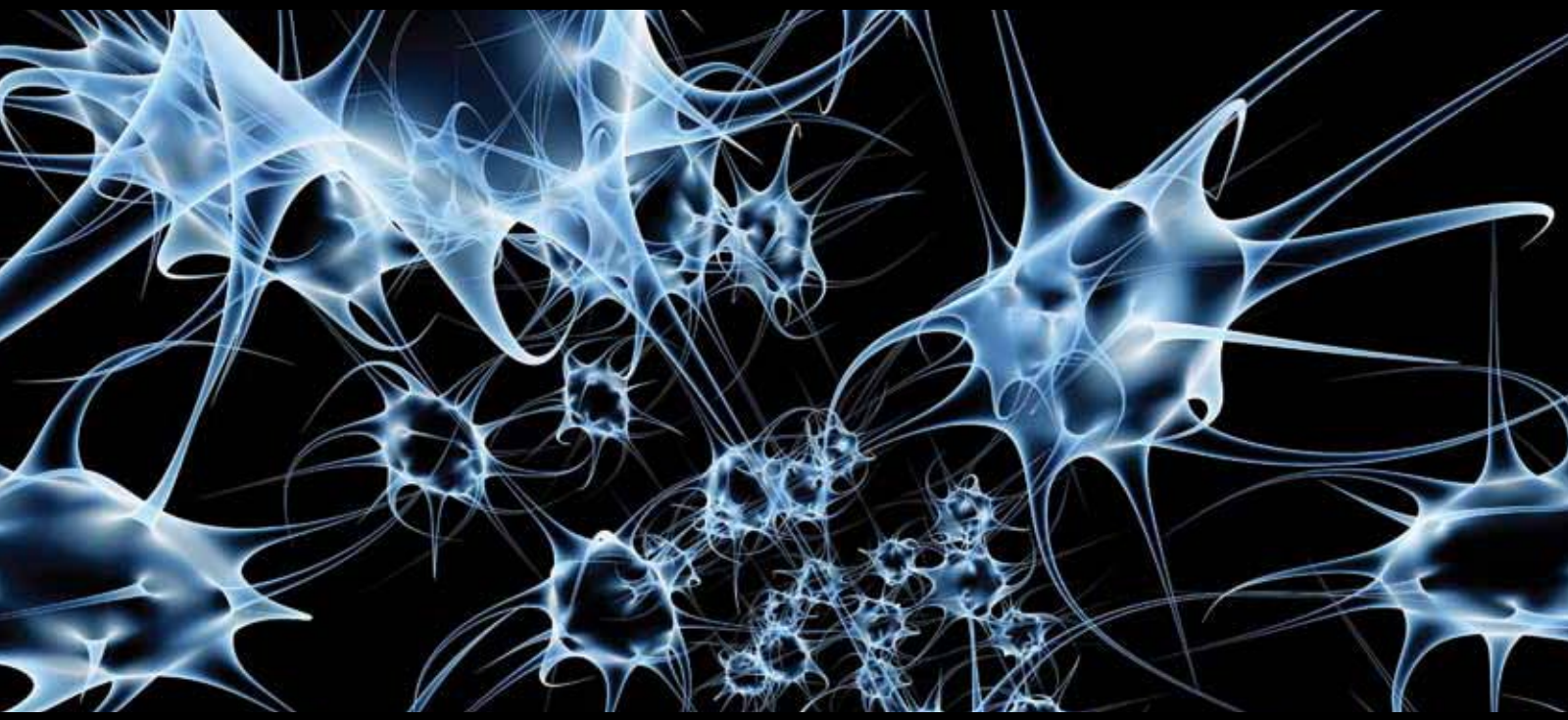
Neurology Research International

Mechanisms of Perinatal Brain Injury

Guest Editors: Robin L. Haynes, Tara M. DeSilva, and Jianrong Li



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Editorial Board

Contents

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Editorial

Mechanisms of Perinatal Brain Injury

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The guest editors of this journal are pleased to introduce this special issue on the mechanisms of perinatal brain injury. The purpose of this issue is to highlight recent developments toward our understanding of both grey and white matter injury in the perinatal brain. Over the last decade, there has been significant advancement in our understanding of the mechanisms underlying this injury. The progression of the field stems from many factors including: (1) the development and refinement of experimental models; (2) advances in modern imaging technologies, such as magnetic resonance imaging and diffusion tensor imaging; (3) detailed characterization of the injury, both in human and animals, on the anatomical, cellular, and subcellular levels and (4) identification of the developmental factors that underlie the vulnerability of the perinatal brain to such injury. Here we present nine review and two research articles that together highlight these advancements, as summarized below. It has been our honor to work with each of the contributing scientists, and we thank them for their commitment to this Special Issue.

The review by C. Thornton et al. summarizes our current understanding of the "*Molecular mechanisms of perinatal brain injury,*" with a focus on mitochondrial functional impairment and apoptotic events during the secondary injury phase. The authors also present the most up-to-date intervention strategies targeting the different stages of brain injury.

V. Ten and A. Starkov further explore the role of mitochondria in hypoxic-ischemic (H-I) injury and provide evidence for the mitochondrial production of reactive oxygen species as a pathogenic mechanism underlying this injury. The authors summarize experimental data delineating mitochondrial sources of reactive oxygen species and their specific targets in neonatal animals.

Necrotic cell death is well documented in H-I brain injury and is traditionally thought uncontrollable. In another review, R. Chavez-Valdez et al. introduce the recently identified RIP1/3 kinase dependent programmed necrosis pathway to the field of neonatal brain injury and propose an apoptosis-necrosis cell death "continuum" for cellular degeneration. The authors highlight experimental evidence supporting a prominent role of programmed necrosis in neonatal H-I brain injury.

Another paper by C. Hill and R. Fitch explores the underlying basis of the increased vulnerability of male infants to H-I injury and their increased incidence of cognitive deficits associated with that injury. The authors discuss hormonal factors underlying these differences as well as sex-specific differences in H-I-induced cell death pathways.

In the review by K. Buller et al., the effects of perinatal H-I injury on the serotonergic system are summarized. The potential use of anti-inflammatory interventions to alleviate this injury is also discussed. Disruption of this neurotransmitter system may underlie the cardiorespiratory, cognitive, and attention deficits observed in survivors of prematurity.

Changing the focus from H-I injury to inflammation and infection, C. Mallard and X. Wang summarize clinical and experimental evidence for neonatal sepsis and increased vulnerability of the immature brain and discuss the involvement of Toll-like receptors (TLRs) in perinatal brain injury.

Continuing in the area of inflammation and infection, R. McAdams and S. Juul address the initiation and activation of cytokines and inflammatory cells in the perinatal brain and

their detrimental short and long-term consequences. They highlight the importance of understanding the dynamic structure of the blood brain barrier, as well as the relatively unknown mechanisms that impair its integrity allowing immune cells direct access to the brain.

In the first of two research articles, D. Selip et al. characterize, in a rodent model of H-I, the spectrum of grey matter damage in association with the white matter damage once thought to be dominant in H-I injury. The authors characterize the regional susceptibility to H-I injury in terms of the inflammatory response, white matter injury and myelin loss, neuronal degeneration, and axonal injury.

Our second research article by K. Shrivastava et al. provides an extensive characterization of the glia/inflammatory response following H-I using a common model of injury thought to spare the contralateral hemisphere. This study extends our knowledge of this model by characterizing a subtle inflammatory response in the contralateral brain, and provides important information regarding the timing of the insult in relationship to the initiation of inflammatory signaling cascades.

The paper by V. Biran et al. highlights a novel area of the brain—the cerebellum—that has emerged as an important attribute of perinatal brain injury through imaging studies. This review provides an extensive detailed analysis of cerebellar development, and the implications of disrupting its normal maturation as a consequence of perinatal brain injury.

Finally H. Kinney and J. Volpe stress the importance of developing and using animal models with a careful consideration of human perinatal brain development and injury. This paper emphasizes the need to model perinatal brain injury as an encephalopathy of prematurity characterized by a combination of both grey and white matter lesions.

Acknowledgment

Lastly we would like to remember a colleague in the field and the senior author of the article by Shrivastava et al. described above. Dr. Laia Acarin made many significant contributions to our field of perinatal brain injury. Her work in excitotoxicity and H-I has yielded valuable insight into the glial and inflammatory responses following neonatal brain damage, as well as the mechanisms controlling this inflammation. We would like to respectfully dedicate this special issue to her memory.

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Research Article

Short and Long-Term Analysis and Comparison of Neurodegeneration and Inflammatory Cell Response in the Ipsilateral and Contralateral Hemisphere of the Neonatal Mouse Brain after Hypoxia/Ischemia

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Understanding the evolution of neonatal hypoxic/ischemic is essential for novel neuroprotective approaches. We describe the neuropathology and glial/inflammatory response, from 3 hours to 100 days, after carotid occlusion and hypoxia (8% O_2, 55 minutes) to the C57/BL6 P7 mouse. Massive tissue injury and atrophy in the ipsilateral (IL) hippocampus, corpus callosum, and caudate-putamen are consistently shown. Astrogliosis peaks at 14 days, but glial scar is still evident at day 100. Microgliosis peaks at 3–7 days and decreases by day 14. Both glial responses start at 3 hours in the corpus callosum and hippocampal fissure, to progressively cover the degenerating CA field. Neutrophils increase in the ventricles and hippocampal vasculature, showing also parenchymal extravasation at 7 days. Remarkably, delayed milder atrophy is also seen in the contralateral (CL) hippocampus and corpus callosum, areas showing astrogliosis and microgliosis during the first 72 hours. This detailed and long-term cellular response characterization of the ipsilateral and contralateral hemisphere after H/I may help in the design of better therapeutic strategies.

1. Introduction

With the improvement of perinatal care, the frequency of infant death has reduced considerably, but the incidence of neurological disabilities related to perinatal brain damage has not decreased in Western countries over the last decades [1–3]. Perinatal brain injury due to asphyxia, cerebral ischemia, cerebral hemorrhage, or intrauterine infection is the major contributor to perinatal morbidity and mortality as the immature brain is highly susceptible to damage. Injury to the newborn during the perinatal stage is the underlying etiology for a host of developmental disabilities that includes spastic motor deficits such as cerebral palsy [4, 5] and cognitive, behavioral, attentional, socialization and learning difficulties [6–9]. As brain development substantially influences the progression and hallmarks of brain injury [10, 11], it is not possible to apply therapeutic procedures used for adult ischemia to newborns.

In term newborn infants, hypoxic/ischemic (H/I) brain injury is the most common cause of encephalopathy and seizures. Presently, optimal management of H/I brain injury involves prompt resuscitation, careful supportive care, and treatment of seizures. Although hypothermia is a promising new therapy, and recent studies suggested that head or whole-body cooling administered within 6 hours of birth reduces the incidence of death or moderate/severe disability at 12 to 22 months [12], there is undeniable need for the identification of new therapeutic targets for the implementation of clinical trials to address treatment of H/I encephalopathy [13]. Accordingly, epidemiological and experimental data have allowed researchers to identify a number of potential targets for neuroprotective strategies.

Animal models have led to the elucidation of biochemical events involved in neurodegeneration and neuroprotection [14–18]; however, important differences among species have been described [19, 20].

The initiation and development of injury to the neonatal brain is complex, with multiple contributing mechanisms and pathways resulting in both early and delayed injury [21]. As in other types of acute central nervous system (CNS) injuries, tissue damage and neurodegeneration initiate a cascade of inflammatory response depending on the nature and extent of damage, which is characterized by the involvement of damaged neurons, microglial, astrocytes, endothelial cells, and recruited blood leukocytes [22–25]. Microglial cells are the main nervous component of the innate immune system, playing a key role in the phagocytosis of cell debris to repair damage and maintain tissue homeostasis, but active producers of inflammatory mediators [26]. Astrocytes rapidly respond to extracellular changes and are the main cell type responsible for the restoration of blood-brain barrier, new glia limitans formation, and the establishment of a long-term glial scar [27]. In addition, vascular damage induces massive influx of blood leukocytes, particularly monocytes and neutrophils, which are also actively involved in inflammatory processes [28].

It is important to note that the glial and inflammatory response after perinatal brain damage differs from the mature brain [25] due to key ongoing postnatal developmental processes. Importantly, neuronal dendritic arborization, establishment of synaptic contacts, axonal growth, myelination, and glial differentiation take place during the first two-three postnatal weeks in rodents [29]. At the molecular level, several studies have described a distinctive expression of growth factors [30], adhesion molecules [31], inhibitors of axonal growth [32], and cytokines [33–35], determining the neonatal brain's particular response to injury, showing increased susceptibility to excitotoxicity [11, 36, 37] and to proinflammatory molecules [38, 39]. In this regard, it becomes evident that descriptions of the glial and inflammatory cell changes in adult injury models cannot be extrapolated to animal models of perinatal brain damage.

In the present study we have used the experimental model of H/I-induced neonatal injury initially described by Vannucci and coworkers [17, 40] for the rat, and adapted to the mouse in several laboratories [14, 18, 41] with the advent and increased usage of transgenic and knock-out mice. As most studies describing detailed neuropathological and glial and inflammatory cellular changes after neonatal H/I have used the rat model, the goal of our study was to provide a neuropathological followup of tissue damage and detailed morphological and quantitative analysis of astroglial, microglial and leukocytic response following H/I to the postnatal day 7 mice at nine different survival times ranging from 3 hours after hypoxia to 100 days, focusing both on the ipsilateral and the contralateral hemispheric changes. This short- and long-term temporal description aims to help in the future design of novel experimental approaches towards the development of neuroprotective strategies.

2. Materials and Methods

2.1. Animals. Ninety-nine C57BL6 mice (from twenty litters bred in Harlan Labs, France) of different postnatal ages were used in this study. Experimental animal work was conducted according to Spanish regulations following European Union directives. Animals were housed under controlled temperature (22°C ± 2°C), with a 12 hour light cycle period and pelleted food (Global diet 2014) and water *ad libitum*. The dams and pups were kept on enriched environment. Experimental procedures were approved by the ethical commission of Autonomous University of Barcelona (CEEAH protocol no. 811). All efforts were made to minimize the number of animals and animal suffering in every step.

2.2. Hypoxia/Ischemia. Hypoxic/ischemic (H/I) brain damage was induced in postnatal day 7 (P7) C57/BL6 mice by permanent left carotid occlusion and exposure to hypoxia as previously described [42]. Briefly, a midline ventral skin incision was made under isoflurane anesthesia (4.5% v/v for induction and 2.5% v/v for maintenance, and 0.6 L/min of O_2); the left carotid artery was exposed and sutured with a 8/0 silk surgical suture. After surgery, pups were returned to their dam for at least 1.5 hours to recover. Later, litters were placed for 55 minutes in a hypoxic chamber containing 8% of oxygen balanced with nitrogen, with controlled humidity and temperature maintained at 37°C. Pups were then returned to their dam until sacrifice. The mean index of postnatal mouse mortality due to surgery or hypoxia was 19.31%, with 18.46% for males and 20.00% for females, showing no statistical differences between genders. As 18 animals died during surgical procedure or hypoxia, only 81 animals were analyzed in this study.

2.3. Groups and Sample Processing. Intact control mice were sacrificed at P7, P10, P14, P21, and adult. Lesioned pups were sacrificed at 3, 12, 24, 48, and 72 hours, and at 7, 14, 30, and 100 days after hypoxia. All survival times included pups from at least 3 different litters. Animals were grouped as follows for comparison and analysis with controls: Group I—P7, 3 hrs, 12 hrs, 24 hrs; Group II—P10, 48 hrs, 72 hrs; Group III—P14, 7 days; Group IV—P21 and/or adult, 14 days, 30 days, and 100 days. For histological and immunohistochemical analysis, mice were i.p. anaesthetized (ketamine and xylazine 80/10 mg/Kg) and perfused intracardially using 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). Subsequently, brains were removed, postfixed for 4 hours in the same fixative, cryoprotected in 30% sucrose, frozen with dry CO_2, and finally stored at −80°C until use. Brains were serially cut in a cryostat (Leica CM3050 S) in 30 μm thick sections and stored in −20°C mounted on Flex IHC slides (Dako).

2.4. Nissl Staining: Evaluation of Injury Score. To determine the injury score, slides were processed for Nissl staining. One series of parallel sections from each animal (6–10 mice/survival time) was air dried at room temperature for an hour, rinsed and incubated with Nissl solution (0.1%

Table 1: Injury score grading system. Survival times from 3 to 72 hours after hypoxia.

Hippocampal CA field
(0) No damage
(1) Only one/two patches of neurodegeneration
(2) More than 3 neurodegeneration patches
(3) Most CA1 or CA3 damaged
(4) All CA1 and CA1 damaged
Hippocampal DG
(0) No damage
(1) <40% of DG neurons damaged
(2) Approximately 50% of DG neurons damaged
(3) >60% of DG neurons damaged
Corpus callosum
(0) No changes seen
(1) Increased cellularity in ipsilateral corpus callosum
(2) Increased cellularity in ipsilateral corpus callosum and swelling
Caudate-Putamen
(0) No damage
(1) <40% of striatal area damaged (usually with increased cellularity in white matter patches)
(2) Approximately 50% of striatal area damaged
(3) >60% of striatal area damaged
Neocortex
(0) No damage
(1) Scattered neurodegeneration columns in cortex
(2) Neurodegeneration columns in most cortical areas
(3) General neurodegeneration in several areas, all layers
Thalamus
(0) No damage
(1) <40% of thalamic area damaged (only rostral thalamus)
(2) Approximately 50% of thalamic area damaged
(3) >60% of thalamic area damaged (extending to caudal thalamus)

Table 2: Injury score grading system. Survival times from 7 to 100 days after hypoxia.

Neuronal density in hippocampal CA fields
(0) No reduction
(1) More than 60% of CA neurons remaining
(2) Approximately 50% of CA neurons remaining
(3) Between 10 and 40% of CA neurons remaining
(4) Less than 10% of CA neurons remaining
Hippocampal CA field
(0) No damage
(1) Only one/two patches of neurodegeneration
(2) More than 3 neurodegeneration patches
(3) Most CA1 or CA3 damaged
(4) All CA1 and CA1 damaged
Neuronal density in hippocampal DG
(0) No reduction
(1) More than 60% of DG neurons present
(2) Approximately 50% of DG neurons present
(3) Between 10 and 40% of DG neurons present
(4) Less than 10% of CA neurons remaining
Corpus callosum atrophy
(0) No reduction
(1) More than 60% of tissue remaining (less than 40% atrophy)
(2) Approximately 50% of tissue remaining
(3) Less than 40% of tissue remaining (more than 60% atrophy)
Corpus callosum cellularity
(0) No changes seen
(1) Increased cellularity in corpus callosum
(2) Increased cellularity in corpus callosum and swelling
Caudate-Putamen atrophy
(0) No reduction
(1) More than 60% of tissue remaining (less than 40% atrophy)
(2) Approximately 50% of tissue remaining
(3) Less than 40% of tissue remaining (more than 60% atrophy)
Neocortical atrophy
(0) No reduction
(1) More than 60% of tissue remaining (less than 40% atrophy)
(2) Approximately 50% of tissue remaining
(3) Less than 40% of tissue remaining (more than 60% atrophy)
Thalamic atrophy
(0) No reduction
(1) More than 60% of tissue remaining (less than 40% atrophy)
(2) Approximately 50% of tissue remaining
(3) Less than 40% of tissue remaining (more than 60% atrophy)

toluidine blue in walpole buffer 0,2 M and pH 4,5) at room temperature for 3 minutes and washed with distilled water. Sections were dehydrated, cleared in xylene, and coverslipped with DPX. The degree of tissue damage was calculated following the injury score detailed on Table 1 (for 3 to 72 hrs) and Table 2 (for 7 to 100 days).

2.5. Immunohistochemistry. Three animals from each control age group and four representative animals from each postlesion survival time (injury scores = mean ± 2 S.D.) were processed for the immunohistochemical demonstration of astrocytes (by glial fibrillary acidic protein, GFAP labeling), microglia/macrophages (by Iba-1 labeling), neutrophils (by Ly-6B.2 labeling), and T-cells (by CD3 labeling). Single immunohistochemistry was initiated by blocking the endogenous peroxidase (2% H_2O_2 in 70% methanol for 10 min) and incubation of sections mounted on slides for 1 h in blocking buffer (BB) containing 10% fetal calf serum and 3% bovine serum albumin in tris-buffered saline (TBS, pH 7.4) with 1% Triton X-100 (TBST) at room temperature (RT). Slides were then incubated overnight at 4°C and 1 h at RT with one of the following primary antibodies diluted in BB: hamster monoclonal anti-CD3 (AbD Serotec no. MCA2690, dilution 1 : 250), rabbit polyclonal anti-GFAP (DAKO no. Z0334, dilution 1 : 1500),

rabbit polyclonal anti-Iba-1 (Wako no. 019-19741, dilution 1 : 3000), and rat monoclonal Ly-6B.2 (AbD Serotec no. MCA771G, dilution 1 : 500). Later, sections were washed with TBST and incubated at RT for 1 h with respective biotinylated secondary antibodies: anti hamster (Vector Labs no. BA9100, dilution 1 : 500), anti-rabbit (Vector Labs no. BA1000, dilution 1 : 500), and anti-rat (Vector Labs no. BA4001, dilution 1 : 500), followed by washes with TBST and incubation for 1 h with streptavidin–peroxidase (Vector Laboratories no. SA-5004, dilution 1 : 500). The peroxidase reaction was visualized by incubating the sections in 3,3-diaminobenzidine and hydrogen peroxide using the DAB kit (SK-4100; Vector Laboratories, USA) for GFAP, Iba-1 and Ly-6B.2. For CD3, slides were treated by the glucose oxidase-DAB-nickel method [43], and the reaction was terminated by washing with 0.1 M acetate buffer (pH 6.0). Finally, sections were dehydrated and coverslipped in DPX. Sections were analyzed and photographed with a DXM 1200F Nikon digital camera joined to a Nikon Eclipse 80i microscope, and plates were arranged using Adobe Photoshop CS.

2.6. Quantitative Analysis of Immunohistochemical Labelling. ImageJ software (National Institute of Health) was used for quantitative analysis of immunoreacted sections. At least 4 animals/lesioned groups were analyzed. Images from 5 sections/animal were taken, representing the following regions: corpus callosum (CC), caudate putamen (CP), hippocampus (H), neocortex (N), and thalamus (T) (Figure 1). Micrographs were captured using the 40x objective (for the CC and the hippocampus at 72 hours after hypoxia) or the 20x objective (rest of areas and survival times). In group I, II and III sections were 240 μm apart, and bregma levels (BLs) analyzed included (approx.): *Anterior*—BL1, 0.26 mm & BL2, 0.02 mm; *Posterior*—BL3, −1.82 mm; BL4, −2.06 mm; BL5, −2.30 mm. In Group IV, sections were 300 μm apart, and BL analyzed included: *Anterior*—BL1, 0.32 mm & BL2, 0.02 mm; *Posterior*—BL3, −1.82 mm; BL4, −2.12 mm; BL5, −2.42 mm. Image analysis was used to obtain the area occupied by glial cells, using a modification from a previously described method [44]. Initially, in each section, the mean intensity of grey (immunoreactive labeling) in the contralateral region was measured. Subsequently, by using the mean intensity of grey as the threshold value, we measured in both hemispheres the percentage of the total area occupied by immunoreactive staining showing an intensity of grey above the threshold (i.e., representing reactive cells). All samples for demonstration of atrocytes and microglia were done simultaneously in order to reduce variability on DAB intensity. Data of both ipsilateral and contralateral hemispheres are shown as mean values ± S.E.M.

