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Brain Injury in Chronically Ventilated Preterm Neonates: Collateral Damage Related to Ventilation Strategy

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Synopsis

Brain injury is a frequent co-morbidity in chronically ventilated preterm infants. However, the molecular basis of the brain injury remains incompletely understood. The focus of this paper is the subtler (diffuse) form of brain injury that has white matter and gray matter lesions, without germinal matrix hemorrhage-intraventricular hemorrhage, posthemorrhagic hydrocephalus, or cystic periventricular leukomalacia. The purpose of this review is to synthesize data that suggest diffuse lesions to white matter and gray matter are collateral damage related to ventilator strategy. Evidence is introduced from the two large-animal, physiological models of evolving neonatal chronic lung disease that suggest an epigenetic mechanism may underlie the collateral damage.

Keywords

White matter injury; gray matter injury; bronchopulmonary dysplasia; neonatal chronic lung disease

Introduction

The brain of chronically ventilated preterm infants is vulnerable to injury during the days, weeks, or months of ventilation support with oxygen-rich gas that are necessary to keep them alive [98]. Familiar lesions are germinal matrix hemorrhage-intraventricular hemorrhage, posthemorrhagic hydrocephalus, or periventricular leukomalacia, particularly with parenchymal cysts [6]. These gross histopathological lesions are not the focus of this review. Instead, we focus on more subtle diffuse lesions that lead to abnormal neural function and subsequent suboptimal neurodevelopmental outcome.

Diffuse lesions to white matter and gray matter are recognized among chronically ventilated preterm infants [72]. Diffuse white matter lesions within the first week of life are characterized histopathologically at autopsy as palely-stained and soft regions of degeneration of white matter and thinning of the corpus callosum. Gray matter lesions also occur [75]. Gray matter lesions are characterized by diffuse neuronal loss in deeper cerebral cortical layers, the

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hippocampus, thalamus, globus pallidus, and cerebellar Purkinje cell layers in the dentate nucleus [75]. A mixture of diffuse white and gray matter lesions presumably contributes to subtler delays and/or deficits in neurodevelopment and impairments in motor skills, learning disabilities, attention deficit/hyperactivity disorders, and/or anxiety disorders in former preterm children [72, 90].

Subtler adverse neurodevelopmental outcomes impact health and quality of life of the survivors and their families. The outcomes also increase the cost for health care borne by the families and society. Therefore, brain injury in chronically ventilated preterm neonates should be viewed as a significant national public health issue.

In spite of increasing recognition of diffuse lesions to white matter and gray matter in chronically ventilated preterm infants, the molecular basis of the lesions remains incompletely understood. The purpose of this review is to synthesize data that suggest ventilator strategy leads to collateral white and gray matter lesions. Evidence will be introduced to suggest an epigenetic mechanism may underlie the collateral damage.

Prematurity as the setting for collateral damage to the brain

Prematurity contributes to about a third of all infant deaths in the United States [59]. Mortality rate is greatest among infants born at or before 25 weeks of gestation (http://www.nichd.nih.gov/about/org/cdbpm/pp/prog_epbo/epbo_case.cfm).

Infants born prematurely are at risk of acute respiratory distress or failure because the future gas-exchange regions of the lung are not developed structurally. The relative or absolute absence of surfactant contributes to collapse of the distal airspaces (atelectasis), which contributes to functional mismatch of ventilation and perfusion that exacerbates gas exchange [4].

