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Modeling premature brain injury and recovery

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Abstract

Premature birth is a growing and significant public health problem because of the large number of infants that survive with neurodevelopmental sequelae from brain injury. Recent advances in neuroimaging have shown that although some neuroanatomical structures are altered, others improve over time. This review outlines recent insights into brain structure and function in these preterm infants at school age and relevant animal models. These animal models have provided scientists with an opportunity to explore in depth the molecular and cellular mechanisms of injury as well as the potential of the brain for recovery. The endogenous potential that the brain has for neurogenesis and gliogenesis, and how environment contributes to recovery, are also outlined. These preclinical models will provide important insights into the genetic and epigenetic mechanisms responsible for variable degrees of injury and recovery, permitting the exploration of targeted therapies to facilitate recovery in the developing preterm brain.

PREMATURE BIRTH AND DEVELOPMENTAL OUTCOME

Very low birth weight (VLBW) preterm infants comprise approximately 2% of all live births and are a major pediatric public health problem (Hack et al., 2002; Als et al., 2007). Despite improved survival rates of approximately 85% for preterm infants weighing less than 1500 grams (Fanaroff et al., 2007), half of preterm children sustain cognitive and neurodevelopmental disabilities. However, recent long-term studies demonstrate progressive improvement over time in children without grade 3/4 intraventricular hemorrhages or significant white matter injury (Ment et al., 2003; for a review, see Saigal and Doyle, 2008), suggesting some capacity for recovery, which has yet to be fully investigated.

Magnetic resonance imaging (MRI) studies have demonstrated structural and functional differences in brain development of preterm children compared to term controls from infancy to early adolescence (Ment et al., 2009; Inder et al., 2005a; Gimenez et al., 2006a; Gimenez et

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al., 2006b; Nosarti et al., 2008; Srinivasan et al., 2007). At all ages studied, preterm children have smaller volumes in various brain regions including cerebral gray matter, basal ganglia and cerebellum (Inder et al., 2005a; Nosarti et al., 2008).

Premature birth also predisposes children to structural disturbances in white matter that may alter processing of information. Serial MRI studies have shown that even with no evidence of gross brain injury, the volume of white matter is less than that of term controls (Dyet et al., 2006; Ment et al., 2009). Recent advances in imaging technology such as diffusion tensor imaging (DTI), allowing for the evaluation of microstructural alterations, have revealed changes in white matter organization and integrity. DTI provides quantitative measurements of fractional anisotropy (FA), or the degree by which water diffusion is restricted in one direction compared to others. As compared to control children, the prematurely born demonstrate significant FA differences at birth through adolescence (12–15 years of age) (Constable et al., 2008; Skranes et al., 2007). There is a correlation between gestational age and degree of white matter injury measured by FA (Dudink et al., 2007) and microstructural volume loss (Nosarti et al., 2008). Infants born at earlier gestational ages have less white matter volume and decreased FA compared to preterm infants born later in gestation. This underscores the importance and intrinsic vulnerability of oligodendrocyte precursor cells, which predominate during the earlier stages (Back et al., 2002; Riddle et al., 2006).

Despite these early differences, serial neurocognitive evaluations have demonstrated progressive improvement such that by adolescence, language scores for preterm subjects approach those of term controls (see review by Saigal and Doyle, 2008; Ment et al., 2003). This long-term behavioral outcome is paralleled by significant structural plasticity in the preterm brain. For example, while there are decreases in the volume of gray and white matter structures at birth (Peterson et al., 2003; Inder et al., 2005a), there appears to be no significant difference in total volume measures as these children reach adulthood (Fearon et al., 2004; Allin et al., 2007). However, subtle differences in the volume of white matter in subcortical structures and ventricular enlargement persist into adolescence (Allin et al., 2004; Fearon et al., 2004) and a recent voxel-based morphometry study (Nosarti et al., 2008) found that there were not only regions of white matter and gray matter loss but also areas of relatively greater volumes when preterm adolescents were compared to term controls at age 14–15 years of age. Interestingly, this increase in both gray and white matter volume is more prominent in the preterm children that had evidence on neonatal ultrasound of periventricular hemorrhage and ventricular dilation, suggesting that in cases of more severe brain injury, the brain tries to compensate by increasing cell number (see next section) in order to adapt. However, even in preterm infants without neonatal ultrasound evidence of periventricular hemorrhage and ventricular dilation, the gray matter in the cingulate, temporal and parahippocampal gyri and white matter in the frontal, parietal and occipital cortices were increased, but not as evident as the more injured preterm group (Nosarti et al., 2008). It is not clear whether these increases are a compensatory mechanism for injury or a delay in the natural maturation process. In addition, the cellular mechanisms responsible for these long-term alterations in gray and white matter need further investigation.