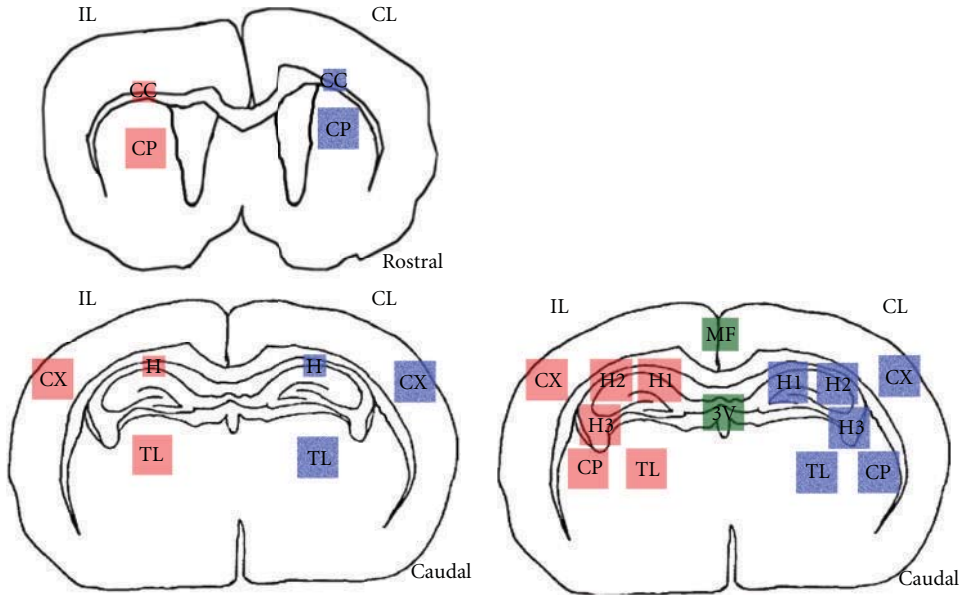
2.7. Neutrophil Cell Counting. Neutrophils were counted using ImageJ software (National Institutes of Health). The regions analysed are shown in Figure 1(b) and included the hippocampus (H1, H2, H3), neocortex (N), caudate-putamen (CP), medial third ventricle (M3V), lateral third ventricle (L3V) median fissure (MF), and thalamus (T) in at least 4 representative animals of each lesioned group and 3 animals/control group, with 3 sections/animal, was analysed. In groups I, II and III sections were 240 μm apart, and counted bregma levels (BLs) included: BL1, −1.82 mm; BL2, −2.06 mm; BL3, −2.30 mm. In Group IV, sections were 300 μm apart, and counted BL included: BL1, −1.82 mm; BL2, −2.12 mm; BL3, −2.42 mm. All data was corrected by Abercrombie correction method [45], with an average of length (t) = 0,848. Data is presented as mean number of cells/mm^2.

2.8. Statistical Analysis. All experiments were performed so as to reduce variations, and data are presented as mean ± S.E.M. The data was considered significant at P-value <0.05. Two-way ANOVA followed by Bonferroni *posthoc* analysis, along with t-test, was used to determine statistical significance as required (Graphpad, Prism 3).

3. Results

3.1. Tissue Damage and Injury Score. Analysis of toluidine blue-stained sections (Figures 2 and 4) was used to evaluate the extent of brain damage in both hemispheres at 3, 12, 24, 48, and 72 hours and at 7, 14, 30, and 100 days after hypoxia. In general, microscopic evaluation showed mild changes in the contralateral hemisphere [mainly in hippocampus (HP) and corpus callosum (CC)], and extensive tissue damage and neuronal loss in the ipsilateral HP and CC at all survival times analyzed, although the caudate putamen (CP) was also usually affected. Damage in the cortex (CX) and the thalamus (TL) was not always seen and showed the highest variability. In order to better characterize lesion progression, a semiquantitative injury score was calculated for each region and animal (Tables 1 and 2, Figures 3 and 5). From 3 to 72 hours after hypoxia, damage was characterized by neurodegeneration and increased cellularity due to gliosis, and the description of the injury score rating is depicted in Table 1. At 7 days after hypoxia, damage was mainly characterized by atrophy of gray and white matter areas, and therefore a different injury score rating was defined, which is depicted in Table 2.

3.1.1. Tissue Damage in the Contralateral Hypoxic Hemisphere. From 3 hours to 7 days after hypoxia, no apparent tissue damage or ventricle swelling in the contralateral hemisphere was observed using the Nissl staining (Figures 2 and 4; right side of the panel). Interestingly, at 14 days after hypoxia, scattered patches of neurodegeneration with a mild reduction in cellular density when compared to intact age-matched control brains were observed in the CA field of the HP (Figure 4), showing a mean injury score in the contralateral HP of 0.92 ± 0.2 (Table 2, Figure 5). In addition, the contralateral CC was also damaged in the 30- and 100-day survival groups, showing approximate 40% of atrophy (mean CC atrophy scores of 1.31 ± 0.59 and 0.86 ± 0.38, resp.) (Table 2, Figure 5), accompanied by evident ventricle swelling (Figure 4). No apparent changes in the contralateral



(a) Regions analyzed for glial reactivity (b) Regions analyzed for leukocyte infiltration

Figure 1: Drawings modified in Adobe Photoshop CS showing brain areas analyzed for quantification of different antibodies used in the study. (a) Regions analyzed for quantification of glial reactivity in rostral and caudal side of the brain (see Section 2 for details). (b) Regions analyzed for quantification of leukocyte infiltration. The regions in red are from the ipsilateral (IL) side and the regions in blue are from the contralateral (CL) side while the medial regions are shown in green. CC: corpus callosum; CP: caudate-putamen; CX: cortex; H: hippocampus; MF: medial fissure; TL: thalamus; 3V: third ventricle.

dentate gyrus (DG), caudate-putamen, neocortical layers and thalamus were seen.

3.1.2. Tissue Damage in the Ipsilateral Hypoxic/Ischemic Hemisphere

H/I Injury in Hippocampus. As early as 3 hours after hypoxia, hippocampal tissue disruption with disorganization of CA cytoarchitecture and the presence of patches of neurodegeneration CA pyramidal neurons was observed in the ipsilateral hemisphere (Figures 2(b), 2(e) and 2(f)), but showing a high degree of variability between animals (Figure 3). From 12 to 72 hours after hypoxia, the hippocampal CA field was visibly damaged in all animals, displaying a degenerating pyramidal cell layer with massive neuronal cell loss in CA1 and CA3 (Figures 2(i)–2(af), left panel), showing a mean injury score of CA field of 3.27 ± 0.74 between 12 and 72 hours after hypoxia (Table 1, Figures 2 and 3). In addition, at 12 hours, the dentate gyrus (DG) also showed neuronal injury and layer disruption, which was most evident at the 12- and 24-hours survival times (Figure 2(i)). At 7 days after hypoxia, massive atrophy of the hippocampus was observed, showing mean total hippocampal injury scores ranging from 5 ± 2.2 to 10.42 ± 1.46 (out of 12, Table 2), where the 30-day survival group showed the lowest score (Figures 4 and 5). Hippocampal damage induced approximately a 10–40% of remaining CA pyramidal neurons, but less than 50% reduction in DG neuronal density (Figures 4 and 5). Interestingly, only in the 33% of the animals, the ipsilateral hippocampus was observed 100 days after hypoxia.

H/I Injury in Corpus Callosum. From 3 hours post-hypoxia, the ipsilateral corpus callosum showed increased cellularity (Figure 2(f)) and the presence of scattered apoptotic cells (data not shown). The density of cells in the ipsilateral corpus callosum was notably increased at 48 and 72 hours post-hypoxia (Figures 2(v), 2(ad) and 3), when ventricle swelling started to become evident (Figures 2(r) and 2(z)). At 7 and 14 days post-hypoxia, increased cellularity was still observed (Figures 4(f) and 4(n)), but this was minimum from 30 days (Figure 4(v)). Important atrophy of the white matter accompanied by ventricle swelling was seen in all animals at 7 days after hypoxia, but it was more remarkable at 14 days after hypoxia, showing mean corpus callosum atrophy score (14–100 days) of 2.33 ± 0.84, which represented an approximate 50% tissue loss (Table 2, Figure 5).

H/I Injury in Caudate-Putamen, Neocortex, and Thalamus. At 3 hours after hypoxia, we observed increased cellularity and disorganization of white and gray matter areas, mainly in the dorsal part of caudate-putamen (Figure 3), showing a mean injury score (3–72 hours) of 1.02 ± 0.81 corresponding to less than 40% of striatal area damaged (Table 1, Figure 3), but showing important variability between animals (Figure 3). At 7 to 100 days after hypoxia, there was apparent caudate-putamen atrophy (Figures 3 and 5).

The neocortex and the thalamus showed mild changes, that were only apparent in a minority of animals at all times analyzed, giving very variable results (Figures 3 and 5). Neocortical damage, when present, was characterized by scattered radial columns of neurodegeneration and tissue

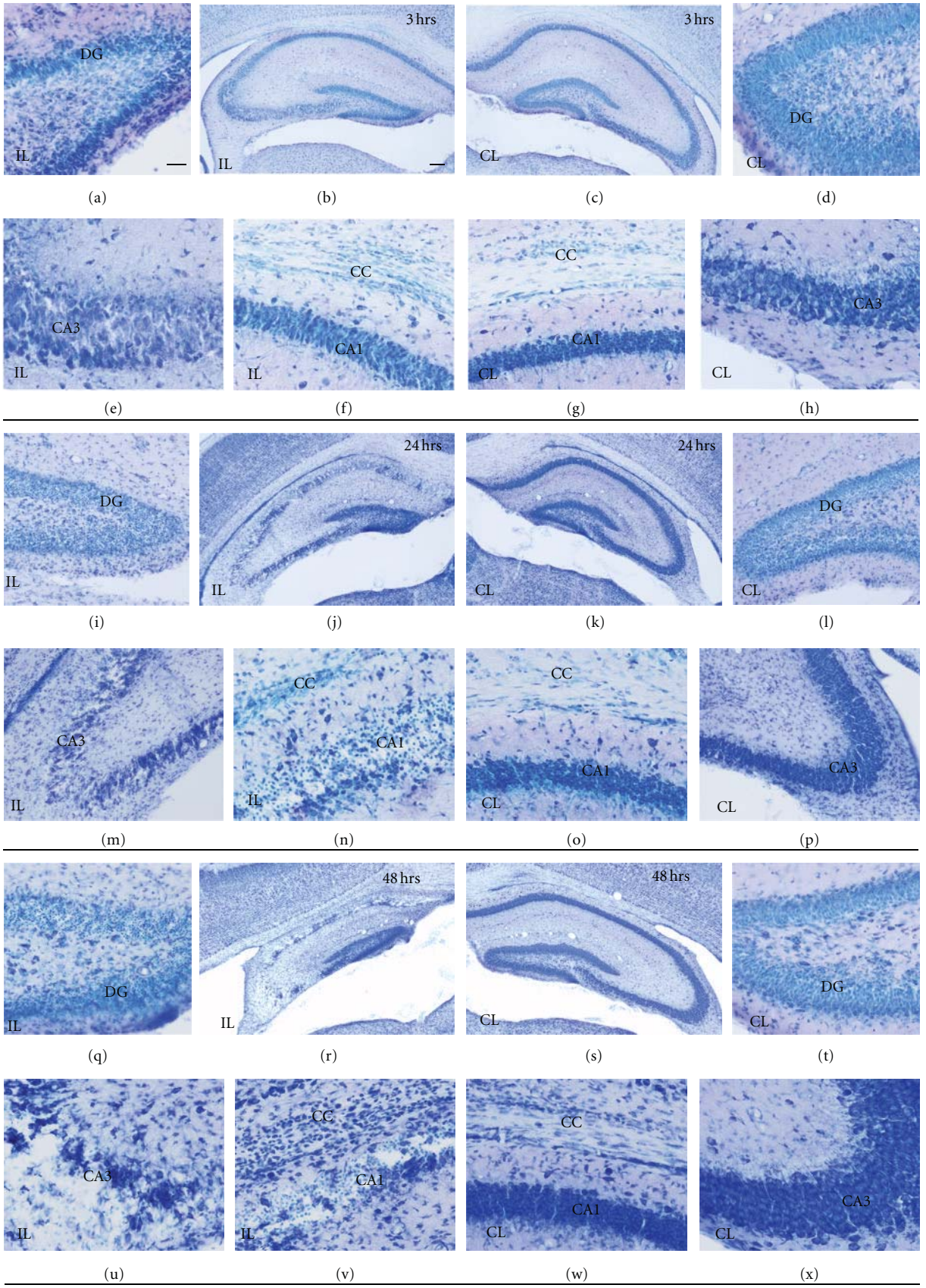


Figure 2: Continued.

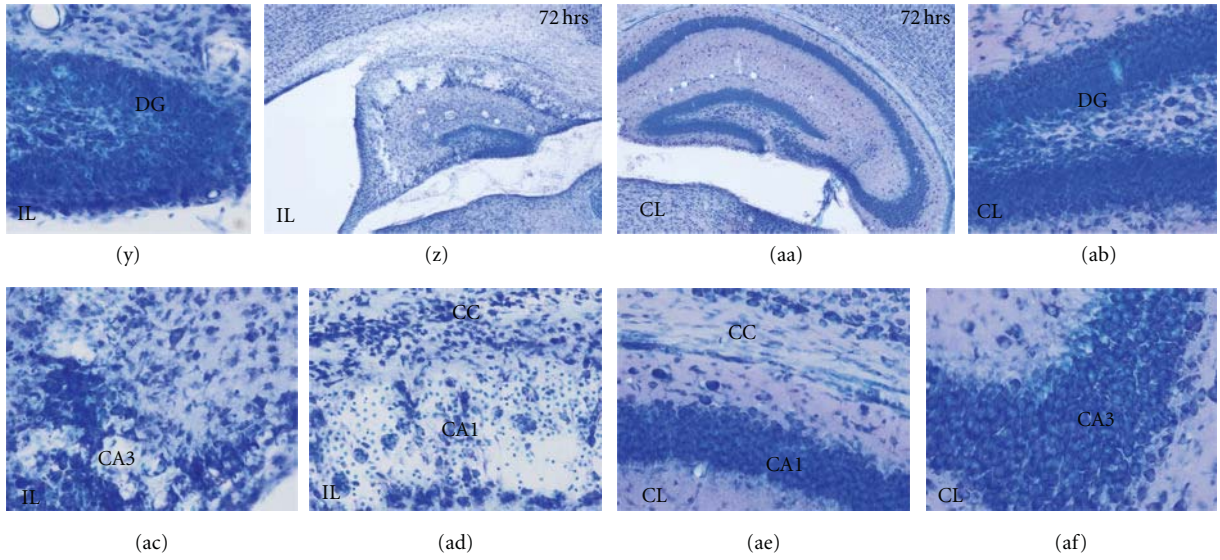


Figure 2: Nissl staining showing hypoxia/ischemia (H/I) effects on the hippocampus and corpus callosum of the contralateral (CL) (right side of the panel), and ipsilateral hemisphere (IL) (left side of the panel), from 3 to 72 hours (hrs) after hypoxia. At 3 hrs (a–h), layer disruption is seen in ipsilateral CA3 (e) and increased cellularity in the ipsilateral corpus callosum (f). At 24 hrs (i–p) neuronal degeneration is widespread in hippocampus (i, j, m, and n). At 48 hrs (q–x) and 72 hrs (y–af), hippocampal atrophy (compare r to s, z to aa) and massive neuronal loss is seen in CA1 and CA3 (r, u, and v for 48 hrs, z, ac and ad for 72 hrs) although the DG is also disorganized (compare q to t, y to ab). Increased cellularity in the corpus callosum is also seen (v and ad). Scale bars (low magnifications: b, c, j, k, r, s, z, aa) = 100 μm; scale bar in all other micrographs = 25 μm. CA1: cornu ammonis 1; CA3: cornu ammonis 3; CC: corpus callosum; DG: Dentate gyrus.

damage, mainly until 12 hours after hypoxia. At 7 days after hypoxia mild atrophy was seen in some cases (Figure 5). Cellular damage in the thalamus was even less frequent but could be observed in some animals, affecting the rostral thalamic nuclei (Figure 3). However, probably as a consequence of ventricle swelling, different grades of thalamic atrophy were seen in most animals at 7 days (Table 2, Figure 5).

3.2. Astroglial Response. Astrocytes were analyzed by GFAP immunostaining and studied in control intact brains from P7, P10, P14, P21 and adult mice, and in the contralateral and ipsilateral hemisphere of hypoxic/ischemic brains from 3 hours to 100 days after hypoxia.

3.2.1. GFAP+ Cells in the Control Postnatal Brain. The distribution and immunostaining intensity of GFAP+ cells changed during postnatal development (Figures 6(a)–6(c)), showing increased GFAP levels at earlier ages, as has been previously reported [46–49]. Briefly, in addition to the GFAP+ radial glial processes still observed at P7 (Figure 7(g)), at the P7–P10 age range, the most intense GFAP+ astroglial cells were found in cortical layer I, the hippocampal fissure (Figure 6(a)) and white matter areas including the corpus callosum (Figure 6(a)), and the fimbria. At P14, GFAP immunoreactivity was generally decreased but it was maintained in cortical layer I, the hippocampal fissure and white matter tracts (Figure 6(b)). By P21 in the adult pattern of GFAP+ cell distribution was established, showing the strongest immunoreactivity in the astroglial endfeet surrounding blood vessels (as in the hippocampal fissure, Figure 6(c)) and in the white matter.

3.2.2. Astroglial Changes in the Contralateral Hypoxic Hemisphere. An astroglial response in the contralateral hemisphere was generally observed, mainly from 3 to 72 hours after hypoxia, and being importantly decreased by 7 days and longer survival times. Increase in GFAP immunoreactivity due to astrogliosis was mainly seen in the hippocampal region (mainly in the hippocampal fissure and the fimbria) and in the cingulum region of the corpus callosum (Figures 6(d)–6(f) compared to age-matched controls in 6(a)–6(c)). Astroglial changes in the contralateral hippocampus were maximal at 24–48 hours after hypoxia (Figure 6(e)). In addition, mild changes were also noted in the neocortex (Figures 7(h) and 7(i)), but no apparent changes were observed in the contralateral caudate-putamen (Figures 7(b) and 7(c)) and thalamus. At 7 days after hypoxia, contralateral hemispheres showed no changes in GFAP+ cell distribution when compared to age-matched controls. In this sense, it is important to note that the contralateral hippocampal and corpus callosum atrophy observed from 14 days post-hypoxia (Figure 5) was not accompanied by noticeable astroglial changes in these areas at late survival times.

3.2.3. Astroglial Changes in the Ipsilateral Hypoxic/Ischemic Hemisphere. Increased GFAP immunostaining and changes in astroglial distribution and astrogliosis were seen in the ipsilaterally damaged hemisphere from 3 hours to the last survival time analyzed (Figures 6–8). The most intense astroglial response was found in the damaged hippocampus although the corpus callosum, the caudate-putamen, the neocortex and the thalamus also showed noticeable astroglial reactivity.

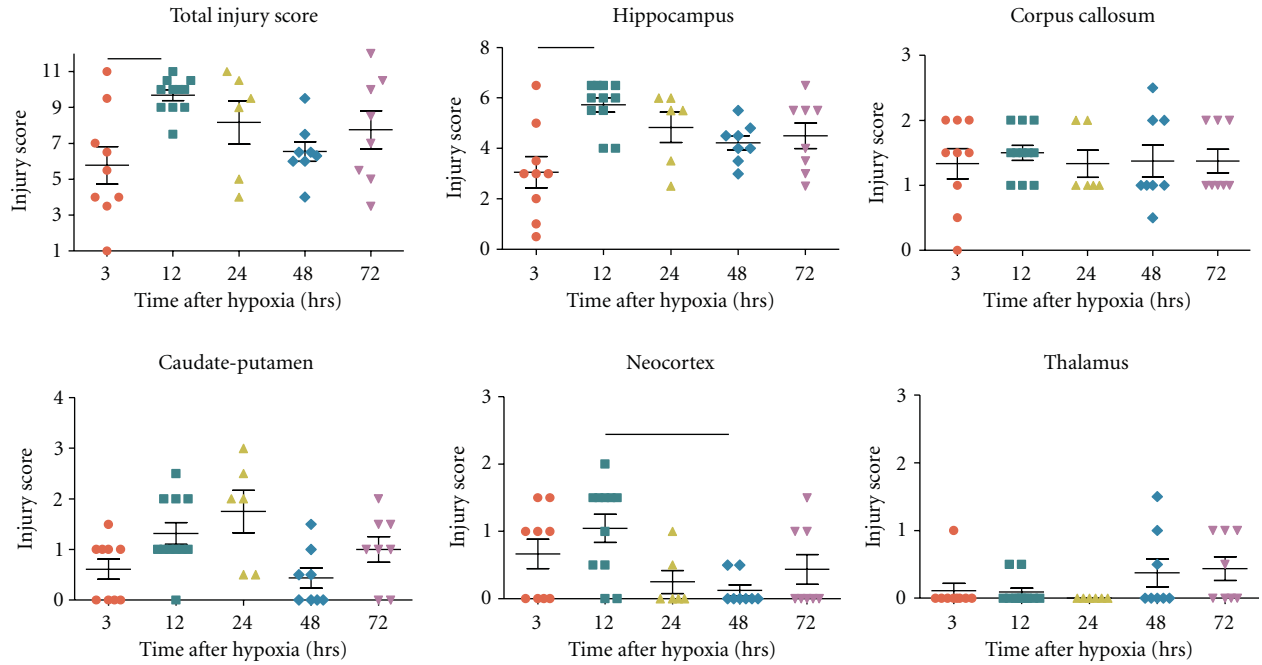


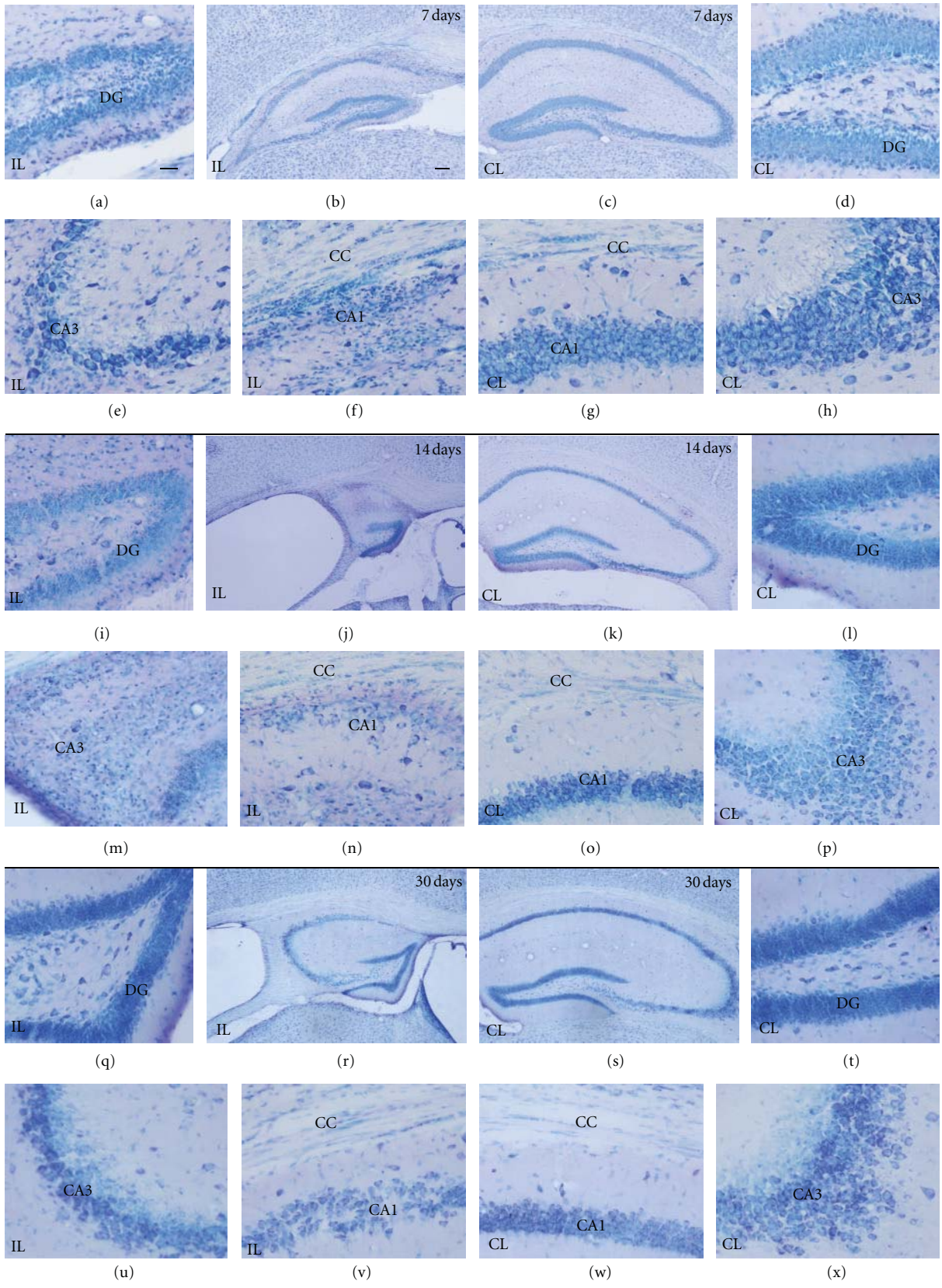
FIGURE 3: Graphs showing the changes in the total injury score along with the injury score in different regions analyzed after 3 to 72 hours (hrs) after hypoxia in Nissl-stained coronal sections. Kruskal Wallis test was done followed by Dunn's multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$ was considered significant.