Two treatments are used routinely for anticipated preterm birth and subsequent respiratory distress. One treatment is antenatal corticosteroid administration to the mother who is in premature labor. The objective of administering corticosteroids antenatally is to stimulate production of endogenous surfactant in the fetus [50]. The other treatment is postnatal surfactant replacement to the preterm infant [62]. The intended consequence of these treatments is to reduce surface tension and thereby increase lung compliance and gas exchange [21, 36, 39, 48] Preterm infants with larger surfactant pools are likely to be supported by nasal continuous positive airway pressure (nasal CPAP). The rationale for using nasal CPAP is to avoid or minimize endotracheal intubation and positive-pressure ventilation support [5, 17, 26, 92]. However, when nasal CPAP is insufficient, the remedy is endotracheal intubation and positive-pressure ventilation using an oxygen-rich gas mixture. High inflation pressure and mean airway pressure may be necessary to recruit the collapsed distal airspaces to achieve ventilation and oxygenation targets. Infants who do not recover from acute respiratory distress and require prolonged positive-pressure ventilation with oxygen-rich gas are predisposed to develop neonatal chronic lung disease (also called bronchopulmonary dysplasia, BPD or the “new” BPD) [2, 68].

Vulnerability of the immature brain in chronically ventilated preterm neonates

Vulnerability for collateral damage to the brain is related in part to the width of the developmental window and types of developmental processes that occur within the window. The developmental window of vulnerability is 22 to 36 weeks of gestation (Table 1). The types of developmental processes during this window include proliferation, differentiation,

and migration of neurons and glia. During the same period, neuronal circuits develop as synapses form and synaptic connections are optimized through a process of synaptic stabilization. Functional circuits become evident as spontaneous electroencephalographic bursts [10, 30, 43, 45, 63]. A structural manifestation of these processes occurs in the subplate layer of neurons, which attains maximal thickness around the 36th week of gestation. Cortical folding also occurs during this developmental window. From 28 weeks of gestation to postnatally after birth at term gestation, the earlier exuberant proliferation of neurons and glia is pruned by apoptosis to optimize their numbers. Myelination occurs from the 36th week of gestation to 2 to 3 years postnatally.

Details regarding development of the cerebral circulation are incomplete. The cerebral circulation expands during the period of 22 to 36 weeks of gestation (Table 1). However, blood vessel formation lags in deep regions, including the germinal matrix, periventricular white matter, and corpus callosum, and deep gray matter structures such as the basal ganglia and hippocampus [32]. The lag in blood vessel formation in deep regions of the brain is part of the basis of the 'watershed' explanation of vulnerability of germinal matrix, periventricular white matter, and deep cortical gray matter. Developmental immaturity of the blood-brain barrier also creates vulnerability for circulating toxic molecules to access the brain parenchyma [64Stonestreet, 2000 #9358].

The immature brain is vulnerable to fluctuations in systemic blood pressure [31, 55, 74]. Fluctuations are dangerous because of the immaturity of autoregulation, which may expose the brain to increased or decreased blood pressure, a characteristic that is referred to as "pressure-passive". For example, systemic hypotension combined with a pressure-passive cerebral circulation may lead to hypoxic ischemia (hypoperfusion) of white matter and/or gray matter [23, 69, 99]. A subsequent vulnerability may be reperfusion injury, particularly in association with systemic hypertension [3, 24, 31, 42, 53, 55, 56, 59, 72, 76, 77, 91, 102]. Another cerebrovascular vulnerability is increased intrathoracic pressure in preterm infants who are intubated and ventilated or who develop a pneumothorax. Increased intrathoracic pressure may affect cerebral perfusion pressure and/or flow [16, 71].

Molecules that participate in brain injury are numerous. Inflammatory cytokines and chemokines, with or without infection, are participants [18, 20, 34, 81]. Their participation, in part, is mediated by platelet and neutrophil adhesion in cerebral blood vessels [35, 51]. Other molecular participants are reactive species of oxygen or nitrogen that are generated during reperfusion following hypoxia-ischemia [35, 70]. In addition, expression of growth factors is reduced, notably, insulin-like growth factor-1 (IGF-1) [8, 29]. IGF-1 is emphasized here because its regulation of expression is relevant to an epigenetic hypothesis that will be proposed subsequently in this review.

Developmental processes that are vulnerable in the brain are summarized in Table 2. The vulnerabilities include germinal matrix injury, diffuse white matter injury, and diffuse gray matter injury.

