

# Towards improved animal models of neonatal white matter injury associated with cerebral palsy

John C. Silbereis<sup>1</sup>, Eric J. Huang<sup>2</sup>, Stephen A. Back<sup>3,\*</sup> and David H. Rowitch<sup>1,4,\*</sup>

Newborn neurological injuries are the leading cause of intellectual and motor disabilities that are associated with cerebral palsy. Cerebral white matter injury is a common feature in hypoxic-ischemic encephalopathy (HIE), which affects full-term infants, and in periventricular leukomalacia (PVL), which affects preterm infants. This article discusses recent efforts to model neonatal white matter injury using mammalian systems. We emphasize that a comprehensive understanding of oligodendrocyte development and physiology is crucial for obtaining new insights into the pathobiology of HIE and PVL as well as for the generation of more sophisticated and faithful animal models.

## Neonatal white matter injury: the clinical problem

In 1861, William John Little reported on a series of 68 cases of difficult birth, which he related to later developments of neurological deficits, such as spastic diparesis (a movement disorder that primarily affects the lower limbs). This condition, originally known as Little's disease, became generally known as spasticity or cerebral palsy (CP) (Little, 1861). CP denotes a condition of the brain reflected by movement limitation that is often associated with some degree of cognitive impairment. It is generally considered a fixed, non-progressive condition resulting from neurological injury in the antenatal or perinatal period ([http://www.ninds.nih.gov/disorders/cerebral\\_palsy/cerebral\\_palsy.htm](http://www.ninds.nih.gov/disorders/cerebral_palsy/cerebral_palsy.htm)). It is now known that in utero hypoxic-ischemic (HI) events (e.g. placental insufficiency, chronic fetal-to-maternal hemorrhage, stroke, infection and inflammation), perinatal events (e.g. placental abruption, respiratory failure) and neonatal disorders (e.g. chronic lung disease) are associated with acquired brain injuries that lead to CP. In recent years, damage to the cerebral

white matter has emerged as an increasingly common cause of CP.

Rates of CP in the United States are increasing. In the 1960s, approximately 2.2/1000 live births resulted in CP. With the advent of advanced resuscitation techniques practiced by neonatologists, rates were reduced to 1.3/1000 in the late 1970s and early 1980s. However, the rate of CP in the United States is currently >3/1000 births (<http://www.cdc.gov/ncbddd/dd/cp3.htm#common>), owing to the increasing survival of extremely low birth weight (ELBW) premature infants born at gestational ages <28 weeks, who are at higher risk for cognitive and motor disabilities. Related to this is the notion that infants who are born prematurely are subject to inflammatory conditions that predispose them to brain injury (Hagberg and Mallard, 2005).

## Common forms of neonatal brain injuries associated with later development of CP

Fig. 1 shows precursor lesions that are associated with CP in full-term and preterm infants. Full-term infants (Fig. 1D) can

suffer from global hypoxic-ischemic encephalopathy (HIE), resulting in both damage to neuronal populations, such as those in the basal ganglia and cortex, as well as significant cellular necrosis and axonal damage in cerebral white matter injury (WMI). Neonatal stroke causes HI in focal areas of the brain (Fig. 1C) (Nelson and Lynch, 2004). Other types of brain injury related to HI or maternal-fetal infection are more common in ELBW infants. Fig. 1A shows intraventricular hemorrhage, which is thought to result from rupture of fragile blood vessels in the germinal matrix (also known as the ventricular zone) with resultant hemorrhage into the ventricles, which sometimes extends into the brain parenchyma. We define WMI as a spectrum of pathology that includes: (1) the classic lesion of periventricular leukomalacia (PVL), which involves macroscopic cystic or microscopic non-cystic necrotic lesions with pan-cellular degeneration, and (2) focal and diffuse non-cystic lesions that selectively trigger oligodendrocyte lineage degeneration and subsequent disturbances in myelination (Khawaja and Volpe, 2008). Neuronal loss and axonal damage are often observed in patients with PVL; these events can reflect primary injury or arise as a secondary response to WMI (Volpe, 2009). However, evidence is lacking for prominent neuronal loss or axonal degeneration in the non-cystic lesions that predominate in most patients.

## A focus on WMI as a common component of full-term and preterm neonatal brain injuries

The development of therapies to prevent neonatal WMI leading to CP, and that promote regeneration and repair, is hindered by a poor understanding of the underlying cellular, molecular and genetic mechanisms. There is a crucial need for further neuropathological studies that will help to address these remaining issues and provide guidance for generating improved animal models of neonatal WMI (Johnston et al., 2005). For

