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Controversies in Preterm Brain Injury

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THE CHALLENGES OF DEFINING HUMAN PRETERM BRAIN INJURY

Preterm infants are a high risk of brain injury and their injuries have been studied for many decades, but there are many unresolved questions regarding the etiology of this injury. Current debate in the field revolves around the relative contribution of impaired or delayed maturation versus specific injury, a debate reviewed here by focusing on: the contribution of specific cell types in gray and white matter; how these gray and white matter alterations change neuronal connections and circuit function; and the role of altered environment or injury in these changes.

There are several major reasons why human preterm brain injury is not a single well-defined entity. First, the clinical management of preterm infants varies widely leading to striking variability in neurological outcome even within a given geographic region (Bodeau-Livinec, Marlow et al. 2008). Additionally, most neuropathology or neuroimaging studies are based upon subjects derived from tertiary/complex care hospitals that typically treat the most critically ill neonates who are at much higher risk for worse outcomes, thus skewing the data. By contrast, studies based upon subjects drawn from community-based hospitals may reflect a broader spectrum of outcomes that is more representative of the population. Second, preterm brain injury may be triggered or exacerbated by multiple factors that may

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be harmful for the preterm brain. These include hypoxemia, hypoxia-ischemia, maternal fetal infection, postnatal sepsis, inflammation, drug and toxicant exposures, pain, neonatal stress and malnutrition (Back and Miller 2014). Third, pathophysiological triggers may be modified by additional factors unique to each preterm baby. These individual factors reflect the confluence of effects exerted by gender, genetics, epigenetics, socio-economic status, the integrity of the family unit and a whole host of other maternal-fetal factors (e.g., maternal smoking, drug or alcohol abuse) that influence the in utero environment and which may have triggered preterm birth. Fourth, there are significant technical challenges to study the human preterm brain. Access to human autopsy brains is very limited and the value of the tissue may vary widely depending on postmortem interval and the modes of tissue preservation (e.g., fresh, frozen, formalin fixed or paraformaldehyde fixed), limiting the application of many modern histological or molecular biology techniques. In contrast to pathology studies, neuroimaging studies can enroll large numbers of subjects, which allows for greater population sampling but may not detect certain types of early or small lesions that are beyond the current resolution of clinical MRI scanners (Back and Miller 2014).

Compared to human studies, experimental animal approaches are invariably reductionistic and typically focus on a single insult (e.g., hypoxia-ischemia, chronic hypoxia, infection, inflammation or drug exposure), although there may be significant cross-talk between insults (e.g. inflammation resulting in hypotension and hypoxia). Most experimental studies rely upon rodents, because of the access to transgenic approaches, the greater feasibility of achieving replicates and the greater access to molecular reagents. However, there are substantial concerns with rodent studies that include significant developmental differences from human at the levels of brain anatomy, physiology, response to pharmacologic agents and triggers of injury, more accelerated postnatal brain maturation and fundamental differences in the biology of the major neural cell types (Back, Riddle et al. 2012). Large preclinical animal models (e.g., fetal rabbit, sheep and non-human primate) offer some distinct advantages, but are costly, challenging to undertake, lack transgenic approaches and also retain some developmental differences from human. Thus, the question of whether a single factor causes the patterns of injury seen in preterm brain or whether a convergence of events is necessary has not yet been answered, but has profound implications for potential therapeutic strategies.

DEVELOPMENTAL DELAY VERSUS INJURY

Preterm brain injury occurs during a phase of rapid brain development outside of the normal *in utero* environment leading to a combination of delays in normal maturation plus specific injuries associated with acute or chronic insults. Developmental delays after premature birth may arise from two major causes. First, a broad range of injuries can cause significant disruption to ongoing endogenous developmental events in the brain - either before or after delivery - thereby affecting fetal and/or postnatal developmental programs and their normal physiological timing. Second, abnormal exposure to specific factors or chemical compounds (e.g. inflammation, external stimuli, drugs) can cause abnormalities in developmental trajectories. These two main causes of developmental delays can occur concomitantly or in sequence, further worsening neurological outcome. The relative contribution of these two

mechanisms—delay of normal maturation and injury— remains a major area of debate and investigation in the field.

The contribution of each of these changes is discussed here (see Table 1 for summary), highlighting critical unresolved questions. Factors that may alter preterm brain development are also discussed to highlight the injurious potential of endogenous and exogenous exposures and losses that also interrupt normal processes. The structural and functional correlates of interrupted development and injury in the premature brain are under active investigation, with the hope that the cellular and molecular mechanisms underlying developmental abnormalities in the human preterm brain can be understood, prevented or repaired. As emphasized here and recently discussed by Salmaso et al. (Salmaso, Jablonska et al. 2014), the information from a variety of animal models needs to be integrated with both modern anatomical and histological analysis of human tissue to more fully define the neurobiology of preterm brain injury and to develop new therapeutic interventions.

Evidence of Delayed Maturation: Cell types and Molecular Mechanisms

A large body of research in human survivors of preterm birth and in animal models has recently pointed to a delay or interruption of development as one of the major consequences of prematurity and a major contributor to neonatal brain injury. After premature birth, many mechanisms appear to converge to cause maturational delay of both neurons and glia – with both predictable and unexpected consequences in circuit formation and function.

Gray Matter—Premature infants display reduced cerebral cortex volume and loss of subcortical gray matter, including thalamus and basal ganglia (Peterson, Anderson et al. 2003, Counsell and Boardman 2005, Ment and Vohr 2008, Ball, Boardman et al. 2012). A direct correlation has been established between degree of prematurity, extent of reduction in cortical volume and neurodevelopmental outcome. Key questions – still largely unresolved – are: i) how does impaired postnatal cortical growth correlate with altered microstructure of the developing cerebral cortex?; ii) what are the associated cellular changes?; and iii) which cellular mechanisms act in concert to cause cortical abnormalities and dysfunction in preterm infants?

Gray Matter Growth and Microstructure: Advanced imaging techniques, particularly diffusion tensor MRI, are being used to address the first question: how does impaired postnatal cortical growth correlate with altered microstructure of the developing cerebral cortex (Ball, Boardman et al. 2012, Ball, Srinivasan et al. 2013, Vinall, Grunau et al. 2013)? Analyzing changes in the cortical microstructure of preterm infants from delivery through term corrected age has demonstrated a direct association between lower gestational age and changes in fractional anisotropy (FA) in the developing cerebral cortex. Vinall et al. showed that reduced birth weight, length and head growth correlated with delayed microstructural development of cortical gray matter. Detailed Diffusion Tensor Imaging (DTI) analysis demonstrated that the changes observed in FA were mostly in the radial, but not axial, diffusion axis, strongly suggesting a developmental delay in growth of neuronal processes within the cerebral cortex. Ball et al. demonstrated that the microstructural maturation rate correlated with local cortical growth, and this growth predicted better neurodevelopmental

test scores when measured at 2 years of age. Both studies indicate that preterm infants at term-corrected age show less mature cortex than term-born infants by MRI measurement. While establishing a direct association between neonatal somatic growth and gray matter development, these studies could not elucidate the underlying mechanisms leading to abnormal microstructure.

