The maturational stage of the brain at the time of injury plays a key role in the pattern of brain damage in humans, including regional and cell-type–specific susceptibility. In this minireview, we will summarize available models of preterm and at-term ischemic brain injury in rodents and in larger species and discuss how maturation stage of the brain at the time of an insult affects the underlying excitotoxic and inflammatory injury signatures. We will review pros and cons of animal modeling for advancing the understanding of the mechanisms of hypoxic–ischemic encephalopathy (HIE) and stroke in human infants.

Ischemia-Related Brain Damage in Preterm and Term Infants

In preterm human babies (23–32 weeks of gestation), periventricular white matter injury (WMI) and its most severe form, periventricular leukomalacia, as well as intracerebral hemorrhage and intraventricular hemorrhage are the most common types of ischemia-related injuries (Figure). Vulnerability of oligodendrocyte progenitor cells, loss of subplate neurons, and a weak germinal matrix are thought to contribute to the pathology.

The pathophysiology of at-term brain damage is multifactorial, with the patterns of ischemic injury differing from those in preterm newborns; injury is no longer diffuse and is manifested focally in gray matter regions, most commonly in the striatum, thalamus, and cortex (Figure). Infection and inflammation have been shown as the major predisposing or modulatory factors in ischemic injury in both preterm and at-term infants.

Perinatal arterial ischemic stroke, defined as stroke that occurs between 20 weeks of gestation and 28 days postnatal, happens in ≤1 in 2300 live infant births and produces significant morbidity and severe long-term neurological and cognitive deficits including cerebral palsy, epilepsy, neurodevelopmental disabilities, and impaired vision and language function.

Increasing evidence suggests that the placenta plays a significant role in HIE and perinatal stroke, and that the presence of at least 1 prothrombotic factor substantially increases the incidence of stroke in term neonates.

Animal Models for Ischemia-Related Preterm and Term Injury

Numerous models of hypoxia, hypoxia-ischemia (HI), intraventricular hemorrhage, and focal stroke have been developed in rodents and in larger species to mimic the different types of injuries seen in the human infant. Studies discussed in this review focus on ischemic and HI animal models and are summarized in the Table.

Large Animal Models

A unique model of birth asphyxia has recently been developed in nonhuman primates by transiently occluding the umbilical cord before birth. The animals develop severe asphyxia and die or present with cerebral palsy-like motor abnormalities. However, the cost limits the number of study subjects in this model. The most commonly used nonrodent large mammal species to induce HI in the immature brain are sheep, rabbits, and pigs, species that have a white/gray matter ratio similar to the human brain.

Sheep are precocial, and thus studies are performed during pregnancy to correlate to relevant maturation stages in the human. Cerebral ischemia models in fetal sheep, induced by bilateral transient occlusion of the carotid arteries, were first developed in the near-term fetus, and, later, during midgestation. The chronically instrumented fetal sheep umbilical cord occlusion model is global and allows examination of intrauterine pathophysiology and the contribution of other organs on the brain, without the influence of anesthesia. In these models, the preterm fetuses are more prone to WMI and deep gray matter injury, with an increased vulnerability of cortical gray matter with advancing gestation; neuropathology that correlates well with human pathology. However, fetal models are complicated by maternal/placenta metabolism, which is not present in the human situation of HIE.

Newborn pigs subjected to various combinations of hypoxia and ischemia show changes in metabolism (magnetic
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In general, injury is characterized by neuronal loss in the sensorimotor cortex and basal ganglia and postnatal seizures. The HI models in newborn pigs, in parallel with fetal sheep studies, have been instrumental in development of therapeutic hypothermia for term infants with HIE.

In rabbits, intrauterine ischemia is induced ≈22 days of gestation as equivalent to the preterm, and at the end of gestation (at 29 days) to mimic at-term injury. HI in preterm rabbits results in injury mainly in subcortical areas, such as basal ganglia and thalamus. Importantly, the preterm rabbit HI model is one of few large animal set-ups, which has demonstrated a correlation between abnormalities on magnetic resonance imaging, neuropathology, and cerebral palsy-like hypertonic motor deficits in the newborn pups.