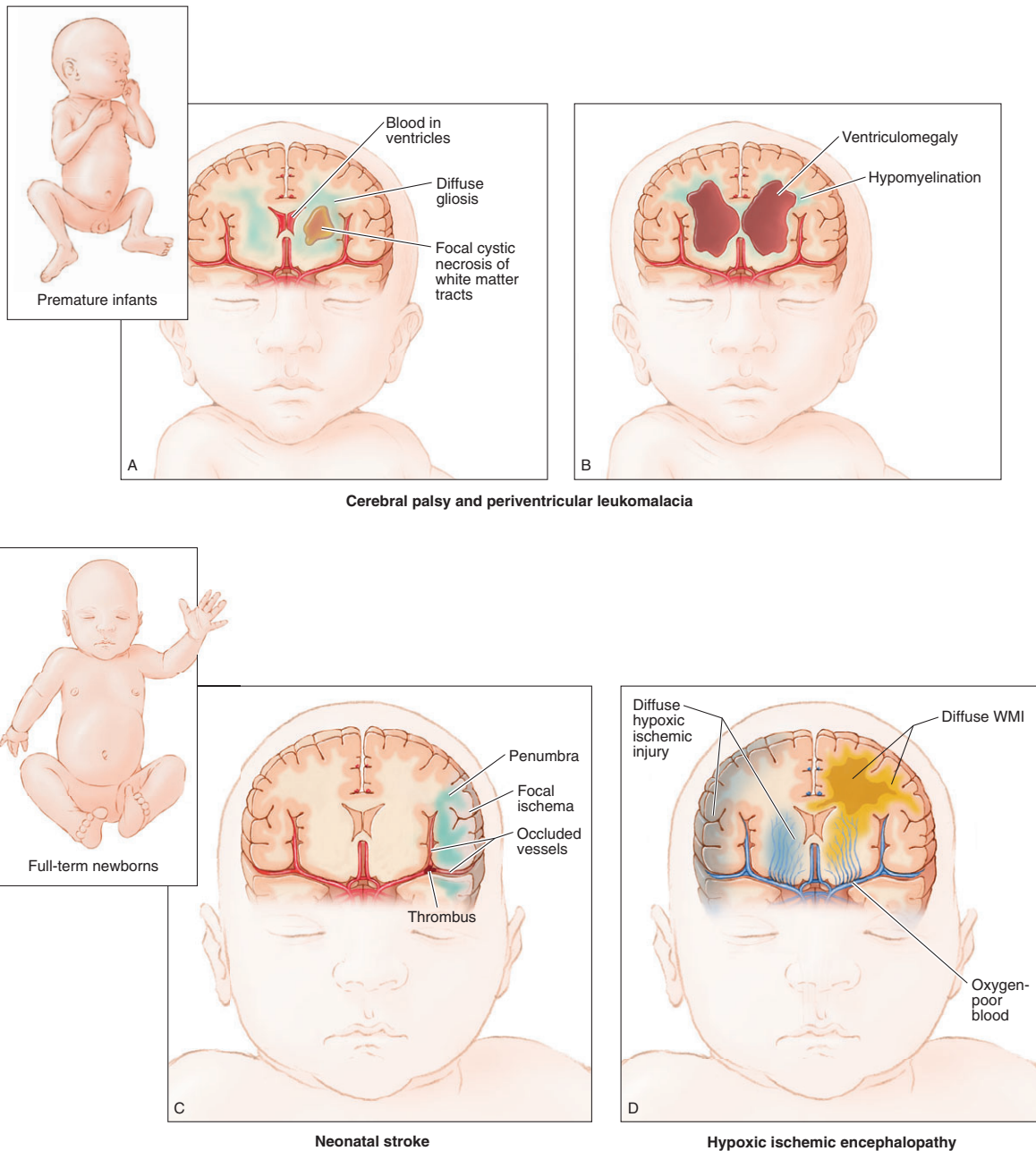
<sup>1</sup>Departments of Pediatrics and Neurosurgery, Eli and Edythe Broad Institute for Stem Cell Research and Regeneration Medicine and Howard Hughes Medical Institute, University of California San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA

<sup>2</sup>Department of Pathology, University of California San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA

<sup>3</sup>Departments of Pediatrics and Neurology, Oregon Health and Science University, Portland, OR 97239, USA

<sup>4</sup>Division of Neonatology, University of California San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA

\*Authors for correspondence (backs@ohsu.edu; rowitchd@peds.ucsf.edu)



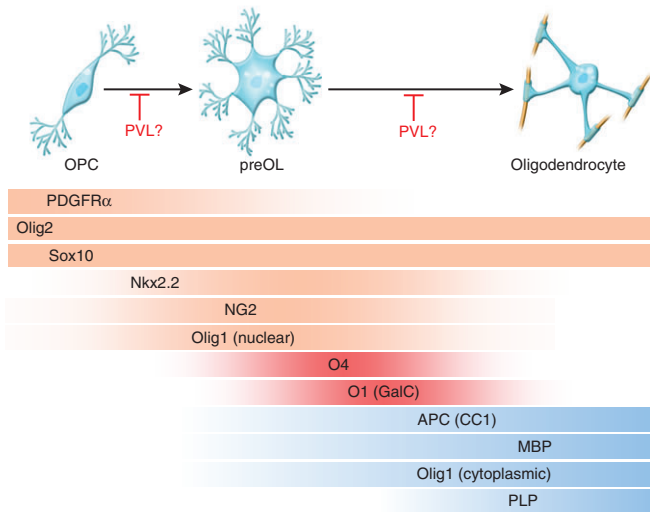
**Fig. 1. Common types of injury associated with development of CP in ELBW and term infants.** (A,B) Illustration of brain injuries commonly affecting ELBW infants. (A) Key characteristics of intraventricular hemorrhage, which results from germinal matrix hemorrhage into the ventricles, sometimes extending into the brain parenchyma, are shown. Additionally, there is a high incidence of PVL (a type of WMI), which can result in a cystic necrosis of white matter tracts and/or diffuse gliosis. (B) Long-term sequelae of brain injury in ELBW infants are shown, including hypomyelination resulting from failure of lesion repair, as well as ventriculomegaly, which represents an ex-vacuo change resulting from significant loss of brain parenchyma. (C,D) Common brain injuries in full-term infants. (C) Neonatal stroke in which a focal region of cortex is affected. (D) HIE results in global HI injury to the brain, more specifically to neurons of the cortical plate and basal ganglia, as well as white matter tracts.

instance, what is the role of inflammatory mediators in enhancing or reducing the risk of injury? Is the risk of injury incremental or non-linear with recurrent insults? Is there a critical window after which normal myelination of injured white matter is not possi-

ble owing to degeneration or irreversible alterations in oligodendrocyte lineage cells or axons? Which are the key inhibitors of myelination in the lesion environment?