Multiple mechanisms may contribute to changes in human cortical microstructure. Although animals can never fully mimic all aspects of preterm brain injury, they allow direct investigation of potentially causal mechanisms underlying well-controlled, reproducible injury. Neuronal cell death due to primary or secondary retrograde degeneration (Kinney, Haynes et al. 2012), anatomical and physiological maturational delay of neurons (Dean, McClendon et al. 2013), and abnormal subcortical growth causing secondary abnormalities in cortical development (McQuillen, Sheldon et al. 2003) have been directly examined in animal models.

One model of stress to the preterm CNS is exposure of preterm equivalent rodents to chronic sublethal hypoxia during early postnatal development (Ment, Schwartz et al. 1998). Studies in this model have helped to define the potential postnatal effects of hypoxemia on gray matter neurons and their developmental program. In one early study, an extensive transcriptome analysis in the whole brain revealed that expression of genes involved in presynaptic function was enhanced, whereas genes involved in synaptic development, postsynaptic function and neurotransmission were significantly downregulated (Currstin, Cao et al. 2002). The authors hypothesized that differential regulation of these sets of genes would cause “dysynchrony” (loss of coordination of developmentally regulated processes) and a delay in neuronal maturation. A limitation of the study was the relatively more mature state of the neurons in the neonatal mouse at the time of exposure to hypoxia, when compared with the state of cortical neuronal maturation in preterm infants.

Recent studies in a sheep model of preterm brain injury used a combination of MRI, cell morphometry and patch clamping studies to link changes in neuronal arborization, but not neuronal loss, with impaired cortical growth and changes in FA (Dean, McClendon et al. 2013). A brief moderately severe episode of global cerebral ischemia in preterm fetal sheep resulted in progressive reductions in cortical growth and disrupted the progressive decrease in FA that is normally seen as the cerebral cortex matures. Detailed morphometric analysis of cortical and caudate projection neurons revealed significantly reduced dendritic arborization and spine density at 4 weeks after birth. Decreased arborization occurred in the absence of detectable neuronal loss and was seen in all cortical layers. In parallel, FA was increased above normal. The authors established a causal relationship between abnormalities in neuronal morphological development and increased cortical FA after ischemia, and – based on time course analysis – concluded that the observed reduction in dendritic arborization resulted from disrupted maturation, rather than from degeneration. These findings highlight the value of cortical FA measurements as a useful non-invasive approach to monitor preterm brain maturation. Furthermore, they beginning to address the mechanisms underlying impaired preterm brain development and emphasize the potential role of delayed neuronal maturation. These morphological and structural abnormalities could severely impact synapse development and neuronal function, and ultimately contribute to the

cognitive and learning disabilities found in survivors of preterm birth. Taken together, there is growing evidence that impaired gray matter microstructure and delayed neuronal maturation contribute significantly to preterm brain injury.

Altered Neurogenesis: In addition to impaired arborization, there may also be a reduction in normal neurogenesis in the proliferative regions of the cortex (Malik, Vinukonda et al. 2013). Immunohistochemical analysis in human tissue revealed that the density of dividing and non-dividing Sox2⁺ radial glial cells as well as Tbr2⁺ intermediate progenitors significantly decreased between 16 and 28 gestational weeks and the proliferation index of neuronal progenitors was greatly reduced in preterm tissue. The authors proposed that preterm birth might suppress neurogenesis. This finding was confirmed in a rabbit model of preterm birth, where glutamatergic neurogenesis was suppressed, as shown by a reduced number of Tbr2⁺ progenitors and an increased number of Sox2⁺ radial glia (Malik, Vinukonda et al. 2013).

Suppressed neurogenesis is likely not specific for glutamatergic neurons as similar effects have been also observed on inhibitory GABAergic interneurons. Postmortem studies in preterm infants and children revealed a significant decrease in GABAergic interneurons in both the subplate and cortex (Iai and Takashima 1999, Robinson, Li et al. 2006). In the first study, parvalbumin -expressing neurons were analyzed in human tissue from 21 GW to 11 years of age, as developmental changes in parvalbumin expression at early developmental stages reflect maturation of thalamocortical connections, followed by functional development of cortical neurons. Parvalbumin expression was decreased in the cerebral cortex of preterm infants with diffuse necrotic white matter injury, indicating impairment of thalamocortical connectivity. Similarly, Robinson et al. focused on human samples of telencephalon tissue from infants born at 25 to 32 weeks of gestation with severe necrotic lesions. Premature infants that exhibited white matter injury displayed a large reduction in GAD-67- and calretinin-expressing cells. Given the severity of the lesions in the human tissues, multiple cell types were lost, however. The degree of contribution from GABAergic neuron loss in the preterm infant population now--with mild diffuse pathology rather than large cystic lesion--remains unclear and there would be significant benefit in a reexamination of the specific neuronal types in more contemporary human pathological specimens with diffuse microcystic lesions.

Until such human studies are available, animal models must continue to inform our understanding of the neuronal subtypes that are susceptible in the preterm brain. For example, using the rodent chronic hypoxia model (Ment, Schwartz et al. 1998), inhibitory interneuron development has been specifically analyzed. For example, a significant decrease in parvalbumin and somatostatin (SST)-expressing GABAergic interneurons was observed in the adult cortex following postnatal hypoxia (Komitova, Xenos et al. 2013). This reduction did not appear to be due to cell death/loss of interneurons following hypoxia, as the total number of GAD-expressing cells did not change. Rather, a specific decrease was noted in the subpopulations of parvalbumin and somatostatin-expressing interneurons, which normally form a dense network of synapses on pyramidal cells (Fino and Yuste 2011). This decrease is likely to be due to interrupted maturation of the interneurons, as parvalbumin expression is strictly dependent on excitatory input and neurotrophin signaling (Patz,

Grabert et al. 2004). Consistent with the role of parvalbumin interneurons in cortical information processing, mice reared in hypoxia display a deficit in spatial memory in the Morris water maze task and increased seizure activity (Schwaller, Tetko et al. 2004). In contrast, other models of preterm injury including the sheep model (McClendon, 2014) have not shown loss of GABAergic neurons in the caudate. While stimulating GABAergic neuron maturation and enhancing GABAergic neurotransmission represents an appealing therapeutic strategy in the premature brain, the balance of benefit to risk will depend on the degree of interneuron loss that is actually occurring, an area that needs further investigation.