3.2.4. Hippocampus. At 3 hours after hypoxia, the ipsilateral hemisphere already showed an increase in astroglial GFAP labeling as well as astrogliosis when compared to the contralateral side (Figures 6(d) and 6(g)). At this survival time, and at 12 hours after hypoxia, reactive astrocytes mainly covered the hippocampal fissure, and the molecular and polymorphic layers of the CA field, but no reactive astrocytes were seen within CA pyramidal cell layer or in the DG. At these early survival times, the area occupied by reactive astrocytes was significantly increased in the IL side (Figure 8). At 24 hours, but mainly at 48–72 hours after hypoxia, astroglial processes started to cover the degenerating CA1 and CA3 pyramidal layers and reactive astrocytes concentrated in the hippocampal fissure, the molecular layer and the polymorphic layer of CA1, adjacent to the white matter (Figures 6(h)–6(m)). Astroglial cell response was at this time also evident, to a lower extent, in the DG, mainly in the hilus (Figure 6(j)). As depicted in Figure 8, the percentage of GFAP+ area in the hippocampus was high and significant in IL hippocampus at all survival times. At 7 days after hypoxia, an intense glial scar formed in the degenerated pyramidal layer, around the blood vessels in the hippocampal fissure and in the hippocampal limits (Figure 6(n)). At 14 days after hypoxia, astroglial response in the DG was noticeably decreased although increased GFAP+ cells were often seen in the hilus (Figures 6(o), 6(s)–6(t)). The glial scar was maintained until 100 days after hypoxia (Figures 6(p) and 6(q)).

3.2.5. Corpus Callosum. An increase in GFAP immunostaining and cell density when compared to the contralateral side was already seen at 3 hours after hypoxia (Figure 6(d) and 6(g)), however maximum response was observed at 24–72 hours after hypoxia (Figures 6(h)–6(k)), when reactive astrocytes presented a marked increase in GFAP intensity, showing hypertrophy and increased process thickness. By 7 days, astrogliois clearly diminished (Figures 6(n) and 6(q)), and at 14 days after hypoxia, GFAP immunostaining was strongly decreased and was indistinguishable from controls (Figures 6(o)–6(r)). It should be noted that no striking changes were observed in the quantification of the astroglial response when compared to the contralateral side (Figure 8).

3.2.6. Caudate-Putamen, Neocortex, and Thalamus. An increase in astroglial GFAP immunoreactivity was noted in the caudate-putamen at 3 hours after hypoxia (Figure 7(d)) although no changes in astroglial distribution were seen until later. From 24 hours, astroglial response was mildly increased until 72 hours, when maximum GFAP labeling was reported (Figure 7(e)). Astroglial GFAP expression was close to control values by 14 days after hypoxia (Figure 7(f)), although glial scarring in the caudate-putamen remained in some animals at longer survival times, showing variability (Figure 8). Notably, the area occupied by reactive astroglial cells in the ipsilateral caudate-putamen was above contralateral values at all survival times analyzed even though variability was found in some time points (Figure 8).

In the neocortex, increased GFAP expression and mild astrogliosis were first observed in layers V-VI at 3–12 hours after hypoxia (Figure 7(j)), and it spread to upper layers from 24 to 72 hours (Figure 7(k)), showing significant increases in astroglial response area (Figure 8). At longer survival times,



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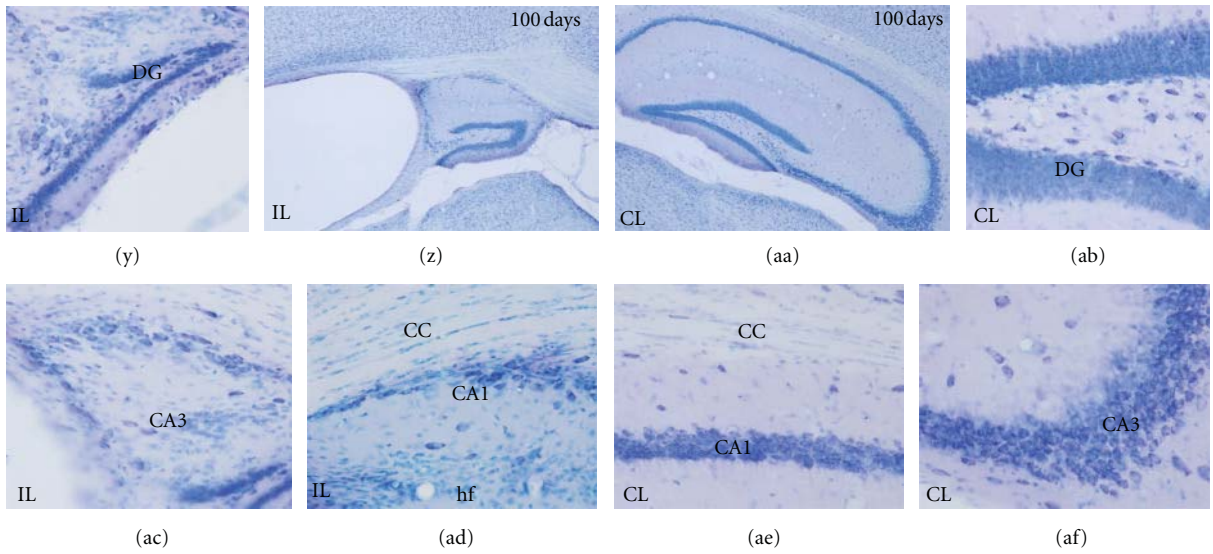


Figure 4: Nissl staining showing H/I effects on the hippocampus and corpus callosum of the contralateral (CL) (right side of the panel), and ipsilateral hemisphere (IL) (left side of the panel), from 7 to 100 days (d) after hypoxia. At 7 d (a–h), overall hippocampal atrophy (b), layer disruption in ipsilateral CA3 (e), and increased cellularity in the ipsilateral corpus callosum (f) are seen. At 14 d (i–p) neuronal degeneration is widespread in hippocampus (i, j, m, and n). At 30 d (q–x) and 100 d (y–af), hippocampal atrophy (compare r to s, z to aa) and massive neuronal loss is seen in CA1 while CA3 is almost disorganized (r, u, and v for 30 d, z, ac, and ad for 100 d), along with the disorganized DG (compare q to t, y to ab). Increased cellularity in the corpus callosum is also visible (v and ad). Scale bars (low magnifications: b, c, j, k, r, s, z, aa) = 100 μm; scale bar in all other micrographs = 25 μm. CA1: cornu ammonis 1; CA3: cornu ammonis 3; CC: corpus callosum; DG: Dentate gyrus.

astrocytic response was clearly diminished (Figures 7(l) and 8) and was practically absent by 14 days after hypoxia. In the thalamus, changes in astrocytes were not observed until 24 hours after hypoxia, showing strong variability between animals (Figure 8). Astroglial response was characterized by patches of reactive astrocytes mainly in the rostral thalamus and only until 7 days after lesion, when glial scarring was noticed. At longer survival times, it was clearly diminished.

3.3. Microglia/Macrophage Response

3.3.1. Iba1+ Microglia/Macrophages Cells in the Control Postnatal Brain. Intense microglial Iba-1 staining was observed at P7 and gradually decreased until adulthood. In postnatal animals, primitive ramified microglial cells were mainly found in the gray and white matter (Figures 9(a), 10(a) and 10(g)) [50] although some amoeboid microglial cells were seen in the cingulum of the corpus callosum, as previously reported [51]. In the hippocampus, the number of microglial cells gradually increased from medial to lateral regions. In addition, round-shaped Iba-1+ macrophages were observed in the pia, very prominently in the medial fissure and in the ventricle linings, as has already been reported [52]. At P10, microglial cells were slightly more ramified, and an increase in cell density was noted, specifically in the corpus callosum, where microglial cells showed a parallel orientation to axon fibers. By P14, microglial cells showed decreased Iba-1 immunostaining (Figure 9(b)) and ramified resting morphology as described for the adult brain [50]. At this age, Iba-1+ macrophages were strongly diminished in the meninges and ventricles. By 21 days after birth, only highly ramified resting microglial cells were observed in the brain parenchyma, showing very low Iba-1 staining (Figure 9(c)).

3.3.2. Microglia/Macrophage Changes in the Contralateral Hypoxic Hemisphere. Microglial activation was generally observed in several areas of the contralateral hemisphere from 3 to 48 hours after hypoxia (Figures 9(d) and 9(e)). Increased expression of Iba-1 and changes in microglial cell morphology towards reactive ramified cells mainly, but also amoeboid cells to a lower extent, were seen in most areas analyzed, but mainly in the hippocampus (very prominently in the hippocampal fissure, Figures 9(d) and 9(e)) and the corpus callosum (Figures 9(d)–9(f)) and other white matter tracts like the anterior commissural and external capsule, where microglial response was seen until 48–72 hours after hypoxia. After 14 days fter hypoxia, only in the hippocampal fissure and corpus callosum of some animals, mild-activated microglia was observed. In the caudate-putamen (Figures 10(b) and 10(c)), neocortex (Figures 10(h) and 10(i)) and thalamus (data not shown), activated ramified microglial cells were seen mainly until 48 hours after hypoxia.

3.3.3. Microglia/Macrophage Changes in the Ipsilateral Hypoxic/Ischemic Hemisphere

Hippocampus. At 3 hours after hypoxia, microglial response in the ipsilateral hippocampus closely resembled that seen in the contralateral side; however, reactive microglial cells

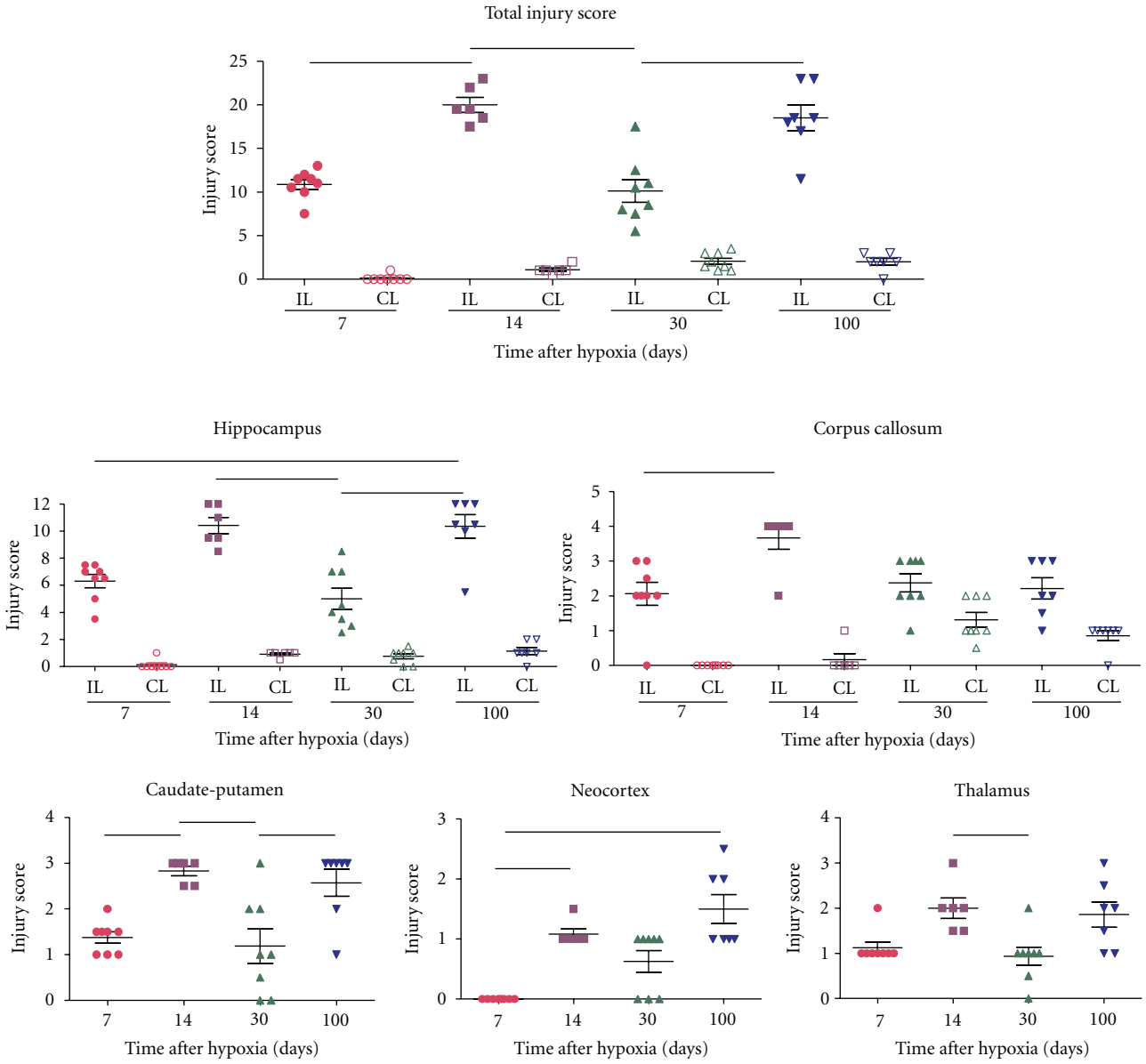
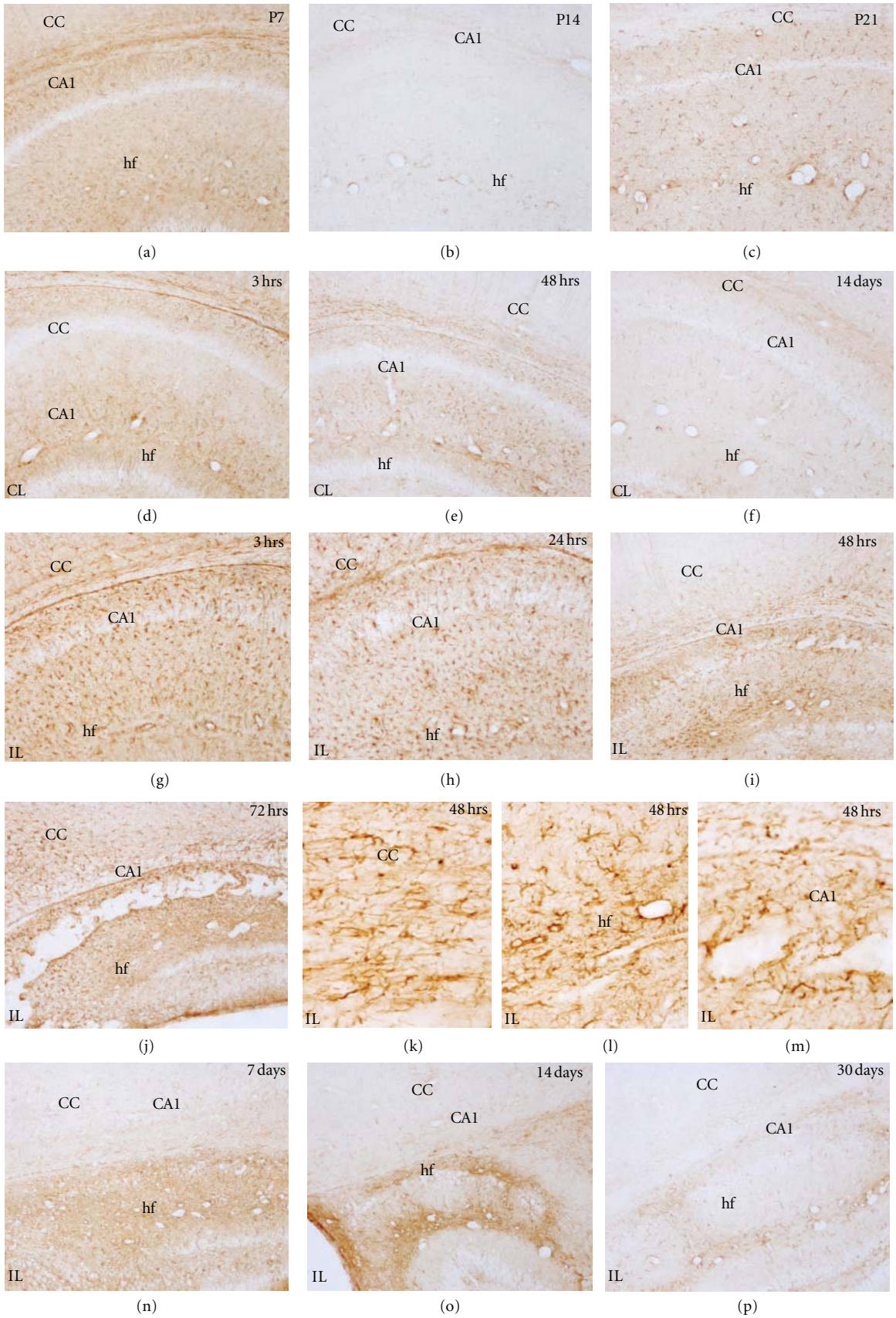


FIGURE 5: Graphs showing the changes in the total injury score along with the injury score in different regions analyzed after 7 to 100 days after hypoxia following Nissl staining. Kruskal Wallis test was done followed by Dunn's multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ is considered significant.

tended to accumulate surrounding the blood vessels in the hippocampal fissure only in the ipsilateral hippocampus (Figure 9, compare 9(d) and 9(g)). By 12 hours, reactive microglial cells changed to pseudopodic/ameboid morphologies and persisted in the fissure, significant differences between IL and CL hippocampus were observed (Figure 11). At 24 hours, increased Iba-1+ macrophages were observed in the third ventricle, and the microglial response was maintained in the hippocampal fissure (Figure 9(h)), but Iba-1+ round-shaped microglia/macrophages started to cover the degenerating CA fields (Figure 9(h)). Notably, at this time, although morphological and distribution changes in the microglial response versus the contralateral hippocampus were evident (Figure 9, compare 9(e) and 9(h)), the area occupied by reactive microglial cells did not differ significantly from the contralateral side (Figure 11), probably as a consequence of the reduced total cell area of pseudopodic/ameboid cells versus ramified cells. From 48 hours to 7 days after hypoxia, a massive increase in microglia/macrophage cell intensity was evident in the fissure and CA field (Figures 9(i), 9(j), 9(l)–9(n)), showing a 5–7-fold increase in the area occupied by reactive microglia/macrophages when compared to the contralateral hippocampus (Figure 11). At longer survival times, microglial response was strongly decreased, showing scattered reactive ramified and macrophages in the fissure and CA only until 14 days (Figures 9(o), 9(s), and 9(t)), but no presence of reactive microglia/macrophages at 30 and 100 days



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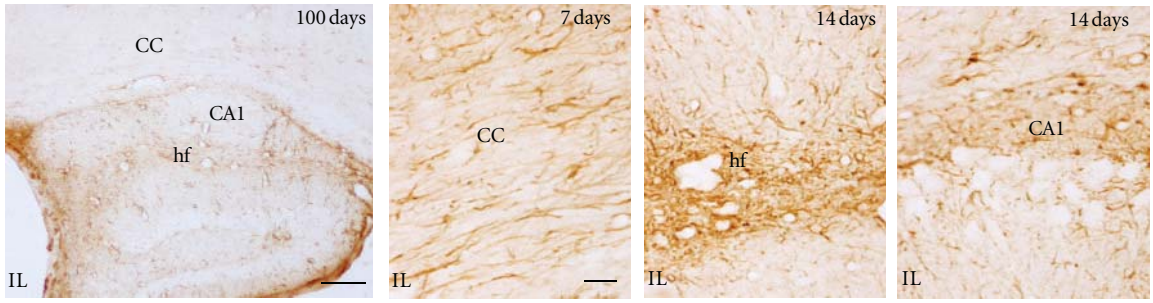


Figure 6: GFAP immunostaining showing age-matched controls and effects of H/I on the hippocampus and corpus callosum. Developmental changes in astrocytes are observed in control animals (a–c), showing a progressive change from more activated astrocytes (a) to resting astrocytes at P14 (b) and P21 (c). Activated astrocytes increase in the contralateral (CL) (d–f), and ipsilateral hemisphere (IL), from 3 hours (hrs) to 100 days after hypoxia (g–t). At 3 hrs (g), astrocyte activation can be seen in the hippocampal fissure, CA and corpus callosum. At 48 hrs (i, m) and 72 hrs, (j) CA layer degeneration is observed. From 7 days (n) to 100 days (t), there is a decrease of the astrocytes activation in the corpus callosum, but in the hippocampus the reduction in astrogliosis starts at 30 days after hypoxia (p). Scale bars (low magnifications: a–j, n–p and t) = 100 μm; scale bar in all other micrographs = 20 μm. CA1: cornu ammonis 1; CC: corpus callosum; hf: hippocampal fissure.

(Figures 9(p), 9(q)). It should be noted that only scattered activated microglial cells were present in the DG, and always located in the hilus, correlating with the above described astroglial response in this area which is mostly spared in this neonatal injury model as a consequence of its late development [53, 54].

Corpus Callosum . The corpus callosum, like other white tracts including the internal and external capsules, showed microglial response characterized by the presence of reactive ramified cells elongated in parallel to axonal tracts, from a few hours after the insult (Figure 9(g)), and some ameboid microglia/macrophages observed at 24–72 hours after hypoxia (Figures 9(h)–9(k)) and until 7 days (Figure 9(n)), when response diminished (Figures 9(o) and 9(r)), almost returning to basal level at 14 days after hypoxia. However, it should be noted that in this region only mild differences in relation to the contralateral side were seen, with no statistically significant differences shown in the Iba-1+ area at any timepoint (Figure 11), This pattern of microglial response in the ipsilateral versus contralateral white matter correlated with the mild response of astroglial cells described above although the changes in glial cells of the contralateral corpus callosum, which also results mildly atrophied, may be masking the increases in glial response in the ipsilateral side.

Caudate-Putamen, Neocortex, and Thalamus. In general, in these areas, microglial response was also seen as early as 3 hours after hypoxia and lasted until 7 days although it showed a high degree of variability and very few significant differences in compared to the contralateral hemisphere (Figure 11). Reactive microglial cells mainly showed an activated ramified morphology and increased Iba-1 labeling (Figures 10(d)–10(f) and 10(j)–10(l)) although some pseudopodic/amoeboid microglial cells were seen from 12 to 72 hours after lesion, when maximum responses were seen (Figures 10(e) and 10(k)). In the caudate-putamen, Iba-1+ cells have shown the higher activation in the ventral-lateral region. At 7–14 days after hypoxia, in all three regions, microglial response remained as patches of reactive ramified microglial cells (Figures 10(f) and 10(l)).

3.4. Neutrophil Recruitment

3.4.1. Distribution of Neutrophils in the Control Postnatal Brain. Neutrophils were generally not present in control brain parenchyma. Only scattered neutrophils were seen in the medial or lateral third ventricle at P7–P21, in decreasing numbers with hardly countable cells at P21. At these ages we also observed a few cells in blood vessels located in hippocampus and neocortex of both hemispheres (Figures 12(a)–12(c)). Scattered neutrophils were also seen in the meninges/median fissure. In comparison to adults, neonates are known to have weakened neutrophil response and reduced tendency to extravasate from blood vessels [55–57].