This article focuses on the coordinated use of human neuropathological studies

and animal models in the study of perturbations in oligodendrocyte development in WMI as a means to address the questions and challenges outlined above. Although we argue below that studies of human pathology will be required to address many of



**Fig. 2. Markers of oligodendrocyte lineage specification and maturation.** The schematic shows oligodendrocyte lineage progression from OPCs to preOLs and then to myelinating oligodendrocytes. In the past decade, various markers have been identified that show lineage- and stage-specific expression (indicated by colored gradients). The markers PDGFR $\alpha$ , Olig2, Nkx2.2, Sox10, NG2 and Olig1 (nuclear) (indicated in orange) are characteristic of OPCs. O4 and O1 [also known as galactocerebroside (GalC)] (indicated in red) mark intermediate preOLs, whereas APC (also known as CC1), myelin basic protein (MBP), myelin proteolipid protein (PLP) and Olig1 (cytoplasmic) (indicated in blue) are typical of mature myelinating oligodendrocytes. Several laboratories studying MS and PVL have reported abnormal oligodendrocyte differentiation in these disorders, suggesting that these abnormalities are associated with a failure to repair demyelinated lesions.

these questions, because of the inherent difficulties in defining the influence of comorbid conditions and the timing of insults, there remains a crucial need for improved animal models to provide clues about the pathways (both cell intrinsic and extrinsic) that regulate the responses of the oligodendrocyte lineage during the initial and progressive phases of injury and myelination failure. Understanding the crucial impact of neonatal insults on neuronal populations has been the subject of many prior reviews (Fatemi et al., 2009; Ferriero, 2004; Vannucci and Hagberg, 2004) and will not be discussed in detail here.

### Oligodendrocytes in WMI Oligodendrocyte development

To comprehensively assess the effects of WMI on oligodendrocytes in the neonatal human central nervous system (CNS), it is necessary to understand their development. Oligodendrocytes enable the formation of myelin and Nodes of Ranvier, and consequently allow optimal conduction of the nervous impulse (Bradl and Lassmann, 2010). In the last decade, a greater under-

standing of the developmental regulation and ontogeny of the human oligodendrocyte lineage in vivo was obtained through the discovery of genes that are necessary for oligodendrocyte development, such as *Olig1* and *Olig2*, which encode basic helix-loop-helix (bHLH) transcription factors. Oligodendrocyte development can be characterized according to four distinct stages of maturation. First, neural stem cells (NSCs) give rise to oligodendrocyte precursor cells (OPCs) during embryonic development [beginning at embryonic day 12.5 (E12.5) in mice and at gestational age 13 weeks in humans]. OPCs proliferate and migrate throughout the brain before differentiating into late oligodendrocyte progenitors [premyelinating oligodendrocytes (preOLs)] (beginning around E16.5 in mice and gestational age 20 weeks in humans), which undergo extensive cellular growth and process elaboration and mature into myelinating oligodendrocytes (Fig. 2) (Jakovcevski and Zecevic, 2005; Kessaris et al., 2006; Jakovcevski et al., 2009; Rowitch, 2004). Recent studies have also provided a panel of new markers that distinguish suc-

cessive stages of oligodendrocyte development and can be used to interrogate oligodendrocyte lineage status in model organisms and in humans (Fig. 2) (Arnett et al., 2004; Ligon et al., 2004; Ligon et al., 2007; Rhee et al., 2009). Amongst others, signaling pathways involving sonic hedgehog (Shh), bone morphogenetic protein (BMP), Wnt, Notch or platelet-derived growth factor receptor- $\alpha$  (PDGFR $\alpha$ ) are known to regulate various steps of oligodendrocyte development and have been the topic of many comprehensive reviews (Althaus et al., 2008; Fancy et al., 2010; Ogata et al., 2006; Rosenberg et al., 2007).

In addition to demonstrating distinct capabilities in the migration, proliferation and/or myelination of different populations of oligodendrocyte lineage cells, studying them at specific stages of maturation has revealed differences in their susceptibility to cellular stress. For instance, both OPCs and preOLs that express the chondroitin sulfate proteoglycan NG2 receive synaptic inputs and display currents that in some white matter tracts are both glutamatergic and GABAergic (De Biase et al., 2010). OPCs also play an important role in brain repair by proliferating, migrating to and then remyelinating lesion sites in response to various forms of neurological injury (Chandran et al., 2008; Franklin and Kotter, 2008; Nishiyama, 2007). Interestingly, mouse fate mapping studies indicate that oligodendrocytes are produced in several successive waves that commence in the embryo in ventral regions and progress to dorsal regions of the cerebrum as the animal matures (Kessaris et al., 2006) (Fig. 3). Thus, developmental-stage-dependent effects of injury might reflect cell-intrinsic differences in oligodendrocyte lineage cells specified at different times and in different regions of the brain.

### Vulnerability of the oligodendrocyte lineage in neonatal brain injury

A large amount of data supports the notion that the oligodendrocyte or its precursors are vulnerable to injury (Kinney, 2009). In both human patients and rodent injury models, white matter is most susceptible to injury at the ages at which preOLs predominate in the forebrain (Volpe, 2009). Although it is not well understood why oligodendrocytes at this stage of development are so vulnerable, it is thought that at least four main factors play a role: (1) their

greater sensitivity to oxidative stress and excitotoxicity, (2) cytokine-induced cell death related to inflammation, (3) perturbations of oligodendrocyte development coupled with ineffective repair mechanisms, and (4) axonal damage.

In addition to being highly susceptible to excitotoxicity (discussed above), preOLs might be acutely sensitive to free radicals generated by HI and inflammation (Boullerne and Benjamins, 2006; Butts et al., 2008). White matter lesions in the brains of premature infants demonstrate significant levels of oxidative damage that are accompanied by depletion of preOLs (Haynes et al., 2003). In addition, preOLs express low levels of superoxide dismutase, suggesting that these cells have limited antioxidant defenses (Folkerth et al., 2004).