Collateral damage to the brain related to ventilation strategy: an epigenetic hypothesis

A trend over the last decade is initial use of nasal CPAP or early extubation to nasal CPAP. The rationale is to reduce the primary injury to the preterm infant's lungs and secondary injury to other organs, notably the brain. However, the impact of ventilation strategy on mechanisms of pathogenesis of brain injury in preterm neonates remains uncertain. To this end, insights are being made using large-animal, physiological models of neonatal chronic lung disease in which brain injury is an accompaniment. The models share the common

feature of brain injury without intraventricular hemorrhage or cystic periventricular leukomalacia.

Preterm baboon model

Brain injury occurs in preterm baboons that have evolving neonatal chronic lung disease. An informative experiment compared brain injury outcomes when the preterm baboons were weaned from mechanical ventilation to nasal CPAP [52] One group was weaned at 24 hours of life (early nasal CPAP); the other starting at 5 days of life (delayed nasal CPAP). The principal results showed brain injury in both nasal CPAP groups compared to the fetal lambs that were not ventilated. In both groups, brain injury was diffuse, without hemorrhage or cystic infarction, and affected white matter and gray matter. Secondarily, the results showed that injury severity was less in the early nasal CPAP group compared to the delayed nasal CPAP group. The latter result suggests that duration of mechanical ventilation is directly related to brain injury. The molecular mechanisms of these pathological changes were not a part of the study.

Preterm lamb model

We are using chronically ventilated preterm lambs to identify molecular mechanisms that are involved in injury to multiple organs, notably the lung and brain. Our studies led us to propose an epigenetic hypothesis for the pathogenesis of neonatal chronic lung disease and its associated co-morbidities, including brain injury.

We focus on epigenetics because of its role in fetal and perinatal adaptation [58, 103]. Importantly, epigenetic regulation of gene expression in the perinatal period is associated with re-adjustment in gene expression in response to changes in environment [40]. For example, intrauterine growth restriction, which predisposes to preterm labor and delivery, is associated with reduced levels of insulin-like growth factor-1 (IGF-1) in the liver of rat pups [22]. In addition, many of the affected epigenetic characteristics persist postnatally, in conjunction with persistently less IGF-1 mRNA and protein levels. Does the same hold true in chronically ventilated preterm lambs? Before answering that question, concepts about epigenetics will be provided.

Epigenetic regulation of gene expression uses modifications to chromatin, the unit of which is the nucleosome. Nucleosomes have 146 bp of DNA wrapped around an octomeric core of histone proteins [54]. The modifications constitute an epigenetic code for regulation of gene expression by directing interactions between transcription complexes with DNA. Because the interactions are dynamic, transcriptional levels of proteins are adjusted and re-adjusted over time, including long-term (life-long).

Epigenetic regulation of gene expression can use several mechanisms, including histone modifications, DNA methylation, microRNAs, and nucleosome positioning [47, 83]. Histone modifications consist of acetylation, methylation, phosphorylation, and ubiquitination. Enzymes that add histone modifications include histone acetylases and methyltransferases [57]. Enzymes that take away histone modifications include histone deacetylases (HDACs) and demethylases. DNA methylation occurs on a cytosine base where cytosine precedes and is linked to a guanosine by phosphate. Therefore, the modification is referred to as methylation of CpG. Methylation involves DNA methyltransferases [47]. Methyl-cytosine demethylation involves the ten-eleven translocation (Tet) gene family [38, 88]. Biologically, these enzymatic processes appear to be coordinated dynamically [11]. MicroRNAs, short RNA molecules that do not code for a protein, silence target genes by binding to their 3' untranslated regions [84]. Nucleosome positioning regulates gene transcription by exposing or not exposing transcription start sites to transcription

complexes and RNA polymerase, as will be described near the end of this review. Understanding how these processes are regulated and dysregulated will be necessary to identify their roles in health and disease.