An important variable that may also influence injury, development and recovery is gender. It has been shown that gender has a strong influence on the neuroanatomical and cognitive outcome observed (Reiss et al., 2004; Kesler et al., 2008). The role of gender and degree of injury and recovery is now being investigated in the laboratory. Evidence suggests that these factors do influence the outcome in models of immature brain injury (Yager et al., 2005; Nijboer et al., 2007; Renolleau et al., 2007), however the mechanisms are as yet unknown.

The cognitive improvement identified in some prematurely born children may be attributable to the development and engagement of alternative pathways, particularly in the language and

memory domains (Schafer et al., 2009; Ment et al., 2006; Ment and Constable, 2007). One methodology to study this non-invasively is functional Magnetic Resonance Imaging (fMRI). With fMRI, scientists and clinicians are capable of exploring differences in neuronal activation while the subject performs different tasks involving memory or language. In a recent study (Gimenez et al., 2005) testing hippocampal activation during a word-face association task, adolescents that were born prematurely exhibited greater activation of the right hippocampus compared to an age-matched term control group. There was a positive correlation between right hippocampal activation, face-name recognition, and volumetric analysis of the hippocampus. That is, the preterm group had greater activation of the right hippocampus during the memory task and there was greater hippocampal volume on the right versus left. There was no difference in handedness between the term and preterm groups. These data suggest that the alternative pathways that preterms engage may underlie the improvement in performance and compensate for the cerebral structural and microstructural changes evident on imaging. An alternate hypothesis suggests that there is a reserve or developmental persistence of an immature pattern of activation (Gaillard et al., 2007). Injury prior to the establishment of dominance may result in opposite hemisphere compensation (Rabin et al., 2004; Mbwana et al., 2009).

Numerous factors including the environment and the genome may contribute to both cognitive development and microstructural changes in the developing brain. Als et al (2004) found that low risk preterm infants enrolled in a Newborn Individual Developmental Care and Assessment (NIDCAP) Intervention Program had greater FA in the internal capsule and frontal region white matter than those who did not receive this care, suggesting that activity-dependent mechanisms may have a significant impact on the degree of structural and functional recovery. These studies suggest that environmental and perinatal events have a strong influence on white matter microstructure in the developing brain [Figure 1].

Although not yet investigated in the preterm population, genetics may also play a role. For example, overall white matter fiber architecture is genetically programmed, however environmental stimuli can alter the programmed trajectory. In a study of monozygotic and dizygotic twins using FA and DTI, white matter integrity (FA) was highly heritable with a strong correlation with performance intelligence quotient and full-scale intelligence quotient (Chiang et al., 2009). It has also been shown that gray matter structures in frontal, sensorimotor and language related cortices are highly heritable in the monozygotic compared to dizygotic twins (Thompson et al., 2001).

It is difficult to disentangle the respective contribution of degree of injury, genes and environmental variables to the long-term outcome. Additionally, it is likely that these factors interact with each other [Figure 1] to condition the variable response to injury. In order to better understand those environmental and genetic factors contributing to the outcome of preterm birth, it is critical to study the impact of these variables independently and their reciprocal interactions in preclinical models which faithfully replicate premature birth.

ANIMAL MODELS OF PREMATURE BRAIN INJURY

The study of premature brain injury is based in large part on research utilizing animal models. The aims of these studies are to increase our knowledge of the underlying mechanisms and evolution of injury, the alterations of brain development and possible benefits of therapeutic strategies. For an animal model to be relevant it must accurately reflect the histopathological spectrum of a specific injury to the developing brain, correlate with developmental changes observed in humans and display the functional outcomes seen in human pathology. Investigators have studied neonatal brain injury using different methods to induce injury,

resulting in neuropathological changes that resemble, in various degrees, those found in humans. This section will offer a brief overview of relevant animal models.

HYPOXIA-ISCHEMIA

The preterm infant has an immature cerebral blood supply and impaired cerebral autoregulation (Boylan et al., 2000). These vascular problems injure grey and white matter structures as well as the germinal regions around the ventricles, which contain the richest supply of blood and thus are the most vulnerable. Astrocytes are increasingly recognized as the most important players in the local regulation of blood flow in brain tissue (Gordon et al., 2008). Low oxygen concentrations alter metabolic substrates including lactate and prostaglandin E2 (PGE2), resulting in astrocyte induced vasodilation of the arterioles (Gordon et al., 2008). These astroglial cells are likely to be immature in the preterm brain and their role in regulation of blood flow is not fully understood.

It has been hypothesized that ischemic damage due to inappropriate vasoconstriction contributes to the pathology in the most severely affected infants, particularly those with intraventricular hemorrhage (IVH). This ischemic component is recapitulated in the Rice-Vannucci model, a well-established neonatal rodent model of hypoxic-ischemic (HI) brain injury. The HI is induced by unilateral ligation of the common carotid artery, followed by exposure to systemic hypoxia for a variable period of time, resulting in a significant reduction in cerebral blood flow and oxygen delivery for the ligated hemisphere compared to the control hemisphere (for a review, see Vannucci and Vannucci, 2005). Blood flow in the ligated hemisphere returns to control levels when the animal is returned to normoxia. The time between ligation of the artery and initiation of hypoxia and the age of the experimental animal are critical. Hypoxia induction 24 hours post ligation does not induce much cell loss compared to onset of this environmental perturbation at 4 hours post surgery (Dwyer et al., 1988). Ligation followed immediately by hypoxia results in a spectrum of focal necrotic injury on the ipsilateral cerebral cortex, white matter, striatum, hippocampus and thalamus followed by local glial cell reaction and prominent scar formation.