White matter—While recognition of gray matter abnormalities in preterm brain injury is relatively recent, the contribution of white matter injury has long been recognized. In earlier decades, cystic periventricular leukomalacia (PVL) was common, but improved perinatal management has shifted the majority of preterm injury to non-necrotic, diffuse white matter injury (diffuse WMI) (Ment, Hirtz et al. 2009, Back and Miller 2014). Neuroimaging and neuropathological studies of postmortem human tissue have demonstrated that either PVL or diffuse WMI in the premature brain lead to changes in white matter volume myelination disturbances. Importantly, the extent of injury and apparent MRI-defined hypomyelination correlate with degree of prematurity (Ment, Hirtz et al. 2009). In humans, development of white matter occurs during the last part of gestation and continues postnatally until adolescence. Therefore, genesis and maturation of glial cells begin during a particularly critical time of vulnerability to premature injury, i.e. between 24 and 32 gestational weeks. Both human and animal studies indicate that dysregulation of glial cell development may contribute to a maturational delay in white matter (Back and Rosenberg 2014). However, the specific contribution of different white matter elements (glia and/or axons) remains an open question and critical periods for enhancing white matter recovery have not yet been defined.

Oligodendrocytes: Studies in postmortem human brain have begun to define the cellular and anatomical abnormalities associated with diffuse WMI. Hypomyelination in premature WMI is associated with a delayed or disrupted developmental program of oligodendrocyte (OL) lineage cells, in particular OL progenitor cells (OPCs) (Back and Miller 2014). Under normal developmental conditions, OPCs give rise to the mature oligodendrocytes that myelinate axons. In postmortem human brain, arrested maturation of O4⁺ preOLs was observed, together with expansion of the cellular pool of early OL progenitors and preOLs (Buser, Maire et al. 2012). In parallel, significant astrogliosis was also found, together with accumulation of the proteoglycan hyaluronic acid in the regions of hypomyelination. Based on similar results obtained on animal models (Back, Tuohy et al. 2005), it was hypothesized that WMI causes OL death and compensatory proliferation of OPCs, which only partially mature to O4⁺ pre-OLs. However, in an animal model of maternal-fetal infection, no OL death or increased OPC proliferation were observed, but only arrested OPC differentiation (Favrais, van de Looij et al. 2011), suggesting different mechanisms may exist depending on the type or severity of the insult. As recently reviewed (Back and Miller 2014), several mechanisms could be potentially involved in accumulation of arrested preOLs in the white matter of premature infants, including – but not restricted to - accumulation of the OL maturation inhibitor hyaluronic acid in reactive astrocytes (Back, Tuohy et al. 2005), microglial activation with subsequent inhibition of preOL maturation (see below), or

changes in proteins involved in OPC cell cycle exit/differentiation (Jablonska, Scafidi et al. 2012). Dysregulation of WNT-beta catenin signaling in OL progenitors also promotes preOL arrest, delays normal myelination and disrupts remyelination (Feigenson, Reid et al. 2009, Ye, Chen et al. 2009, Fancy, Harrington et al. 2011, McClain, Sim et al. 2012).

The notion of delayed maturation leading to hypomyelination is further supported by immunohistochemical analysis in very preterm infant brains, where preserved Olig2⁺ cell density in axonal crossroads areas (C1 and C2) was observed (Verney, Pogledic et al. 2012). In these very preterm brains, enhanced microglia-macrophage densities and reduced astroglial reactivity were observed, as compared with preterm cases with periventricular WMI (Verney, Pogledic et al. 2012). These findings indicate that delayed maturation of the OL lineage significantly contributes to WMI observed in the premature brain, and that glial activation patterns might be different between very preterm and preterm brains.

Axons: A crucial unresolved question is whether disturbances in premature white matter maturation exclusively derives from failure of OL maturation, or also involves axonal abnormalities (Nave and Werner 2014). Changes in anisotropy observed in white matter signal abnormalities on MRI might also reflect different degrees of axonal abnormalities and axonal injury, which could be due to either direct injury or to indirect effects associated with defective OL metabolic support of axons. These FA changes may be related to multiple factors, including hypoxia, ischemia, inflammation and infection that may contribute to white matter microstructural disturbances through OL loss and delayed maturation, as well as axonopathy. Additionally, abnormal patterns of electrical activity in axons due to neuronal injury could significantly alter axon-OL interactions during critical developmental windows, resulting in defective myelination, together with malformed nodes of Ranvier.

Imaging and neuropathological studies have provided evidence for axonal degeneration in focal necrotic WMI in the preterm brain, but axons appear to be spared in diffuse WMI (Riddle, Maire et al. 2012). Neuropathological studies revealed a significant incidence of microscopic necrosis in human lesions, which is not detected by MRI, but involves axonopathy (Pierson, Folkerth et al. 2007, Haynes, Billiards et al. 2008). In necrotic WMI, immunomarkers of axonal pathology – beta-APP and the apoptotic marker fractin – were used to detect these foci of axonal injury (Haynes, Billiards et al. 2008, Buser, Maire et al. 2012).

In vitro studies have identified glutamate-mediated maturation-dependent mechanisms of susceptibility of immature axons to oxidative stress and hypoxia-ischemia (Alix and Fern 2009, Alix, Zammit et al. 2012). In one model, larger caliber axons, which are about to initiate myelination, may be particularly susceptible to injury in contrast to smaller caliber unmyelinated axons, which are more resistant (Alix, Zammit et al. 2012). However, in other models, both myelinated and unmyelinated axons may be lost and function primarily impaired in the unmyelinated axons (Drobyshevsky, Jiang et al. 2014). Focal axonal degeneration is related to severe energy failure and does not appear to be a major component of diffuse WMI prior to active myelination (Riddle, Maire et al. 2012), but structural and functional alterations may show differences.

DTI analysis showed a significant and specific decrease in the relative anisotropy (RA) of injured WM regions, suggesting abnormalities in the normal developmental program of axonal fibers (Huppi, Murphy et al. 2001, Miller, Vigneron et al. 2002). In fact, during normal development, RA increases in different fiber tracts, but this change does not occur in areas of WMI. These findings are consistent with the notion that axonal growth is extremely active during brain development over the last trimester of gestation (Haynes, Billiards et al. 2008), and that prematurity might also slow or impair this process.