The dynamic nature of epigenetics is complex, making generalizations difficult when comparing one cell type to another, one organ to another, one species to another, along the continuum of developmental processes, and among diseases. Difficulty is even greater in the *in vivo* context, where interplay among epigenetic, genetic, physiologic, and pathophysiologic processes occurs dynamically in the setting of a whole organism [1]. Although daunting, testing epigenetic hypotheses *in vivo* using large-animal models of human disease, and in humans, is necessary to translate epigenetic concepts and principles to understand the epigenetic basis of human health and disease.

Our recent work suggests that epigenetic characteristics in the brain are affected in chronically ventilated preterm lambs with evolving neonatal chronic lung disease (unpublished data). Highlights of some of these studies will be summarized from two perspectives of epigenetic characteristics: (1) genome-wide and (2) candidate-gene-specific epigenetic characteristics. Comparative results will be highlighted for the brain and lung from the same preterm lambs.

Genome-wide epigenetic characteristics—Some clinical evidence suggests that nasal CPAP reduces the risk for premature infants to develop neonatal chronic lung disease and its co-morbidities [92]. However, the biological basis for the different outcomes is not known. To address this unknown, we are pursuing studies in chronically ventilated preterm lambs. One study is assessing genome-wide epigenetic characteristics in the brain (and lung) of preterm lambs that are supported by either positive-pressure ventilation or a version of bubble nasal CPAP that delivers high-frequency ventilation at the level of the nose (nasal HFV) [80].

We measure levels of several histone modifications in homogenates of brain and lung tissue. For example lower levels of genome-wide acetylation of histone 3 lysine 14 (H3K14ac) occurred in the brain and lung of mechanically ventilated preterm lambs compared to preterm lambs supported by nasal HFV. Another histone modification that we measured is trimethylated H3K36 (H3K36me3). Lower levels of H3K36me3 are detected in the mechanically ventilated group compared to the nasal HFV group. These initial results suggest that ventilation strategy affects genome-wide histone covalent modifications.

Gene-specific epigenetic characteristics—A limitation of our aforementioned studies is that the results do not identify an epigenetically regulated gene as a molecular culprit. An alternative approach is to test the participation of a candidate gene *in vivo*.

Our current research efforts are focusing on IGF-1 as a prototypic, epigenetically-regulated gene [22, 40]. Our rationale is a follows. First, IGF-1 is involved in brain and lung development [49]. Second, IGF-1 expression is increased in the lung of preterm infants who died during acute respiratory distress or neonatal chronic lung disease [13]. Whether IGF-1 expression is affected in the brain of chronically ventilated preterm infants remains to be determined. Of note, IGF-1 expression is decreased in the brain in other models of perinatal insult in which growth is disrupted and adverse long-term neurodevelopmental outcomes are detected [44, 100]. Together, these characteristics make the IGF-1 gene a candidate to study in the context of brain vulnerability to injury in chronically ventilated preterm lambs.

Our initial results suggest that IGF-1 expression is affected in the brain and lung of ventilated preterm lambs. For example, the brain of mechanically ventilated preterm lambs appears to have lower IGF-1 protein levels compared to preterm lambs supported by nasal

HFV. Lower levels of IGF-1 protein in the brain are consistent with the reduction that occurs in other models of perinatal insult, such as intrauterine growth restriction [22, 44]. In the same preterm lambs, the lung appears to have higher levels of IGF-1 protein in the mechanically ventilated group compared to the nasal HFV group. Elevated IGF-1 in the lung of the mechanically ventilated preterm lambs is consistent with results obtained at autopsy of preterm infants who died with respiratory failure [13].