Many have used HI to study metabolic alterations, white matter injury and neurogenesis in rodent pups between postnatal days (P) 6–12. This period of rodent development corresponds to near term neurodevelopment in humans and thus does not faithfully replicate injury to very preterm brain. It has been shown that increased white matter injury in premature infants is related to maturation-dependent vulnerability of the oligodendrocyte lineage cells during a specific period of prenatal brain development (i.e., 23–32 weeks post-conceptional age), suggesting that it is important to consider the age at which the insult occurs (Back et al., 2001; Back et al., 2002).

Recently, Segovia et al (2008) performed the Rice Vannucci HI procedure on P3 rodent pups, a model more consistent with acute focal stroke. Their findings of delayed pre-oligodendrocyte degeneration and arrest of maturation are similar to the neuropathological findings in premature human brain specimens. However, the premature infant displays diffuse bilateral neuronal and white matter injury in pathological specimens and neuroimaging. As an alternative to the unilateral Rice-Vannucci HI model, Cai et al (2006) occluded both carotid arteries (BLCAO) followed by a brief period of systemic hypoxia. Using this model in P4 rodent pups, there is preferential white matter injury resulting in hypomyelination with decreased expression of myelin basic protein (MBP) and other markers of mature oligodendrocytes such as Adenomatous polyposis (APC/CC1) 3 weeks after insult (Cai et al., 2006). In addition, there are significant increases in activated microglia, astrogliosis as indicated by increased glial fibrillary acidic protein (GFAP) immunostaining and evidence of increased apoptosis (Cai et

al., 2006). The bilateral earlier injury results in less injury to neuronal cell populations compared to the traditional Rice-Vannucci HI model.

The long-term effects and cognitive behavioral sequela have been also investigated. Utilizing the BLCAO model in P4 rodents, Fan et al (2005) found significantly decreased performance in locomotor activity, memory tasks and passive avoidance 3 weeks after injury. This study also found that increasing the duration of hypoxia resulted in worsening performance. To date, there are only a few long-term behavioral studies in the Rice-Vannucci HI model, possibly due to the variability of injury among pups, unilateral ligation and inevitable compensation from the undamaged hemisphere, and other unidentified factors that complicate the interpretation of this model.

The use of HI has also been applied to other animal species such as piglets, sheep and non-human primates. A majority of these studies were conducted in newborn animals and the age at the time of insult approximates full term neonates (for review Roohey et al., 1997; Yager and Ashwal, 2009). To model prematurity, fetal sheep have been used extensively. The brain development of a preterm sheep fetus corresponds to that of the 24–28 week human fetus with regards to completion of neurogenesis, sulcation patterns and increased predilection for white matter injury due to pre-oligodendrocyte predominance (Penning et al., 1994; Reddy et al., 1998). The cerebral hemodynamics of this model is similar to humans and allow for repeated unanesthetized physiological and cerebral blood flow measurements (Riddle et al., 2006; McClure et al., 2008). However, large animal experiments are expensive and complex, and generally not amenable to systematic behavioral evaluation and drug discovery. Furthermore, at this time genetic manipulations are not feasible, thus limiting their utility in modeling the interactions between the genetic and environmental variables depicted in Figure 1.

HYPOXIA

The premature infant is subject to failure in oxygenation due to immature lungs and respiratory disturbances. Hypoxia is an important contributor to brain injury in premature infants and results in altered neuronal differentiation and synaptogenesis as well as a loss in neurons, glia and their progenitors due to increased apoptosis. There are two main models of hypoxia, which are not mutually exclusive, chronic and intermittent (Fagel et al., 2006; Douglas et al., 2007). Perinatal rodents subjected to chronic sublethal hypoxia during the first 10 days after birth, a period of rapid brain development, suffer global injury and long-term behavioral impairment. Fagel et al (2006) exposed mice pups to hypoxia (9.5–10.5% O₂) from P3 to P11 followed by recovery in normoxic conditions until P49. These mice initially suffer a reduction in corpus callosum and subcortical white matter volume, exhibiting ventriculomegaly and a 30% loss of cortical neuron number, cortical volume and brain weight (Fagel et al., 2006). Similarly to the findings in long-term human studies on preterm infants, subsequent recovery in rodent gray matter cortex occurs. After 5 weeks, cortical neuron number, volume and brain weight were similar to that of normoxic control mice. However, mice subjected to chronic perinatal hypoxia suffer long-term neurobehavioral sequelae including hyperactivity, increased anxiety (Weiss et al., 2004) and impairment in learning tasks and discrimination (Dell'Anna et al., 1991; Nyakas et al., 1996). While the hyperactivity subsides after few weeks, working memory and other impairments appear to be permanent (Chahboune et al., 2009). Taken together, these data suggest that this model causes alterations in brain development similar to those found in humans.