Subplate Neurons: The potential loss or impaired maturation of subplate neurons (SPNs) in the pathogenesis of preterm brain injury also remains an open question. SPNs are a heterogeneous population of excitatory and inhibitory neurons that play critical roles in the establishment of cortical lamination and thalamocortical connectivity (McConnell, Ghosh et al. 1989, Ghosh and Shatz 1993, Ghosh and Shatz 1994, McConnell, Ghosh et al. 1994, Bystron, Molnar et al. 2005, Kanold and Shatz 2006). SPNs reside in subcortical white matter and are critical in development of the cerebral cortex and deep nuclei (reviewed extensively in (Hoerder-Suabedissen and Molnar 2015)). Because SPNs are present during the high-risk period for WMI, it was proposed that loss of SPNs might coincide with injury to preOLs (Volpe 1996). Deletion of SPNs at a critical window in cortical development might disrupt corticofugal and thalamocortical white matter axons, as well as disrupt cortical neuronal connectivity. The hypothesis that injury to SPNs might have significant secondary effects on maturation of thalamocortical connections and white matter development was first addressed in a neonatal rodent model of hypoxia-ischemia where the SPNs were reported to be more vulnerable than cortical neurons (Volpe 1996, McQuillen, Sheldon et al. 2003, Kinney, Haynes et al. 2012). However, in these more severe white matter lesions, SPNs were just one of several classes of neurons shown to be vulnerable (Andiman, Haynes et al. 2010), which leaves unresolved the question of selective SPN vulnerability.

The Potential Consequences of Human Preterm WMI—The role that WMI plays in the developmental disabilities of preterm survivors requires considerably more study. Most experimental studies have relied upon rodent models that have significant limitations to replicate human pathology (Back and Rosenberg 2014). Mechanistic findings from rodents have not been replicated in large preclinical animal models, which has hampered progress toward translation of potential therapies. Moreover, critical questions remain unresolved in human WMI regarding the cellular mechanisms that underlie the MRI-defined disturbances in connectivity defined by DTI studies. Despite recent progress to employ high field ex vivo MRI to define the pathological features of early and subacute WMI in preterm fetal sheep (Riddle, Maire et al. 2012), the cellular basis of chronic human WMI remains largely unresolved. The magnitude and distribution of myelin loss and axonal dysfunction remain poorly defined, which limits our ability to define the relevance of findings from animal models. Moreover, the extent to which endogenous regeneration and repair mechanisms promote partial or complete repair of myelination disturbances remains unclear.

Although disruption of human OL maturation at a critical window in cerebral development may disrupt a wide array of development events critical to maturation of axons as well as neurons, the long term consequences for human motor and neurobehavioral disabilities

remains to be defined. Going forward, it will be critical to focus on models of WMI that do not generate extensive white matter necrosis and neuro-axonal degeneration, since this form of pathology is presently uncommon in human WMI. Rather, a focus on the cellular and molecular events associated with diffuse WMI is necessary to define specific contributions of axons and glia to the chronic pathology and to the functional abnormalities. To reach this goal, it will be important to: i) develop better imaging approaches and more complete neuropathological assessment of human preterm WMI; ii) define specific early biomarkers for axonal vs. glial injury in the premature brain, and iii) define physiological approaches to determine the functional status of myelinated and unmyelinated axons as one predictor of long-term cognitive dysfunction associated with premature birth.

Functional Connectivity: How are Gray and White Matter Disturbances

Linked?—An emerging approach to define the long-term consequences of preterm cerebral injury is to analyze functional connectivity during normal postnatal development and after injury in preterm survivors (Partridge, Mukherjee et al. 2005, Miller and Ferriero 2009, Smyser, Inder et al. 2010, Lubsen, Vohr et al. 2011, Lee, Morgan et al. 2013, Smyser, Snyder et al. 2013, Doria, Arichi et al. 2014, van den Heuvel, Kersbergen et al. 2014). These studies have sought to establish a relationship between structural abnormalities in cortical growth, using MRI-defined measures of volume and fractional anisotropy, and functional disturbances defined by resting state and task-based connectivity. The results suggest that both gray and white matter structures are affected, and demonstrate that functional neurodevelopmental impairment caused by premature birth, particularly in language, is related to disturbances neural connectivity (Schafer, Lacadie et al. 2009, Myers, Hampson et al. 2010, Mullen, Vohr et al. 2011, Constable, Vohr et al. 2013, Reidy, Morgan et al. 2013). Importantly, correlations between changes in microstructural connectivity and cognitive/behavioral outcome measures have been established (Brown, Inder et al. 2009, Bora, Pritchard et al. 2014). These changes are of special interest from a therapeutic perspective, as cognitive interventions in animal models have recently been shown to improve microstructural connectivity in the developing brain (Komitova, Xenos et al. 2013).

KEY FACTORS CONTROLLING DELAYED MATURATION AND RECOVERY

The molecular etiology of gray and white matter injury and the factors that contribute to maturational disturbances are still largely undetermined, but it is essential to develop strategies that minimize disruption or key developmental events while maximizing more normal maturation. A critical first step is to define which endogenous factors are required for normal development during the preterm period and what exogenous factors may interfere with their actions. Animal studies in injury models (Scafidi, Fagel et al. 2009, Back and Miller 2014) and in normal development (Gallo and Deneen 2014) have begun to suggest some cellular and molecular mechanisms that might cause this maturational delay. However, the relevance of these molecular pathways identified in animal models to the developing human brain is still undefined, limiting rapid development of therapeutic interventions. Major unanswered questions include: i) how to minimize loss or dysregulation of key intrinsic factors critical for preterm cerebral maturation?; ii) which extrinsic or iatrogenic factors (e.g., infection, inflammation, drugs, pain, stress, malnutrition) are most important to

minimize?; iii) what is the role of genetic and epigenetic factors as modifiers of injury progression; and iv) what environmental interventions and rehabilitative strategies will best promote more normal developmental function after preterm cerebral injury?

Endogenous factors that expand progenitor pools, promote maturation and recovery

Which endogenous factors are most necessary for normal maturation of the brain during the third trimester of gestation and which have proven, at least in animal models, to lead to improved functional recovery following injury? Here we briefly outline factors that are most likely to provide key insights into these proliferative and maturational pathways and have potential as therapeutic agents, as suggested by analysis in animal models of preterm injury.

Fibroblast growth factors—Fibroblast growth factor (FGF) regulates neurogenesis and gliogenesis during embryonic and postnatal development (Vaccarino, Schwartz et al. 1999), and contributes to cortical gyrification (Rash, Tomasi et al. 2013) as well as survival of oligodendrocyte progenitors (reviewed in (Back 2006)). FGF2 levels increase in parenchymal GFAP-expressing astrocytes during postnatal development (Vaccarino, Schwartz et al. 1999). Several studies have identified the FGFs as an endogenous activator of gray matter recovery arising from chronic sublethal hypoxia in preterm equivalent postnatal mice. In the cerebral cortex, FGF1 And FGF2, together with their receptor, Fgfr1, are significantly increased during the recovery phase from chronic hypoxia (Ganat, Soni et al. 2002, Fagel, Ganat et al. 2009). Genetic ablation of Fgfr1 in GFAP⁺ astrocytes prevents recovery in cortical volume and excitatory neuron number that occurs after hypoxia, particularly of Tbr1⁺ pyramidal neurons (Fagel, Ganat et al. 2009). Interestingly, Fgfr1 exerts opposite effects on interneurons, as its deletion accentuates the decrease of parvalbumin-expressing GABAergic interneurons in cerebral cortex (Ganat, Soni et al. 2002, Fagel, Ganat et al. 2009). Finally, Fgfr1 ablation also prevented the increase in stem cells/neural progenitors in the SVZ caused by hypoxia (Fagel, Ganat et al. 2009). Together these results suggest that FGF2 plays an important role in cortical cell recovery in this rodent model of preterm brain injury, specifically on excitatory pyramidal neurons.