Finding less IGF-1 protein in the brain, and more in the lung, in mechanically ventilated preterm lambs prompted us to ask how the changes may occur. As a start, we are determining the pattern of histone modifications (the histone code) along the length of the IGF-1 gene locus in sheep. The rationale for assessing the histone code is because its pattern plays a prominent role in regulating gene transcription [22, 40, 44]. Briefly, chromatin is condensed in the heterochromatic state. While chromatin is condensed, access of transcription complexes to transcription start sites is blocked physically by close-packing of nucleosomes (Figure 1A). For transcription to be initiated, the chromatin has to open, forming euchromatin. Opening chromatin, and therefore permitting transcription, is mediated in part by acetylation of histones. As acetylation occurs, open space is created upstream of a transcription start site or sites by unraveling a nucleosome, creating a nucleosome-free zone (Figure 1B). The open space provides access for transcription complexes to bind to the exposed transcription start site. Conversely, eventual silencing to reduce or stop transcription occurs when other histones are methylated, especially tri-methylated. A consequence of increasing tri-methylation of histones is decreasing acetylation of histones. This reciprocal shift is believed to help condense the chromatin along the body of the gene to prevent inappropriate transcription initiation from occurring downstream of the designated transcription start site.

With the aforementioned outline of the process of epigenetic regulation of gene expression in mind, how might we explain the results in the brain and lung of preterm lambs, depending on ventilation strategy? First, we must admit that the scenario is hypothetical. That is, we do not have direct cause-and-effect evidence. Taking into account that epigenetic regulation is organ-specific and that every cell has the same genetic information, yet each organ develops uniquely, we hypothesize that uniqueness is conferred by cell-specific or cell- and organ-specific epigenetic regulation. We specifically hypothesize that genome-wide hypoacetylation in the brain shifts the balance toward more apoptosis, and less proliferation of astrocytes and oligodendrocytes (Alvord abs). Reduced numbers of both types of glia would decrease IGF-1 locally in the brain because astrocytes and oligodendrocytes synthesize and secrete IGF-1 [7, 25, 67, 101]

How might we explain more IGF-1 in the lung of the same chronically ventilated preterm lambs, given that the lung also has genome-wide histone hypoacetylation? We propose that lung-specific epigenetic mechanisms may lead to more expression of pulmonary IGF-1. One mechanism is the source of IGF-1 mRNA and protein in the lung is mesenchymal cells (Heber's refs). We showed that mesenchymal cell proliferation exceeds apoptosis in the walls of the distal airspaces of preterm lambs that are supported by mechanical ventilation compared to nasal HFV [80]. Another possibility is that mechanical ventilation may lead to promiscuous transcription of IGF-1 in the lung. To explain this hypothesis, attention is drawn again to Figure 1. Figure 1C depicts several transcription start sites that are uncovered because numerous nucleosomes are absent (nucleosome-free zones). We have new preliminary data to suggest that ventilation of preterm lambs exposes multiple transcription start sites for IGF-1 in the lung. By comparison, only one site appears to be used in the lung of fetal lambs that are not allowed to breathe. We propose that exposure of more transcription start sites across a longer length of the gene locus upstream of promoter 1 may contribute to more IGF-1 transcription in the lung of chronically ventilated preterm lambs.

Of course, other epigenetic mechanisms may also influence gene expression, such as regulation of elongation and/or termination of transcription. Such potential epigenetic mechanisms will need to be investigated.

A hypothesis that remains to be tested is whether early changes in the histone code provide survival advantage (adaptation) in the short-run, but also exact a cost later in life, the cost being vulnerability to subsequent insults and/or onset of adult diseases. This hypothesis will be important to test because survival advantage in the short-term may create vulnerability to disease (*e.g.*tumor formation), to subsequent insults later in life (*e.g.*recurrent respiratory tract infections), and/or to adult-onset diseases (*e.g.*obesity, diabetes, cardiovascular disease). To this end, our newest studies use a modified protocol whereby preterm lambs are weaned from ventilation support and fostered for the equivalent of 2 years or 6–8 years of postnatal life in humans. The intention of these new studies is to correlate epigenetic characteristics of gene regulation with long-term neurodevelopmental delays and deficits among former preterm, ventilated lambs.

Ultimately, a histone modification-specific transgene construct will be necessary to definitively show that a specific epigenetic modification causes a specific phenotype.