A role for inflammation- and cytokine-mediated brain injury in preterm survivors with WMI or CP has been suggested by numerous reports, but this notion is not without controversy (Dammann et al., 2002). Certain studies found no increased risk of CP in preterm survivors by examination of either intrauterine exposure to infection or inflammatory cytokines in neonatal blood (Grether et al., 2003; Nelson et al., 2003). Although elevated levels of TNF $\alpha$  and IL1 $\beta$  were observed in cystic PVL lesions or cerebrospinal fluid, the expression of these cytokines has not been studied in the more common diffuse WMI form of PVL. Microglial activation is characteristic of WMI and supports a proinflammatory response; however, it is a non-specific finding that is also observed in acute HIE and other neurological pathologies that do not have a primary inflammatory component (Rezaie and Dean, 2002).

The aforementioned findings suggest that the myelination disturbances associated with WMI arise from the acute degeneration of oligodendrocyte lineage cells, particularly preOLs (Khwaja and Volpe, 2008). However, this classical pathogenic model deserves reconsideration in light of more recent studies in human and rodent models of chronic cerebral WMI caused by HI that support a new model: that the myelination disturbances arise from a combination of factors leading to an arrest in oligodendrocyte maturation that could result in fixed demyelinated lesions. Billiards et al. used Olig2 and other early oligodendrocyte markers, and found that OPCs were present

in acute and chronic human PVL lesions (Billiards et al., 2008), suggesting a block in differentiation. Findings in a neonatal rat model confirmed that remyelination after chronic HI injury was delayed owing to a failure of preOL differentiation (Segovia et al., 2008). Thus, failure in myelin regeneration seems to be due to both early preOL degeneration and a later phase of persistent preOL maturation arrest. This seemingly nuanced revision of the classical model actually has profound implications for our approach to PVL pathogenesis, raising questions about the nature of the oligodendrocyte differentiation block and about how such inhibition might be overcome.

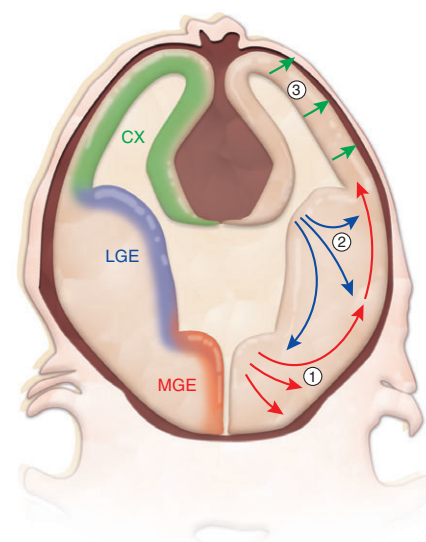
The contribution of neuroaxonal injury to myelination failure has received relatively little attention. Injury to axons is a prominent feature of white matter lesions in the cystic-necrotic form of PVL (Haynes et al., 2008); however, it remains unclear whether axonal injury is primary or secondary. Axons release important growth factors, such as PDGF and brain-derived neurotrophic factor (BDNF), that promote oligodendrocyte survival and myelination (Ng et al., 2007; Simons and Trajkovic, 2006). Additionally, few oligodendrocytes in the optic nerve survive after transection of retinal ganglion cell axons, and co-culture of oligodendrocytes and neurons promotes oligodendrocyte survival and differentiation (Barres and Raff, 1993; Barres and Raff, 1999; Ng et al., 2007; Simons and Trajkovic, 2006). Conversely, studies of injury to developing axons show that as-yet-unmyelinated axons are acutely sensitive to excitotoxic stress, leading to damage and perturbations of their development (Alix and Fern, 2009). Thus, it is possible that a lack of normal signaling between axons and oligodendrocytes contributes to myelination failure.

### Examples of existing models of WMI

In this section we summarize existing models of developmental WMI. Selected pathological features as well as relative advantages and caveats to several models are described in Table 1.

### Models of rodent neonatal HI

The most widely studied model of neonatal brain injury is induction of HI by unilateral ligation of the common carotid artery followed by a period of hypoxia ranging from 30 minutes to 4 hours. In neona-



**Fig. 3. Oligodendrocytes are generated in multiple waves and in various locations of the CNS.** Results from the Richardson laboratory and others have shown that, in mice, oligodendrocytes of the forebrain are initially specified in ventral regions, such as the medial ganglionic eminence (MGE; corresponding to region 1 in the figure), a ventral domain of embryonic proliferative precursor cells, from which the oligodendrocytes migrate to more dorsal areas of the brain (Kessaris et al., 2006). At subsequent developmental stages, later waves of oligodendrocytes are produced in successively more dorsal regions, such as the lateral ganglionic eminence (LGE, corresponding to region 2) in late embryonic development and the cerebral cortex (CX, corresponding to region 3) in the early postnatal period. The first three waves of oligodendrocyte production are shown. In addition, NG2-positive precursor cells continue to cycle in the adult brain and are thought to contribute to oligodendrocyte turnover, as well as the response to injury (not shown). It is unclear whether oligodendrocytes from different regions of the brain have cell-intrinsic differences in their ability to repair myelin, or in their vulnerability to HI or other toxic insults.

tal rat pups at postnatal day 9 (P9), this procedure induces gray matter injury resembling that seen in full-term infants with HIE (Rice et al., 1981; Vannucci and Vannucci, 2005). The application of the same procedure to mice causes less-extensive focal gray matter injury (Han et al., 2001). The procedure was later adapted in the preterm equivalent rat at P2 to cause injury to both cerebral gray and white matter. At this time, preOLs are present in high numbers and are highly vulnerable to injury (Back et al., 2001). By contrast, OPCs and myelinating oligodendrocytes are more resistant to single and