Rodent Models

Like in humans, in rats, and in mice much of brain development occurs after birth and, like in humans, individual regions of the rodent brain mature at a different pace, thus making it difficult to adhere to a single postnatal day as a comprehensive representation of brain development in human. Cross-comparisons of gross neuroanatomy, the timing of neurogenesis, synaptogenesis, gliogenesis, maturation, and myelination, as well as age-dependent molecular and biochemical changes in rodents and humans, have demonstrated that the rodent brain at postnatal day 1 (P1) to P5 corresponds to 23 to 32 weeks of gestation in the humans and is thus suitable for studies of preterm injury, whereas the rodent brain at P7 to P10 corresponds to 36 to 40 weeks of gestation in humans, thus suitable for studying brain injury at-term (Figure; Table).

Several models of WMI have been developed, including HI in P1 to P3 rat, a model that consists of unilateral ligation of the common carotid artery followed by a hypoxic episode, ibotenate injection in P5 rats, and prolonged low-grade gestational hypoxia. The inception of the experimental HI model in P7 rat by Rice et al and extension of the HI model to P7 to P9 mice has allowed mimicking HIE in the at-term human infant and obtain seminal information about the pathophysiology of the disease, including CBF regulation and energy metabolism, inflammation, excitotoxic and oxidative injury, intracellular injury mechanisms and neuronal death, and mechanisms of brain repair. HI studies have also demonstrated that genetic background and sex affect mechanisms of neuronal cell death and injury severity. A limitation of this model is animal-to-animal variability of injury.

Several models have been established to study the underlying mechanisms of perinatal arterial stroke, including models of permanent middle cerebral artery (MCA) ligation and suture transient MCA occlusion (tMCAO) in P7 to P10 rats and P9 mice. Varying the length of tMCAO has allowed induction of injuries of different severity. The demonstrated presence of recirculation in the tMCAO model mimics reperfusion, which frequently occurs in arterial stroke in term infants.

Excitotoxicity and Oxidative Injury

Glutamate receptors are prevalent in the developing brain, and glutamate-induced excitotoxicity is an important factor that contributes to ischemic injury in both the preterm and the at-term brain. Maturation-dependent expression of different glutamate receptors has been linked to specific injury patterns. Thus, high expression of NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors on neurons in the deep gray matter and cortex is thought to play important roles in at-term brain injury,
whereas AMPA/kainate receptors are more important in the preterm brain. Also, dysregulation of glutamate transporters on oligodendrocytes and microglia likely contribute to excessive extracellular glutamate in the immature brain.

The developing brain is sensitive to oxidative injury. Excitotoxicity and inflammation are major processes that lead to generation of reactive oxidants, which can damage DNA and proteins, both directly and indirectly.

### Table. Animal Models to Study Neonatal HI and Stroke

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ADC indicates apparent diffusion coefficient; BBB, blood–brain barrier; CBF, cerebral blood flow; CCAL, common carotid artery ligation; CCAO, common carotid artery occlusion; CP, cerebral palsy; DTI, diffusion tensor imaging; EEG, electroencephalogram; Gal-3, galectin-3; HI, hypoxia-ischemia; HIF, hypoxia-inducible factor; IC, internal capsule; i.c.v., intra-cerebroventricular; KO, knockout; LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; MRI, magnetic resonance imaging; NFκB, nuclear factor kappa beta; OL, oligodendrocyte; P, postnatal day; SOD, superoxide dismutase; Tg, transgenic; TRIF KO mice, C57BL/6J–Ticam1Lps2/J; UCO, umbilical cord occlusion; VEGF, vascular endothelial growth factor; WMI, white matter injury; and WT, wild type.
Maturation-Dependent Factors That Affect the Inflammatory Signatures in Preterm Versus At-Term Ischemic Brain Damage

Inflammatory Responses

Clinical data have demonstrated that proinflammatory cytokines are elevated in cerebrospinal fluid of asphyxiated term infants, and that the levels of these cytokines reflect the degree of HIE.30 Cytokines are also elevated in newborns with severe intraventricular hemorrhage and in brains of neonates with periventricular leukomalacia.28 There is a robust and continued inflammatory response in the developing brain after HI, including reactive microglia and mast cells, as well as infiltration of neutrophils, monocytes and γ-delta T cells.26 Initially, this response is predominantly characterized by production and release of proinflammatory cytokines, but over the following days to weeks, an anti-inflammatory/restorative milieu is established.