3.4.2. Distribution of Neutrophils in the Contralateral Hypoxic Hemisphere. At 3 and 12 hours after hypoxia, some neutrophils were observed inside the blood vessels in the neocortex, caudate-putamen, and in the hippocampus, but also in the lateral side of third ventricle. By 24–72 hours, neutrophil cell numbers decreased in the blood vessels of neocortex and in the third ventricle (Figure 13). At 7–14 days after hypoxia, some neutrophils were observed in the medial third ventricle (Figure 13), the neocortex, and the thalamus. At 30 and 100 days after injury, there was hardly any cell found in the brain blood vessels or the parenchyma (Figure 13).

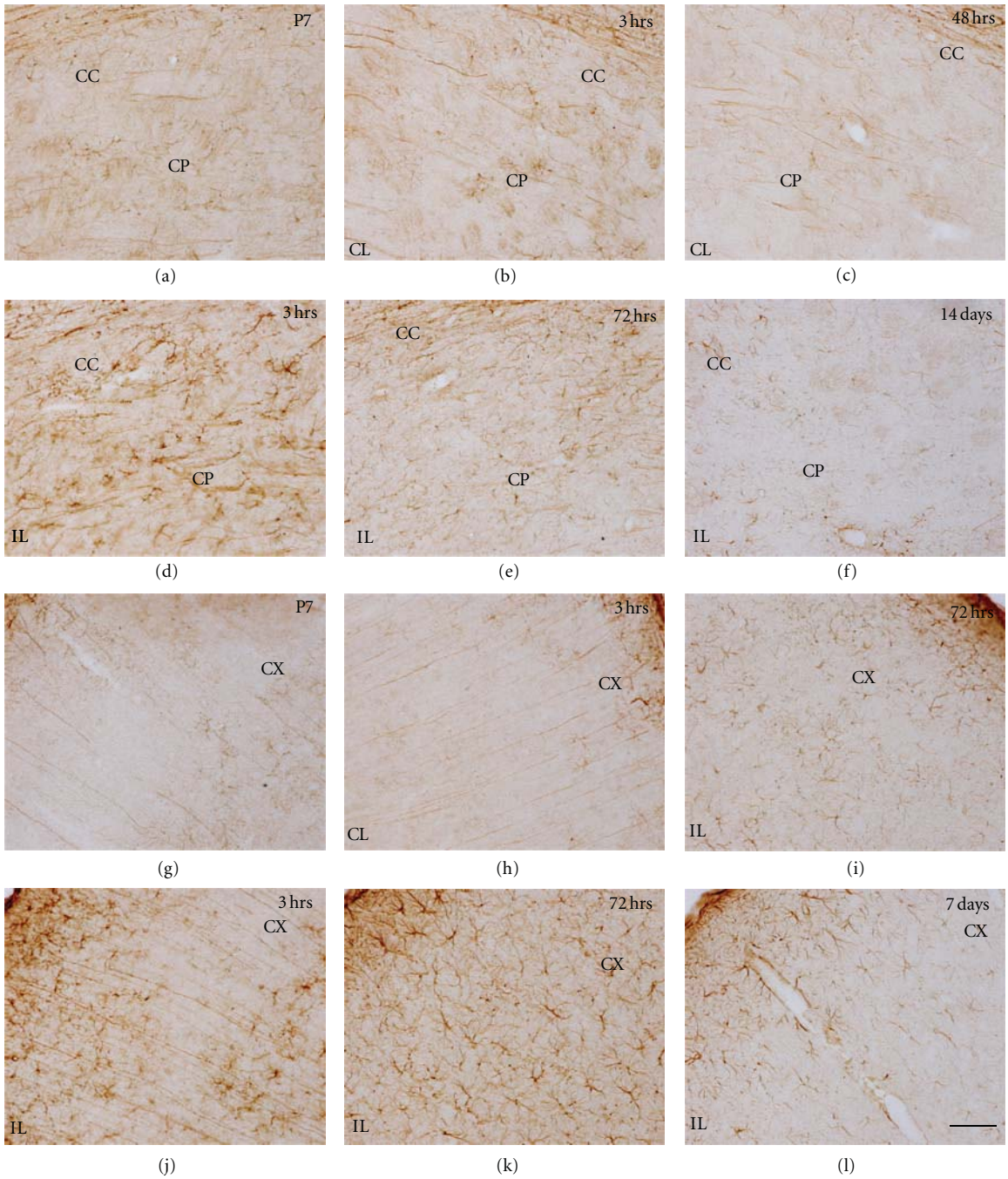


Figure 7: GFAP staining showing effects of H/I on the caudate-putamen (a–f) and cortex (g–l) of the contralateral (CL) (b, c and h, i) and ipsilateral hemisphere (IL) from 3 hours (hrs) to 14 days after hypoxia (d–f and j–l). Reactive astrocytes are seen in ipsilateral side from 3–72 hrs (d, e) as compared to the P7 control (a) or the contralateral side (b, c). At 14 days after hypoxia the astrocytes reactivity decreases (f). The cortex at 3 hrs (j) shows increase in reactive astrocytes and radial glia-like structures (only at this age) as compared to P7 control (g) and contralateral side (h) with a maximum reactivity at 72 hrs (k) when compared to the respective contralateral side (i); and a decrease in astroglial reactivity is seen at 7 days after hypoxia (l). Scale bar = 100 μm. CP: caudate-putamen; CC: corpus callosum; cx: neocortex.

3.4.3. Distribution of Neutrophils in the Ipsilateral Hypoxic/Ischemic Hemisphere

Hippocampus. Neutrophils were observed in the ipsilateral hippocampus as early as 3 hours after hypoxia (Figure 13). Cells were usually found distributed in the hippocampal fissure, the dentate gyrus, or the fimbria. At 12 hours after hypoxia, the number of cells increased and was localised in the CA3 region, in the parenchyma as well as inside the blood vessels. In the hippocampal fissure, the dentate gyrus and in the fimbria, most of the neutrophils were inside the blood vessels (Figure 13). At 24 hours after hypoxia, neutrophils

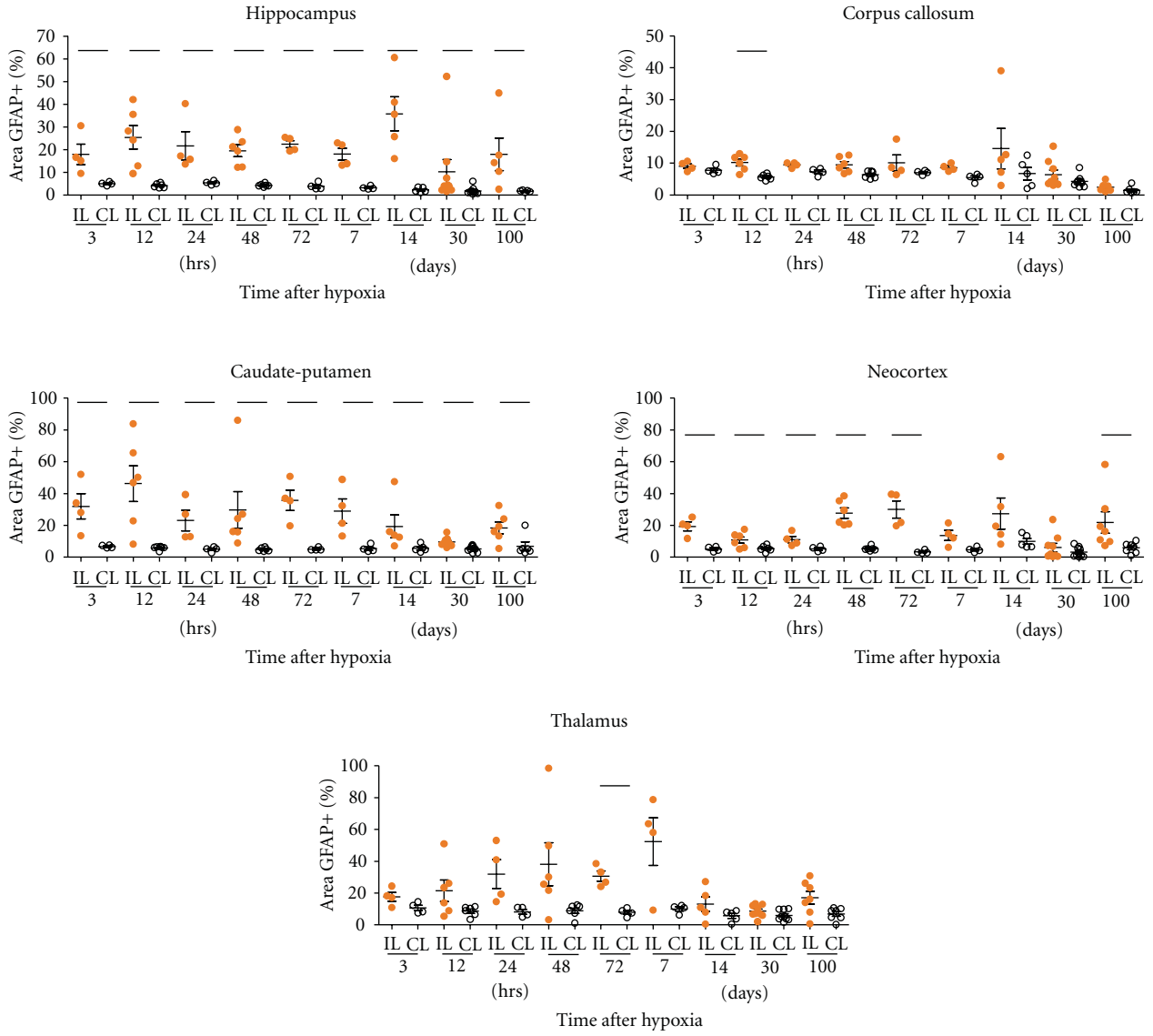
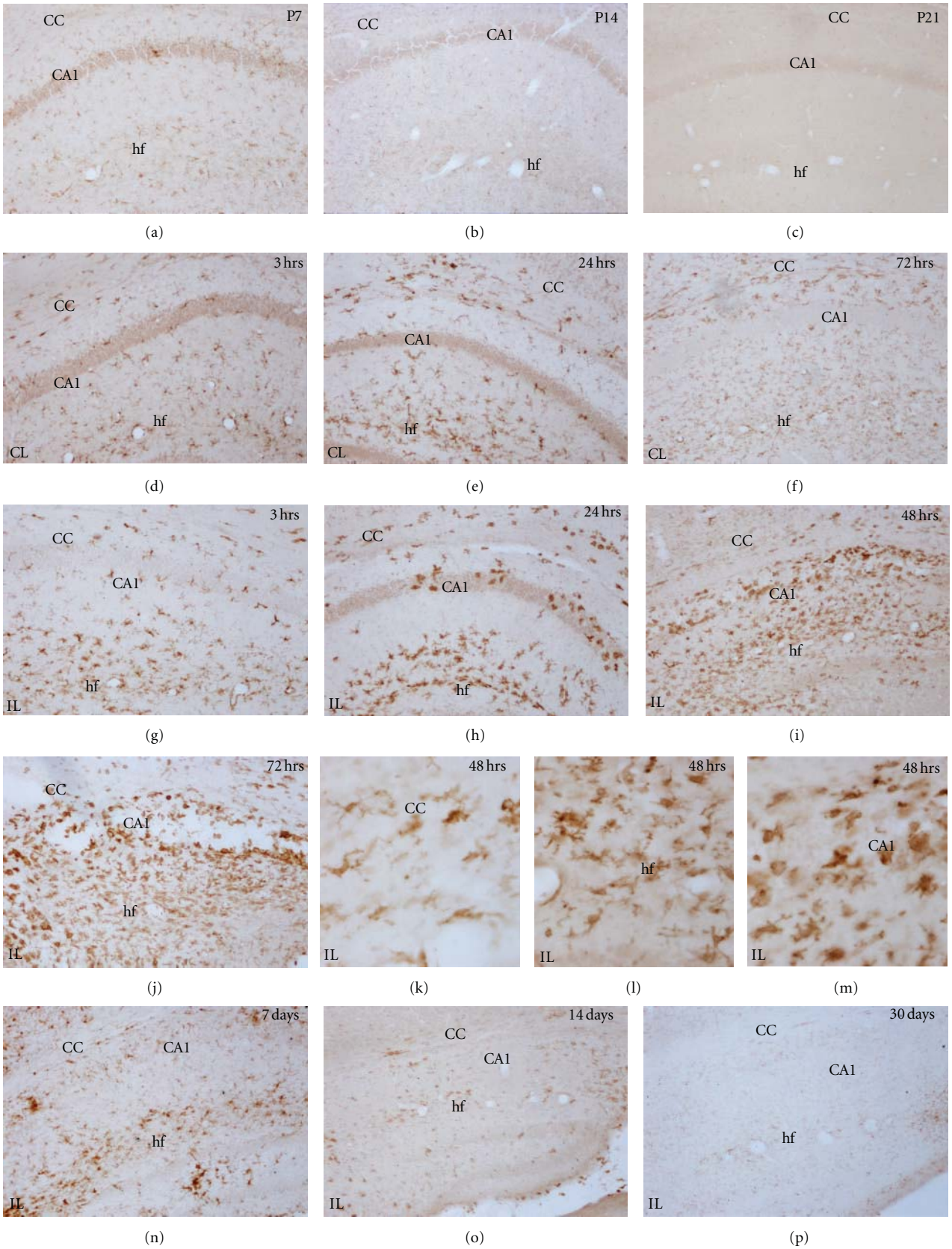


Figure 8: GFAP+ area on the hippocampus, corpus callosum, caudate-putamen, neocortex, and thalamus is evaluated from 3 hrs to 100 days after hypoxia in the ipsilateral (IL) and contralateral (CL) hemispheres. Astrogliosis is shown as the percentage of the GFAP+ area (see Section 2 for details). The hippocampus and caudate-putamen are the most affected regions and significant differences between IL and CL hemispheres are found at all time points. The astroglial reactivity in corpus callosum is observed at 12 hrs after hypoxia between IL and CL. Higher astrogliosis is observed in the ipsilateral neocortex at almost all times analyzed compared to CL side. A significant increase in astroglial activation after 72 hrs is seen in the IL thalamus. Significant differences between IL and CL hemisphere are shown using unpaired t tests, with Welch's correction if suitable ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Individual data and mean ± S.E.M, are represented to show the dispersion in each group.

were observed throughout the hippocampus but mainly localised in CA1 region and the fimbria (Figure 13). By 48 hours after hypoxia, neutrophils were not observed in the dentate gyrus though a few cells were present near CA3 and the fimbria (Figure 13). Neutrophils appeared to be evenly distributed throughout the hippocampus after 72 hours after hypoxia, but significantly higher density of cells were observed at 7 days after hypoxia (Figures 12(d)–12(f) and 13). At this time of maximum neutrophil numbers, the cells were mostly observed near the hippocampal fissure, CA1 and CA3 region, with the majority of cells in the parenchyma, but usually concentrated near the blood vessels (Figures 12(e) and 12(f)). At 14 days after hypoxia, the amount of cells rapidly decreased although a few cells were still found, in close opposition to blood vessels in the hippocampal fissure and around the CA3 region. At 30 days after hypoxia, very few neutrophils inside the blood vessels could be identified, and at 100 days after hypoxia no neutrophils were seen inside the hippocampus.

Ventricles. An elevated number of cells were also present in the third ventricle, both medially and in the ipsilateral



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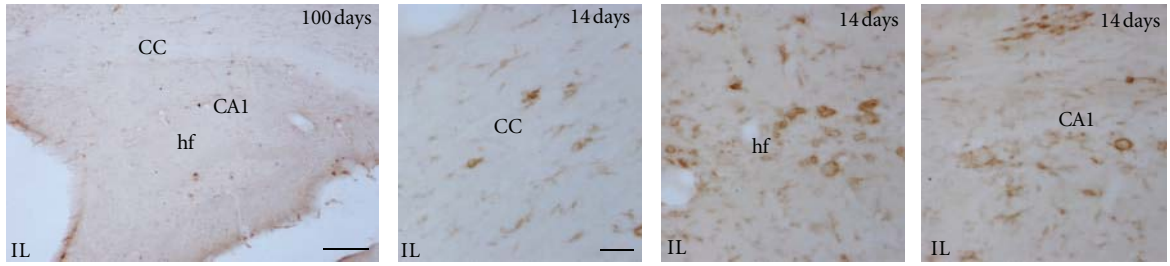

(q) (r) (s) (t)

Figure 9: Iba-1 immunostaining showing age-matched controls and H/I effects on the hippocampus and corpus callosum. Developmental changes in microglia are observed in control animals (a–c), showing a progressive change from mainly primitive ramified and amoeboid cells (a) to a resting morphology at P14 (b) and P21 (c). Activated microglia increases in the contralateral (CL) (d) and ipsilateral (IL) hemisphere at 3 hrs (g). At the CL side, Iba-1 shows the maximum labeling at 24 hrs (e) in the hippocampal fissure, returning to control level at 72 hrs (f). In the IL side, higher proportion of amoeboid cells is seen from 24 hrs (h) to 72 hrs (j). At 7 days after hypoxia (n), a reduction on the level of microglia morphologically activated is observed, returning to the basal level at 14 days (o). Resting morphology is observed at 30 (p) and 100 days (q). Detailed morphology of Iba-1+ cells in the cc (k), hf (l), and CA1 (m) observed after 48 hrs and 14 days after H/I (r, s and t, resp.) are shown. Scale bars: in low magnifications: (a) to (j) and (n) to (q) = 100 μm; scale bar in (k, l, m r, s, and t) = 20 μm. CA1: cornu ammonis 1; hf: hippocampal fissure; CC: corpus callosum.

side of the third ventricle as early as 3 hours after hypoxia (Figures 12(g) and 13). At 12–48 hours, the quantity of cells progressively decreased, but they were mostly distributed in the medial part (Figure 13). By 7 days after hypoxia, correlating with increased numbers also in hippocampus, an increase in neutrophils both in the medial and ipsilateral side of the ventricle could be seen (Figure 13). Finally, by 14 to 100 days, no neutrophils were seen in the lateral side of the third ventricle although scattered cells were located in the medial part.

Caudate-Putamen, Neocortex, and Thalamus. From 3 hours to 72 hours, only a few neutrophils were located in the caudate-putamen region (Figure 13). An increase in the number of cells was seen at 7 days (Figures 12(h) and 13), correlating with previously described areas. At longer survival times, no neutrophils were seen in this region.

At 3 hours after hypoxia, some neutrophils were distributed in the blood vessels of different layers of the neocortex (Figure 12(i)), being the time showing the highest density (Figure 13). From 12 to 72 hours a reduction in neutrophil cell counts was generally observed although by 72 hours a few cells remained in the upper layers of neocortex. At 7 days after hypoxia, there was a mild increase in neutrophils located inside the cortical blood vessels in both hemispheres. At 30 and 100 days, almost no neutrophils were present in the neocortex, and if so, they were located inside the blood vessels (Figure 13).

In the thalamus, very few cells were observed as compared to the other regions analysed. No neutrophils were observed from 3 to 48 hours after hypoxia, and only a few cells were seen at 72 hours, 7 and 14 days (Figures 12(j) and 13). From 30 days, neutrophils were no longer present in the thalamus (Figure 13).

3.5. Lymphocyte Distribution in the Control and the Hypoxic/Ischemic Brain. In the control brain and at all ages analysed, scattered lymphocytes were only located in the ventricles and meninges, although scattered single cells were sometimes seen in the hippocampus, neocortex, always inside the blood vessels (Figures 12(k), 12(l) and 12(m)). At all time points analysed after hypoxia, no changes were seen in the contralateral or the ipsilateral hemisphere when compared to control.

4. Discussion

In this study we have performed a detailed short and long-term analysis of neuropathological changes, astroglial, microglial response, and leukocyte recruitment following H/I to the neonatal mouse brain, describing massive damage and cellular changes in the ipsilateral hemisphere, but also not negligible changes in the contralateral side. Several of these results will be discussed in separate sections.

4.1. Neuropathological Changes in the Ipsilateral H/I Hemisphere. Our description of neuropathological changes in the ipsilateral hemisphere is in agreement with previous reports [18, 42, 58, 59], showing hippocampal damage as the most striking feature of hypoxic/ischemic damage in the neonatal mouse, whereas damage to caudate-putamen, neocortex, and thalamus is highly dependent on the postnatal age and the duration of the hypoxia. Hippocampal damage with tissue disruption, neuronal damage, and disorganization of the CA cytoarchitecture was observed as early as 3 hours after hypoxia followed by milder damage to DG at later survival times, which is maintained relatively spared due to its postnatal development. At 7 days after hypoxia, significant atrophy of hippocampal area is evident. This temporal pattern of neurodegeneration is consistent with the observation from other studies showing that H/I damage in an immature brain evolves more rapidly than its adult counterpart [40, 60].

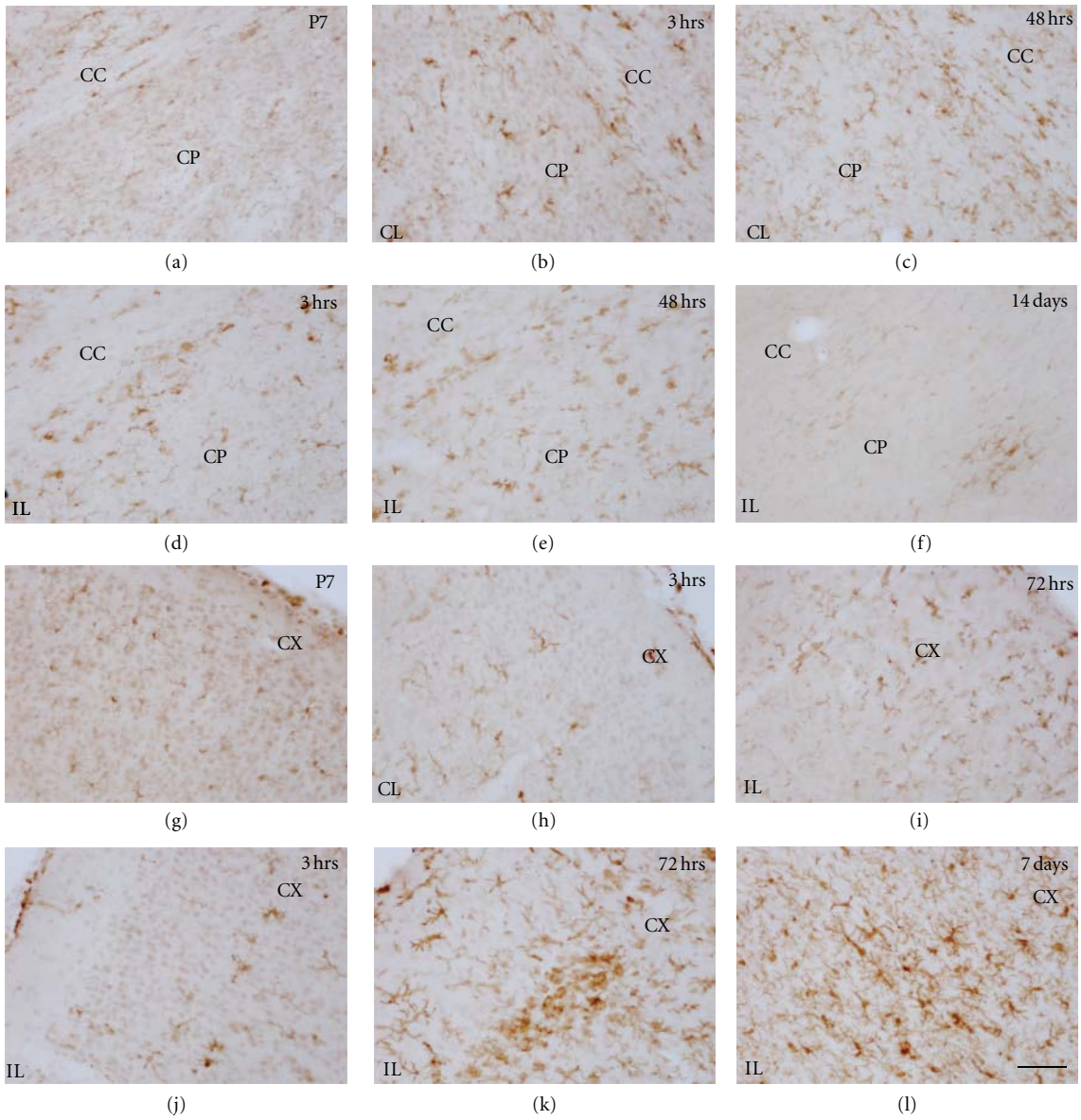

Figure 10: Iba-1 immunostaining in control animals and H/I effects on the caudate-putamen (CP) (a–f) and neocortex (CX) (g–l). Control brain P7 shows basal expression of microglial Iba-1 in the CP (a) and CX (g). In the CP, activated microglia increase in the contralateral (CL) hemisphere at 3 hrs (b) up to 48 hrs (c). Higher activation is observed in the ipsilateral (IL) hemisphere at 3 hrs (d), showing a maximum at 48 hrs (e), and slowly returns to resting morphology with some patches of activated microglia after 14 days (f). Similar pattern is observed in the neocortex, CL hemisphere shows differential expression with respect to control animals from 3 hrs (h) to 72 hrs (i). Higher activation is observed in the ipsilateral (IL) hemisphere at 3 hrs (j), a clear amoeboid patch pattern is observed in the neocortex after 72 hrs (k), which slowly returns to resting morphology with some patches of primitive ramified microglia after 7 days (l). Scale bars: 50 μm.