**Table 1. Overview of common animal models for studying developmental white matter injury**

Model	Species	Pathological features of WMI	Advantages	Disadvantages	References
Rice-Vannucci (HIE)	Mouse, rat	Oligodendrocyte cell death Alterations of oligodendrocyte development Necrotic cysts of white matter and cortex Axonal damage Forebrain neural cell death Microglial activation Ventriculomegaly	Widely used and well characterized Use of transgenic mice	Not adapted to large animals Variability between animals Stroke and global ischemia rare in premature infants	Vannucci and Vannucci, 2005; Segovia et al., 2008
Chronic hypoxia	Mouse, rat	Oligodendrocyte cell death is minimal Alterations of oligodendrocyte development Moderate neural cell death Ventriculomegaly	Generates chronic global hypoxia that is common in premature human infants Consistency between specimens Use of transgenic mice	Lacks gliosis Inflammatory response poorly characterized Relatively mild WMI	Back et al., 2006a; Fagel et al., 2006; Chahboune et al., 2009; Scafidi et al., 2009
In utero ischemia	Rabbit	Lesions to multiple forebrain structures (cortex, thalamus and basal ganglia) and white matter tracts Intraventricular hemorrhage	Rabbits exhibit hypertonic motor deficits similar to those observed in CP	Not amenable to genetic experiments Requires specialized infrastructure and expertise	Cai et al., 1998; Drobyshevsky et al., 2005
	Sheep	Relatively selective graded cerebral white matter lesions that resemble the spectrum seen in human Cortical and subcortical gray matter injury is a major feature if ischemia is severe	Brain development and neurovasculature more similar to human Chronic instrumentation allows physiological monitoring, EEG and analysis of fetal cerebral blood flow		McClure et al., 2008
Premature delivery	Baboon	Cystic necroses, diffuse white matter gliosis Ventriculomegaly Intraventricular hemorrhage	Highest similarity to human development Includes premature birth and neonatal intensive care with no need for experimental insult Allows for chronic instrumentation	High cost Requires highly specialized infrastructure and expertise Animals do not survive long term	Dieni et al., 2004; Inder et al., 2005a; Inder et al., 2005b
LPS-induced inflammation	Mouse, rat, sheep, rabbit	Microglial activation Diffuse white matter gliosis Oligodendrocyte cell death Deep cortical layer neuronal cell death More severe when coupled with HI	Specifically creates an inflammatory insult to the developing brain Can be coupled with HI-based models Feasible in transgenic mice	Infection of LPS-containing bacteria rarely causes inflammation in clinical CP Unclear whether LPS crosses the blood-brain barrier	Wang et al., 2006
Gliotoxic	Mouse, rat	Demyelination and oligodendrocyte cell death in focal lesions	Allows for well-controlled study of OPC repair response and mechanisms of remyelination Can be used in adult transgenic mice	Not adapted to neonatal stage Lacks inflammatory component	Blakemore and Franklin, 2008

EEG, electroencephalogram.

recurrent episodes of HI (Segovia et al., 2008). Thus, the point during oligodendrocyte development at which this procedure is carried out is crucial for differential effects of gray versus white matter injury, and can lead to variability in results.

**Chronic-intermittent perinatal and intrauterine HI models**

ELBW infants with chronic lung disease as a complication of premature birth suffer multiple hypoxic episodes and are at higher risk of developing CP. Neonatal rodent

models involving either continuous or intermittent hypoxic rearing have been proposed as models of this type of brain injury and display some pathological features of developmental WMI (Back et al., 2006a; Chahboune et al., 2009; Ment et al., 1998;

Scafidi et al., 2009). Intrauterine models of transient global ischemia have been described in the rat and rabbit by housing of pregnant dams in hypoxic conditions or by abruption of placental blood flow by inflation of a balloon catheter inserted into the uterine artery, respectively (Cai et al., 1998; Cai et al., 1995; Hersey et al., 1995). In both rat and rabbit, this model recapitulates many histopathological aspects of developmental WMI (Derrick et al., 2007). In rabbits, in addition to lesions throughout the cerebral gray and white matter, some animals develop spontaneous intraventricular hemorrhage with ventriculomegaly and periventricular white matter loss. Detailed neurobehavioral assessments have demonstrated that the surviving rabbit kits display a wide range of hypertonic motor deficits that resemble CP. This is currently the only model that permits such detailed clinicopathological correlations with neurobehavioral outcome and neuro-radiological assessment (Drobyshevsky et al., 2005). This model has also been used extensively to study the cellular mechanisms of WMI. For example, it was recently shown that the susceptibility to WMI during development closely coincided with the timing of appearance of preOLs (Buser et al., 2010).

### Large animal models of preterm WMI

Large animal models of WMI offer several advantages over rodent models, including (1) a gyrencephalic brain with an abundance of cerebral white matter; (2) the feasibility of studying preterm animals at a gestational age that is relevant to humans; (3) a neurovasculature with cerebral blood flow responses that are similar to those in humans; (4) the potential to monitor cerebral blood flow, metabolism and electrophysiological activity in precisely defined brain regions; and (5) some large animal models closely mimic clinical features of premature birth and neonatal intensive care in humans. Therefore, large animal models of WMI have significant translational potential.