The premature infant is often subjected to apneic events, composed of alternating periods of normoxia and hypoxia at a time of rapid cortical, hippocampal and white matter development (Finer et al., 2006). Exposure to intermittent hypoxia results in significant neuronal injury as measured by N-acetyl aspartate (NAA)/creatinine ratios in the hippocampus and thalamus

immediately after injury using proton nuclear magnetic resonance spectroscopy (Douglas et al., 2007). Interestingly, this ratio normalized following 4 weeks of normoxic recovery. As in the chronic hypoxia model, there is also decreased myelin production. Behavioral testing of rodents exposed to intermittent hypoxia from P1 to P3 exhibited attention deficit hyperactivity (ADHD) like hyperactivity but no attention deficit (Oorschot et al., 2007).

The paradigms of chronic and intermittent hypoxia induce significant and distinct alterations in neocortical and hippocampal gene expression. In the neocortical region, chronic hypoxia altered genes responsible for cell signaling, development, and metabolism, while intermittent hypoxia altered genes responsible for CNS function and cell death (Zhou et al., 2008). In the hippocampus, chronic hypoxia effected oxygen transport and adaptation while intermittent hypoxia effected regulation of inflammatory and immune responses (Zhou et al., 2008). Further, the two models may result in different outcomes. Chronic hypoxia leads to adaptation, whereas intermittent hypoxia results in pathological responses that activate reactive oxygen species (ROS) generation leading to increased cell death (for review, see Nanduri and Nanduri, 2007). Long-term behavioral studies and more detailed analysis of neural progenitor cell activation and maturation are needed in these models.

PREMATURE DELIVERY

As illustrated above, these animal models all require experimental interventions to produce the insult. While they may mimic histopathological changes and functional deficits seen in humans, they do not address the issues surrounding medical management in the neonatal intensive care unit and its effects on the development of premature infants. Recently, a premature delivery model has been developed using baboons. Similar in many respects to that of the human infant, the mothers receive antenatal doses of corticosteroids and the premature baboon receives similar interventions to those of premature humans. Baboons delivered at 125 days of gestation have marked similarities in ontogeny to human brain development. Dieni et al (2004) and Inder et al (2005b) found that the neuropathological sequelae of baboons born prematurely at 125 days are similar in humans and baboons without direct cerebral insult being inflicted. Using histopathological examinations and magnetic resonance technology to examine the brain, they found diffuse gliosis, subarachnoid hemorrhage, grey matter injury, cystic PVL and moderate ventriculomegaly. The long-term behavioral sequelae and potential for recovery have not been investigated to date in this model, but again, these studies are likely to be difficult in large animals requiring intensive care.

RECOVERY: NEUROGENESIS AND GLIOGENESIS

The early postnatal brain has the capacity to recover from various types of developmental injuries. This plasticity can be attributed to the reorganization of existing circuitry, through neurogenesis, synaptogenesis and differential axon growth and guidance. Studies utilizing various injury models have shown that the postnatal brain contains an endogenous pool of multipotent progenitor cells with the ability to proliferate and differentiate into new neurons and glial cells following insult (Plane et al, 2004; Felling et al, 2006; Fagel et al, 2006; Yang et al, 2007; Fagel et al, 2009). The germinative zones where these cells reside are the subgranular layer of the hippocampal dentate gyrus and the subventricular zone (SVZ) adjacent to the lateral ventricles of the forebrain. Precursor cells may also exist in the latent stage in white matter and diffusely in grey matter (Palmer et al., 1999). The SVZ is of particular interest because in humans it is prominent during mid to late gestation (corresponding to the first perinatal week in rodents). Animal studies have demonstrated that during this critical period the SVZ is comprised of immature cell types that are at different stages of lineage restriction with the capacity to provide neural precursors in the event of perinatal injury (Yang and Levison, 2006). In both humans and rodents, slowly dividing GFAP+ multipotent progenitor

cells in both the hippocampus and the SVZ neurogenic niches give rise to proliferating neural progenitors which in turn produce new neurons and oligodendrocytes, thus enabling recovery (Doetsch et al, 1999).

The number of SVZ progenitor cells is larger in the newborn brain, and furthermore, parenchymal astrocytes may retain stem cell properties at this stage as compared to the adult (Laywell et al., 2000), suggesting that the immature brain may have a greater capacity to recover from injury than the adult (often referred to as the Kennard Principle). The newborn rodent shares many key developmental characteristics with preterm infants in mid to late gestation, specifically in regards to the susceptibility of pre-oligodendrocyte lineage cells, and proliferation of neuronal and glial progenitors. Premature birth and the stressors that occur during this perinatal period are likely to alter the environment of these cells their natural course of development.

