involved in cell death with inflammation and that interferon γ especially, through induction of iNOS, may be an important mediator of pre-OL death in PVL. TNF α from microglia likely plays a critical potentiating role. Additionally, however, studies with mixed glial (microglial and astrocytic) cultures derived from TNF α and interferon γ knockout animals show that TNF α signaling is essential for LPS-induced toxicity to pre-OLs

and that this toxicity is independent of interferon γ (Li et al., 2008). Finally data in experimental models suggest that interferon γ may be involved in inhibiting subsequent differentiation of surviving or replenished pre-OLs, thereby accentuating hypomyelination (LaFerla et al., 2000; Lin et al., 2006b).

A role for PAMP-activated microglia on proliferation of OL precursors is suggested by studies of mouse pre-OLs in primary culture showing that LPS-activated microglia cause attenuation of proliferation of OL precursor cells, without inducing pre-OL death (Taylor et al., 2010). This work could help explain the failure in PVL of remaining or replenished pre-OLs from differentiating to myelin-producing OLs (see Neuropathology earlier). These investigations also showed that LPS directly activated TLR4 on *pre-OLs* to inhibit proliferation, an effect that could accentuate the failure of viable pre-OLs in PVL to produce sufficient myelin-producing OLs to prevent hypomyelination. However, it is noteworthy that other work has failed to detect TLR4 on pre-OLs (see later) (Lehnardt et al., 2003). More data are needed.

3.3.4.3 Microglia as convergence point in potentiation of hypoxic-ischemic and infectious/inflammatory insults: Deleterious interactions between the two major upstream mechanisms in pathogenesis of PVL, i.e., hypoxia-ischemia and systemic infection/inflammation, may be mediated in considerable part by microglia. Thus, several studies of immature animals have demonstrated that pretreatment with the Gram-negative bacterial product, LPS, at subthreshold doses (insufficient to cause brain injury), caused a subthreshold hypoxic-ischemic insult to produce marked degrees of injury, including white matter injury (Eklind et al., 2001; Eklind et al., 2004; Coumans et al., 2003; Lehnardt et al., 2003; Ikeda et al., 2004; Larouche et al., 2005; Wang et al., 2007b; Wang et al., 2007a; Lyng et al., 2005; Rousset et al., 2008; Girard et al., 2009; Wang et al., 2009; Wang et al., 2010). In most of these experimental models the potentiation of hypoxic-ischemic injury by LPS involved pretreatment several hours before the insult, although in one study LPS was administered many *days* before *in utero*. In one report the potentiating effect was apparent when LPS was administered in the several days *after* hypoxia-ischemia (Barks et al., 2008). In a study of LPS followed by hypoxia-ischemia that assessed pre-OLs directly, pre-OL cell death occurred acutely and decreased myelin basic protein-expressing OLs, later (Wang et al., 2010).

Importance for innate immunity in the genesis of potentiation of white matter injury by hypoxia-ischemia and infection/inflammation is suggested by two sets of observations. Firstly, TLR4, the TLR for LPS, and CD-14, essential for the action of TLR4, were up-regulated following LPS treatment (see earlier) (Eklind et al., 2001). Secondly, in mice lacking TLR4 or MyD88, the downstream adaptor protein for TLR4 action), the combination of LPS and hypoxia-ischemia produced *no* injury (Lehnardt et al., 2003; Wang et al., 2009). These data strongly suggest that microglial cells are central to the sensitizing effect of LPS, and that the two potent activators of microglia, infection (with its associated PAMPs) and hypoxia-ischemia, may converge on the microglial cell to provoke a deleterious series of effects (Fig. 12). The key molecular events likely involve ROS/RNS, cytokines, and glutamate.

It is worthy of emphasis that the precise molecular mechanisms by which microglial activation leads to cell injury are not entirely clear but are likely multiple (Degos et al., 2010). Although beyond the scope of this review, these mechanisms will require more precise delineation for formulation of optimal therapeutic interventions.