Epidermal growth factors—Epidermal growth factor (EGF) ligands and their receptor, EGFR, appear to play a significant role in white matter development, specifically in normal OL maturation (reviewed in (Gonzalez-Perez and Alvarez-Buylla 2011)). The role of EGF expression changes and alterations in signaling after neonatal brain injury is even more striking. In the chronic sublethal hypoxia model, EGF expression in white matter is significantly up-regulated within the first week after hypoxia (P11–P18), suggesting that this growth factor might contribute to the recovery process after hypoxic injury (Scafidi, Hammond et al. 2014). This hypothesis is supported by the finding that selective overexpression of the human EGF receptor (hEGFR) in the oligodendroglial lineage promotes OPC proliferation and expansion, oligodendrocyte regeneration, myelination and behavioral recovery associated with white matter function (Scafidi, Hammond et al. 2014). Strikingly, while endogenous EGF is increased after hypoxia, this effect was not saturating, as treatment with exogenous EGF was still effective and promoted complete recovery. Delivery of heparin binding-EGF intranasally immediately *after* hypoxia (7 applications within 3 days following the injury, P11–P14) enhanced OPC proliferation and generation of

new OLs, and it prevented OL death caused by hypoxia. This resulted in higher myelin production and improved axonal function – as shown by electron microscopy, electrophysiology and DTI - reversing the injury phenotype. Importantly, specific behavioral deficits caused by hypoxia that were associated with WM abnormalities were also prevented by heparin binding-EGF. Diverse molecular mechanisms likely mediate the EGF effects, but a major contributor is the suppression of Notch activation in OPCs caused by hypoxia, as Notch signaling is a well-established inhibitor of OPC maturation and myelination (Aguirre, Rubio et al. 2010) and is regulated by hypoxia inducing factor 1-alpha (Gustafsson, Zheng et al. 2005). Understanding the molecular mechanisms that contribute to the multiple effects of EGF on OL recovery and differentiation may lead to additional therapeutics for repair of diffuse WMI.

Thyroid hormones—Like many of the growth factors, the thyroid hormone (TH) thyroxine (T4) has long been recognized as critical for neurogenesis, glial maturation and myelination (Patel, Landers et al. 2011). Much preclinical and clinical research has focused on the potential for T4 to promote preterm neurodevelopment (Osborn and Hunt 2007, Osborn and Hunt 2007, Schang, Gressens et al. 2014), particularly with the recent recognition of the continued contribution of maternal thyroid hormone to brain development in the third trimester of pregnancy (Berbel, Navarro et al. 2010). In preterm human brain tissue and in an animal model of preterm brain injury, levels of TH-activating enzyme deiodinase-2 are reduced (Vose, Vinukonda et al. 2013). These findings, coupled with the transient hypothyroidism of prematurity (Osborn and Hunt 2007) suggest that cerebral injury may subject the developing brain to abnormal T4 levels. However, success with T4 supplementation in animal models as well as in preterm infants has been mixed. In animal models, T4 supplementation enhanced OPC maturation in WMI associated with intraventricular hemorrhage but not WMI caused by severe inflammation (Vose, Vinukonda et al. 2013, Schang, Van Steenwinckel et al. 2014). In humans, no evidence yet exists for specific rescue of diffuse WMI (Osborn and Hunt 2007, Osborn and Hunt 2007), although the ongoing TIPITS trial suggests that there may be a weak correlation between free T4 levels and DTI disturbances (Ng, Turner et al. 2014). It remains unknown, whether specific populations at greater risk for preterm injury, such as those with IVH, might benefit from T4 supplementation alone or in combination with other endogenous factors that might further activate protective pathways.

Steroid Hormones—Steroid hormones are also factors to which the developing brain is normally exposed that may prove to be novel neuroprotective agents that promote cell survival and possibly maturation (reviewed in (Hirst, Kelleher et al. 2014).

Allopregnanolone (ALLO) is the neurosteroid studied in the greatest detail. It reduces cell death, increases cell proliferation and promotes myelination (Mellon 2007, Wang, Singh et al. 2010, Schumacher, Mattern et al. 2014), making it an excellent candidate for treating preterm brain injury. ALLO is a progesterone derivative, made by the placenta and later the brain. As a consequence of preterm birth, infants are withdrawn prematurely from placentally-derived steroids, such as ALLO, which may enhance the risk for cerebral injury. ALLO's role as an endogenous neuroprotective agent for the developing brain is suggested by the fact that ALLO levels peak just prior to delivery (Hill, Parizek et al. 2000). Studies in

several animal models found that ALLO reduces apoptosis following *in utero* hypoxia-ischemia (Yawno, Yan et al. 2007, Yawno, Hirst et al. 2009, Kelleher, Palliser et al. 2011). ALLO can also limit apoptosis and neurite outgrowth, while promoting myelination (Hirst, Kelleher et al. 2014), but the mechanism is largely unknown. Other experiments show that ALLO, later made by the brain itself, can protect developing neurons and glia from injury (Brunton, Russell et al. 2014, Hirst, Kelleher et al. 2014). Despite these pre-clinical findings, there is limited data on ALLO levels in human newborns and even less correlation with neurological outcomes, particularly in the most preterm newborns (< 28 weeks gestation) whom abruptly lose ALLO exposure months earlier than normal.

Can replacement of endogenous factors promote maturation or decrease injury?

Preterm birth can disrupt exposure to endogenous factors in multiple ways. Endogenous factors regulating neurodevelopment may be produced locally in the developing brain, by the fetus, by the placenta itself or transported across the placenta from the maternal circulation. Replacement of missing endogenous factors, while an appealing therapeutic strategy, is complicated by our current limited data on normal ranges of exposure in utero and longitudinally after preterm delivery. The timing and route of delivery for many factors and dosage related effects are likely to play a role in efficacy and therapeutic potential.