HI using the Rice-Vannucci model in rodents at earlier ages than what is typically studied (P2 to P3) resulted in caspase-3 independent acute pre-oligodendrocyte degeneration followed by caspase-3 dependent delayed degeneration (Segovia et al., 2008). Using Ki-67 as a nuclear marker of cell cycle proliferation, the white matter on the lesioned side displayed significant increases in O4+ (a marker of immature oligodendrocyte)/Ki-67+ and Platelet derived growth factor receptor-alpha (PDGFR α)/Ki67+ labeled oligodendrocyte progenitor cells 7 to 11 days post injury.

However, these investigators found no significant increases in cell density of oligodendrocyte progenitors or pre-oligodendrocytes in the ipsilateral SVZ. It is possible that these cells are locally proliferating NG2+/PDGFR α + oligodendrocyte precursors that by this stage are present in the white matter and even in the parenchyma (see review by Nishiyama et al., 2009). These mice also experienced a decrease in myelination and an increase in the number of GFAP labeled cells in the ipsilateral hemisphere. The reduced myelination was not due to oligodendrocyte lineage cell loss, but rather, the increased premyelinating cells failed to initiate myelin production even though axonal integrity did not appear disrupted. Although these investigators did not provide long-term follow-up, these data suggest that the impairment in these progenitor cells to mature may be a result of intrinsic failure or altered environment, i.e., trophic factors (Ong et al., 2005).

In an HI study in older rodents (P7), electron microscopy was used to analyze the brain ultrastructure approximately one month after injury (Skoff et al., 2007). This study showed that there is structural reorganization in the ipsilateral striatum with neurite extension, axon growth cones and myelination of many small axons (Skoff et al., 2007). Newly generated mature oligodendrocytes (BrdU+/MBP+) after HI insult are found in the ipsilateral side, however it is not currently known whether functional recovery occurs (Zaidi et al., 2004).

Other studies looked at neuronal cell populations and examined the long-term developmental and morphological outcomes. Mice subjected to chronic perinatal hypoxia model from P3 to P11 suffered a decrease in brain weight, cortical volume and neuron specific neuronal nuclei (NeuN)+ neuron number in the cerebral cortex immediately after cessation of injury. However, after 1 week of recovery in normoxic conditions, the hypoxic reared mice showed a substantial increase in the number of proliferating cells, marked by the incorporation of 5-bromo-2-deoxyuridine (BrdU), many of which co-expressed astroglial markers, including vimentin, brain lipid binding protein (BLBP) and astrocyte-specific glutamate transporter (Fagel et al., 2006). Cells of the radial glial lineage express these proteins during embryogenesis, giving rise to neural precursors (Feng et al., 1994; Shibata et al., 1997). By P29, putative neuroblasts expressing Mash1, Polysialic Acid-NCAM (PSA-NCAM), Distal-less homeobox 2 (DLX-2) and doublecortin (Dcx) were visualized streaming from the rostral migratory stream through

the white matter to the subcortical layers in hypoxic mice (Fagel et al., 2006). To examine the fate of the proliferative precursors, BrdU was incorporated into these cells one week after the cessation of hypoxia and a detailed analyses of the long-term fate of BrdU+ cells was performed. This study revealed a two-fold increased in the number of BrdU+ cells in the cortex four weeks after the insult, and a parallel increase in neurogenesis as well as gliogenesis, with no changes in the proportion of differentiated neurons, astrocytes and oligodendrocytes (Fagel et al., 2006). More recent studies examining the genesis of different neuronal subtypes revealed a preferential generation of -brain-1 (Tbr1)+ excitatory as opposed to Parvalbumin+ and Calretinin+ inhibitory neurons (Fagel et al., 2009). This mismatch may correlate with the long-term cognitive deficits that these hypoxic mice continue to exhibit and may provide a clue for targeting future interventions.

GENES AND MOLECULAR PATHWAYS

In order to understand the neurobiological substrates responsible for premature brain injury, altered developmental trajectories, and the potential for recovery, molecular manipulation of key regulatory proteins and genes are necessary. This is difficult in large animal models, thus necessitating investigation using mouse models. Advances in murine genetics and in the understanding of the molecular mechanisms that regulate gene expression have allowed scientists to develop null-mutant or transgenic mouse strains to examine the role of specific genes in the response to injury. For example, it is known that during embryogenesis, key secreted factors play a critical role in brain development such as Fibroblast Growth Factor (FGF), Insulin like growth Factor (IGF), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Brain-derived neurotrophic factor (BDNF), and Sonic Hedgehog (Shh). In the postnatal brain, these factors continue to be expressed in the SVZ. Many of these factors show altered expression after injury and may play an important role in recovery [Table 1].