Summary

Structural and functional immaturity of a preterm neonate's lung necessitates use of antenatal steroids, postnatal surfactant replacement therapy, postnatal ventilation support with oxygen-rich gas, and other measures to keep the neonate alive. Acute lung injury often ensues. If recovery from acute lung injury does not happen, endotracheal intubation and prolonged positive-pressure ventilation support with oxygen-rich gas may be necessary, leading to neonatal chronic lung disease. A frequent co-morbidity of evolving neonatal chronic lung disease is brain injury. However, the molecular basis of the brain injury remains incompletely understood. This void is addressed by use of large-animal, physiological models of brain injury in the setting of evolving neonatal chronic lung disease. An advantage of the chronically ventilated preterm baboon and lamb models is that the setting of preterm birth is uncomplicated by antenatal conditions, such as intrauterine infection or asphyxia, that may potentiate brain injury. Also, neither chronic model is associated with intraventricular hemorrhage or cystic lesions of periventricular white matter. Consequently, drawing mechanistic conclusions is more straightforward. On the other hand, the less complicated setting for the large-animal models may be viewed as a disadvantage because the models lack antenatal conditions that may potentiate injury to the lung and brain. Nonetheless, new mechanistic insights are being provided by the chronic animal models. In particular, involvement of epigenetics in the pathogenesis of lung and brain injury opens new doors for mechanistic studies that may provide opportunities for interventions. Endotracheal intubation with prolonged positive-pressure ventilation support and oxygen-rich gas appears to change epigenetic determinants of gene expression. Such ventilation support appears to scramble the histone code, at least based on results for IGF-1. This effect does not appear to occur when nasal HFV is used. A caveat, of course, will be whether altering epigenetic regulatory patterns provides short-term adaptations that improve survival during the neonatal period, but creates unintended consequences later in life. This caveat makes the pursuit a high-risk/high-potential-benefit for understanding and improving health and outcomes of chronically ventilated preterm infants.

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Key Points

- Brain injury is a frequent co-morbidity in chronically ventilated preterm infants. However, the molecular basis of the brain injury remains incompletely understood.
- This article focuses on the subtler (diffuse) form of brain injury that has white matter and gray matter lesions, without germinal matrix hemorrhage-intraventricular hemorrhage, posthemorrhagic hydrocephalus, or cystic periventricular leukomalacia.
- This clinical review synthesizes data that suggest diffuse lesions to white matter and gray matter are collateral damage related to ventilator strategy.
- Evidence is introduced from the two large-animal, physiological models of evolving neonatal chronic lung disease that suggest an epigenetic mechanism may underlie the collateral damage.