We observe subcortical white matter damage and long-term atrophy, which has been described as a hallmark of neonatal H/I in preterm infants, where the oligodendrocytes in the periventricular white matter are considered one of the most vulnerable cell types to H/I damage [61, 62]. In rodent models, neonatal H/I injury has been shown to cause axonal degeneration [63] and disturbances in myelination [64, 65]. Following H/I in the P9 mouse, several authors have reported decreased expression levels of myelin basic protein (MBP) and proteolipid protein (PLP), decreased neurofilament expression, and the presence of apoptotic cells in the corpus callosum within 24 to 72 hours after injury [66, 67]. White matter damage has been related to the loss of immature oligodendrocytes in the tracts as well as the loss of subventricular zone (SVZ) progenitors after H/I, inducing a depletion of oligodendrocyte precursors [68, 69].

Another area showing consistent damage and atrophy in the mouse model of H/I is the caudate-putamen, and the neocortex to a lesser extent and showing higher variability. In this regard, a recent study by Selip and coworkers using the

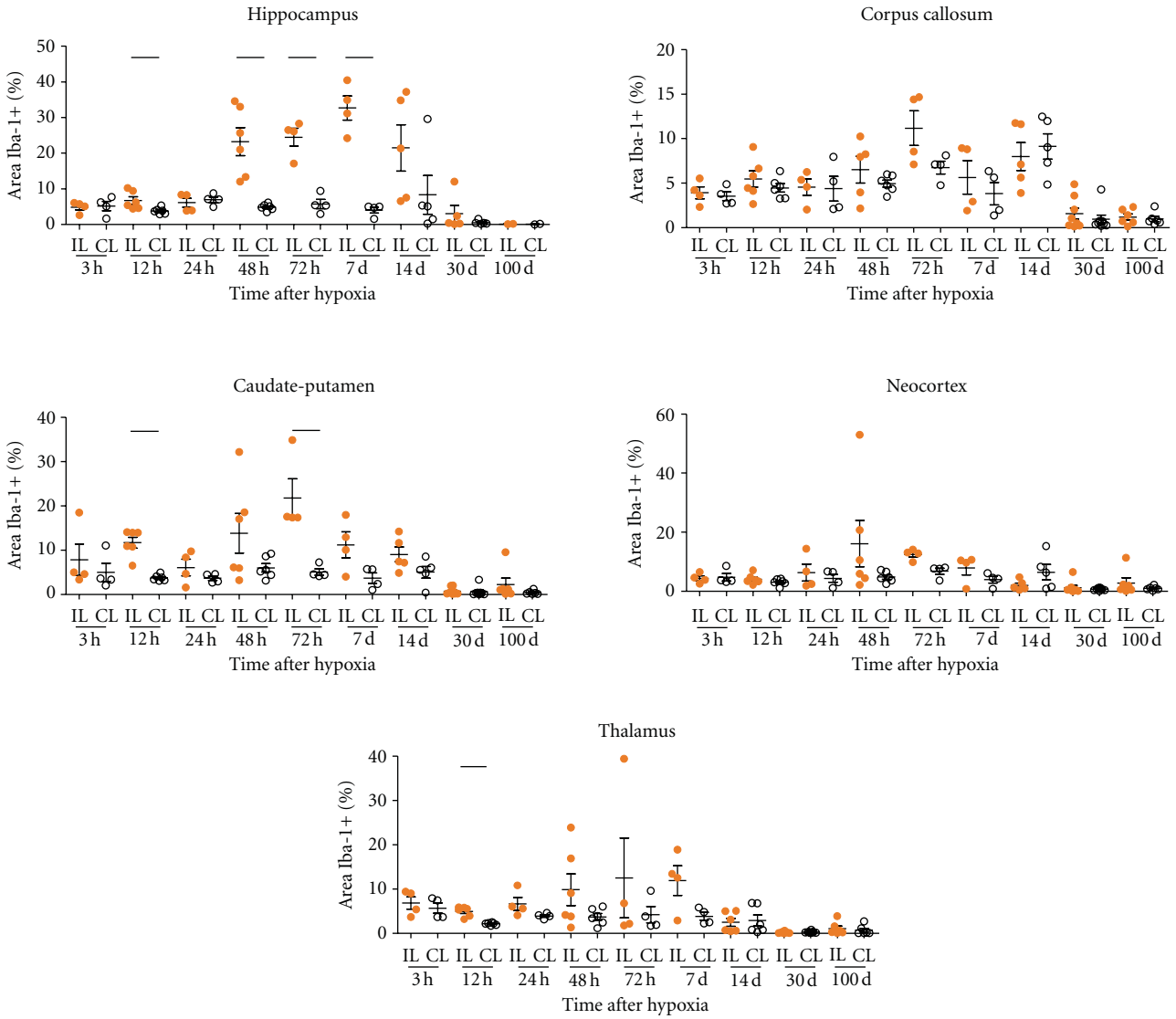

Figure 11: Iba-1+ area on the hippocampus, corpus callosum, caudate-putamen, neocortex, and thalamus is evaluated from 3 hrs to 100 days after hypoxia in the ipsilateral (IL) and contralateral (CL) hemispheres. Reactive microglia is shown as the percentage of the Iba-1+ area (see Section 2 for details). The hippocampus is the most affected region by H/I at 12, 48, 72 hrs and 7 days, as can be observed between IL and CL hemisphere. Significant differences on Iba-1+ area are observed at 12 and 72 hrs after hypoxia in the caudate-putamen and after 12 hrs in the thalamus. Significant differences between IL and CL hemisphere are shown using unpaired t tests, with Welch's correction if suitable ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Individual data and mean ± S.E.M, are represented to show the dispersion in each group.

neonatal rat model [70] have shown that rats with moderate or severe loss of MBP having significantly increased axonal degeneration in the temporal-parietal cortex, caudate-putamen, thalamus, and internal capsule. Moreover, pups without evidence of severe white matter loss exhibited mild selective grey matter injury, as evidenced by mild axonal injury and neuronal degeneration, in the cortex, internal capsule, and caudate-putamen; structures central to language processing and understanding, and motor and sensory function. Injury in these regions, even if mild, may be implicated in the neurocognitive disturbances noted in preterm survivors who do not demonstrate other clinical or radiological evidence of overt periventricular white matter injury [71]. It is interesting to note that we here describe in the mouse that caudate-putamen and cortical atrophy are mainly noted as a long-term effect but show very disperse injury scores at early survival times.

4.2. Contralateral Hippocampus and Corpus Callosum Show Mild Long-Term Atrophy. Moreover, the effect of H/I in the contralateral hemisphere has been studied extensively to suggest that it cannot be used as an efficient control for histological assessment of brain damage in mice, in contrast to what has been described previously in the neonatal rat [60, 72, 73], providing an significant difference in these species response to H/I.

Previous studies using the rat model of H/I have demonstrated that the blood flow to the contralateral cerebral

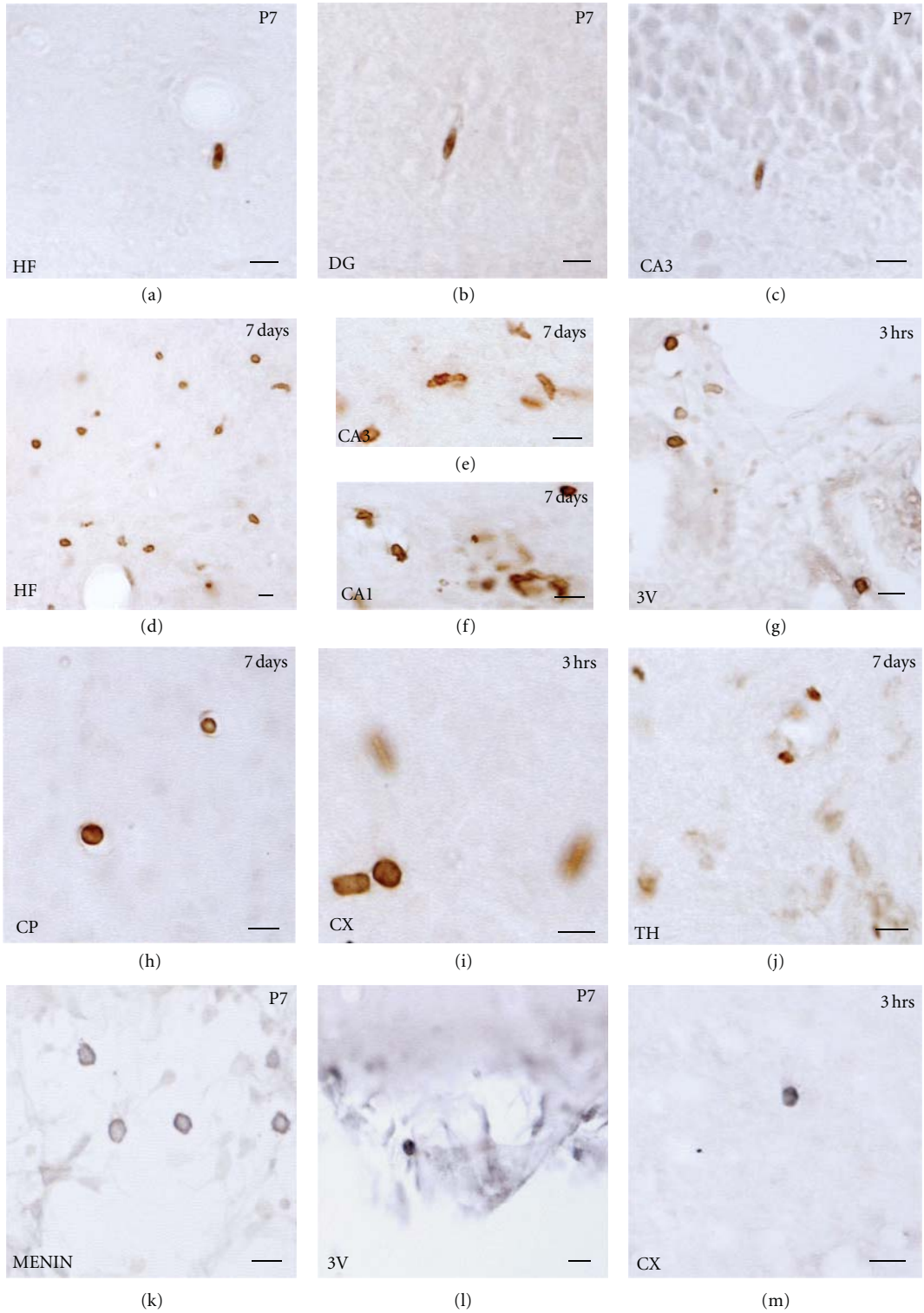

Figure 12: Leukocyte infiltration is monitored by analysing neutrophils (Ly6B2 staining) and T-lymphocytes (CD3 staining). Ly6B2 staining in the control animals at P7 shows a few neutrophils inside the blood vessels in the hippocampal fissure (a), in DG (b), and in the CA (c) regions. In the hippocampus the maximum density of cells is observed at 7 days after hypoxia especially in the hippocampal fissure (d); CA3 (e) and CA1 (f). In caudate-putamen an increase is observed at 7 days after hypoxia (h). In the ventricles (g) and the neocortex (i) the maximum quantity of neutrophils is observed at 3 hours after hypoxia. No significant difference was observed in the thalamus (j). CD3 staining in the control animals shows the presence of lymphocytes in the meninges (k) and in the ventricles (l). Finally, scattered cells are observed in the neocortex (m). Scale bars: 10 μm. Hf: hippocampal fissure; DG: Dentate gyrus; CA: cornu ammunis.

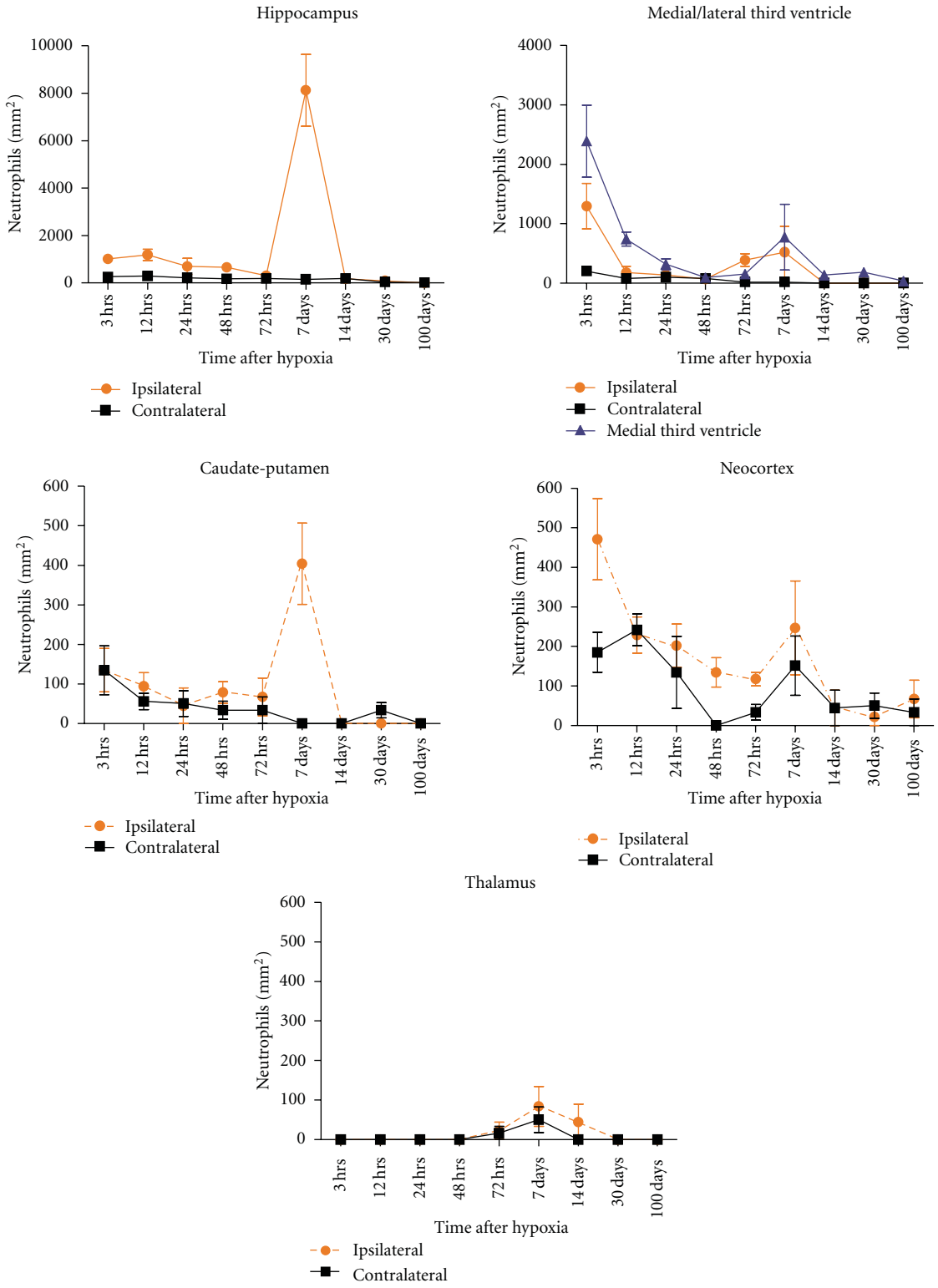

Figure 13: Neutrophils quantification is done using image J software (NIH) and is evaluated at 3–72 hours and 7–100 days. The regions analysed were the hippocampus, medial/lateral third ventricle, caudate-putamen, neocortex, and thalamus. The quantity in the ipsilateral side is compared to the contralateral hemisphere. Significant changes are observed in the ipsilateral side of the hippocampus and caudate-putamen at 7 days after hypoxia while a decrease in number is observed after 3 hours in the ventricles and neocortex. There is no change in the thalamus. All values are represented as mean ± S.E.M and are corrected using Abercrombie correction method. Two-way ANOVA with Bonferroni post-hoc analysis is used to compare ipsilateral versus contralateral hemisphere at all time points and $^{*}P < 0.01$, $^{**}P < 0.001$ was considered significant.

hemisphere structures is relatively unchanged during hypoxia [74], and that the contralateral hemisphere, when evaluated several weeks after the injury, shows no tissue alterations or atrophy, suggesting that the contralateral hemisphere can be used as a "control" reference for the evaluation of the extent of damage in the ipsilateral hemisphere in the rat [73]. Some molecular changes in kinases and proinflammatory molecules have been described in both hemispheres in H/I neonatal rats [75, 76]. Also Jansen and Low [72] histologically assessed a hypertrophy of the contralateral hemisphere in adult rats that had undergone perinatal H/I. In the mouse brain, several laboratories have shown that it does not suffer apparent changes during the first week following H/I [18] as is also commonly used as a reference to evaluate the ipsilateral hemisphere. Interestingly, inflammatory gene profiling in P9 mouse brain after H/I shows more than 140 genes involved in the tissue response during the first 72 hours; however, only microglial expression of osteopontin showed an increase in contralateral subcortical white matter [77]. While mice and rats show distinguished regional features in tissue damages, it would be interesting to analyze the molecular changes in HI-neonatal mice. Nevertheless, it should be noted that we here report that analysis of the H/I mouse brain up to 3 months after the injury shows some degree of atrophy in the contralateral hippocampus and the corpus callosum, accompanied by ventricle swelling, an event that has been reported earlier [78].

The immature brain has a tendency of considerable compensatory reorganization following injury. There are reports stating compensatory reorganizational changes occurring in the contralateral hemisphere in some animals following neonatal H/I brain injury and that this plasticity may be functionally advantageous [72]. Moreover, the presence of significant cognitive deficit in apparent unilateral focal brain injury also indicates towards the involvement of contralateral hemisphere [79]. In this sense, it should be noted that H/I animals undergo systemic hypoxia, which has been shown to induce changes in gene expression and cell activity by itself. As an example, change in the expression of certain cytokines, like Hypoxia Inducing Factor alpha (HIFα), and P-Akt to the same extent in both the ipsi—as well as contralateral hemisphere showed that hypoxia is sufficient to regulate multiple mediators that may contribute, but may not be sufficient to induce long-term neuronal damage [76].

4.3. Glial Response

4.3.1. Transient Astroglial Response in the Contralateral Hippocampus and White Matter and Long-Term Glial Scar Formation in the Ipsilateral Hemisphere. As reviewed by Sofroniew and Vinters [80], many gray matter astrocytes in healthy CNS do not express GFAP at immunohistochemically detectable levels or express low levels as in neonates. In our immunohistochemically processed sections, although GFAP expression was seen in control neonatal brains, an increase in GFAP immunostaining was observed after H/I from early time points (3–12 hours) in comparison to P7 age-matched control, implying an onset of astrogliosis.

Notably, changes in astroglial morphology by GFAP immunostaining were first seen at 3 hours post-hypoxia both in the ipsilateral as well as contralateral hemisphere. In the contralateral hemisphere, increased GFAP and astroglial hypertrophy showed a maximum response at 24–48 hours after hypoxia but decreased at longer survival times. In the contralateral side, astroglial response was very restricted to the corpus callosum and the area of the hippocampal fissure, but never covering the CA-neuronal layer. However, in the ipsilateral H/I-damaged hemisphere, the increase in GFAP expression and cell hypertrophy peaked at 14 days after hypoxia and was evident in the corpus callosum, the caudate-putamen, the neocortex, and the hippocampus, where the long-term glial scar persisted till 100 days after hypoxia.

As the radial glia mature, they show GFAP expression and some give rise to GFAP-expressing radial neural stem cells (NSCs) that persist in juvenile and adult forebrain, while others become astrocytes [81–83]. Some of these radial NSCs remain constitutively active throughout life in the subventricular zone of the lateral ventricles and in the subgranular zone of the hippocampal dentate gyrus, where they are the predominant source of adult neurogenesis. This might be the reason of concentration and persistence of glial scar or GFAP+ cells at 14–100 days after hypoxia in hilus and hippocampal fissure in our study. Notably, GFAP expression after H/I was also found most highly concentrated in layers showing high content of synaptic contacts including the hippocampal fissure in the neonatal brain as seen in our study, which is in concordance with reports where astrocytes appear to influence developmental synaptic pruning by releasing signals and thereby tag them for elimination by microglia [84, 85].

The role of reactive astrogliosis in the evolution of ischemic brain lesions especially in neonates is at present not clear, but recent studies have suggested that reactive astrocytes provide essential metabolic support to neurons during transient ischemia and that failure of astrocyte functions may contribute to neuronal degeneration [86, 87]. Additionally, in adult transgenic mice, experimental disruption of astroglial scar formation following stroke is associated with loss of barrier functions along the margins of infarcts, resulting in increased spread of inflammation and increased lesion volume [88]. Moreover, adult mice lacking GFAP [GFAP$(-/-)$] show attenuated reactive gliosis, reduced glial scar formation after focal brain ischemia as compared to injured developing brain where there is only an increase in the survival of newborn neurons [89].

Astrocytes also play a vital role in white matter, regulating molecules such as glutamate in the extracellular space and preventing excitotoxic damage to neighbouring oligodendrocytes and axons. GFAP knockout mice exhibit degeneration of myelin with progressing age [90]. Consistent with previous reports, we noted an increase in GFAP expression in white matter astrocytes accompanied by hypertrophy and process thickening in ipsilateral hemispheres [91].

4.3.2. Transient Microglial Response in the Contralateral Hemisphere and Widespread in the H/I Damaged Side. In

control postnatal mice, we observed amoeboid and ramified microglia throughout gray and white matter from P7 to P14 mice, as has been described previously [92]. As the brain development continues after birth, microglial cells need to adapt to the changes in the microenvironment [92]. Until P14, we observed groups of amoeboid microglial cells which are present in the developing corpus callosum, cingulum, and fimbria. These cells are proposed to be involved in the phagocytosis of cellular debris and contribute to the axonal nerve fiber remodeling and synapsis during normal development [93–95].

In the present study, we have observed morphologically activated microglia from 3 to 72 hours after hypoxia in the contralateral corpus callosum, with a peak of response at 24 hours. This microglial response to hypoxic conditions in the subcortical white matter has been extensively studied by the group of Ling and coworkers, who have demonstrated that hypoxia-activated microglial cells in the developing white matter produce several inflammatory mediators including cytokines, chemokines, and reactive oxygen species which are detrimental for white matter development and oligodendrocyte survival (reviewed in [96]), which may account for the long-term contralateral corpus callosum atrophy we observe, although the microglial response in the contralateral corpus callosum is transient, in agreement with the findings of Zaidi and coworkers [97] in the P7 rat model, that did not observe activated microglia after 14 days of hypoxia in the contralateral hemisphere. Interestingly, in agreement with our observations, Cowell and coworkers [98] have shown a transient contralateral microglia activation in the cortex, white matter and hippocampus after an unilateral transection of MCA in neonatal rat brain.