The neuropeptide FGF2 is part of large family of growth factors required for normal progenitor proliferation, the induction of neural fate and the genesis of excitatory cortical neurons, particularly Tbr1+ pyramidal neurons (Vaccarino et al., 1999; Shin et al., 2004). Studies in chronic hypoxia have demonstrated that FGF gene expression is upregulated in the various brain regions, possibly leading to the appearance of radial glial type cells, a subset of which co-express FGF receptor 1 (FGFR1) (Ganat et al., 2002). This led to speculation that astroglial cells respond to perinatal injury either by reverting back to immature radial glia or that injury during this critical period hinders normal maturation of radial glial into GFAP+ astrocytes, diverting their differentiation away from glial and towards neuronal progenitor cells. To investigate the role of FGFR1 in chronic perinatal hypoxia, Fagel et al (2009) utilized a conditional knock-out of the *Fgfr1* gene in GFAP+ cells of the developing dorsal telencephalon (including cortical radial glia and their progeny), demonstrating that the loss of *Fgfr1* precluded the recovery of NeuN+, Tbr1+ or neuron specific Neurofilament H non-phosphorylated protein (SMI-32)+ neuron number in the cerebral cortex as compared to wild-type hypoxic mice (Fagel et al., 2009). One week after cessation of hypoxia, wild-type hypoxic mice showed a 60% upregulation of FGFR1 immunoreactivity, an 80% increase in cell proliferation in the SVZ and increases in neuronal progenitors expressing the transcription factors Sox2, Pax6 and Tbr2 in the SVZ compared to their normoxic counterparts. The FGFR1 knock-out hypoxic mice, however, did not exhibit the increase in BrdU+ proliferative cells in the SVZ and failed to mount a sufficient cortical recovery, demonstrating a persistent 30% loss of excitatory neurons as well as inhibitory interneurons. However, this knock-out model did not preclude all neurogenesis as there was still some generation of olfactory bulb neurons and to a lesser extent even cortical neurons. This was to be expected, as the knock-out occurred well before the induction of hypoxia, thus allowing for compensatory mechanisms to play a role in recovery.

While chronic hypoxia results in an increased expression of FGF1 and FGF2 (Ganat et al., 2002), HI in the immature brain resulted in decreased expression of FGF-1 in the SVZ using microarray analysis (Felling et al., 2006). The effect of FGF administration in HI was investigated in the bilateral common carotid artery occlusion model in P3 rats. Intraventricular infusion of Fgf2 resulted in significant increases in proliferation in the SVZ (BrdU+ cells) as well as the number of BrdU+ cells co-expressing markers such as NeuN (mature neurons), NG2 (immature oligodendrocyte progenitors), Nestin (immature progenitors) and GFAP (astrocyte lineage) (Jin-qiao et al., 2009). Interestingly, most of the BrdU positive cells expressed NeuN in the bFGF treated HI groups. The infusion of bFGF intraventricularly may be impractical in the immature animal. However, it has been shown that peripheral (subcutaneous) administration of bFGF rapidly crosses the blood brain barrier in young and aged rodent pups and causes increased DNA synthesis (Wagner et al., 1999).

Another important neurotrophic factor which promotes neural growth, differentiation and survival is the Insulin like growth factor I (IGF-I). In the immature brain, IGF-I mRNA is decreased in HI (Clawson et al., 1999). The ipsilateral intra-ventricular administration of human recombinant IGF-I after HI resulted in 40% reduction in brain injury (Brywe et al., 2005). It was found that the IGF-I treatment 3 days after HI increased the phosphorylated serine/threonine kinase Akt activity in the cytosol and decreased caspase-3 and caspase-9 activity compared to controls (Brywe et al., 2005; Wood et al., 2007). The protective effects of IGF-1 in the immature brain may be a result of preventing glutamatergic excitotoxicity, blocking glutamate mediated death of the late oligodendrocyte progenitor cells, and preventing the loss of the basic helix-loop-helix oligodendrocyte lineage transcription factor 2 (Olig2) positive cells in the white matter after the HI insult (Ness et al., 2004; Wood et al., 2007).

Sustained neuro- gliogenesis in the SVZ after injury requires other strong mitogens such as Epidermal Growth Factor (EGF) and its receptors (EGFR). EGFR and its ligands have a broad range of functions including the stimulation of cell division, cell migration and survival. The infusion of EGF into the lateral ventricles of adult rodents has been shown to activate EGF receptors found on Type C transit amplifying cells and cause this cell type to become highly proliferative (Doetsch et al., 2002). In neonatal HI, the SVZ was found to up-regulate EGFR gene expression (Felling et al., 2006). Other signaling molecules such as FGF and bone morphogenetic protein (BMP) are also important in regulating the way the EGFR expressing progenitor cell responds when stimulated by EGFR ligands. BMP inhibits EGFR while FGF2 antagonizes the BMP inhibitory effects, potentially exerting an indirect activating effect on EGFR (Lillien and Raphael, 2000). The role of EGFR overexpression or loss of function in chronic hypoxia or HI in the neonatal brain has not been investigated.