Innate immune mechanisms may be involved not only in the effects of infection/inflammation but also in the effects of hypoxia-ischemia per se, with the microglial cell

again the point of convergence. Thus, it is becoming increasingly recognized that insults, such as hypoxia-ischemia, result in tissue injury and the release of “danger signals” or danger associated molecular patterns (DAMPs) that serve as endogenous ligands for TLRs (Arumugam et al., 2009; Lehnardt, 2010; Sloane et al., 2009) (Fig. 12). The resulting activation of microglia has the same deleterious effects as activation by PAMPs, and indeed this dual impact on TLRs and innate immune mechanisms may be critical in the potentiation of hypoxia-ischemia and infection/inflammation. The endogenous ligands include a variety of proteins, nucleic acids, and glycosaminoglycans, such as RNA, DNA, heat shock proteins and such extracellular matrix components as heparan sulfate and hyaluronan (Sloane et al., 2010). The principal microglial TLRs involved are TLR2 and TLR4. This mechanism could lead to *amplification of injury*, since the injurious effects of TLR/microglial action could result in greater release of DAMPs and a vicious cyclical state. More data are needed on these issues.

Endogenous ligands for TLRs also may play a role in the failure of differentiation of pre-OLs and myelination with human PVL. Thus, recall that in addition to pre-OL cell death, *failure of subsequent differentiation of OL progenitors* that respond to the initial injury is an important feature of the pathology of PVL (see earlier). A potential role for endogenous TLR ligands in this context was suggested initially by the finding of accumulation of hyaluronan in demyelinated lesions of multiple sclerosis (Back et al., 2005b). Typically OL progenitors are present around the areas of demyelination but these progenitors do not remyelinate axons. Back and coworkers showed in a lysolecithin-induced demyelination model that hyaluronan and pre-OLs accumulate but the pre-OLs do not mature to myelin-producing cells (Back et al., 2005b). In culture hyaluronan prevented OL progenitor maturation. *The likely mechanism of this maturation disturbance* was shown by Sloane and coworkers to be mediated by TLR2 on *OL progenitors* (Sloane et al., 2010). Thus, OL progenitor maturation was prevented by activation of TLR2 via a MyD88-mediated effect. Other TLRs did not show this response. Low molecular weight hyaluronan produced the maturation disturbance, and the initial high molecular weight hyaluronan required the action of hyaluronidases, also present on OL progenitors. Thus, this important TLR effect was mediated directly by receptors on pre-OLs and not on microglia (which also contain TLR2) (Fig. 13). Studies in human PVL are needed.

3.3.5 Interrelations between pathogenesis of pre-OL injury and subsequent failure of differentiation of pre-OLs – importance of recurrent insults—The pathogenesis of pre-OL injury, i.e., cell death or survival with loss of cell processes, has been the focus of the previous discussion. However, as noted earlier (see Neuropathology), PVL is characterized also by replenishment of the total OL population but with apparent failure of these new OL progenitors to differentiate to myelin-producing OLs. The bases for this differentiation failure are unknown, but current epidemiologic, clinical and neuroimaging data suggest that postnatally VLBW infants are exposed to *recurrent* hypoxic-ischemic and infectious/inflammatory insults (Volpe, 2008b). Indeed, the prolonged and repetitive nature of insults to which sick premature infants are exposed have implications re: the timing of potential therapeutic interventions. The likely mechanisms operative with these recurrent insults are summarized in Table 3 and are supported by the data recorded in the previous sections. With both recurrent upstream insults inhibition of pre-OL development could occur by persistent toxicity to pre-OLs or by direct activation of receptors on pre-OLs to inhibit development.