Dose—The best studied endogenous factor currently in clinical trials, erythropoietin (Epo), needs to be given in pharmacological, not physiological, doses to produce a neuroprotective effect in animal models of full term hypoxia-ischemia. Epo normally acts as an endogenous hematopoietic signal, but both Epo and its receptor (EpoR) are expressed in the developing brain. Analysis of mice that do not express Epo or EpoR show similar defects in neurogenesis and mild abnormalities of neural tube closure (Tsai, Ohab et al. 2006). Furthermore, transgenic mice missing EpoR only in the neuronal lineage have reduced proliferation in the SVZ during development and adulthood (Tsai, Ohab et al. 2006). While endogenous levels of Epo appear to have minimal effect in the face of profound neural injury, pharmacological doses of exogenous Epo are neuroprotective in multiple injury models, including a preterm baboon model (Brines, Ghezzi et al. 2000, Traudt and Juul 2013). Epo is under active investigation as a neuroprotective agent for both preterm brain injury (Juul, McPherson et al. 2008) and as an adjunct to hypothermia treatment of term HI (Wu and Gonzalez 2015). Whether it will decrease diffuse WMI, improve myelination or improve behavioral outcomes awaits clinical trial completion (McAdams, McPherson et al. 2013, Ohls, Kamath-Rayne et al. 2014, O’Gorman, Bucher et al. 2015).

Delivery—Those factors that are made in situ in the brain (FGF, EGF) are less likely to easily cross the blood-brain barrier than those made primarily by the placenta (steroids) or those that are provided, at least partially, by the maternal circulation (T4). Thus, the growth factor treatments described above involve for the most part intracerebral administration of exogenous growth factors. Although future therapeutic approaches could potentially be based on these paradigms, there is a need for more feasible and non-invasive clinical treatments. Intranasal delivery of drugs and growth factors has been used for different types of brain disorders and neurological conditions (Dhuria, Hanson et al. 2010). A few studies have reported successful interventions in animal models of premature brain injury based on

intranasal delivery of factors (Guardia Clausi, Paez et al. 2012, Scafidi, Hammond et al. 2014). As mentioned above, intranasal delivery of heparin binding-EGF was recently reported to ameliorate rodent preterm WMI (Scafidi, Hammond et al. 2014). Interestingly heparin binding-EGF was detected in the white matter within 5 minutes from the first intranasal application and its levels continued to increase up to 30 minutes after delivery. In parallel, EGFR phosphorylation also increased with a similar time-course both in white matter and in specifically in OPCs.

Timing—The timing of exogenous factor delivery is also a critical area of investigation. For example, it was found that heparin binding-EGF was ineffective in preventing behavioral abnormalities when treatment began a week after hypoxia, demonstrating that early interventions selectively promote neural plasticity and better functional recovery (Scafidi, Hammond et al. 2014). Some factors, especially those immediately lost upon preterm delivery because they are provided by placenta, may need to be replaced even before injury occurs to maintain an optimal environment in which normal development can proceed or to provide a window in which additional neuroprotective agents can act.

In conclusion, cellular factors that promote OL maturation under normal physiological conditions may also promote regeneration, and ultimately functional recovery under pathological conditions, when non-invasively administered to the developing mammalian brain. Similar treatment paradigms may allow us to explore the potential role of other factors or pharmacological agents that do not easily cross the blood-brain barrier. Importantly, these studies will also open new avenues of investigation to define critical periods during and after injury.

WHAT EXTRINSIC INJURIOUS EVENTS INTERFERE WITH MATURATION?

Preterm birth itself and other related events that trigger prematurity can disrupt intrinsic processes the direct brain development and maturation. To this point, we have focused on the critical unanswered questions related to cellular and molecular mechanisms of brain dysmaturation, focusing on the contributions of different cell types and the factors that may play a role in their normal maturation. We now focus on mechanisms independent of normal development that contribute to the disruption of developmental processes through acute or chronic injury.

The impact of infection and/or inflammation

Epidemiological studies support an association between chorioamnionitis, fetal inflammatory syndrome, preterm birth, and subsequent neurological impairment (Dammann and Leviton 2014). In animal models, a causal link between systemic inflammation and WMI can be demonstrated (Rousset, Chalon et al. 2006, Favrais, van de Looij et al. 2011). In these models, systemic inflammation, through yet to be fully elucidated mechanisms, lead to neuroinflammation as demonstrated by microglial activation and increased production of cytokines/chemokines.

Microglial activation has been hypothesized to be one of the key steps leading to white matter dysmaturation. Recent postmortem human studies have shown a strong association

between diffuse WMI and robust microglial activation (Verney, Pogledic et al. 2012), especially in regions such as axonal crossroads where microglia accumulate during normal brain development during the third trimester (Verney, Monier et al. 2010). Of note, microglial activation can be triggered by a large variety of brain insults as they express a large repertoire of toll-like receptors making them able to respond to numerous pathogen-associated molecules as well as damage-associated molecules.

The demonstration that specific abnormalities occur in cortical neuron maturation in human tissue and in animal models of preterm birth raises the interesting hypothesis that inflammatory events associated with premature brain injury may also play a role in these neuronal changes. Indeed, microglial cells have been recently shown to play an important functional role during normal development of the cerebral cortex. In macaques and in rodents, microglia regulate the number of cortical neurons by phagocytosing neural precursor cells during the final critical phases of neurogenesis (Cunningham, Martinez-Cerdeno et al. 2013). Microglia also affect cortical maturation, as neurons in layer V require support from microglia for survival. Insulin growth factor 1 was identified as the cellular factor mediating the effects of microglia on these neurons (Ueno, Fujita et al. 2013). Finally, synaptic pruning that occurs during normal cortical development also depends on microglial function, as abnormalities induced by genetic manipulation of these cells result in excess dendritic spines and in immature synapses, together with functional properties typical of immature brain circuitry (Paolicelli, Bolasco et al. 2011). In conclusion, based on its roles during normal development, it is possible that activated microglia participate in causing abnormalities in cortical neuronal development and synaptic maturation that could be present in the premature brain.

The role of hypoxia- ischemia

Preterm infants are particularly prone to hypoxic or hypoxic-ischemic (HI) events due to their immature lungs, episodic hypotension and potential for loss of cerebral blood flow autoregulation leading to pressure-passive perfusion. Acute transient episodes of moderately severe hypoxia and ischemia give rise to diffuse WMI in several animal models of preterm brain injury (recently reviewed in (Back and Rosenberg 2014)). However, cerebral blood flow studies in fetal sheep demonstrate that the distribution pattern of ischemia does not fully account for the regional topography of diffuse WMI (McClure, Riddle et al. 2008). Rather, the distribution appears to be determined by the distribution of preOLs and their particular susceptibility to HI injury, which leads to subsequent preOL maturational arrest (Back and Rosenberg 2014). Loss of cerebral blood flow autoregulation can occur even when systemic hypotension is not present (Soul, Hammer et al. 2007) resulting in loss of cerebral perfusion even with small blood pressure fluctuations. Episodes of ischemia combined with either chronic low-grade hypoxia or with the intermittent hypoxia seen in preterm infants may thus combine to injure the white matter and arrest the differentiation of preOLs to myelinating OLs.

Potential impact of pharmacological agents

Many of the life-saving and commonly administered medications used in preterm infants have known CNS effects that may compromise normal neurodevelopment. The uncertain neurodevelopmental impact of most medications used is even more unsettling.