The effect of hypoxia in neural stem cells is complex, as these cells must integrate the effects of different signaling pathways. The hypoxia inducible Factor α (HIF-1 α), a transcription factor that increases in low oxygen conditions, directly upregulates a number of downstream genes, including the Vascular Endothelial Growth Factor (VEGF), Brain Derived Neurotrophic Factor (BDNF), FGF2, and Notch-1 (Gustafsson et al., 2005) (Figure 2). The stimulation of Notch downstream genes has been implicated in the down-regulation of neuronal fate and inhibition of neural stem cell differentiation in the developing nervous system (see review by Louvi and Artavanis-Tsakonas, 2006). Growth factors and extracellular matrix proteins such as Beta-1 integrin work in concert with Notch-1 to regulate cell fate (Campos et al., 2006). Furthermore, it has been suggested that Fgf1 and Fgf2 upregulate *Notch1* expression during embryogenesis which, in turns, inhibits neuronal differentiation (Faux et al., 2001). Consistently with the known increase in FGFR1 expression in the hypoxic SVZ (Ganat et al., 2002; Fagel et al., 2009), Felling et al (2006) found an up-regulation of Notch-1 and EGFR in the SVZ after a HI insult in the neonatal brain, ipsilateral to the side of injury, concurrent with an increase in proliferation in neural stem cells. Recently, Covey and Levison (2007) found a

significant increase in the SVZ of Leukemia inhibitory factor (LIF), which in turn, by acting on the LIF receptor and the activation of the JAK/STAT pathway, resulted in increased Notch-1 activity (Covey and Levison, 2007). Thus, the increase in activated Notch-1 may contribute to the increase in SVZ neural progenitor cells, however the question becomes whether these progenitors will migrate, differentiate and become viable after injury. Fate mapping studies are necessary to help answer this question.

More recent studies involve transgenic mouse models which provide not only the opportunity to analyze the identity and lineage potential of postnatal neural cell populations, but more importantly, the ability to target candidate genes at specific time periods to preclude compensatory mechanisms. Ganat et al (2006) utilized mice carrying an inducible Cre recombinase controlled by the human GFAP promoter, which permanently tags astroglial cells with reporter genes upon injection of tamoxifen. This study demonstrated that even under normoxic conditions, GFAP+ cells tagged by cre-induced reporter genes at P15 generated neurons in the cerebral cortex; this was a low-level of neurogenesis compared to the embryonic period, but consistently observed (Ganat et al., 2006).

Our group has utilized this model to tag and follow the fate of cells of the GFAP lineage after cessation of hypoxia. These mice will provide the opportunity to inactivate candidate genes in these cells at the start of the recovery phase. This will provide a better understanding of the cellular and neurobiological substrates involved in perinatal injury and the genetic mechanisms that enable neuronal and glial recovery.

ENVIRONMENTAL ENRICHMENT

Human studies of children born prematurely have demonstrated that social, environmental and family dynamics contribute in determining long-term outcomes (Ment et al., 2003). In a review of published randomized trials of early developmental intervention, a meta-analysis concluded that these programs improved cognitive outcomes at preschool age (Spittle et al., 2007). Intervention stimulates various cognitive, social and sensory-motor components in these infants.

In preclinical models of disease, this paradigm is referred to as environmental enrichment, and it has been shown to increase hippocampal stem cell proliferation (Kempermann et al., 2002), enhance learning and memory and improve sensory-motor tasks in different animal models of human disease, including stroke and premature birth (Komitova et al., 2005; for review Johansson, 2004). The experimental paradigm of environmental enrichment in rodents varies between laboratories but what is consistent is that objects which have different colors, textures, odors, shape and sizes are placed in a larger than standard rodent cage. The rodent has free access to food and water as well as voluntary exercise in the form of a running wheel. A key aspect to this paradigm is novelty as objects in the cage are changed regularly.

In adult rodents, a post-ischemia enriched environment results in enhanced progenitor cell proliferation in the SVZ (Komitova et al., 2005). The recruitment of these new precursors in the peri-infarct region however did not result in mature neurons (Komitova et al., 2005) but rather glial cells expressing the proteoglycan NG2. There was a significant increase NG2+/BrDU+ and NG2+/phosphorylated histone H3+/mitotically active cells in both the ipsilateral lesioned cortex and the contra lateral non-lesioned cortex several days after the ischemic event (Komitova et al., 2005). Furthermore, these newly generated NG2+ cells expressed BDNF throughout the cortex. In these enrichment studies, only a small percentage (approximately 0.5%) of newborn glial cells surrounding the lesion matured to express MBP (Komitova et al., 2006).