Obviously, microglial response in the ipsilaterally damaged corpus callosum is very striking, showing reactive ramified and ameboid/macrophagic forms from 3 hours to 14 days after hypoxia, with a peak of response at 48–72 hours. It is now evident that the developing brain is highly susceptible to hypoxic damage because of its high oxygen and energy requirements [99, 100], and that white matter at this developmental stage is vulnerable. Moreover it have been described that the myelin from the degenerating axons is phagocytosed by microglia [101]. In this sense, as long-term atrophied white matter is observed after microglia returns to a resting state, we may suggest that activated microglial cells may not be sufficient to complete phagocytosis and avoid the inhibition of oligodendrocyte precursors differentiation. As most of this knowledge is mainly obtained from results in rat models and several differences has been described between rodents, a more detailed description on the late effects on oligodendrocytes, their precursors, myelination and axonal degeneration in neonatal mice brain hypoxic ischemic injury is needed.

Interestingly, microglial response in the contralateral gray matter areas was more evident that the astroglial response, and activated microglial cells were seen as early as 3 hours after hypoxia in the hippocampus, but also in the caudate-putamen and cortex (see Figures 9 and 10); however, contralateral microgliosis was very transient and only persisted until 48–72 hours depending on the regions. In the contralateral hippocampus, microglial response was mostly evident in the hippocampal fissure, and not so widespread as in the ipsilaterally damaged side, where we describe a layer-specific activation of microglia as early as 3 hours after hypoxia, with a maximum response from 48 hours to 7 days, followed by a patchy pattern at later time points. This has also been demonstrated at early time points in rat model as mentioned previously by Cowell and coworkers [98]. Remarkably, hippocampal microglial response was first observed surrounding the blood vessels in the hippocampal fissure, which have been suggested to be more vulnerable to ischemic episodes than those from other hippocampal areas [102]. Interestingly, this is known to be one of the sources of microglia progenitors during late embryonic life in the rat, showing, during early postnatal development an outside-to-inside microglia distribution pattern towards the pyramidal or granular cell layers [92]. From 24 hours onwards evident neuronal damage when evident neuronal damage takes place in the ipsilateral hippocampus and then ameboid/macrophagic phagocytic microglia populate the neurodegenerating CA areas.

The association between microglia activation and injury development raises the question whether this reaction is detrimental or beneficial [103–105]. Traditionally, microglia activation was considered harmful [19, 58]. However, it is now established that, as macrophages do in the periphery, microglia has two different patterns of activation and function in response to CNS injury (revised by [26, 103, 106]). Then, selective ablation of proliferating microglial cells exacerbates ischemic injury [107]. Moreover, opposite effects have been described in neonatal H/I mice and rats using minocycline, a tetracycline derivative that nonspecifically blocks all microglia activation. In rat brain, this treatment protects the brain tissue in some reports [19, 20] but only have a transient protective effect in others [108]. In contrast, tissue damaged increases in minocycline-treated H/I mice, especially in cortex, caudate-putamen and thalamus without significant effects on hippocampus [20]. Additionally, selective depletion of microglia before a transient MCAO in a P7 rats does not change the volume of injury but enhances cytokines production compared to not depleted animals [109], suggesting a beneficial role of microglial cells.

These evidences made a complete characterization of neonatal mice microglial response essential, in order to define the better window and target for protective therapies. New insides in the physiological activity of microglia (called "surveillance" instead of "resting"), joined to adult MRI and behaviour assessment [78, 110], would be beneficial to promote phagocytic and anti-inflammatory response of microglia than a complete blocking of their activation in order to obtain better outcomes of therapies applied to injured developing brain.

4.3.3. H/I Induces Neutrophil Recruitment but Very Low Presence of Lymphocytes. The neonates are known to have weakened neutrophil response and reduced tendency to leukocyte extravasation from blood vessels [55–57]. Previous studies have demonstrated that neutrophils contribute to the

long-term hypoxic/ischemic brain injury in the neonatal rat brain [111, 112]. We here report that neutrophils appeared as early as 3 hours after hypoxia in blood vessels of most of the regions studied, especially in the neocortex and third ventricle, in agreement with previous reports showing that neutrophils are seen in brain blood vessels rather early [111, 113, 114]. However, there are limited studies reporting neutrophils in the neonatal parenchyma after hypoxia, and the results are variable; we observed neutrophil recruitment to the injured mouse parenchyma (mainly hippocampus and caudate-putamen) after 72 hours to 7 days after hypoxia, whereas other studies have shown neutrophils accumulated in the injured rat parenchyma at 12–24 hours after hypoxia, peaking at 72–96 hours [113, 115]. Notably, neutrophils accumulate in the same areas of microglia/macrophage accumulation, contributing in the removal of cellular debris and the release of cytokines to further attract more immune cells to the injury site [114, 116]. The negligible lymphocytic infiltration reported here is in accordance with previous reports where no CD3+ cells were detected in the neonatal P1 rat brain at 48 hours after hypoxia and LPS induction [117]. Furthermore, there are reports of very low expression of CD3γ chain of the T-cell receptor in P3, P7, and P14 mice brain in contrast to adult [33].

Since many investigators are using transgenic and knockout mice to determine the importance of specific molecules in the evolution of damage after brain injury, there is an urgent need to perform comparative studies on the relative vulnerability of the mouse brain in comparison to other species. A mouse model of hypoxic-ischemic encephalopathy has paved a way for the description of the specific molecular mechanisms associated with this destructive disease, by the use of genetically modified animals. Our major finding describing the short- and long-term effects as well as the involvement of the contralateral hemisphere may serve as a valuable resource for functional definition of neuroprotection or damage as well as will aid in selecting the time and mode of intervention in the broad therapeutic window.

5. Conclusion

To summarize, this study describes qualitatively and quantitatively the tissue damage, glial response, and inflammatory cell recruitment after brain injury induced by carotid occlusion and systemic hypoxia (8% O2, 55 minutes) to the postnatal day 7 mouse brain, analyzing changes from 3 hours to 100 days after hypoxia. In general, massive tissue injury and atrophy in the ipsilateral hippocampus, corpus callosum and caudate-putamen are consistently shown, with neutrophil recruitment and earlier microgliosis, but persistent long-term glial scarring until 100 days after hypoxia. Remarkably, in the contralateral hippocampus and corpus callosum, milder atrophy is delayed in areas that show the activation of astrocytes and microglial during the first 72 hours. This study highlights that care should be taken when using the contralateral hemisphere as control while studying ipsilateral H/I injury in postnatal mouse brain.

Acknowledgments

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Review Article

Modeling the Encephalopathy of Prematurity in Animals: The Important Role of Translational Research

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Translational research in preterm brain injury depends upon the delineation of the human neuropathology in order that animal models faithfully reiterate it, thereby ensuring direct relevance to the human condition. The major substrate of human preterm brain injury is the encephalopathy of prematurity that is characterized by gray and white matter lesions reflecting combined acquired insults, altered developmental trajectories, and reparative phenomena. Here we highlight the key features of human preterm brain development and the encephalopathy of prematurity that are critical for modeling in animals. The complete mimicry of the complex human neuropathology is difficult in animal models. Many models focus upon mechanisms related to a specific feature, for example, loss of premyelinating oligodendrocytes in the cerebral white matter. Nevertheless, animal models that simultaneously address oligodendrocyte, neuronal, and axonal injury carry the potential to decipher shared mechanisms and synergistic treatments to ameliorate the global consequences of the encephalopathy of prematurity.

1. Introduction

Translational research in the brain injury of premature infants involves the delineation of basic mechanisms and therapeutic strategies in animal models and their subsequent transformation into human clinical trials to improve neurological outcome. Yet, from the outset, advances in our understanding of preterm brain injury are directly contingent upon neuropathologic studies in humans. Indeed, translational research depends upon the initial delineation of the basic neuropathology in the human brain and then development of animal models that faithfully reiterate this pathology, thereby ensuring direct relevance to the human condition. The major neuropathologic substrate of human preterm brain injury is the encephalopathy of prematurity (EP), a term coined to characterize the multifaceted gray and white matter lesions in the preterm brain that reflect acquired insults, altered developmental trajectories, and reparative phenomena in various combinations [1–4]. The encephalopathy of prematurity also is associated with hemorrhages, notably in the germinal matrix of the ganglionic eminence and cerebellum and with focal micro or macroinfarcts [5–7]. Because EP occurs at a time of rapid brain growth, the insult may impact a host of developmental programs, resulting in maturational defects that compound the acquired lesion, for example, hypoxic-ischemic injury leading to loss of pre-OLs in turn leading to impaired myelination. The cause of EP is multifactorial, and includes cerebral hypoxia ischemia and systemic infection/inflammation that results in glutamate, free radical, and/or cytokine toxicity to pre-OLs, axons, and neurons [8]. In addition, other maturation-dependent biochemical derangements likely contribute to EP caused by the multiple extrauterine insutls that the preterm infant experiences and is not developmentally equipped to defend against [3]. One example is bilirubin toxicity that may contribute to the (nonspecific) neuronal loss and gliosis seen in the basal ganglia in EP [3, 7, 9]. Given the heterogeneity and diverse combinations of the lesions that comprise EP, it is not surprising that the spectrum of neurodevelopmental abnormalities in preterm survivors is wide and includes, often in combination, deficits in executive functions [10, 11], autistic

Table 1: Key developmental events in the cerebral white matter, cortex, and subplate region in the last half of human gestation for considering in the design of animal models of the encephalopathy of prematurity.

(1) Cerebral white matter
(a) Development of vasculature and autoregulation
(b) Dominance of pre-OLs
(c) Overexpression of pre-OLs of calcium-permeable, GluR2-deficient AMPA receptors
(d) Expression of pre-OLs of NDMA receptors
(e) Transient expression of glutamate transporter EAAT2
(f) Transient abundance of microglia
(g) Oligodendrocyte expression of cytokine (interferon-γ) receptors
(h) Radial glial fiber transformation and disappearance
(i) Late formation of fibrous astrocytes
(j) Lag in the expression of superoxide dismutases
(k) Active axonal elongation
(2) Cerebral cortex
(a) Gyration
(b) Lamination
(c) Neuronal differentiation
(d) Late migration of GABAergic neurons
(e) Late formation of protoplasmic astrocytes following neuronal migration
(3) Subplate region
(a) Ingrowth of axons and "waiting period"
(b) Involution

Table 2: Major histopathology features of the encephalopathy of prematurity in the human brain.

(1) White matter
(a) Periventricular leukomalacia of the telencephalic white matter
(i) Periventricular focal necrosis in different stages (acute, organizing, and macro- and/or microcysts)
(ii) Gliosis and microglial activation in the surrounding white matter
(iii) Early loss of pre-OLs
(iv) Expression of markers of oxidative and nitrative stress by pre-OLs
(v) Possible maturation arrest of OLs
(vi) Impaired myelin formation
(vii) Upregulation of cytokines in macrophages, activated microglia, and reactive astrocytes
(b) Widespread axonal damage within and distant from the necrotic foci
(c) Deficit of neurons within necrotic foci, surrounding white matter distant from the necrotic foci, and subplate region
(d) Postmitotic migrating neurons as possible reparative event
(e) Gliosis of the cerebellar white matter
(2) Gray matter
(a) Neuronal loss and/or gliosis of the cerebral cortex, thalamus, globus pallidus, hippocampus, cerebellum, and brainstem in different combinations and to different degrees, with preferential involvement of thalamus and basal ganglia
(3) Hemorrhages
(a) Subpial
(b) Subarachnoid
(c) Germinal matrix (with suppression of cell proliferation)
(d) Cerebellum
(4) Infarcts
(a) Microinfarcts of the thalamus
(b) Focal infarcts of the cerebral cortex

behaviors [12], cerebral palsy [13], and visual cognitive impairments [14].

The goal of the following review is to highlight key features of EP that we believe are critical to model in animal paradigms. The patterns and mechanisms of injury in EP are highly dependent upon the specific maturational stages of OLs, neurons, and axons over the last half of gestation, that is, the time frame of EP. We begin with a brief overview of events in human preterm brain development that are particularly relevant to animal modeling (Table 1), given that the vulnerability of pre-OLs, axons, and neurons to injury in EP is critically dependent upon specific maturational stages. We then define the major components of the neuropathology of EP that animal models need to consider (Table 2). We conclude with a consideration of the interplay between human and animal analyses in translational research and the need for the two types of analysis to inform and build upon each other towards the complete elucidation of EP and its treatment.

2. The Development of the Brain in the Last Half of Human Gestation

The encephalopathy of prematurity spans the last half of human gestation, a spectacular and complex period in brain growth and development. From midgestation to term, the brain weight increases dramatically by 90%, and the cerebral cortex changes from a smooth surface with only the Sylvain fissure to a complex pattern with all primary, secondary, and tertiary gyri [7, 15]. The ganglionic eminence is thickest at 20–26 gestational weeks and involutes by 34–36 weeks; until 18 weeks, proliferating cells as identified by Ki67 immunoreactivity are present throughout the ganglionic eminence, after which they persist ventrally until about 28 weeks and then markedly decrease [5]. Among the many interrelated developmental events that occur during this critical period of brain growth, several are particularly germane to modeling EP in animals because they relate to cellular vulnerabilities to glutamate, free radical, and cytokine toxicities, as illustrated in the cerebral white matter and cortex (Table 1).

2.1. Vascular Development in the Cerebral White Matter in the Preterm Period. Immature vascular end zones irrigate distal

fields in the preterm cerebral white matter [3, 8, 16–18], and thus the deep and periventricular regions are susceptible to very low basal blood flow, documented in human preterm brain by multiple techniques [8, 17, 19, 20]. This vascular immaturity is characterized by decreased numbers of vascular perforators from the leptomeninges in the white matter compared to the cortex [16]. Moreover, the intrinsic capillary plexus of the white matter has fewer and longer capillaries and larger intercapillary spaces than that of the cortex [16]. Functional cerebral vascular autoregulation is also underdeveloped in the premature infant, with a propensity for a pressure-passive cerebral circulation [8, 17, 19, 20]. Thus, the "margin of safety" for blood flow of the deep and periventricular cerebral white matter is compromised due to its developmental anatomic and physiological features, and these regions are vulnerable to fluctuations in blood pressure, as well as overt hypotension, common complications of pulmonary immaturity, respiratory distress syndrome, and accompanying mechanical ventilation in premature infants.

2.2. Oligodendrocyte (OL) Development in the Cerebral White Matter in the Preterm Period. The peak window of vulnerability to PVL, that is, 24–36 weeks, coincides with the period of dominance of pre-OLs [21, 22]. Oligodendrocyte maturation from an OL progenitor into a myelinating OL involves a sequence of developmental stages, each characterized by a progressively complex morphology and the expression of stage-specific markers. This progression includes the following OL-developmental stages in increasing order of maturation as defined by cell-specific antibodies: (1) A2B5-expressing OL progenitors; (2) O4-expressing precursor OLs; (3) O1-expressing immature OLs; (4) myelin basic protein (MBP-) expressing mature OL [21–24]. Neural stem cells give rise to OL precursor cells around 13 gestational weeks that proliferate and migrate throughout the brain, and then differentiate into pre-OLs around 20 weeks [23, 24]. The O4-positive cell comprises approximately 90% of the total OL population in the preterm brain until about 28 gestational weeks and accounts for at least 50% of the total population until term [21]. In contrast, the O1-positive OL population accounts for only about 10% of the total OL population in the preterm infant and does not approach 50% of the total OL population at term [21]. OLs producing MBP first appear around 30 gestational weeks in the cerebral white matter [21], but active myelin sheath production, as detected by Luxol-fast-blue which stains myelin sheath phospholipids, does not begin until 3 postnatal months [25]. Once an axon makes synaptic contact with its target cell, wrapping of the axon by the myelin sheath begins, a process which depends upon both axonal and OL maturation and multiple signaling factors between them, many of which are yet unknown [26]. In the human cerebral white matter, OL contact with the axon involves the extension of immature, O4+/O1+/MBP-, "pioneer" processes that extend longitudinally along the length of the axon [22]. Once axonal contact is made and myelination initiated, OLs transport myelin proteins and lipids to the myelin membrane [26].

2.3. The Development of Glutamate Receptors in Cerebral White Matter in the Preterm Period. Given the critical role of glutamate receptors in mediating excitotoxicity, their regional and cellular development is of major interest in the preterm brain. Several studies of glutamate receptor subtypes have been performed in perinatal human brains [27–29], including a comprehensive cellular localization of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor subtypes in the human cerebral cortex and white matter from the preterm period into infancy [29]. Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propioinic acid receptor subtypes lacking GluR2 are especially permeable to calcium and therefore likely to convey increased susceptibility to hypoxia ischemia. From 20 to 37 weeks, pre-OLs, radial glial fibers (RGFs), and subplate neurons express AMPA receptors that lack GLuR2 expression [29]. The glutamate transporter EAAT2, on the other hand, is transiently expressed in the cerebral white matter in the period of vulnerability to PVL and likely increases the susceptibility of this site to excitotoxicity because it is a major source of extracellular glutamate complicating ischemic injury [30]. This transporter is expressed by OLs but not astrocytes or axons in the preterm white matter [30].

2.4. Development of Microglia in the Cerebral White Matter in the Preterm Period. Microglia are abundant in the human forebrain around 16–22 gestational weeks [31–33]. They are involved in a variety of normal developmental events, such as axonal development, angiogenesis, and synaptic pruning [33]. Their density in the cerebral white matter reaches a peak around the early third trimester and then declines rapidly after 37 gestational weeks [33]. As the microglial density declines in the white matter, it increases in the cortex, perhaps reflecting continuing migration of microglia [33]. Thus, microglia are transiently elevated in the peak window of vulnerability to PVL, well situated to become activated and lead to free radical and cytokine injury to pre-OLs [8]. Of note, O4 OLs in the human cerebral white matter express interferon-γ receptors, indicating that they are vulnerable to receptor-mediated mechanisms by cytokines [34].

2.5. Development of Radial Glial Fibers and Astrocytes in the Cerebral White Matter in the Preterm Period. The last half of human gestation is a crucial time in astrocyte formation in the cerebral cortex and white matter. The radial glial cell originates in the ventricular/subventricular zone and retains connections with the ependyma and pia [16]; it is capable of generating neurons and astrocytes [35]. Its long, thin, and linear processes, that is, RGFs, serve as a guide for migrating neuroblasts and glial cells [16]. Glutamatergic neurons form in the dorsal telencephalic pallium and migrate along RGFs early in gestation [36]. In the human brain, in contrast to the rodent brain, approximately two-thirds of GABAergic neurons arise from the dorsal telencephalic zone and migrate along RGFs; the remaining one-third originates in the ganglionic eminence and migrates tangentially to the cortex [37]. From 19 to 30 weeks, RGFs are abundant; around 30-31 weeks, they begin to transform into fibrous astrocytes

in the white matter and from 30 weeks to term gestation (37–41 weeks), they progressively disappear as the white matter becomes increasingly populated with transformed astrocytes [38–40]. By term, RGFs completely disappear, thereby definitively marking the end of radial migration. Fibrous astrocytes in the white matter also form from glial precursors that migrate outward from the ventricular/subventricular zone independent of RGFs [16]. Reactive gliosis with gemistocytic morphology and GFAP-positive immunostaining begins around midgestation in the human brain [7].

2.6. The Development of Antioxidant Systems in the Cerebral White Matter in the Preterm Period. The human preterm brain is susceptible to free radical injury in hypoxia ischemia because of a relative developmental deficiency in antioxidant enzymes. These enzymes include the superoxide dismutases-1 and -2 for the conversion of superoxide anion oxygen to hydrogen peroxide and catalase and glutathione peroxidase for the breakdown of hydrogen peroxidase. In the cerebral white matter of the preterm infant, the expression of both superoxide dismutased significantly lags behind that of catalase and glutathione peroxidase [41]. These enzymes are all expressed by OLs and astrocytes in the preterm brain and exceed adult levels at 2–5 postnatal months, the peak period of active myelin sheath synthesis in cerebral white matter [41]. Indeed, human myelination with the rapid production of cellular membrane is associated with the "physiological" generation of free radicals resulting in lipid peroxidation [42].

2.7. Axonal Development in the Cerebral White Matter in the Preterm Period. The second half of human gestation is characterized by axonal elongation from the early migrating pyramidal neurons, as well as from cortical afferents from the thalamus and other cortical and subcortical structures [43]. Central axons elongate, search out their proper targets, and establish synaptic contacts; initial axonal excess is followed by axonal elimination or pruning, leading to refinements in connectivity [26]. The expression levels of growth-associated protein-43 (GAP-43), a neuronal membrane phosphoprotein and marker of axonal elongation, are low in the human cerebral white matter at 19-20 weeks, increase approximately 3-fold within two weeks, peak at term at approximately 5-times adult levels, and decrease dramatically at approximately 17 postnatal months, with adult levels attained in the second year [44]. From 14 gestational weeks onward, so-called "crossroads" of intersecting projection and associative axons are present in the periventricular zones, directly adjacent to the lateral ventricles, coinciding with the sites of predilection for focal necrosis in PVL [45]. Because of the complexity of the axonal pathways in these discrete and restricted loci, discrete lesions within them are postulated to result in diverse and multiple functional impairments simultaneously in preterm survivors [44].

2.8. Neuronal Development of the Cerebral Cortex in the Preterm Period. During the last half of gestation, the neocortex transforms from an undifferentiated cortical plate to a highly specialized structure [7, 16, 46–48]. Around 30 gestational weeks, the cortical plate becomes comprised of six layers in which each layer is characterized by a specific composite of differentiating pyramidal and nonpyramidal neurons [46]. The cortex increases in thickness due to striking increases in the neuropil, for example, neuronal cell size, dendritic arborization, spine formation, and arrival of preterminal afferents [7, 16, 46–48]. Indeed, the number of specific gene expression and alternative splicing patterns are large and associated with distinct regions and developmental processes [49]. Relative to excitotoxicity, GluR2 is low in the pyramidal and nonpyramidal neurons in the cerebral cortex during the term neonatal period [29].

2.9. Late Development of the GABAergic System in the Cerebral Cortex in the Preterm Period. A defining feature of cortical development in the human preterm period is the late development of the GABAergic interneurons that play a key role in cortical specification, output, and synaptic plasticity [16, 36, 50, 51]. At least 20% of GABAergic neurons migrates through the white matter to the cerebral cortex over late gestation [50]. This migration peaks around term and then declines and ends within the first 6 postnatal months; in parallel, the GABAergic neuronal density increases in the cortex over late gestation, peaks at term, and declines thereafter [50]. From midgestation to infancy, the pattern of $GABA_A$ receptor binding also changes from uniformly low across all cortical layers to high levels concentrated in the middle laminae [50]. This developmental profile may reflect the ingrowth of glutamatergic thalamocortical fibers during this time period with a parallel upregulation of inhibitory (GABAergic) modulation in the middle laminae to counterbalance the increase in excitatory inputs during the preterm period. The $GABA_A$ receptors are likely excitatory because of relatively high intracellular levels of chloride. The latter occurs because of a developmental imbalance of the chloride importer (NKCC1) and the chloride exporter (KCC1). In the human cerebral cortex, the expression of NKCC1, causing chloride influx, rapidly increases from 20 gestational weeks to term and then dramatically decreases, reaching a plateau after 4 postnatal months [52]. The expression of KCC2, causing chloride influx, on the other hand, is low during the preterm period but increases dramatically postnatally to adult levels [52]. Thus, in the preterm period, GABA agonists lead to chloride efflux and depolarization/excitation, rather than the normal chloride influx and hyperpolarization/inhibition.

2.10. Development of Protoplasmic Astrocytes in the Cerebral Cortex in the Preterm Period. In the cerebral cortex, astrocyte precursors that have migrated upward along the RGFs to Layer I, differentiate and send processes towards the developing cortical blood vessels, and gradually transform into the protoplasmic astrocytes of the cortex [16]. This event occurs "late", that is, after the completion of the migration of neuroblasts destined to form cortical neurons. These astrocytes express the glutamate transporters EAAT1 and EAAT2 but not glial acidic protein (GFAP) in the late preterm

period [53]. Discrete EAAT1/EAAT2 astrocytic "patches" appear in the developing cortex in the late preterm period [53]. The patches may reflect nonoverlapping astrocytic territories that potentially contribute to the synchronization of neurons [53].