With *recurrent hypoxia-ischemia* all three downstream mechanisms would be expected to persist (Table 4). Excitotoxicity is likely to be enhanced with *recurrent* hypoxia-ischemia. Thus, with the latter, there is downregulation of the GluR2 subunit on AMPA receptors, thus rendering pre-OLs still more vulnerable to hypoxia-ischemia (Deng et al., 2003; Jensen,

2005). Accumulation of DAMPs with recurrent hypoxia-ischemia would result in both microglial activation and inhibition of pre-OL maturation via TLRs, as discussed earlier. Similarly, activation of the downstream mechanisms of excitotoxicity and microglial activation by recurrent hypoxia-ischemia would cause persistence of ROS/RNS and free radical attack.

Additionally, however, as noted earlier activation of specific pre-OL receptors to inhibit differentiation could occur via TLRs (DAMPs) and interferon- γ . One mechanism not discussed earlier involves the potential role of adenosine and adenosine receptors on pre-OLs (Table 4). In one well-established neonatal rat model, *chronic* hypoxia from P3 to P12 caused selective white matter injury and chronic hypomyelination due to failure of pre-OL differentiation (Back et al., 2006; Segovia et al., 2008). In this model acute pre-OL death occurred and was followed by a robust regenerative pre-OL response but failure of pre-OL maturation. This sequence appears to replicate the human neuropathology. Notably, administration of caffeine in the experimental model was shown to prevent the cerebral hypomyelination. The postulated mechanism for the caffeine benefit is considered to relate to the presence of A1 adenosine receptors on pre-OLs, activation of which inhibit maturation (Back et al., 2006). Caffeine, which blocks the A1 adenosine receptors, may remove the maturation block. This effect is of particular interest also because a recent clinical report shows improved neurological outcome in premature infants treated with caffeine (for respiratory stimulation) (Davis et al., 2010; Schmidt et al., 2007).

With *recurrent infection/inflammation*, pre-OL toxicity would occur via microglial activation by PAMPs and generation of ROS/RNS and cytokines (Fig. 12). Additionally, however, activation of specific pre-OL receptors to directly affect differentiation could occur via TLRs (PAMPs) and perhaps interferon- γ (Table 4).

4. Preventative interventions for pre-OLs vs. hypoxic-ischemic and inflammatory injury

The insights into the cellular and molecular mechanisms that underlie the pre-OL injury in PVL provide a rational basis for formulation of preventative interventions. Indeed, a variety of studies in experimental models have shown pronounced benefit for such pathogenesis-based interventions (Fig. 14). Many of these interventions are likely to be clinically safe, but careful safety studies and design of clinical trials will be needed before assessment in living premature infants. Indeed, the optimal timing and duration of interventions remain to be established. As delineated in detail elsewhere (Volpe, 2008b), sick premature infants are subjected to recurrent hypoxic-ischemic and systemic infectious/inflammatory insults often for many weeks after birth. Not unexpectedly, human neuropathological and imaging data indicate that the incidence of PVL increases with postnatal age of premature infants (Volpe, 2008b). Thus, therapies will likely be required for prolonged periods, and this requirement increases concerns for safety of agents employed.

4.1 Prevention of upstream mechanisms – hypoxia-ischemia and systemic infection/inflammation

Prevention of the two crucial upstream mechanism in pre-OL injury in PVL, hypoxia-ischemia and infection/inflammation, has been discussed in detail elsewhere (Volpe, 2008b; Khwaja and Volpe, 2008) and will not be reviewed here. Of potential importance are detection in the premature infant of a pressure-passive cerebral circulation (e.g., with near-infrared spectroscopy), avoidance of hypotension, avoidance of hypocarbia, and administration of antenatal corticosteroids (for acceleration of lung maturation).

4.2 Downstream mechanisms – microglial activation, excitotoxicity and free radical attack

The principal downstream mechanisms are microglial activation, excitotoxicity and free radical attack (Fig. 14). The former two mechanisms cause their deleterious effects principally by leading to the last of these, free radical attack.