Steroid Hormones—Glucocorticoids are frequently the first pharmacological agent to which a preterm infant is exposed, since women in preterm labor are routinely treated with synthetic glucocorticoids. Under normal circumstances, the fetus is protected from high maternal glucocorticoid levels by the actions of the placental barrier enzyme, 11 β -hydroxysteroid dehydrogenase (HSD2), which inactivates endogenous glucocorticoids (Chapman, Holmes et al. 2013). Some synthetic steroids however can cross the placenta and antenatal glucocorticoid treatment greatly reduces acute preterm neonatal mortality and major morbidities, including IVH (Ballabh 2014). Postnatal use of the synthetic glucocorticoid, dexamethasone, greatly declined after an association between prolonged early exposure and later cerebral palsy was found (Doyle, Ehrenkranz et al. 2010), although short courses remain in use for treatment of severe broncho-pulmonary dysplasia. Hydrocortisone is now in use as a more physiological steroid choice for both bronchopulmonary dysplasia and treatment of the adrenal insufficiency frequently seen in extremely preterm infants (Fernandez and Watterberg 2009). Both human and animal data suggest that the beneficial effects of steroids may be counter-balanced by potential disturbances in neurodevelopment, depending on the timing, duration and type of steroid used (Bennet, Davidson et al. 2012). For example, betamethasone before delivery may decrease WMI (Agarwal, Chiswick et al. 2002), but may increase risk of attention deficit disorders later in life (Khalife, Glover et al. 2013). Steroids in the neonatal period may impair cerebellar growth (Tam, Chau et al. 2011), but several additional studies suggest that hydrocortisone does not reduce brain volumes or cause neurological deficits (Rademaker, Rijpert et al. 2006, Kersbergen, de Vries et al. 2013) while dexamethasone exposure has been linked to long-term adverse neurodevelopment (Doyle, Ehrenkranz et al. 2010). Complicating the conclusions that can be drawn from these observational studies, experiments in rodents and sheep show a wide range of effects when steroid exposure is used in preterm HI models (Bennet, Davidson et al. 2012). In addition, long-term reprogramming of the developing hypothalamic-pituitary axis by both endogenous and exogenous glucocorticoids is an area of active investigation with implications for neuropsychological as well as cardiovascular and metabolic health (Reynolds 2013).

Narcotics—Opioids, primarily morphine and fentanyl, are frequently used for analgesia and sedation in preterm neonates. They are used acutely for painful procedures such as arterial blood draws or for prolonged periods of mechanical ventilation. Much like steroids, these drugs also show conflicting effects depending on timing and duration of treatment (Durrmeyer, Vutskits et al. 2010). Recent meta-analysis of several large randomized trials of morphine treatment for ventilated infants showed no differences in long-term behavioral outcomes, even when analysis was limited to a very preterm population (Bellu, de Waal et al. 2008). While animal and in vitro studies suggest an apoptotic effect of morphine on developing neurons and microglia, as measured by activated Caspase-3 expression (Hu, Sheng et al. 2002), and potential exacerbation of WMI, available studies are limited and few

have examined long-term behavioral outcomes (Durrmeyer, Vutskits et al. 2010). Pain, either directly or indirectly through elevated glucocorticoids, may also disrupt preterm brain growth (see below).

Benzodiazepines and barbiturates are sedative GABA_A receptor agonists that can alter the balance of neural excitation and inhibition, potentially effecting activity-dependent development of neural circuits. In the immature brain, GABA may have even broader effects because it is initially a trophic factor controlling cellular proliferation and migration (Cellot and Cherubini 2013). This multiplicity of roles makes the use of these drugs in preterm infants particularly worrisome. Benzodiazepines (midazolam, diazepam) are frequently used for sedation in combination with opioids, while barbiturates are used as anti-epileptics, with phenobarbital remaining the first-line drug of choice for neonatal seizures. Administration of benzodiazepines or barbiturates at pharmacological levels to neonatal rodents results in widespread cortical neuronal apoptosis (Durrmeyer, Vutskits et al. 2010). A very recent report measuring GABA levels by magnetic resonance spectroscopy and documenting resting-state neuronal networks detected significant alteration in GABA levels in the right frontal lobe of preterm infants assessed at term-equivalence and an inverse relationship between these levels and connectivity when compared to term newborns (Kwon, Scheinost et al. 2014). Further large-scale studies are needed to understand the impact of frequently used GABAergic drugs on preterm brain development and injury, particularly with regard to the effects of cumulative dosing and the timing of exposure.

Additional Agents—There are many additional medications in common use in preterm infants that may have deleterious effects on CNS development. For example, the prostaglandin inhibitor indomethacin was suggested to potentially improve neurodevelopment, at least in male preterm survivors (Ment, Vohr et al. 2004), but whether this effect is due to the closure of the patent ductus with prevention of IVH or due to anti-inflammatory pathways remains unknown. Given the current focus on inflammation as a potential contributor to preterm brain injury (Kuban, O'Shea et al. 2014), it is tempting to suggest that decreasing inflammation will limit developmental disruptions. However, glucocorticoids, which are potent anti-inflammatories, would also be expected to show protection, but no such protection has yet been demonstrated. Additional medications such as diuretics, which alter sodium-potassium balance or antibiotics, some of which have known neurotoxic properties, may also modify outcomes in unanticipated ways. As our understanding of the cellular changes and molecular pathways that interact to cause preterm brain injury increases, the medications to which these patients are exposed should be continually reassessed for potential impacts on developing brain.

Contribution of Pain and Stress

An unfortunate element of neonatal intensive care is repeated procedural pain and related stress. By 24 weeks gestation, nociceptive circuitry for pain perception exists, but is not yet fully functional, such that painful stimuli appear to produce a more generalized response in preterm infants (Lucas-Thompson, Townsend et al. 2008). Despite ongoing concerns about the impact of pain on the preterm brain, until very recently there have been relatively few studies directly linking pain and abnormal preterm brain development (recently reviewed in

(Ranger and Grunau 2014)). New MRI studies of very preterm infant cohorts at term-equivalent (Smith, Gutovich et al. 2011, Brummelte, Grunau et al. 2012) and during childhood (Ranger, Chau et al. 2013) have begun to define associations between the number of painful or stressful procedures experienced in the NICU and alterations in brain architecture. Such studies are complicated by the need to control for multiple clinical confounding factors related to preterm birth, but the convergence of recent reports is striking. At term-equivalence, brain volumes are reduced in frontal and parietal cortex (Smith, Gutovich et al. 2011) and white matter maturation is delayed (Brummelte, Grunau et al. 2012). In school-age children, cortical thickness, in multiple regions but particularly in the frontal lobes, is significantly reduced in those who had a greater number of early painful procedures (Ranger, Chau et al. 2013). The functional circuitry underlying pain processing and response may also be altered by this early experience (Doesburg, Chau et al. 2013). Pain, and the glucocorticoid response of the hypothalamic-pituitary axis to it, has been hypothesized to directly impact gray and white matter development by increasing apoptosis in vulnerable cell populations such as OPCs or subplate neurons (Vinall and Grunau 2014), but direct evidence is limited. Likewise, support for using common opioid analgesics (morphine, fentanyl) to reduce pain-induced damage is limited (Ranger and Grunau 2014). Indeed, opioid exposure may be deleterious, as discussed above, and suggested by the NEOPAIN trial (Drobyshevsky, Jiang et al. 2014). Increased investigation in animal models is needed to defined the precise mechanisms that lead either directly or indirectly to pain-related cortical changes.