2.11. Development of the Subplate Region in the Preterm Period. Subplate neurons are among the first generated neurons of the neocortex and come to lie immediately beneath the developing cortical plate where they form part of the early neocortical circuitry [54, 55]. During early development, subplate neurons play a critical role in the establishment of connections between the cortex and thalamus and cortical lamination [54, 55]. These neurons are also critical in the establishment of axons destined to the cortex from the contralateral cerebrum (commissural-cortical fibers) and from unilateral cortical sites (corticocortical association fibers). There is no available immunomarker that specifically labels human subplate neurons in tissue sections. Their phenotype is defined by morphology, location, and connectivity with the cortical plate. In the human subplate region, fusiform, granular, unipolar, bipolar, multipolar, and inverted pyramidal neurons have been observed [54, 56]. The specific function of each morphological subtype is unknown. Glutamatergic, GABAergic, and other transmitter-specific neurons, transporters, and receptors have also been observed in the human subplate region [29, 50, 51, 56]. Kostovic and Rakic divide the developmental stages of the human subplate zone into the presubplate stage at around 12-13 gestational weeks, the subplate formation stage around 12–15 weeks, the subplate stage around 15–35 weeks, and the subplate dissolution stage beyond 35 weeks [54]; the latter two stages coincide with the period of EP. In the subplate stage (15–35 weeks), the subplate serves as a "waiting" compartment for the competition, segregation, and growth of afferents originating from the thalamus, brainstem, basal forebrain, and ipsi- and contralateral cerebral hemisphere [43, 54]. During this stage, the histochemical stain acetylcholinesterase highlights cholinergic terminals arising from the basal forebrain [54]; around midgestation, this stain indicates that the subplate zone is four times thicker than the cortical plate [54]. After the incoming fibers enter the cortical plate around 32 weeks, the subplate zone almost completely disappears, leaving only a "vestige" of neurons cells scattered throughout the subcortical white matter, that is, so-called interstitial neurons, which nevertheless contribute to the modulation of adult cortical processing [57].

2.12. The Development of Subcortical Structures in the Preterm Period. The involvement of the basal ganglia, thalamus, hippocampus, cerebellum, and brainstem in EP suggests that all of these gray matter sites have developmental factors that increase susceptibility to glutamate, free radical, cytokine, and metabolic insults. Nevertheless, information is relatively limited about these factors in the human preterm brain. Transient expression of glutamate receptors has been described in the developing human basal ganglia [28] and brainstem [58, 59]. The expression levels and cellular localization of several antioxidant enzymes have also been defined in the basal ganglia and brainstem by immunohistochemistry [60].

3. The Pathology of the Encephalopathy of Prematurity

3.1. Periventricular Leukomalacia (PVL). This lesion is the major white matter component in EP and is defined as focal periventricular necrosis associated with reactive gliosis and microglial activation in the surrounding cerebral white matter [3, 7]. The necrotic foci likely represent a core infarct with destruction of all cellular elements [3, 7, 61], while the astrocytic and microglial response in the surrounding white matter represents the penumbra with less severe and potentially reversible ischemic injury [3]. The necrotic foci progress from coagulative necrosis (characteristic of the histology of tissue ischemia in all tissues [3]), with hypereosinophilia, nuclear pyknosis, and axonal spheroids, followed by organizing necrosis with reactive gliosis, macrophagocytic infiltration and tissue disintegration, and then end-stage cystic formation and gliosis [3, 7]. Importantly, the necrotic foci are not always apparent upon macroscopic examination. In autopsy studies in our hospital from the modern era of intensive care, 46–82% of PVL cases, depending upon the dataset, have only microscopic necrotic foci (with macrophagocytic infiltration) that measure less than 2 mm in diameter [9, 46, 62]. Moreover, neuroimaging studies over the past 10–15 years demonstrate that cystic PVL has declined in incidence and noncystic PVL has become the dominant lesion, accounting for more than 90% of PVL and occurring in approximately 50% of very low birth weight infants [2]. Nevertheless, visually obvious foci of necrosis, so-called "white spots", as well as cysts greater than 2 millimeter in diameter are still detected at autopsy [9, 46, 62]. Diffuse white matter gliosis without periventricular necrotic foci occurs in preterm brains [63] but its relationship to PVL is uncertain, for example, whether or not it represents the least severe end of a spectrum of ischemic injury to the premyelinated white matter, with PVL at the most severe end. Nevertheless, neuronal loss in gray matter sites occurs almost exclusively in association with PVL and not with diffuse white matter gliosis [9], suggesting that PVL is the hallmark of EP and that injury that leads to focal necrosis in the white matter *and* neuronal loss in the gray matter is the defining event of EP.

The pathogenesis of PVL involves acute loss of pre-OLs [64, 65]; some OL cell bodies appear to survive with loss of cell processes [66], others with morphological dysfunction in myelin formation [66], as well as hypomyelination [67]. Immunocytochemical analysis using an antibody to Olig2, a pan-OL lineage marker, indicates no significant difference in Olig2 cell density in the periventricular or intragyral white matter between PVL cases and controls [66]. Nevertheless, early lineage markers are needed to determine if there is arrested OL maturation with dominance of pre-OLs over mature OLs. Qualitative abnormalities of MBP staining in both the diffuse and necrotic foci of PVL occur

despite preserved OLIG2 cell density [66]. They include excessive MBP immunostaining in enlarged OL perikarya that presumably reflects a functional derangement in MBP transport from its site of production in the OL cell body to the OL processes [66]. Free radical injury to pre-OLs in PVL is indicated by immunocytochemical evidence for protein nitration and lipid peroxidation of pre-OLs in the diffusely gliotic component of PVL [64]. In addition, F(2)-isoprostanes, an arachidonate metabolite/lipid peroxidation marker of oxidative damage, is significantly increased in the white matter of early PVL cases [65]. The end-stage of PVL is delayed or hypomyelination of the cerebral white matter and compensatory ventricular enlargement [7].

3.2. Gray Matter Lesions in EP. Neuronal loss and/or gliosis are the histopathologic hallmarks of gray matter injury in EP and occur in virtually all gray matter sites, albeit in variable combinations [9]. Over one-third of PVL cases demonstrates gray matter lesions characterized by neuronal loss and/or gliosis [9]; microglial activation is oftentimes striking. Of note, more refined techniques, such as analysis of dendritic and spine number and morphology, may ultimately detect neuronal deficits at the subcellular (and molecular) levels. The incidence of neuronal loss, as assessed semiquantitatively in tissue sections, is 38% in the thalamus, 33% in the globus pallidus and hippocampus, and 29% in the cerebellar dentate nucleus [9]. Gliosis without obvious neuronal loss is more common than combined neuronal loss and gliosis, occurring in the thalamus (56% of PVL cases), globus pallidus (60%), hippocampus (47%), basis pontis (100%), inferior olive (92%), and brainstem tegmentum (43%) [9]. In a histopathologic survey of brain injury in very low birth weight infants, the frequency of neuronal loss (sites unspecified) is reportedly less than cerebral white matter abnormalities [68]. The basis of neuronal injury in PVL may be heterogeneous, as suggested in the thalamus [69]. At this site, injury occurs in four different patterns, that is, diffuse gliosis with or without neuronal loss, microinfarcts with focal neuronal loss, macroinfarcts in the distribution of the posterior cerebral artery, and status marmaoratous [69]. These different patterns likely each reflect separate mechanisms, including diffuse hypoxia ischemia and focal arterial embolism [69], as well as potential different temporal characteristics of the responsible insults.

The cerebellum in the preterm infant demonstrates bilateral, symmetric deficits in hemispheric volume without overt parenchymal hemorrhage or infarction [60–72]. This reduced volume is commonly associated with intraventricular or subarachnoid hemorrhage [72]. Moreover, cerebellar underdevelopment is associated with supratentorial lesions, especially PVL and posthemorrhagic infarction, suggesting the possibility of transsynaptic mechanisms in its pathogenesis via corticopontocerebellar pathways [70, 72]. Yet, neuroimaging studies also indicate a gradual deficit in cerebellar volume in preterm infants associated with infratentorial hemosiderin deposition in the majority of cases [72]. Thus, it has been postulated that blood products (hemosiderin/nonheme iron) in the cerebrospinal fluid lead to cerebellar underdevelopment due to their toxic effects upon the proliferating granule precursor cells of the external granular layer which are located directly at the interface with the subarachnoid space and which migrate inward to form the internal granular layer [72]. Nevertheless, this idea, based upon neuroimaging studies, has not been verified by quantitative analysis of the cell number of the internal granular layer. Indeed, semiquantitative analysis of the cerebellum has revealed moderate loss of cortical neurons in 24% of cases with PVL and moderate loss of dentate neurons in 29% of cases [9] in association with reactive astrocytes. Thus, the neuropathologic features (notably gliosis) suggest an acquired insult leading to cerebellar atrophy rather than underdevelopment as the basis of the small size of the cerebellum on neuroimaging studies. Yet, the distinction between atrophy and underdevelopment is difficult in the developing cerebellum in which migration from the external to the internal granular layer is protracted over the last half of gestation into infancy. That is, a particular insult may simultaneously lead to *atrophy* with drop out of cells already at their proper address (inciting gliosis) *and underdevelopment* due to disruption of still migrating cells and an incomplete complement of neurons. Indeed, the pathology of the cerebellum epitomizes the so-called "complex amalgam" of EP where developmental and destructive processes intersect [2]. In regards to the cerebellar relay nuclei, it is uncertain if the neuronal loss in the basis pontis and inferior olive, the major cerebellar relay nuclei, which is seen in 21% of PVL cases [9], is primary or secondary to transsynaptic degeneration.

3.3. Deficit of Neurons in the Subplate Zone and White Matter in EP. Not only is there damage to neurons in gray matter sites but also to neurons located in the white matter and subplate region. The density of granular neurons is significantly reduced in the periventricular and central white matter and subplate region in PVL [56]. These neurons are likely late migrating GABAergic neurons and/or non-GABAergic constituents of the subplate region and interstitial white matter [56]. In regard to the former possibility, a reduction in the density of GAD67-immunopositive neurons and neurons expressing the $GABA_A\alpha 1$ receptor has been reported in human perinatal white matter lesions (with and without focal necrosis) [51]. The granular neurons expressed GAD67/65, a marker of the GABAergic phenotype, but not markers of neuronal and glial immaturity (Tuj1, doublecortin [DCX], or NG2) [56]. Notably, in contrast to granular neurons, there is not a consistent deficit in unipolar, bipolar, multipolar, or inverted pyramidal neurons in the white matter or subplate region in PVL [56]. The finding of reduced density of white matter neurons in the necrotic foci in PVL is not unexpected since necrosis involves destruction of all cellular elements. The deficit in the granular neurons distant from the focally necrotic lesions, that is, in the subplate region, on the other hand, is of major interest because it occurs presumably in zones of less severe insult. The preferential damage to granular neurons, including distant from the necrotic foci, suggests that this particular

subtype is exquisitely sensitive to hypoxia ischemia. In humans, approximately one-third of GABAergic neurons arises from the ganglionic eminence [37]. In the study of reduced granular cells in the white matter and subplate region, approximately one-third of the PVL cases had germinal matrix hemorrhages in the ganglionic eminence, raising the possibility that the reduction in neuronal density in the white matter in these PVL cases was accentuated by mechanical damage to the GABAergic neurons originating in this site. It has recently been reported that germinal matrix hemorrhage is associated with a marked decrease in proliferating cells, as identified by Ki67 immunoreactivity and not an increase in apoptosis, in survivors over 12 hours [5]. Yet, there was no significant difference in the granular neuronal density in the white matter between PVL cases with and without ganglionic hemorrhages.

3.4. Damage to RGFs in EP. Radial glial fiber damage could adversely affect radial neuronal migration with secondary maldevelopment of the vertical columns of the cerebral cortex. This idea has not been rigorously tested, however, in the preterm brain with the necessary tissue methods to define quantitative derangements in cortical mini- and macrocolumn formation in postmortem brains. Damage to RGFs may also potentially impair astrocytic development, as fibrous astrocytes in the white matter develop from the transformation of RGFs, and protoplasmic astrocytes in the cortex transform from Layer I astrocytes following RGF migration [16]. A deficit in fibrous and/or protoplasmic astrocytes in EP may be potentially masked by gliosis, as there are no quantitative criteria for an "adequate" astrocytic response. Nevertheless, reactive astrocytes in EP demonstrate evidence of oxidative and nitrative stress, which is potentially primary and could lead to an "inadequate" glial response [64, 73]. Indeed, so-called "acutely damaged glia" in PVL [63] may represent astrocytes undergoing cell death. Given the role of astrocytes in protecting against ischemic injury via glutamate uptake and in orchestrating cytokine responses, damage to them secondary to potential RGF injury in EP potentially is likely to be especially deleterious. The delineation of RGF pathology in EP is an important direction for future research.

3.5. Diffuse Axonal Injury in the Cerebral White Matter in EP. With β-amyloid precursor protein, axonal spheroids are detected within the necrotic lesions of PVL, whether focal or large [74]. With the apoptotic marker fraction, on the other hand, diffuse axonal injury is detected in the white matter distant from acute or organizing necrotic foci, suggesting a widespread axonopathy in PVL [62]. This diffuse axonal damage may reflect secondary degeneration of thalamocortical afferents complicating primary thalamic neuronal loss. Alternatively, it may be primary due to hypoxic ischemic or inflammatory injury directly to the axon, with secondary impairments in axonal-OL interactions in the initiation and maintenance of myelination. Irrespective of its pathogenesis, widespread axonal damage likely contributes to the reduced white matter volume and callosal thinning in end-stage PVL. Axonal injury throughout the diffuse and focal components of PVL may also lead to architectonic changes in the overlying cerebral cortex [46, 75].

3.6. Reactive Gliosis and Activated Microglia in the Cerebral White Matter in EP. Reactive gliosis and activated microglia are the two major inflammatory components of PVL [3, 4, 7]. Presumed to be initially protective against pre-OL cell damage, they carry the potential for compounding tissue injury when the insult is prolonged and/or severe. Reactive gliosis in PVL is preferentially located in the deep as compared to intragyral white matter [64] and thereby defines injury in the vascular distal fields of the cerebral white matter. Activated microglia likewise conform to this regional distribution, while macrophages are prominent in the organizing necrotic foci of the periventricular regions [3, 5, 7]. Both astrocytes and microglia/macrophages produce inflammatory cytokines, and immunocytochemical studies in PVL demonstrate increased cytokine expression within them as a distinctive feature of the histopathology [34, 76]. Notably, reactive astrocytes in PVL express interferon-γ and thus are a potential source for this toxic cytokine, particularly to pre-OLs compared to mature OLs [77]. Reactive astrocytes and microglia/macrophages also help protect pre-OLs from excitotoxic injury by the upregulation of the glutamate transporter EAAT and uptake of excessive tissue glutamate, as suggested by the finding that the percentage of EAAT2-immunopositive astrocytes is increased in PVL compared to control white matter, and macrophages in the necrotic foci express EAAT2 [78]. Yet, reactive astrocytes and microglia may contribute to free radical injury in PVL, as indicated by intense expression of inducible nitric oxide synthase (iNOS), a marker of nitrative stress, in reactive astrocytes in the acute through chronic stages of PVL, and in activated microglia primarily in the acute stage, the latter observation suggesting an early role for microglial iNOS in the pathogenesis of PVL [73]. In addition, the density of iNOS-immunopositive cells is significantly increased in the diffuse component [73].

3.7. Neural Repair in EP. Evidence is mounting that tissue repair is underway in EP within the neonatal period, that is, within the period of the inciting insult(s). In this regard, Olig2 cell density at the necrotic foci is increased in PVL cases compared with that in sites distant from these foci, suggesting that OLs are migrating to the ischemic core to replenish OL cell number [66]. In PVL, the stem cell immunomarker to nestin demonstrates its increased expression in glia and neurons, attributed to nestin upregulation in response to injury rather than regeneration of new cells [79]. Using DCX immunopositivity as a marker of postmitotic migrating neurons, we found significantly increased densities of DCX-immunopositive cells in PVL cases compared to controls in the subventricular zone, necrotic foci, and subcortical white matter in the perinatal time window, that is, 35–42 postconceptional weeks [80]. These increased DCX-immunopositive neurons may be *en route* to replenish the loss of white matter neurons. Their increased density in the subventricular zone suggests that

the regenerative capacity originates in this germinal site [81]. Successful incorporation of the DCX-immunopositive cells into the neuronal circuitry of the white matter in PVL will ultimately depend upon timing and extent of injury, as well as the availability of neurotropic factors necessary for cellular differentiation and the formation of functional circuits.

4. The Bridge between Human and Animal Research in the Encephalopathy of Prematurity

4.1. Strengths of Human Neuropathologic Studies in Translational Research in EP. Insights from such studies are crucial to translational research because they shape the relevant hypotheses for animal models by defining the vulnerable cell populations and brain regions, pathogenic molecules, and cellular features of the inflammatory and reparative responses. Human studies have taught us that: (1) pre-OLs, neurons, and axons *in combination* are the key cellular substrates at risk in EP; (2) the cerebral white matter, cerebral cortex, thalamus, basal ganglia, cerebellum, and brainstem are the key brain regions involved; (3) cerebral white matter damage involves micro- and/or macrofoci of necrosis with macrophages in combination with diffuse reactive gliosis and microglial activation and axonal damage; (3) reactive astrocytes and activated microglia are critical components of both gray and white matter injury. Moreover, human neuropathologic studies provide insights into the anatomic substrate of the cognitive, emotional, and behavioral disorders in preterm survivors that are not always forthcoming in animal models, given the profound species differences in executive functions and higher affective processing. Indeed, the spectrum of neuronal and axonal lesions in EP elucidates the basis of the complex *cognitive* deficits in preterm survivors and indicates that these deficits are not based upon white matter damage alone, but rather, likely result from simultaneous damage to diverse nodes in cognitive processing, that is, the corticothalamic-commissural-associative-subplate network [9, 46, 51, 56, 69, 75]. The finding of thalamic damage in the mediodorsal nucleus and reticular nucleus associated with PVL, for example, may help explain the clinical observations of deficits in working memory and state regulation, respectively, in preterm survivors [69], the finding of cerebellar damage helps explain the autistic behaviors [12, 72], and the finding of secondary cerebral cortical changes overlying necrotic white matter lesions, the seizure disorders, and cortical-based cognitive impairments [75]. In addition, the tissue demonstration of neuronal loss and/or gliosis in gray matter sites provides a starting point for establishing the cellular underpinnings of gray matter volume deficits defined by neuroimaging studies [10–12], with the need for future investigations into the potential contributions of associated neuropil (synaptic) loss. The human neuropathologic studies also indicate the intersection of destructive injury and altered developmental trajectories, for example, acquired damage to axons traversing the cerebral white matter to and from the cortex and subsequent trophic neuronal changes in the overlying cortex [46, 75], cerebellar atrophy/underdevelopment [70–72].

4.2. Strengths of Studies in Animal Models in Translational Research in EP. Despite their many strengths, human neuropathologic studies have several drawbacks that mandate their performance in unison with animal studies in order to establish the "complete picture" of EP. Indeed, the examination of human tissue sections under the microscope provides only a single snapshot in which the dynamic process is frozen at one single time point and the distinction between primary and secondary features is impossible. Moreover, while the patterns of injury in human tissue sections can suggest a mechanism, for example, coagulative necrosis and ischemia [3], the patterns are not always pathognomonic and therefore cannot specify the mechanism(s) precisely. Thus, animal models are essential for the determination of cellular and molecular mechanisms critical for the development of therapeutic interventions in patient care. Examples include the testing of different drugs in the prevention or amelioration of white matter damage in rodent models [81–84]. The strength of animal models in deciphering mechanisms is well illustrated in studies addressing the relative roles of hypoxia ischemia and infection/inflammation in pre-OL cell death in perinatal white matter damage. In a variety of small and large animal models, hypoxia ischemia has been shown to lead directly to pre-OL damage [81–88]. Yet, several animal models indicate that hypoxia ischemia alone is not always sufficient to cause brain injury, but rather, results in significant injury only when combined with an infectious/inflammatory insult, notably pretreatment with lipopolysaccharide (LPS) [8, 88, 89]. When LPS administration is followed by hypoxia ischemia in a perinatal murine model, for example, pre-OL death occurs acutely and is then followed by decreased mature, MPB-expressing OLs [88]. Chronically administered LPS, however, does not induced hypoxemia in the fetal sheep model but also causes white matter injury, with axonal damage, activated microglia, and OL injury [90], albeit to less severe degrees than when applied acutely [91]. Thus, animal models allow for testing hypotheses about causal factors alone and in combination, the latter more faithfully mimicking the complex clinical course of preterm infants with multiple simultaneous insults.

Nevertheless, animal models also have limitations for mechanistic testing. Cell culture and slice systems are needed to determine the molecular and biochemical effects of injury upon single cell types, as exemplified by the determination of the basis of the vulnerability to glutamate and free radical toxicity of pre-OLs compared to mature (myelinating) OLs [8, 92–95] and the effects of different trophic factors on OL proliferation, differentiation, and myelin sheath synthesis [96].

An additional strength of animal models is that they allow for elucidation of evolution of the histopathologic changes though the sequential examination of brains from a cohort of animals sacrificed at different time points following a common insult [83, 87]. This approach is well demonstrated in the delineation of the sequence of events

following uterine artery ligation in a rat model in which cell death, as defined by TUNEL-positivity at P3, was followed by O4 cell loss at P7, microglial activation, and then reactive gliosis in the cerebral white matter, with the persistence of impaired myelination into adulthood [87]. In this way, we learn that apoptosis is involved in pre-OL loss and precedes cell dropout, and that pre-OL damage precedes and therefore potentially incites the inflammatory responses (microglial and then astrocytic activation) in the early stages. Animal models can also lead to novel insights into molecules critical to human lesions but not originally recognized in the lesions *per se*, thereby providing new leads for human investigation. The role of A1 adenosine receptors in the pathogenesis of preterm white matter damage, for example, was not suggested from human neuropathologic studies but rather, from animal models. In a rat model in which hypoxia ischemia leads to pre-OL injury at P3-P12, cerebral hypomyelination was prevented by the administration of caffeine [97]. The postulated mechanism for the caffeine benefit relates to the presence of A1 adenosine receptors on pre-OLs which when activated inhibit pre-OL maturation; caffeine, which blocks A1 adenosine receptors, may in turn remove the maturation block [97]. The relevance of molecules originally discovered in animal lesions to human pathology is determined by their demonstration in human lesions with immunocytochemistry or other applied tissue methods. In this way, animal models "feed back" to the human condition and expand upon its elucidation in new ways. It is important to demonstrate with human tissue methods the expression of adenosine receptors by human pre-OLs to confirm the relevance and ultimate therapeutic potential of adenosine receptor blockage in human PVL. Similarly, the unanticipated observation in the sheep model that the vulnerability of the deep white matter to hypoxia ischemia is related to the increased spatial concentration of a susceptible population of pre-OLs and not to a preferential reduction in cerebral blood flow compared to the intragyral white matter or cortex [98, 99] needs to be pursued in human white matter by studies of the quantitative distribution of pre-OLs in periventricular, deep, and intragyral white matter zones relative to each other to determine the relevance of the animal discovery to human pathogenesis. Nevertheless, these sheep studies corroborate the role of ischemia in the deep white matter to the genesis of pre-OL injury.

4.3. Types of Animal Models for Translational Research in EP. Multiple animal models of perinatal brain injury are currently available, generally with the studies focused to date upon white *or* gray matter injury, rarely both in combination. Several comprehensive reviews delineate the pros and cons of the different (small and large) animal models relative to preterm brain injury [96, 100–110]. The strengths of rodent models include a comparable timetable of OL lineage in the cerebral white matter, relative ease and low cost of experimental manipulations, and capability to utilize genetically engineered (knockout) mice. Their disadvantages include the paucity of cerebral white matter and the lack of cortical gyration. Moreover, there are key intrinsic differences in aspects of cerebral development between human and rodent. A relevant example in this context is the origin of GABAergic neurons, for example, nearly entirely from the ganglionic eminence in the rodent but principally from the dorsal pallium in the human [37]. The strengths of large animals, on the other hand, include major structural similarities with the developing human brain, including in gyration and the sequence of OL differentiation, comparable scaling of gestational age relative to brain development, the capability for invasive instrumentation relative to measures of cerebral blood flow and cardiorespiratory parameters, and closer analogy of neurological consequences to those in human preterm infants. Their disadvantages include the need for considerable expertise and resources in large animal husbandry and surgical and supportive procedures.