In the only primate model of prematurity, baboons were delivered by induced labor and provided ventilator-support and neonatal intensive care (Dieni et al., 2004; Inder et al., 2005a; Inder et al., 2005b). Although an attractive feature is the lack of an experimentally induced insult, these animals display limited survival, compro-

### Case study

A 31-year-old pregnant woman with two healthy children presents to her obstetrician with contractions and abdominal pain at 24 weeks gestation (normal gestation 40 weeks). She is admitted to hospital with diagnoses of preterm labor and possible chorioamnionitis (infection of the lining of the amniotic sac). Magnesium sulfate is administered in an attempt to inhibit uterine contractions, and antibiotics and a dose of  $\beta$ -methasone (a glucocorticoid used to promote lung maturity in the fetus) are given. Subsequent fetal heart-rate monitoring reveals bradycardia prompting emergent delivery by Caesarian section.

A male infant is born that weighs 0.5 kg. Although vigorous at birth, he rapidly develops respiratory distress requiring intubation and installation of surfactant (a medication to improve respiratory function) into the lungs. After 3 days of ventilator support, he is weaned to supplemental oxygen by nasal cannula. On day 3 of life, an in-dwelling central venous catheter is placed to augment nutrition intravenously. On day 5, he begins to feed on the mother's milk by nasogastric lavage. On day 3, there is a drop in red blood cell count and a head ultrasound is performed, which shows a large right-sided grade intracranial hemorrhage. On day 12, the patient deteriorates, showing hypotension and apnea requiring re-intubation. Antibiotics are initiated and blood cultures are found to be positive for *Staphylococcus epidermidis*. Lumbar puncture results are consistent with past intracranial hemorrhage but not meningitis.

The patient recovers and is weaned off of the ventilator. He advances to full volume enteral feeds and gains adequate weight (approximately 30 g/day). On day 30, a head ultrasound shows extensive echogenicity in the right hemispheric white matter. Later MRI shows right ventricular dilatation and diffuse WMI. In follow-up, the patient is diagnosed with CP and shows left-sided hemiplegia and cognitive deficits with an IQ of 85.

missing the ability to carry out long-term neurobehavioral assessments. Pathological features in this model include cystic PVL, diffuse white matter gliosis, ventriculomegaly and intraventricular hemorrhage.

The fetal sheep is the most widely studied large animal model of developmental brain injury (Back et al., 2006b). WMI is induced by reversible carotid artery occlusion, umbilical cord compression or maternal hypoxemia between ~90 days gestation (65% of the full gestation time) and near-term. Advantages of this model include: (1) neurodevelopment of the preterm sheep fetus (65% gestation) is comparable to that of the preterm human between approximately 24 and 28 weeks of gestation, and (2) the size of the fetal sheep permits longer-term procedures that cause damage, as well as invasive monitoring of fetal physiological status (e.g. brain oxygenation and electroencephalography) (Bennet et al., 2010; Rees et al., 2010). It is feasible to generate selective focal or more diffuse white matter lesions without necrotic PVL-like lesions; this leads to lesions that resemble the types of injury that are now common in surviving preterm infants (Riddle et al., 2006). Hence, fetal sheep are a model in which it is possible to generate graded cerebral WMI that can be studied with reliable measurements of blood flow and metabolism in histologically defined regions of cerebral white matter.

### Model of inflammation and perinatal brain injury

Intrauterine infection is proposed to be a causative factor of premature birth and WMI, whereas maternal infection is correlated with an increased incidence of CP (Clark et al., 2008). Several models of maternal infection use mice, rats and sheep. Mouse models have mainly been applied for studying the neurobehavioral consequences of infection and their relevance to psychiatric disorders [for a comprehensive review, see Meyer et al. (Meyer et al., 2009)]. In ovine and rat models in which bacteria or the lipopolysaccharide (LPS) endotoxin are administered either systemically or uteroplacentally to the mother, robust microglial activation, WMI injury and death of deep layer cortical neurons in the child is observed (Hutton et al., 2007; Rousset et al., 2006).

Induction of an inflammatory response by focal or systemic administration of LPS in neonatal rodents is also a widely used method to study the effect of infection and inflammation on the developing brain (for review, see Wang et al., 2006). Other studies have coupled LPS injection with HI to demonstrate an additive effect on the extent of infarct (Wang et al., 2006). An important caveat to this system is that LPS-containing bacteria represent only one (and a rare) cause of inflammation in the human fetus and neonate. A mouse model of antenatal chorioamnionitis induced by infection of the uterus with the much more common

bacterium *Ureaplasma parvum* has recently been developed. In this model, pups demonstrated microglial activation, myelin deficits and reduced numbers of interneurons (Normann et al., 2009).

### Focal and systemic gliotoxins are used to study the effects of damage to oligodendrocytes and remyelination

Understanding the repair response after demyelination and oligodendrocyte death might be relevant to understanding the failure of myelination during developmental insults. In this respect, gliotoxic models, which are traditionally used to study demyelinating diseases such as multiple sclerosis (MS), might prove useful for understanding developmental WMI. The two main toxin-based models of demyelination in adult white matter are (1) systemic administration of cuprizone, which induces widespread CNS demyelination, or (2) focal injection of lysolecithin or ethidium bromide, which typically induce focal lesions in spinal cord white matter or caudal cerebellar peduncle, respectively (Blake-More and Franklin, 2008). Although such models lack an inflammatory basis, which might be an important consideration in neonatal WMI, they might allow targeted investigation of the responses of oligodendrocyte progenitors to WMI and define mechanisms involved in regeneration and repair. In addition, they might be useful for assessing mechanisms of myelin regeneration that are conserved in both neonatal and adult white matter disorders, such as MS.

### Conventional transgenic knockout mice for modeling lineage-specific roles of genes in the oligodendrocyte repair response

The discovery of genes controlling normal oligodendrocyte development might provide additional insight into pathways that are compromised in developmental WMI. For example, *Olig1* function is essential not only for developmental myelination, but also for the repair of lesions in adult white matter (Arnett et al., 2004). Oligodendrocyte-lineage-specific genes can be studied in conventional knockout mice. However, because many genes of interest are expressed in other CNS cell types or outside the brain, tissue-specific gene targeting will need to be employed in

many cases, as discussed in the next section.