Impact of sensory environment

While the existence of critical periods—time windows during brain development when cellular components and/or circuits are plastic—has long been recognized, human critical periods especially as they relate to brain injury and repair remain poorly defined (Cioni, D’Acunto et al. 2011). Much like the concerns regarding pain, the impact of auditory, visual and olfactory exposures experienced by preterm infants has been of significant concern over the past decades leading to changes in NICU design (Lester, Hawes et al. 2014). However, demonstrations of improvements in neurodevelopment with modification of stimuli (e.g. decreased ambient noise, light/dark cycles) have proved elusive. In fact, an intriguing recent study suggests that preterm infants cared for in private rooms showed delayed neurological maturation compared to those cared for in larger wards of the same institution (Pineda, Neil et al. 2014). Aversive and beneficial sensory exposures may play a modulating role in preterm brain injury, but as this recent report highlights, the appropriate balance of inputs has not yet been identified.

While the optimal sensory environment for preterm infants during their initial months of life remains a matter of debate, animal models and clinical trials suggest that early, enriched activity after injury may be beneficial. Social dynamics, as well as environment and parental interactions, appear to play a significant role in long-term neurological outcome of prematurely born children (Ment, Vohr et al. 2003). The most robust predictors of more optimal long-term neurological outcome of very low birth weight infants are the level of maternal education and existence of a two-parent household (Wong and Edwards 2013). In addition, early interventions in premature infants during late infancy can improve cognitive

outcomes at school age (Spittle, Orton et al. 2007). These findings point to a strong role of the environment on promoting neural plasticity and enhancing recovery during critical developmental stages of premature infants.

Sensory enhancement in rodents can be mimicked by an Enriched Environment where rodents are housed in a larger cage with a number of objects of different color and shapes as well as a running wheel. This enriched environment promotes hippocampal stem cell proliferation and enhances learning and memory (van Praag, Kempermann et al. 2000). Similar paradigms also improve sensory-motor tasks in a variety of animal models of disease (Laviola, Hannan et al. 2008). Recently, specific application of enriched environment to a preterm injury model, the chronic sublethal hypoxia model, showed that 2 weeks of enrichment expanded the endogenous astrocyte pool and prevented behavioral deficits observed in mice reared in a non-enriched environment (Salmaso, Silbereis et al. 2012). This behavioral improvement might be in part due to a specific effect of an enriched environment on inhibitory interneurons (Komitova, Xenos et al. 2013). Thus, in addition to direct effects on stem cell and progenitor proliferation, may also promote maturation of specific neural cell types. These distinct effects are likely mediated through different molecular mechanisms, which could involve some of the signaling pathways activated during normal development, but could also be specifically induced in response to injury (Gallo and Deneen 2014). For example, enrichment enhances expression of several endogenous growth factors (Kuzumaki, Ikegami et al. 2011, Seo, Yu et al. 2013). The specific role of these factors in promoting maturation of distinct neural cell types after neonatal injury is yet to be defined. Identification of cellular factors and signaling pathways activated by the enriched environment is important to develop more selective therapeutic approaches targeted to specific developmental time windows of recovery after premature brain injury. In infants, improvements are likely to depend on both pharmacological and environmental treatments that promote maturation and therefore prevent disruption of normal developmental processes.

CONCLUSIONS

Recent advances in neonatal care have coincided with a pronounced reduction in the overall severity and extent of the destructive lesions that previously were the most commonly associated with preterm cerebral dysfunction. The emergence and application of improved brain imaging has provided unprecedented access to population-based data that has defined key features of the progressive responses to cerebral injury during the period of most rapid changes in brain growth and maturation. The pronounced decline in destructive cerebral injury has moreover required a shift in focus to newly emerging forms and distributions of injury that may be more amenable to regenerative therapies for extended periods of time after insults. Both within the gray and white matter, there is growing appreciation that the chronic disabilities in preterm survivors may arise primarily from processes that disrupt maturation of both neurons and glia during a critical developmental window that coincides with rapid brain growth and enhanced neuronal connectivity related to elaboration of the dendritic arbor, synaptogenesis and myelination.

The timing and nature of regenerative therapies will likely differ for gray and white matter. Given the apparent complexity and potentially disparate nature of the injury responses across different brain regions, it will be particularly important to ensure that interventions do not have adverse consequences for particular brain regions or some neural cell types. Multifactorial interventional strategies appear to be needed in order to promote more normalized cerebral growth through approaches that may include improved infant nutrition, reductions in postnatal infections and neonatal stress and earlier behavioral interventions to enrich the neonatal environment. An important additional challenge will be to recognize and reduce the negative impact on brain development of iatrogenic factors that are now commonly associated with the challenging care of very low birth weight infants.

The timing and nature of all interventions will need to take into consideration the importance of maturation-dependent factors. The future of neonatal brain care may well center around individualization of therapies that will be age and gender appropriate and take into consideration genetic and epigenetic influences on the response to therapies. There are clearly many new opportunities to promote enhanced brain maturation and growth that were not recognized even a decade ago when impaired brain development was largely attributable to irreversible injury related mostly to destructive processes.

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Table 1

Major areas under investigation in preterm brain injury: How do they combine to cause impairments?

Areas of investigation	Impairment Type	Examples
Gray Matter	Dysmaturation	Neuronal loss, reduced arborization, impaired neurogenesis
White Matter	Dysmaturation	Arrest of oligodendrocyte maturation, glial loss
Axons	Dysmaturation	Loss of myelinated or unmyelinated axons, impaired conduction
Subplate Neurons	Dysmaturation/injury	Loss leading to impaired thalamic-cortical connectivity
Endogenous growth factors, hormones	Dysmaturation/repair	Altered steroid or thyroid hormone exposure, recovery via endogenous growth factors
Inflammation, infection	Injury	Microglial activation altering glial and neuronal maturation, cell loss
Hypoxia–ischemia	Injury	Arrest of glial and neuronal maturation, cell loss
Iatrogenic factors	Injury	Exposure to steroids, narcotics, pain, abnormal sensory input altering development