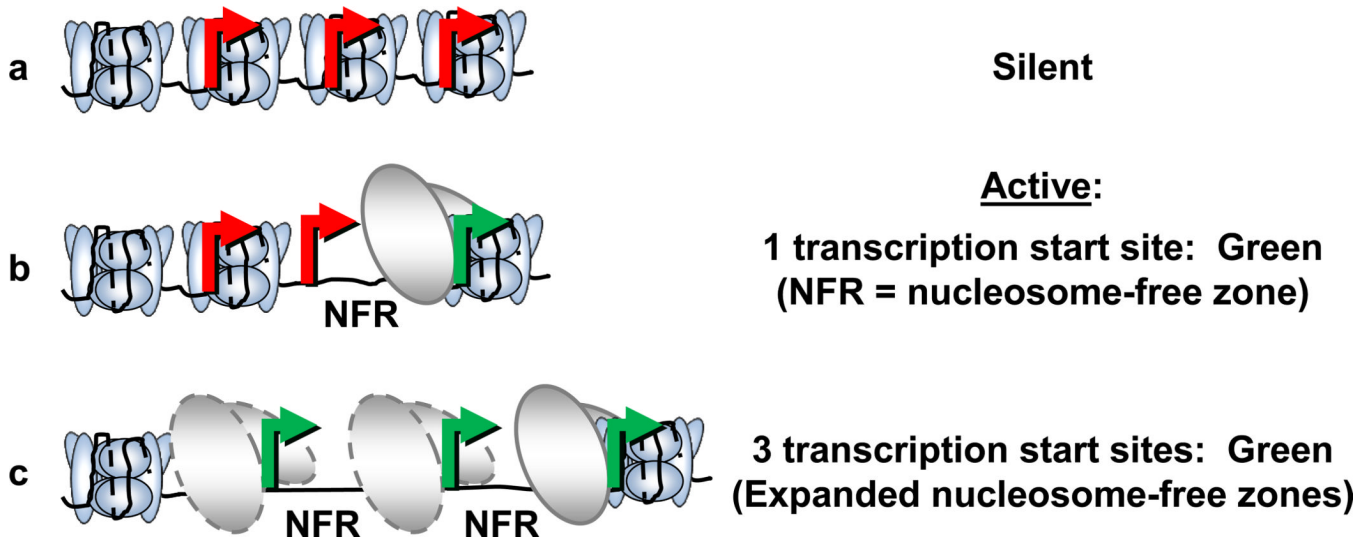

Figure 1.

Schema transcription start sites in a promoter region. (a) Transcriptionally silent promoter. Transcription start sites are shown in red because they are covered by nucleosomes and therefore are not accessible to transcription complexes. (b) Transcriptionally active promoter. A nucleosome-free region (NFR) is shown where a nucleosome is displaced, exposing a transcription start site (green). A transcription complex (ovals) is shown immediately upstream to the exposed (green) transcription start site, where access is available for RNA polymerase. (c) Transcription is potentially enhanced by expanded nucleosome-free zones, which expose 3 transcription start sites to RNA polymerase.

Table 1

Timing of Structural Development of the Human Brain

Time from conception	Developmental Process	References
3–4 weeks	Formation of neuroectoderm	96
3–4 weeks	Primary neurulation	15,86,96
5–10 weeks	Formation of prosencephalon and hemispheres	96
5–10 weeks to ? years postnatally	Cerebral angiogenesis and formation of the blood-brain barrier	66
7–10 weeks	Generation of the subplate layer of neurons (from germinal matrix)	46
10–15 weeks to ? years postnatally	Neurogenesis	96
12–24 weeks	Neuronal migration	27,28,78,79,85,96
16–24 weeks	Blood vessel density in subcortical white matter is low	65
20 weeks to ? years postnatally	Synaptogenesis and synaptic stabilization	12,16,71
20 weeks to ? years postnatally	Gliogenesis	9,14,95,96
22–36 weeks	Maximal thickness of the subplate layer of neurons (from germinal matrix) is reached	46
23–29 weeks	Cortical folding and spontaneous EEG bursts; delta brushes	10,30,43,45,63
28–36 weeks	Blood vessel density in deep white matter is low	37,65
28 weeks to ? years postnatally	Neuronal and glial apoptosis	96
36 weeks to ? years postnatally	Myelination	9,96

Table 2

Vulnerability of the Brain of Preterm Neonates

Time from conception	Developmental Process	Vulnerability	References
10–15 weeks to ? years postnatally	Neurogenesis	Germinal matrix injury[1]	
12–24 weeks	Neuronal migration		
16–24 weeks	Low vessel density in subcortical white matter		
20 weeks to ? years postnatally	Synaptogenesis and synaptic stabilization		160,93,95
20 wk to ? years postnatally	Gliogenesis	Diffuse white matter lesions (periventricular leukomalacia)2	219,33,37,41,60,61,65,73,82,87,90,94
22–36 weeks	Maximal thickness of the subplate layer of neurons (from germinal matrix) is reached		
28–36 weeks	Blood vessel density in deep white matter is low		360,89,97
28 weeks to ? years postnatally	Neuronal and glial apoptosis	Diffuse gray matter lesions[3]	
36 weeks to ? years postnatally	Myelination		