No single study has compared in detail how the maturation stage of the brain at the time of an insult affects the inflammatory injury signatures. However, inflammation is evident in rodents in both preterm HI40 and at-term4 injury models, as well as in fetal sheep15 and in fetal rabbits.15 Developmentally regulated inflammatory responses are important determinants of outcome because several reports demonstrate that anti-inflammatory interventions that are beneficial in the immature brain are not so in the adult24 or vice versa.32 Furthermore, inflammatory interventions that are beneficial in the immature brain are not so in the adult24 or vice versa.32 Initially, this response is predominantly characterized by production and release of proinflammatory cytokines, but over the following days to weeks, an anti-inflammatory/restorative milieu is established.

There is also growing evidence that the response to immune therapy may be sex dependent, as was shown for protection of female but not of male neonates against HI by an iNOS inhibitor, 2-iminobiotin, and poly (ADP-ribose) polymerase.37

Peripheral Leukocytes

Neutrophils are one of earliest leukocyte populations to infiltrate and contribute to injury and blood–brain barrier (BBB) disruption in adult transient cerebral ischemia models.41 Unlike in adults, neutrophil infiltration is limited during acute and subchronic time points after neonatal HI and focal stroke in rats, likely contributing to better BBB integrity in injured neonates. The exact mechanisms that restrict leukocyte infiltration in the ischemic neonatal brain are not completely understood, and it remains unclear whether the higher resistance of the neonatal BBB to stroke is a cause or a consequence of reduced leukocyte transmigration. However, there is a lack of detailed knowledge on leukocyte cell-type–specific infiltration in models of neonatal brain injury. Monocyte infiltration is relatively low after acute tMCAO in neonatal rats. On the contrary, on-going studies in neonatal mice show significant monocyte infiltration during the first days after HI injury (L. Smith, et al, unpublished data, 2015). Neither the possibility that peripheral leukocytes contribute to injury without their infiltration nor the possibility that beneficial leukocyte populations enter injured brain via choroid plexus have been sufficiently explored. There is evidence that T cells infiltrate the neonatal brain during a prolonged period over several months after HI, and a recent study suggests that blocking lymphocyte trafficking is neuroprotective after lipopolysaccharide-sensitized HI injury.22

Microglia

Microglia populate the brain before birth and are distinct from peripheral monocytes. Recent data show that microglial cells play key roles in controlling synaptic pruning and the formation of precise synaptic circuits that occurs during the first 2 weeks of rodent life.44 Improper microglia–neuronal communications during this time and the resultant inappropriate synaptic connections could be the cause of neurodevelopmental disorders.

Specific hot spots of amoeboid microglia are found in the developing human brain and are also present in fetal sheep at midgestation in the white matter, a region susceptible to injury. Because of their active-like state during this developmental stage, microglial cells have been suggested to specifically contribute to WMI in the preterm brain. Furthermore, microglia can produce toxic reactive oxidants and injure the immature brain, which has limited antioxidant defenses.21

However, there is mounting evidence that suggests a protective role for microglia. In the adult, microglial cells can provide neuroprotective effects by producing growth factors although this has to date not been demonstrated in the developing brain. Microglial cells serve as endogenous neuroprotectants in neonatal arterial stroke because microglial depletion greatly enhances the excitotoxic and inflammatory responses and injury.30 Distinct maturation-dependent microglial phenotypes and diversity of the microglial phenotypes at a given time are being increasingly recognized.

The heterogeneity of the microglial pool, the timing of activation, and the type of stimulus critically affect an array of microglial effects. A better understanding of these events in the developing brain is likely to benefit the development of novel protective strategies.