4.2.1 Microglial activation—Although a variety of anti-inflammatory interventions have shown promise in experimental models of combined gray and white matter injury (induced neutropenia (Hudome et al., 1997), platelet activating factor antagonist (Liu et al., 1996; Zhang et al., 1994), cytokine antagonists (Hagberg et al., 1996), the effects of *minocycline* on microglia have been most encouraging (Arvin et al., 2002; Fan et al., 2005; Buller et al., 2009; Lechpammer et al., 2008) (Fig. 14). As noted earlier, in a model of selective white matter injury in the neonatal rat, systemic, post-insult (hypoxia-ischemia) treatment with minocycline led to decreased numbers of microglia, attenuated pre-OL death and improved myelination. Melatonin may exert some of its protection of developing white matter in a hypoxic-ischemic sheep model via an anti-microglial effect (Welin et al., 2007). The beneficial effects of minocycline have been shown in a variety of other relevant models, including excitotoxicity, oxidative stress and cytokine attack (Lee et al., 2004; Morimoto et al., 2005; Tikka et al., 2001; Song et al., 2004; Pi et al., 2004; Kraus et al., 2005; Buller et al., 2009). Nevertheless, minocycline is not without clinical hazard, and further study of this agent and related analogs is needed. To date, anti-TLR agents have not been studied *in vivo*, but these compounds would be of considerable interest in this context (Lehnardt, 2010; Sloane et al., 2009).

4.2.2 Excitotoxicity—Prevention of excitotoxicity with glutamate receptor blockers appears to be a particularly promising approach, because two agents shown to be effective experimentally are likely to be clinically safe. The targets, of course, are non-NMDA, particularly AMPA receptors, and NMDA receptors, (Fig. 14). *Topiramate*, an AMPA blocker, has been shown to markedly attenuate pre-OL injury in a neonatal rat model (P7) of selective hypoxic-ischemic white matter injury (Follett et al., 2004) (Fig. 9). Unlike other AMPA blockers topiramate appears to be safe, at least in developing animals (Follett et al., 2004; Glier et al., 2004). Similarly, the NMDA receptor blocker, *memantine*, also has been protective to pre-OLs in the same model of selective white matter injury (Manning, 2004) (Fig. 10). Unlike MK-801 and other NMDA blockers, memantine also appears to be relatively safe (Manning et al., 2010).

Combination therapy, with non-NMDA and NMDA antagonists could be beneficial in two ways. This approach may provide optimal protection for the pre-OL and also may contribute protection against associated neuronal injury (Volpe, 2009).

4.2.3 Free radical attack—The final common pathway to pre-OL injury is free radical attack (Fig. 14). Protective interventions should be considered in terms of free radical generation, scavenging of free radicals, and blockade of the final pathway to cell death.

Prevention of free radical generation involves ROS/RNS and could occur at three levels. One approach to prevention of generation of ROS/RNS is *inhibition of enzymes that are crucial in the genesis of ROS/RNS*. Although *in vivo* studies of neonatal white matter injury are not available, studies in cultured OLs and in adult stroke models suggest value for 12-lipoxygenase inhibitors (Wang et al., 2004a; van Leyen et al., 2008; Jin et al., 2008). 12-Lipoxygenase has been shown to be a key generator of ROS in pre-OLs (Wang et al., 2004a). NOS inhibitors are likely to be of value, in view of their proven effectiveness in several animal models of neonatal brain injury (Peeters-Scholte et al., 2002; Feng et al., 2002; Tutak et al., 2005; Muramatsu et al., 2000; van den Tweel et al., 2005). However,

selective white matter injury or pre-OLs specifically have not been studied re: NOS inhibitors.