### Towards improved models of neonatal WMI

The sections above reviewed some of the currently available approaches to model neonatal WMI. We discuss below advances and approaches relevant to the development of new models and to the validation of existing models of neonatal brain injury. These include extending the perspective of modern developmental neurobiology to better understand the pathogenesis of human WMI, and the development of new genetic tools that allow the targeting of precise gene functions and signaling pathways in mice.

### Application of insights from developmental neurobiology

Limitations in our understanding of the normal cellular and molecular mechanisms of human white matter development remain a fundamental obstacle in defining mechanisms of myelination failure in preterm infants. More work is also needed to define the impact of WMI on neural progenitor populations that reside in the white matter or that localize to germinal zones such as the subventricular zone (SVZ), which contains a persistent population of NSCs capable of producing olfactory bulb interneurons or migratory OPCs in adulthood (Menn et al., 2006). For example, WMI induced by HI triggers a rapid several-fold expansion in resident populations of OPCs in subcortical white matter (Segovia et al., 2008). Death and depletion of SVZ progenitors followed by robust cellular expansion of the SVZ in the repair phase of injury is a common feature following acute HIE, chronic neonatal hypoxia and toxin-induced lesions of the subcortical white matter (Fagel et al., 2006; Levison et al., 2001; Ness et al., 2001; Silvestroff et al., 2010; Yang and Levison, 2006). Although OPCs make up only ~5% of SVZ progenitors in the healthy brain, this population expands substantially upon WMI and SVZ-derived OPCs migrate to regions of WMI (Menn et al., 2006). These studies thus suggest that activation of local OPCs and the migration of OPCs derived from stem cells in the germinal niches of the brain are important components of the response to WMI. Finally, it is unclear whether the preOLs that arrest in lesions

## Clinical terms

**Cerebral palsy (CP)** – a spectrum disorder defined here as a condition of the brain with a component of movement limitation that is often associated with some degree of cognitive impairment; generally considered a fixed and non-progressive lesion associated with neurological injury that occurs during the antenatal or perinatal period

**Chorioamnionitis** – inflammation of the amniotic sac due to bacterial infection

**Excitotoxicity** – a pathological process by which neural cells are damaged due to excessive extracellular levels of the excitatory neurotransmitter glutamate, which binds its receptors AMPA and NMDA, leading to toxic levels of  $Ca^{2+}$  influx; often caused by hypoxia and other disruptions of cellular metabolism that prevent glutamate reuptake

**Extremely low birth weight (ELBW)** – a birth weight of less than 1000 g; typically observed in premature infants <28 weeks gestational age; these infants are at high risk for WMI and CP

**Hypoxia-ischemia (HI)** – insufficient supply of oxygen and blood flow to cells and organisms leading to deficiencies of aerobic metabolism and disruptions in cell physiology that can lead to cellular damage or death

**Hypoxic-ischemic encephalopathy (HIE)** – a condition characterized by HI of the cerebrum due to asphyxia and leading to cell death and brain injury; a common cause of CP in term infants

**Multiple sclerosis (MS)** – an autoimmune disorder of the adult brain characterized by chronic episodic inflammatory attacks of the myelin sheath and subsequent demyelination and oligodendrocyte cell death

**Periventricular leukomalacia (PVL)** – also known as white matter injury (WMI); involves injury to the cerebral white matter tracts characterized by some combination of the following symptoms: focal necrotic cysts, diffuse white matter gliosis, ventriculomegaly, axonal damage, oligodendrocyte cell death and abnormalities of myelination. PVL is the most frequent cause of CP in premature infants

are equivalent to preOLs in normal white matter. In particular, their potential to disrupt normal glial-neuronal signaling has not been explored. Deciphering the clinical relevance of these findings will also require a better understanding of the influence of WMI on the normal structure and function of NSCs and OPCs of the human fetus and neonate.

Although signaling by Shh, Wnt, BMP and Notch pathways is recognized to regulate oligodendrocyte development in vertebrate systems, the extent to which their

### Clinical and basic research opportunities

- Apply novel biomarkers adapted from developmental neurobiology to better understand the neuropathology of human neonatal WMI and better validate existing animal models of CP
- Use new transgenic approaches to target precise stages of the oligodendrocyte lineage in order to probe the function of genes and signaling pathways involved in the complex process of myelin regeneration
- Incorporate known clinical co-morbidities of CP (e.g. commonly encountered infections, severe lung disease) into animal models to more accurately reflect the neonatal hospital course

functions are conserved in the developing or injured human brain are unclear (Rowitch, 2004). An approach to address these issues would be to identify signaling pathway activation in situ in human brain. Large-scale analyses of the proteome and transcriptome in normal versus injured white matter might also uncover the signaling and genetic pathways affected in lesions and thereby provide new avenues of research. Indeed, progress has been made using proteomic techniques to identify novel factors implicated in pathogenesis (Han et al., 2008). Although such studies have been limited in the fields of human brain development, Johnson et al. performed expression profiling to compare distinct regions of the fetal human cerebral cortex (Johnson et al., 2009).

In summary, there is a crucial need for a comprehensive understanding of the neuropathology of developmental WMI, PVL and HIE that fully integrates our concepts of how normal developmental signaling pathways and cellular mechanisms (specification, expansion, maturation, migration, etc.) might be impacted in specific cellular lineages of the brain. However, there are significant hurdles associated with the human studies proposed above. First, it is difficult to obtain high-quality autopsy tissues. Second, many antibodies developed for model-organism- and cell-based studies are ineffective for human tissue. These obstacles point to the need for coordinated human neonatal brain banking centers with appropriate procedures for tissue processing and optimization of reagents for analysis.