BBB Integrity, Inflammation, and Angiogenesis

The presence of a leaky BBB in the immature brain has been commonly assumed, but endothelial tight junctions are already present during early embryonic development.45 Specific BBB transporters are present in the brain endothelium during midgestation, and no fenestrations are observed at birth.46 Expression of endothelial BBB proteins undergoes major changes from the embryonic period to adulthood, but susceptibility of the BBB to injury does not decrease linearly with age. In fact, comparison of albumin leakage after acute tMCAO between neonatal and adult rats showed a marked leakage in adults but not in neonates. Distinct expression patterns of the extracellular matrix proteins and tight junction proteins, along with lower than in adults leukocyte infiltration in neonates,29 are the likely contributors to BBB integrity after neonatal stroke. In contrast, in P9 mice subjected to HI, BBB permeability for large- and small-size tracers is transiently increased during the first 24 hours, changes associated with modified expression of BBB tight junction proteins.25 It is
presently unknown whether differences in BBB leakage are model or species related. Neurovascular outgrowth (angiogenesis) continues during the first 2 postnatal weeks in the rat brain,29 but after stroke in P7 rats, only a subtle and delayed angiogenic response is observed in the cortex during 2 weeks after injury.30 The higher resistance of the neonatal BBB to ischemic injury may negatively impact the angiogenic response and ultimate endogenous neurogenesis after stroke, but the relationships between the 2 processes are still poorly understood.

Immune Therapies and Cell-Based Therapies to Enhance the Repair Currently, hypothermia is used to treat neonatal HIE. However, the therapy is only partially protective and can only be used in term infants. Thus, there is a great need to find additional/synergistic therapeutic strategies. A variety of drugs targeting neuroinflammation tested in rodent models of ischemia-related brain injury reported variable neuroprotection, depending on the timing and type of intervention and on model and sex of animals used.26 Considering that it is often unknown when injury occurs in human infants, more recently emphasis has shifted to cell therapies or molecules, like growth factors (for example, brain-derived neurotrophic factor and erythropoietin), that can support and enhance the repair.31 Although some cell therapies and growth factors have shown promising long-term effects and some are currently in clinical trial (melatonin and erythropoietin), it remains unclear whether cells/agents have direct effects or, which is likely, act via immune-mediated change in brain microenvironment.

Pros and Cons of Different Ischemia-Related Animal Models for Translation Large animal cerebral ischemia models would be most suitable in translational research considering that the brains are gyrencephalic and that the white/gray matter ratio is similar to the human brain. Among large nonprimate neonatal models, newborn pigs are appropriate because their general brain and organ maturation at-term are similar to that of humans. Different global models have been developed, where global hypoxia and hypotension often is combined, which results in permanent brain injury, organ failure, posthypoxic seizures, and abnormal neurology, similar to humans on survival. However, practical issues, the high cost of maintenance and long-term neurorehabilitation have dramatically limited the use of larger species over the past decade. New hypoxia models in immature ferrets,46 a species with brain gyricization, may provide a potentially transplantable model of preterm WMI. Although parallels are often made between findings in humans and rodents, it is important to realize the limitations and translatability of studies in rodents. Rodents are not gyrencephalic species and their physiology, CBF regulation and white/gray matter ratios are vastly different from those in humans. Age-dependent regional vulnerability that may stem from uncoordinated maturation of individual cells in the parenchyma and in the vasculature should also be considered while interpreting the results from HI or focal stroke studies produced in immature rodents of different postnatal ages.

The role of the systemic effects of neonatal stroke has not been sufficiently addressed in animal models. Stroke is no longer viewed as a disease only of the brain because it exerts a rapid response in the blood and in peripheral organs, including liver, heart, and spleen, and in the bone marrow.38 In addition, studying the systemic effects of perinatal stroke are important because injured newborns need intensive care including organ support (ventilation, cardiovascular, and renal) and because brain repair may stem from the periphery, but such studies are rather sparse.

Conclusions To summarize, ischemia-related injury to prenatal or early postnatal brain impacts many key neurodevelopmental processes that undergo maturation changes during these time frames, leading to differing structural–functional abnormalities later in life. There is no single animal model that completely recapitulates the complexity of the human condition, but utilization of several ischemia-related age-specific models in rodents, and in larger species, and of both sexes, enables the enhanced understanding of brain pathology and development of novel therapies for the immature brain, as was demonstrated for hypothermia.

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References


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Carina Mallard and Zinaida S. Vexler

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