But do the cellular changes and newborn cells following environmental enrichment improve cognitive outcomes? Pereira et al (2007) performed unilateral HI in P7 rats and subjected them to environmental enrichment for 1 hour per day for 9 weeks. Using the Morris Water Maze to test spatial memory, a hippocampal dependent task, they found that HI rats exposed to brief, daily enrichment performed better than HI non-enriched animals. To date, studies of enrichment in groups that underwent HI at earlier time points, mimicking prematurity have not been published. It is not known whether there is a morphological or behavioral difference if enrichment is initiated immediately after injury or later during recovery and whether there is a difference between short daily periods of enrichment compared to continuous enrichment.

CONCLUSION

The premature infant provides both scientists and clinicians with the opportunity to study brain development and how injury alters this normal process. The mechanisms underlying recovery in the developing brain are as yet unknown. The clinical data are variable and the mechanisms underlying this variability are unclear. Preclinical models permit the study of those molecular and epigenetic mechanisms responsible for variable degrees of injury and recovery.

Multipotent neural progenitor cells offer new insight into potential plasticity of the postnatal brain following injury associated with preterm birth. Understanding their development, migration and incorporation into an existing neural network which has been altered by injury is imperative, because of their potential for repair and regenerative medicine. However, many questions remain. It is not known whether these new cells become fully functional or integrated into the surrounding neural circuitry. There is also a need to identify the interactions among neurotrophic factors and signaling pathways activated during perinatal brain injury and responsible for expansion, migration and differentiation of the neural stem cells and progenitor populations in the postnatal germinal zones. Appropriate preclinical models will permit the exploration of targeted therapies and environments to facilitate recovery in the developing preterm brain.

Abbreviations

DTI, diffusion tensor imaging; FA, fractional anisotropy; HI, hypoxia ischemia; P, postnatal day; SVZ, subventricular zone.

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Figure 1.

There are many contributing factors to premature brain injury and recovery. The causes of premature birth, as well as the postnatal environment and genetic (gender) factors result in variable outcomes.

Figure 2.

A working model of the different signaling pathways activated by hypoxia and HI. Hypoxia and HI results in activation of many different growth factors that bind to their respective receptors. It has been shown that after HI there is a significant increase in Leukemia Inhibitory Factor (LIF) mRNA and activation of the LIF receptor in the SVZ, thus contributing to increased Notch-1 activation (Covey and Levison, 2007). Other factors that are increased are BDNF (Li et al., 2008), VEGF (Li et al., 2008), FGF (Ganat et al., 2002) and EGFR (Felling et al., 2006). These receptors activate common signaling pathways that ultimately are responsible for neural progenitor cell proliferation and survival. However, genetic differences exist and may contribute to variable recovery and repair (Li et al., 2008).

Table 1

This table outlines some of the key factors that have been investigated in perinatal HI and/or Hypoxia models. The mechanism of how each one contributes to recovery and interacts with each other in the postnatal brain after injury is not completely understood. Information from this table was pooled from different sources (see text for references).

HIF1 α (Hypoxia Inducible Factor 1- α)	Transcription factor	$\uparrow\uparrow$	Activated by low oxygen Stabilized by NO signaling	Activates VEGF, Epo, glucose transporters and glycolytic enzymes
VEGF (vascular endothelial growth factor)	Growth factor	$\uparrow\uparrow$	Activated by HIF-1 α	Secreted and expressed by endothelial, neuronal, glial cells to modulate neurogenesis; Activates angiogenesis
BDNF (brain derived neurotrophic factor)	Neurotrophic factor	$\uparrow\uparrow$	Synthesis further stimulated by HIF-1 α	Secreted and expressed by endothelial, neuronal, glial cells to modulate neurogenesis; increases NO production
FGF 1, 2 (Fibroblast Growth Factor)	Growth Factor	HI $\downarrow$ (FGF1) Hypoxia $\uparrow\uparrow$ (FGF1.2)	Synthesis further stimulated by HIF-1 α	Growth factor required for normal progenitor proliferation and induction of neural fate.
EGF (Epidermal Growth Factor)	Growth Factor	$\uparrow\uparrow$	Expressed by proliferating cells in SVZ-role in hypoxic injury or recovery unknown	Promotes proliferation of Type C transit amplifying cells in the SVZ.
IGF (Insulin Growth Factor)	Neurotrophic Growth Factor and anti-apoptotic	HI $\downarrow$ Hypoxia ?	lcv administration decreases injury and cell death in HI	Promotes cell proliferation, differentiation and maturation.
BMP (bone morphogenetic protein)	Neurotrophic	?	Thought to antagonize the action of FGFs-role in hypoxia unknown	Promotes the differentiation of neural stem/progenitor cells into GFAP+ cells that exit the cell cycle and lack self renewal.
Notch	Transmembrane receptor activated by Jagged and Delta	$\uparrow\uparrow$	Interacts with HIF1 α ; increases cell proliferation and promotes glial fate	Maintains neural stem cell pool and promotes gliogenesis; stroke and HI increase the intracellular Notch domain (NICD) and the transcription of Hes1; HIF-1 α interacts with NICD to maintain neural stem cells in undifferentiated state.