### Use of new genetic tools to model tissue-specific function of candidate genes and signaling pathways in WMI

The past decade has produced a wealth of transgenic mice that carry conditional (floxed) alleles, enabling knockout of oligodendrocyte lineages in a tissue- or developmental-stage-specific manner. The development of transgenic lines expressing bacteriophage P1 Cre recombinase and tamoxifen-inducible creERT2 under control of oligodendrocyte-lineage-specific enhancers, which enable targeting of oligodendrocytes at precise stages of development (e.g. *PDGFRaCreERT2* and *MBP-Cre*), provide powerful tools for studying WMI at distinct stages of development (Guo et al., 2009; Kessaris et al., 2006; Niwa-Kawakita et al., 2000; Rivers et al., 2008; Rowitch et al., 2002; Zhu et al., 2008). Studies of oligodendrocyte development, regeneration and cellular stress in adult models of WMI, such as toxin-induced demyelination and spinal cord injury, have yielded great advances using these strategies. For instance, genetically targeting the Wnt and BMP pathways specifically in oligodendrocyte lineage cells has provided new insights into remyelination (Fancy et al., 2009; Jablonska et al., 2010). The success of these studies suggests that such approaches can be extended to the study of mouse models of infant WMI. Limitations of the application of these tools in rodents must be considered, including the marked neuroanatomical and developmental differences between humans and rodents, and the difficulty in obtaining selective WMI in existing mouse models. However, a notable advantage of rodent models is that transgenic experiments are not feasible in more clinically relevant large animal models owing to technical limitations and/or high costs.

### New markers for stage-specific study of the oligodendrocyte lineage in vivo and in vitro

Maturation-specific markers of the oligodendrocyte lineage provide a further means of identifying and studying oligodendrocytes in neonatal WMI. For instance, several studies have used NG2 and other maturation markers to specifically identify OPCs and preOLs in adult white matter and to probe their electrical properties. Such studies have revealed that there are spiking and non-spiking OPCs and preOLs, and that the spiking class has a selective sensitivity to

excitotoxicity in adult spinal cord injury (Karadottir et al., 2008). Overstimulating ionotropic glutamate receptors on preOLs specifically leads to preOL death in vitro (Follett et al., 2000; Husson et al., 2005; Manning et al., 2008). The GENSAT project (<http://www.gensat.org/index.html>) has also provided many new mouse strains that express the fluorescent marker green fluorescent protein (GFP) under the control of regulatory sequences that are specific to glial lineages, better enabling their identification in slice culture and in vivo (Anthony and Heintz, 2007).

Finally, cell culture techniques based on immuno-panning provide a powerful means by which to purify oligodendrocyte lineage cells at particular stages of maturation and study changes in gene expression. This approach has identified genes that are necessary for oligodendrocyte development and myelination, and has provided transcriptome-level information about cell-type heterogeneity in the CNS (Cahoy et al., 2008; Dugas et al., 2008; Emery and Barres, 2008). Together, these methods provide powerful opportunities to study cell-type-specific roles of particular genes or signaling pathways in the context of HI and other neonatal brain insults.

### Modeling adverse features of the neonatal hospital course

Although neonatal brain injuries might result from antenatal and neonatal insults (Anjari et al., 2009; Leviton et al., 2010), in other cases CP seems to result from postnatal damage to the brain associated with distinct complications of prematurity. For instance, premature infants with chronic lung disease (also known as bronchopulmonary dysplasia) or congenital heart disease, who might be chronically hypoxicemic, are at the highest risk for poor long-term neurodevelopmental outcome (Sherlock et al., 2009). Yet, few investigators have integrated these co-morbid conditions in animal models of neonatal brain injury.

Standard treatment practices in neonatal care might cause iatrogenic injury to the developing brain. For instance, it was demonstrated that postnatal dexamethasone, which is used routinely for respiratory indications in neonates, is associated with the development of CP (Baud and Sola, 2007). In addition, there is concern about the potential of inhaled anesthetics to cause

neuronal apoptosis in the neonate (Loepke et al., 2009), as well as potential effects of these and other GABA antagonists (e.g. benzodiazepines) on interneuron populations in humans during critical windows of developmental plasticity (Durrmeyer et al., 2010). Ongoing dialogue between clinicians and basic neuroscientists is necessary to develop advanced models that address the current and complex causes of acquired human newborn neurological injury.

## Conclusions

Given the advances in neonatal care, the incidence of neonatal WMI continues to increase worldwide; therefore, it remains the main form of brain injury in preterm survivors and in children with congenital heart disease. Moreover, advances in care have resulted in a pronounced shift in the spectrum of WMI such that the incidence of cystic necrotic PVL has markedly declined and focal or diffuse non-cystic lesions now predominate. There is a crucial need for human neuropathological studies that better define key cellular and molecular events that accompany the progressive phases of WMI and myelination failure. Such studies will provide the basis for the more rational development of small and large animal models that more faithfully reproduce the major forms of WMI observed in the current population of preterm survivors. Small animal models have a paucity of cerebral white matter and typically fail to generate a spectrum of pathology that closely resembles that observed in humans, but they provide initial answers to numerous cellular and molecular questions. By contrast, large pre-clinical animal models have an abundance of cerebral white matter with a developmental profile similar to humans. These models are attractive for pathophysiological studies and clinical-translational studies, which are typically not technically feasible in small animals. Collectively, these models provide unprecedented opportunities for more rapid progress towards defining the pathobiological mechanisms relevant to preventive and regenerative therapies. Insights gained from these neurodevelopmental studies will probably be broadly relevant to other adult disorders, such as stroke, MS and dementia, in which injury to cerebral white matter is also a prominent but understudied feature.

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