Prevention of free radical generation by vitamin K may be an especially potent approach because available data support the value of this intervention, including effectiveness when administered even hours *after* the onset of free radical accumulation (Li et al., 2003). The mechanism of the effect of vitamin K involves inhibition of 12-lipoxygenase (Li et al., 2009). This agent should be safe in human premature infants because vitamin K is routinely administered in newborns for blood coagulation and is protective in vitro in very low concentrations. Thirdly, *counteraction of the deficient antioxidant defenses* and thereby prevention of continued ROS/RNS generation by the use of mimetics of anti-oxidant enzymes could be of particular value (Deng et al., 2004). These mimetics are non-peptidyl and can cross the blood-brain barrier. As yet, no studies of these agents re: pre-OLs or white matter injury in in vivo models have been reported.

Scavenging of free radicals appears promising as a protective approach. *Vitamin E*, a clinically safe agent, has been shown to be effective in cultured pre-OL models (Back et al., 1998). *N-Acetylcysteine*, another compound that has been used in newborns, has been shown to be protective in an in vivo animal model of LPS-induced white matter injury (Wang et al., 2007c; Paintlia et al., 2008). Another free radical scavenging agent, alpha-phenyl-N-tert-butyl nitron, administered systemically following the hypoxic-ischemic insult, attenuated white matter injury in the immature rat (Lin et al., 2006a).

Blockade of the final common pathway to cell death could be the final point of rescue for the pre-OL threatened by hypoxia-ischemia and inflammation. The predominant form of cell death in neonatal white matter injury is not clearly known. Evidence derived from studies of pre-OLs undergoing free radical attack (Back et al., 1998), ischemic white matter injury in the neonatal animal models (Yue et al., 1997; Segovia et al., 2008)] and human neuropathology (Back et al., 2005a; Kadhim et al., 2006; Billiards et al., 2008; Volpe, 2008b) suggest that both apoptosis and necrosis may occur, dependent perhaps on the severity and temporal characteristics of the insult. Thus, prosurvival and antiapoptotic interventions are relevant.

Several growth factors have been effective versus pre-OL death. Thus, IGF-1 has been shown to be protective to pre-OLs in the hypoxic-ischemic neonatal rat (Lin et al., 2005); and in the near-term fetal lamb (Cao et al., 2003). In both models, IGF-1 suppressed apoptotic death and promoted oligodendroglial precursor proliferation. The antiapoptotic effect involves activation of Akt and prevention of both cytochrome c release and caspase activation. Two caveats re: IGF-1 should be noted. First, administration of the protein in the above models has been either intraventricular or intracerebral, i.e., a clinically problematic approach. (A report in a hypoxic-ischemic *adult* model shows appreciable neuroprotection after *intravenous* administration of the N-terminal tripeptide of IGF-1) (Guan et al., 2004). A second concern with IGF-1 is that in the presence of LPS-induced inflammation in a developing rat model, enhancement of inflammation and intracerebral hemorrhage have been observed (Pang et al., 2010). Other growth factors may also be protective versus pre-OL death. In separate animal models, BDNF and CNTF have shown benefit (Husson et al., 2005; Linker et al., 2002).

The recently described protective effect of *estradiol* in pre-OLs subjected to oxygen-glucose deprivation in culture and in neonatal white matter subjected to hypoxia-ischemia in vivo may be antiapoptotic (Gerstner et al., 2009) (Fig. 14). Thus, one mechanism of estrogen neuroprotection involves antiapoptotic effects (Arnold and Beyer, 2009). Moreover, estradiol attenuates hyperoxia-induced injury to developing white matter at least in part by

an antiapoptotic effect (Gerstner et al., 2007). The demonstration of white matter and pre-OL protection against hypoxia-ischemia by estradiol is particularly noteworthy because estradiol may be clinically safe when administered over a relatively brief period. The premature brain *in utero* is exposed normally to a placental supply of estrogens that is many-fold higher than the levels occurring postnatally. However, careful attention to safety studies is needed before consideration in premature infants.

Erythropoietin (EPO), a glycoprotein originally recognized for its role in erythropoiesis has been shown to be involved in several beneficial adaptive responses to perinatal hypoxia-ischemia and to exhibit neuroprotective properties (Sola et al., 2005a; Wang et al., 2004b; Chang et al., 2005; Spandou et al., 2005; Sola et al., 2005b; Kellert et al., 2007; Statler et al., 2007). Among its several beneficial effects are antiapoptotic and survival promoting responses. Recently intravenous EPO has been shown in a fetal sheep model of white matter injury, induced by LPS administration and hypotension, to reduce injury and preserve myelination (Rees et al., 2010). This agent has been utilized in newborns for erythropoiesis and thus may be clinically safe as a white matter protectant. A recent retrospective report of VLBW infants administered EPO in the first six weeks of life showed higher Bayley Mental Developmental Index scores in the treated infants (Brown et al., 2009). Unfortunately, white matter injury was not assessed in this report. More data are needed.

5. Conclusions

Pre-OLs are the principal cellular target in cerebral white matter injury, i.e., PVL, the most important form of brain injury and subsequent neurological disability in premature infants. Primary injury to pre-OLs relates to a confluence of maturation-dependent characteristics that render these cells vulnerable to two principal upstream mechanisms, hypoxia-ischemia and systemic infection/inflammation. These upstream mechanisms converge on three interacting downstream mechanisms, i.e., microglial activation, excitotoxicity and ultimately, free radical attack (Fig. 6). Interruption of these mechanisms has been shown in excellent experimental models to lead to prevention or amelioration of pre-OL injury. A selected summary of the most promising interventions is shown in Fig. 14. It is now clear that the extraordinary insights into pathogenesis in recent years has provided considerable hope that prevention of this serious and common form of brain injury in the premature infant may be possible.

Acknowledgments

The research described from our group was supported by grant P01NS38475. Joseph J. Volpe has been Program Director and the program has included projects on the cellular basis of human PVL (P.I., Hannah C. Kinney), mechanisms of free radical-mediated injury to developing OLs (P.I., Paul A. Rosenberg), mechanisms of excitotoxic injury to developing OLs (P.I., Frances E. Jensen) and innate immunity in the pathogenesis of PVL (P.I., Timothy Vartanian).

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Abbreviations

DAMP	danger-associated molecular pattern
EAAT2	excitatory amino acid transporter 2
EPO	erythropoietin
IVH	intraventricular hemorrhage
LPS	lipopolysaccharide
MBP	myelin basic protein
NO	nitric oxide
OL	oligodendrocyte
PAMP	pathogen-associated molecular pattern
Pre-OL	premyelinating oligodendrocyte
PVL	periventricular leukomalacia
RNS	reactive nitrogen species
ROS	reactive oxygen species
TLR	toll-like receptor
VLBW	very low birth weight

Fig. 1. Periventricular leukomalacia. Coronal section of cerebrum (H+E stain) in a premature infant who died several weeks after a cardiac arrest. Note the two components of the lesion (i.e., deep *focal* areas of cystic necrosis and more *diffuse* cerebral white matter injury). See text for details. (Courtesy of Dr. Hannah C. Kinney).

Fig. 2.

Progression of the oligodendroglial lineage (OL) through the four major stages. The predominant form in cerebral white matter of the premature infant is the 04-positive pre-oligodendrocyte. See text for details. (From Back SA, Volpe JJ, *Ment Retard Dev Disabil Res Rev* 3: 96-107, 1997). Note that in this review the abbreviation pre-OL includes both premyelinating OL forms, i.e., the 04-positive pre-oligodendrocyte and the 01-positive immature oligodendrocytes.

Fig. 3. Cystic (A) and noncystic (B) periventricular leukomalacia (PVL) – schematic diagrams. A. Cystic PVL is characterized by macroscopic (several mm or more) focal necrotic lesions that become cystic and by diffuse astrogliosis and pre-OL injury. B. Noncystic PVL is characterized by focal necrotic lesions that are *microscopic* and evolve principally to small glial scars rather than cysts.

Fig. 4. 04 immunostaining of pre-OLs in control (A) and PVL (B) cases. Pre-OLs in control cases (A) are multipolar with multiple discrete processes (arrow), whereas in PVL (B) some pre-OLs lack processes (arrows). (From Billiards SS, et al, *Brain Pathol* 18: 153-163, 2008).

Fig. 5. Axial MRI (FLAIR) of cerebrum in a 20-month-old infant who was born prematurely and had PVL. Note the marked paucity of cerebral white matter and, as a consequence, enlarged lateral ventricles. The increased signal intensity in white matter is caused by astroglia. (Courtesy of Dr. Linda deVries.)

Fig. 6. Pathogenesis of PVL. The two major upstream mechanisms (pink) are ischemia and systemic infection/inflammation, activating three major downstream mechanisms (blue), microglial activation, glutamate excitotoxicity and ultimately, free radical attack. See text for details.

Fig. 7. Oxidative (upper panel) and nitrative (lower panel) injury in PVL. Hydroxynonenal (HNE) staining (upper panel), a marker for attack by reactive oxygen species, colocalizes (yellow) with a marker of pre-OLs (O4) in the diffuse component of PVL. Nitrotyrosine (NT) staining, (lower panel), a marker for attack by reactive nitrogen species, colocalizes (yellow) with pre-OLs (O4) in the diffuse component of PVL as well. (From Haynes RL, et al, *J Neuropathol Exp Neurol* 62: 441-450, 2003).

Fig. 8. Regression curves of analyses of antioxidant enzyme expressions (Western blots) in cerebral white matter from midgestation to the first postnatal weeks, relative to the adult standard (100% value). Immunocytochemical studies showed similar temporal changes in pre-OLs. The peak period for occurrence of PVL is shown by the black bar. (From Folkerth RD, et al, *J Neuropathol Exp Neurol* 63: 990-999, 2004).

Fig. 9. Protection from hypoxic-ischemic selective cerebral white matter injury in the immature rat (P7) by systemic administration of topiramate. In 9A, the top panels show a pronounced deficit in myelin-basic protein staining in cerebral white matter at P11, 4 days after hypoxemia-ischemia at P7. Compare in 9A the hemispheres ipsilateral (*left*) and contralateral (*right*) to the carotid ligation. (In separate experiments marked loss of pre-OLs was demonstrated in the ipsilateral hemisphere in the 24 hours after the insult.) In 9B, topiramate was administered intraperitoneally over the 48 hours following the insult, beginning immediately after termination of the insult. A marked protective effect is apparent. (From Follett, P, et al, *J Neurosci* 24: 4412-4420, 2004).

Fig. 10. Protection from hypoxic-ischemic selective cerebral white matter injury in the immature (P7) rat by systemic administration of the NMDA receptor blocker, memantine. The paradigm is similar to that described in the legend to Fig. 9, except that memantine rather than topiramate was administered systemically after termination of the insult. *A* (ipsilateral to carotid ligation) and *B* (contralateral) were vehicle-treated and *C* (ipsilateral) and *D* (contralateral) were memantine-treated. Note the pronounced protective effect of memantine (compare *A* to *C*). (From Manning SM, et al, *J Neurosci* 28: 6670-6678, 2008.)

Fig. 11.

Potential differential effects and temporal aspects of excitotoxicity to developing oligodendrocytes. The intact cell (*top*) has AMPA receptors primarily on the cell soma and NMDA receptors primarily on the cell processes. Initially with excess extracellular glutamate, activation of NMDA receptors could lead to loss of cell processes, and if excitotoxicity continues, to activation of AMPA receptors and cell death. Either event could lead to impaired myelination (*solid arrows*) and potentially also to axonal disturbance (*dotted lines*).

Fig. 12.

Microglia and innate immune mechanisms in pre-OL injury. Microglia may act as a convergence point for both upstream mechanisms in PVL, i.e., systemic infection/inflammation and hypoxia-ischemia, and innate immunity is likely involved in both microglial mechanisms. Thus, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), respectively lead to microglial activation and resulting release of products, especially reactive oxygen and nitrogen species (ROS/RNS) and cytokines, that result in pre-OL injury. See text for details.

Fig. 13. Potential mechanism by which endogenous TLR ligands, specifically hyaluronan and TLR2, may lead to inhibition of pre-OL differentiation to mature myelin-producing OLs. See text for details.

Fig. 14. Interventions for prevention of injury to developing OLs resulting from hypoxic-ischemic and inflammatory insults. See text for details.

Table 1

Pre-OL “Injury” in PVL

Pre-OL death
Pre-OL survival with loss of processes and failure of differentiation/myelination
Pre-OL replenishment but with failure of differentiation/myelination

Table 2**Pathogenesis of PVL – Major Interacting Factors**

Cerebral Ischemia
<ul style="list-style-type: none">• Pressure-passive cerebral circulation• Hypocarbica
Systemic Infection/Inflammation
<ul style="list-style-type: none">• Propensity for maternal intrauterine infection or postnatal neonatal infection• Potentiation of cerebral ischemic injury
Maturation-Dependent Intrinsic Vulnerability of Pre-OLs
<ul style="list-style-type: none">• Microglial activation• Excitotoxicity• Free radical (ROS/RNS) attack

Table 3**Major Maturation-Dependent Factors Underlying the Three Downstream Mechanisms in PVL*****Free Radical Attack**

- Vulnerability of pre-OLs to free radical attack
 - Abundant production of both ROS and RNS in PVL (by pre-OLs, microglia, astrocytes)
 - Delayed development of antioxidant defenses in pre-OLs
 - Acquisition of Fe^{++} by pre-OLs

Excitotoxicity

- Vulnerability of pre-OLs to excitotoxicity
 - Exuberant expression of major glutamate transporter (source of glutamate) by pre-OLs
 - Exuberant expression on pre-OLs of AMPA receptors, which are deficient in the GluR2 subunit and therefore are Ca^{2+} -permeable
 - Exuberant expression on pre-OLs of NMDA receptors, which also are Ca^{2+} -permeable
 - Likely mechanism of excitotoxicity is generation of ROS/RNS

Microglial Activation

- Central role of microglia in free radical generation
 - Microglia, especially abundant in PVL; potent sources of ROS/RNS
 - Presence of TLRs on microglia; activation results in release of free radicals
 - Maturation-dependent concentration of microglia in normal cerebral white matter
- Microglial activation releases potentially injurious cytokines
 - $\text{TNF}\alpha$ derived from microglia in diffuse PVL
 - $\text{TNF}\alpha$ potentiates the maturation-dependent toxicity to pre-OLs by interferon γ (interferon γ expressed in astrocytes in diffuse PVL and interferon γ receptor expressed in pre-OLs)
- Microglial activation impairs glutamate transport and accentuates excitotoxicity

* See text for references

Table 4

Likely Mechanisms for Failure of Subsequent Differentiation of Pre-OLs in PVL *

Recurrent Hypoxia-Ischemia

- Toxicity to pre-OLS
 - Excitotoxicity enhanced (GluR2↓)
 - Microglial activation
 - ◆ DAMPs/TLRs
 - ROS/RNS persist
- Pre-OL receptors activated -- inhibit development
 - TLRs (DAMPs)
 - Interferon- γ
 - Adenosine

Recurrent Systemic Infection/Inflammation

- Toxicity to pre-OLs
 - Microglial activation
 - ◆ PAMPS/TLRs
 - ROS/RNS persist
- Pre-OL receptors activated -- inhibit development
 - TLRs (PAMPS)
 - Interferon- γ

* See text for details