4.4. Caveats in Modeling White Matter Injury in the Preterm Infant. As noted above, the clinical picture of preterm white matter injury is changing such that cystic PVL is now uncommon and has been replaced by a "diffuse" lesion in neuroimaging studies in living infants. Studies in sheep suggest that cystic PVL results from severe ischemic insults, whereas diffuse lesions result from lesser degrees of ischemia [98, 99]; thus, the decline in cystic PVL in the neonatal nursery may reflect in part improvements in the management of the cardiorespiratory disorders of prematurity. Because cystic PVL may indeed represent the severe end of the spectrum, it is nevertheless common in fatal human cases that are presumably the most severely challenged. Still, the presence of cystic PVL at autopsy in the current era cannot be ignored because it indicates that the responsible pathogenic factors in the past era (when cystic PVL was the dominant white matter lesion by neuroimaging) are still operative.

What is the neuropathology of the "diffuse" lesion seen by neuroimaging studies in the preterm infant today? Based upon human autopsy studies, this lesion is comprised, in our opinion, of foci of microcystic necrosis in the deep white matter (with these small cysts below the detection capability of modern neuroimaging techniques) in association with diffuse microglial activation, gliosis, and axonal damage (Figure 1). Precise correlations between neuroimaging and autopsy findings in the same infant at the time of death are needed for verification that this microcystic lesion is in fact the diffuse lesion of neuroimaging studies. In a fetal sheep model, however, high-field MRI of chronic perinatal white matter injury indicates correlations between particular patterns of images with microscopic necrosis and reactive gliosis and with pre-OL maturation arrest upon histopathologic examination [111]. In essence, microcystic PVL (with diffuse gliosis and microglial activation) remains a major finding in the preterm brain in active pediatric neuropathology services today. Until proven otherwise, the hallmark of preterm white matter injury remains focal necrosis with macrophages, and its replication in animal models, as well as its relationship to pre-OL injury, should be sought in translational research.

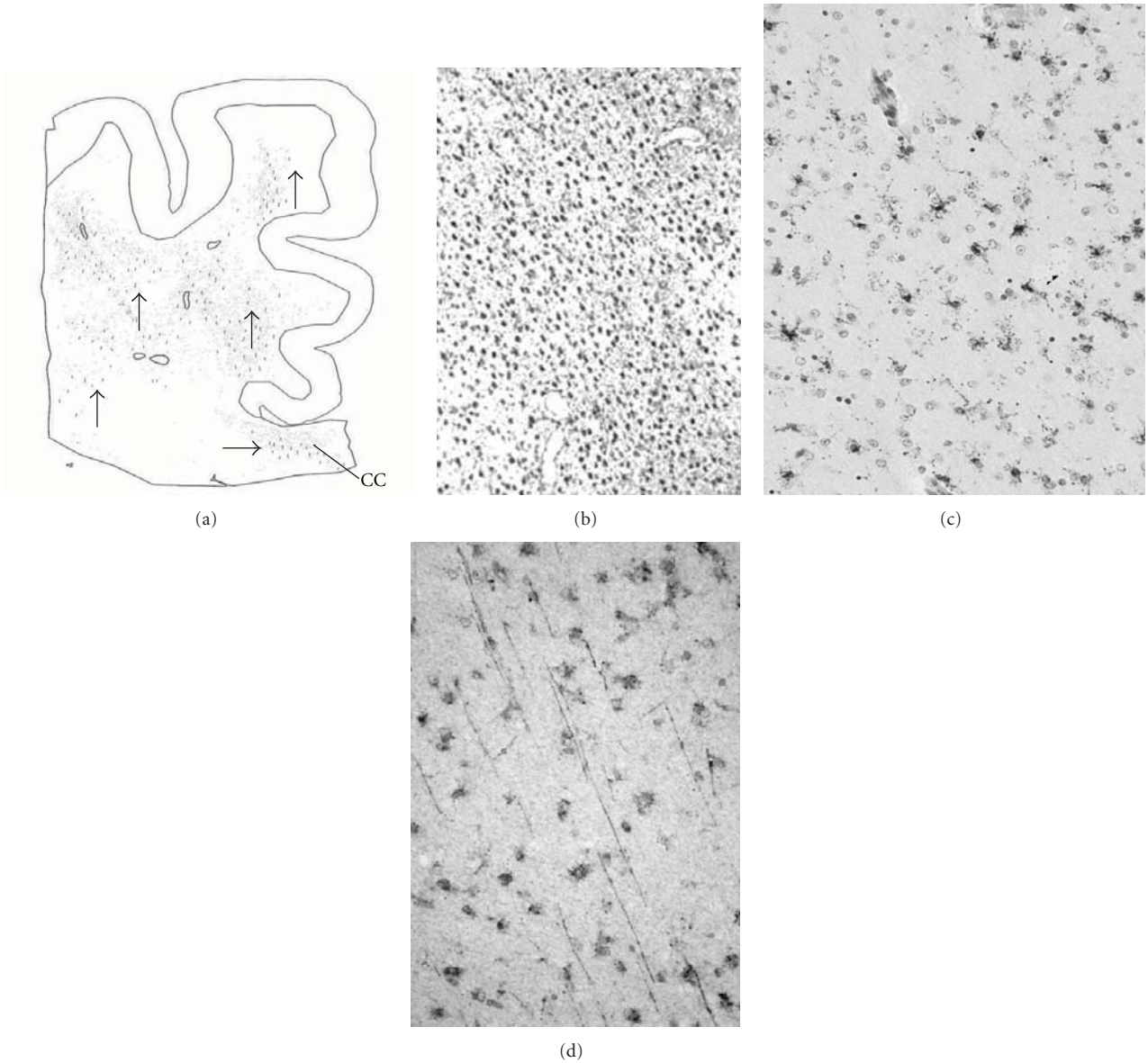

Figure 1: White matter damage in the human preterm brain is characterized by microscopic foci of necrosis and diffuse reactive gliosis, microglial activation, and axonal damage. (a) Camera lucida drawing of the distribution of microcysts (*) and axonal fragments (arrows) in the posterior frontal white matter (level of the body of the corpus callosum [CC]). In the white matter distant from periventricular foci of necrosis is reactive gliosis, as demonstrated by the immunomarker glial fibrillary acidic protein (b), microglial activation, as demonstrated by the immunomarker CD68 (c), and axonal injury, as demonstrated by the immunomarker fraction (d).

4.5. The Selection of an Animal Model in EP. The question arises: should an animal paradigm model the entire neuropathologic spectrum of EP and thereby cell interactions in pathogenesis, or rather, model one feature of the spectrum, for example, pre-OL cell death, in search of a cell-specific mechanism? The answer is obviously that both types of models play valuable and complementary roles. Yet, it is increasingly clear over the last decade from neuroimaging and neuropathologic studies that human preterm injury is a complex spectrum of pre-OL, neuronal, and axonal injury in multiple brain regions, as well as distinct inflammatory responses, reparative events, hemorrhages, and focal infarcts [2, 3, 6], and the interrelationships of these pathologic processes need to be determined to elucidate the shared mechanisms and sequential cascade of the tissue reactions. It is critical, for example, to examine pre-OL and axonal damage in conjunction with each other to determine the molecular influences of injured pre-OLs and axons upon each other in the initiation and progression of myelin sheath wrapping. While human studies indicate both focal and diffuse axonal injury in PVL [7, 62, 73, 74], such injury is often not addressed in animal models in the same brains that undergo intensive investigations of pre-OLs. In a recent neonatal rat model of hypoxia ischemia in which

axonal integrity was assessed with the antineurofilament antibody SMI-312, axonal degeneration or reductions in axonal density were not observed, whereas pre-OLs were present that failed to initiate myelination [86]. The apparent resistance of the axons to hypoxic ischemic injury in this model was likened to that of murine axons at P3 in an oxygen glucose deprivation model [112]. In a large animal (sheep) model, however, axonal degeneration was apparent with prolonged ischemia [98], providing insight into the potential basis of the widespread axonal damage in human preterm infants [73].

Still, the major focus in animal models related to preterm brain damage to date has been upon white matter injury and the cellular mechanisms of pre-OL injury almost in isolation for a variety of historical reasons, as recently reviewed [4]. This focus illustrates in large part the cell-specific approach to animal models, as they address the hypothesis that hypoxia-ischemia causes pre-OL cell death and hypomyelination. The major criteria for an animal model of white matter injury are thus a cellular sequence of OL lineage similar to that of human preterm white matter and an analogous developmental window when pre-OLs dominate. Various hypoxic-ischemic models have demonstrated the death and dropout of pre-OLs with O1 and O4 immunomarkers, the regeneration and arrested maturation of pre-OLs with proliferative and early OL markers, and hypomyelination by loss of MBP staining [81, 85, 86, 88, 97–99]. Thus, these models have successfully delineated the "natural history" of pre-OL damage upon exposure to hypoxia ischemia and have established unequivocally the role of this insult in white matter injury. Moreover, they have provided an important explanation for the observation in human PVL that the density of OLs labeled with the immunomarker OLIG2 is not decreased, that is, this marker labels all stages of OL lineage and therefore this preservation of OL density may reflect proliferation of pre-OLs with arrested maturation with dominance of pre-OLs over mature OLs [66]. Animal models have also solidified the role of glutamate and free radicals in the tissue injury. In a preterm fetal sheep model of bilateral carotid occlusion, for example, extracellular excitatory aminoacids and malondialdehyde (but not 8-isoprostane) were significantly increased in the periventricular white matter with a peak at 2-3 days following occlusion [113].

Yet, the pathology of white matter damage in the human infant is far more complex than pre-OL damage only. Indeed, the hallmark of PVL is focal necrosis with macrophages in association with diffuse reactive gliosis and microglial activation [3, 7], and it is possible that different mechanisms are operative in producing pre-OL damage *and* focal necrosis as opposed to diffuse pre-OL damage *without* focal necrosis, as seen in certain animal models (e.g., [81]). Given that microglial activation is a hallmark of PVL, the role of microglia relative to pre-OL injury is of critical interest. The demonstration that the drug minocycline suppresses microglial activation and substantially attenuates pre-OL injury in perinatal rodent models of hypoxia ischemia is important [83, 84]. It underscores the role of microglia in the pathogenesis of PVL, as suggested by focal macrophagocytic infiltration and diffuse microglial activation in surrounding nonnecrotic white matter [64], and provides a potential effective means of intervention [83, 84]. Microglia mediate pre-OL cell death, at least in part, via pathways of oxidative and nitrative stress [84].

Indeed, it is likely that macro- and microcysts in the white matter result when the ischemic insult is severe enough to cause concomitant gray matter injury, as suggested by the finding that gray matter neuronal loss and/or gliosis in the human preterm infant occurs only in association with necrotic foci and not with gliosis alone [9] and that prolonged ischemia in the sheep model results in white *and* gray matter pathology [98]. In the past, animal models have been sought that demonstrate white matter injury exclusively, and those with both gray and white matter injury were considered undesirable. It is likely, however, that in the effort to "create" only white matter injury (without associated gray matter injury), that is, the historically perceived dominant pathology of the preterm infant [4], animal models were based upon lesser degrees of insult that "stopped short" of (white or gray matter) necrosis, and the insult was not severe enough to recapitulate the entire spectrum of the human pathology. The challenge in animal modeling now is to discover the timing, degree, and type of insult that recapitulate the full human spectrum if further advances, in our opinion, are to be made.

The approach to modeling the whole spectrum of EP is indeed complicated, and may not be possible, given the complexity of the histopathology, the nonspecificity of certain lesions, for example, neuronal loss and gliosis, the likelihood that multiple insults are involved, for example, ischemia, infection, hemorrhage, hyperbilirubinemia, and hypoglycemia, and the variable timing (e.g., intermittent, recurrent) and intensity of the insults. The analysis of the thalamus in association with PVL indicates heterogeneous lesions implicating different mechanisms, for example, diffuse gliosis and neuronal loss consistent with generalized hypoxia ischemia, microinfarcts consistent with small arterial vessel thrombi, and large infarcts consistent with large (posterior arterial) occlusions [69]. The underdevelopment of the cerebellum in the preterm infant may not be directly related to hypoxia ischemia but rather to a secondary consequence of intraventricular hemorrhage, heme deposition in the leptomeninges, and heme toxicity to the external granular layer with secondary cell loss and impaired migration to the internal granular layer [72].

One approach to analyzing the whole spectrum of EP is to focus upon animal models that mimic the circumstances of prematurity in the modern neonatal intensive care nursery without a specific single severe insult. In this regard, the baboon model of preterm delivery and subsequent care with mechanical ventilation, blood gas and electrolyte monitoring, and administration of pressors indicates a spectrum of white matter injury, including focal necrosis, gray matter (hippocampus) injury, focal and leptomeningeal hemorrhages, and ventriculomegaly that in multiple respects mirrors human EP [108]. This model allows for the determination of the natural history of injury to pre-OLs under the nearly identical circumstances that

most closely reflect that of the human preterm infant. While this baboon model does not provide the unequivocal establishment of the specific mechanism of pre-OL cell death, it allows for the determination of the sequence of pre-OL cell injury in the setting of the multiple insults of human prematurity and intensive management.

Animal models of EP need to focus upon *combined* gray and white matter injury to facilitate the discovery of shared cellular and molecular pathways that lead to pre-OL, axonal, and neuronal damage that are all seen in the single "snapshot" at tone time under the microscope, having occurred simultaneously or at different times in the newborn's clinical course. Developing neurons, axons, and OLs, for example, are known to share molecular pathways leading to apoptosis and thus the development of a drug that targets these pathways could potentially prevent neuronal and pre-OL cell death at the same time. Shared pathways may relate also to glutamate receptors and free radical defenses, as these factors involve both pre-OL and neuronal toxicity [8]. Both cell types, for example, express glutamate receptor subtypes in the human preterm brain that mediate excitotoxicity [27–29], and animal models that test glutamate receptor antagonists need to assess protection of both white and gray matter populations. Alternatively, drug testing in animal models may need to provide an agent that targets pre-OL injury and one that targets neuronal injury at the same time. Indeed, combined models that delineate mechanistic commonalities between pre-OLs, neurons, and axons may yield synergistic therapeutic agents that prove to be the most effective in preventing the *global* consequences of EP. The sheep model, for example, demonstrates white matter injury in conjunction with basal ganglia and cortical injury [98], and the elucidation of the mechanisms underlying these combined lesions could indeed be sought in this key model.

4.6. Synergy between Human Neuropathologic and Animal Models Studies in Translational Research in EP. Translational research is advanced by the analysis of human and animal studies in parallel, with each approach informing the other. This vital synergy is well illustrated by the recent study of the role of ceramide, a bioactive sphingolipid pivotal to sphingolipid metabolism pathways, in PVL in which parallel human and animal analyses were presented in a single comprehensive publication [114]. Ceramide, which regulates cell death in response to diverse stimuli, was found to accumulate in reactive astrocytes in the diffuse component of human PVL by immunocytochemical methods, thereby establishing it as a factor in the human pathology [114]. Next, ceramide was reported in cell culture to interact with the cytokine tumor necrosis factor, resulting in apoptotic death of OLs in an astrocyte-dependent manner. Finally, altered sphingolipid metabolism was restored during spontaneous remyelination following toxic-induced demyelination in a whole animal model. Taken together, these studies suggest that the modulation of sphingolipid signaling pathways in reactive astrocytes is a potentially important and novel means to prevent PVL in humans [114]. The demonstration of ceramide accumulation in reactive astrocytes in PVL solidified the relevance of the experimental findings to the human condition. A second example of the synergistic value of human, whole animal, and cell culture models concerns the presence of GluR2 AMPA-deficient receptors and NMDA receptors on pre-OLs and the protection afforded by respectively topiramate and memantine against excitotoxicity [81, 82]. In addition, the discovery of diffuse microglial activation in the white matter surrounding necrotic foci led to a body of experimental data demonstrating the role of microglia in innate immunity in microglial activation, toll-like receptor biology, necrotic reactions, cytokine production, and free radical generation [8, 94, 115, 116], with the potential therapeutic relevance of the amelioration by minocycline of white matter damage in animal models [81, 82]. The synthesis of the human and animal data leads in turn to the provocative insight that microglia are the critical "convergence point" in the potentiation of hypoxic ischemic and infectious/inflammatory insults in PVL, as recently reviewed in depth [8].

5. Conclusions

The pathology of EP is complex and heterogeneous and mandates multiple types of large and small animal models to address all of its many facets in global and cell-specific paradigms. It could be argued that the "best" animal in which to model EP is the animal in which EP occurs in the natural state, as in the report of PVL in neonatal monkeys born prematurely [117]: here all of the "right", human-like, factors must be in place, operative, and spontaneous. While no one experimental model captures all of the complexity of the human disorder, important advances in our understanding of preterm brain injury have resulted from different experimental approaches that focus on different questions, resulting in an increasingly complete picture. In tandem with animal models are the human neuropathologic studies with state-of-the-art methods, including gene expression profiling [49], proteomics [118], western blotting [29, 30, 41, 44, 50, 52], stereology [119], array tomography [120], immunoprecipitation and protein identification [42], tissue receptor autoradiography [27, 28, 50, 58, 59], single- and double-label immunocytochemistry [22, 23, 29, 38, 64], biochemical assays [65], histochemistry [30, 39], electron microscopy [39, 40], and confocal microscopy [22], as well as the ever-valuable Golgi technique [16, 40, 48, 75]. Yet, at this time of unprecedented tools for human brain analysis, the autopsy rates are unacceptably low. We urgently need to develop a culture among those caring for premature infants that place supreme value upon the role of the autopsy in research so that families are readily and routinely approached for consent. Central tissue banks have also been advocated to facilitate preterm brain research given the difficulties for any one single investigator to accrue sufficient sample sizes [96]. In addition, the scientific community at large needs to place a premium on the unique role of human neuropathologic studies in translational research, with an appreciation of the applicability of highly sophisticated and quantitative tools for tissue analysis for which the effects of postmortem can be corrected. Rather than downplaying

human autopsy-based research as not "mechanism driven" or "hypothesis testing", the scientific community should value such investigation for its many strengths, specifically the role in defining the major cell types, brain regions, and molecules in the human condition and generating relevant hypotheses for mechanistic testing in experimental systems. In short, human and animal studies in parallel are essential to inform and build upon each other; one without the other just won't work.

Abbreviations

AMPA: Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
DCX: Doublecortin
EAAT: Excitatory aminoacid transporter
EAAT1: Excitatory aminoacid transporter 1
EAAT2: Excitatory aminoacid transporter 2
EP: Encephalopathy of prematurity
GABA: γ-aminobutyric acid
GAP-43: Growth-associated protein-43
GFAP: Glial fibrillary acidotic protein
GluR2: Glutamate receptor subunit 2
KCC1: Potassium chloride cotransporter 1
LPS: Lipopolysaccharide
MPB: Myelin basic protein
NG2: Proteoglycan 2
NKCC1: Sodium potassium chloride cotransporter 1
NMDA: N-methyl-D-aspartate
OL: Oligodendrocyte
Pre-OL: Premyelinating oligodendrocyte
PVL: Periventricular leukomalacia
RGF: Radial glial fiber.

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Review Article

Programmed Necrosis: A Prominent Mechanism of Cell Death following Neonatal Brain Injury

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Despite the introduction of therapeutic hypothermia, neonatal hypoxic ischemic (HI) brain injury remains a common cause of developmental disability. Development of rational adjuvant therapies to hypothermia requires understanding of the pathways of cell death and survival modulated by HI. The conceptualization of the apoptosis-necrosis "continuum" in neonatal brain injury predicts mechanistic interactions between cell death and hydrid forms of cell death such as programmed or regulated necrosis. Many of the components of the signaling pathway regulating programmed necrosis have been studied previously in models of neonatal HI. In some of these investigations, they participate as part of the apoptotic pathways demonstrating clear overlap of programmed death pathways. Receptor interacting protein (RIP)-1 is at the crossroads between types of cellular death and survival and RIP-1 kinase activity triggers formation of the necrosome (in complex with RIP-3) leading to programmed necrosis. Neuroprotection afforded by the blockade of RIP-1 kinase following neonatal HI suggests a role for programmed necrosis in the HI injury to the developing brain. Here, we briefly review the state of the knowledge about the mechanisms behind programmed necrosis in neonatal brain injury recognizing that a significant proportion of these data derive from experiments in cultured cell and some from in vivo adult animal models. There are still more questions than answers, yet the fascinating new perspectives provided by the understanding of programmed necrosis in the developing brain may lay the foundation for new therapies for neonatal HI.

1. Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is a significant cause of mortality and morbidity in the pediatric population [1]. The therapeutic options for neonatal HIE are limited in part because the mechanisms of cellular degeneration in the immature brain are not fully understood. These mechanisms resulting from ischemia-reperfusion, oxidative stress, excitotoxicity and inflammation among others, activate or coactivate multiple pathways of cell death. Although, necrosis was initially described as the most prominent form of cellular degeneration following neonatal hypoxia-ischemia (HI) [2, 3], research emphasis switched to the study of apoptosis (programmed cell death type I) and autophagy largely due to advances in cell biology and to experimental animal studies on the molecular dissection of pathways for apoptotic and autophagocytic initiation and execution. The significance of necrosis in neonatal HI has been difficult to assess because of the presumed lack of a measurable regulatory pathway; however, the pathological evidence for necrosis has been well documented following HI [4, 5]. We now know that necrosis can be regulated and programmed

and that many components of the regulatory pathways are shared between different types of cell death opening a new window of opportunity for examination/reexamination of the cell death mechanisms in the neonatal brain with the goal of finding novel targets for therapy.

Based on morphological and biochemical data, we conceptualized that neurodegeneration in the neonatal brain is best classified according to an apoptosis-necrosis cell death "continuum" [6] and proposed that programmed cell necrosis (also called necroptosis in cell cultures) has a prominent contribution to neurodegeneration following HI [7]. It is certain that neonatal HI injury evolves through many cell death chreodes influenced by the dynamic injury landscape of the developing brain [8] and the mechanisms of injury in human neonatal HI are more complex than previously anticipated from experimental animal models. The accurate identification of the various cell death chreodes including programmed necrosis and their mechanisms unfolding within the immature brain will, in all likelihood, provide fresh rationale for the development of molecular-based therapies for neonatal brain injury following HI.

2. Programmed Cell Necrosis in Neonatal HI

Programmed necrosis as such has only recently been recognized as an important mechanism of injury in the immature brain following HI [7], however many aspects of programmed necrosis signaling have been comprehensively analyzed by the neonatal brain injury research community over the past decade (Table 1). This work piggybacks on a tremendous body of cell culture data on the mechanisms and contributions of programmed necrosis to cell death since the publication of 3 seminal papers in 1998, 2000, and 2003 [9–11]. This literature has been extensively reviewed recently [12–17].

We proposed that this novel regulated programmed necrosis, lies along the apoptosis-necrosis "continuum" and contributes meaningfully to several forms of acute neonatal brain injury [7, 18]. The death domain containing serine/threonine kinase, receptor interacting protein (RIP)-1, is central to the most well-described forms of programmed necrosis. Its kinase activity is selectively blocked by necrostatins and this affords protection against RIP-1-dependent forms of cell death [19, 20]. Blockade of RIP-1 kinase using necrostatin provides protection in adult animal models of myocardial ischemia and ischemic and traumatic brain injury [18, 21, 22]. Similarly in neonatal HI, blockade of RIP-1 kinase attenuates brain injury at delayed stages in forebrain, hippocampus, and thalamus [7]. The necrostatins have been a major tool for investigation of RIP-1-dependent cell death pathways, however there are other tools that are now being used to explore RIP-1-dependent pathways and these will be discussed below.

The specific allosteric blockade of the kinase activity of RIP-1 has been studied extensively in cell cultures to demonstrate distinct signaling pathways leading to morphologic necrosis; however, many forms of necrosis in cultured cells, appear to proceed with different kinetics and not all are RIP-1 kinase dependent [23]. Some of the known and suspected RIP-1-independent programmed necrosis pathways include (i) caspase recruitment domain (ASC)-mediated necrosis, that is dependent of the non-catalytic activity of caspase-1 [24]; (ii) p53-cathepsin Q-mediated necrosis, that is activated by reactive oxygen species (ROS) and deoxyribonucleic acid (DNA) damage [25]; (iii) apoptosis inducing factor (AIF) and poly(ADP-ribose)polymerase-1-(PARP-1-) dependent pathways (controversy exists over the role of RIP-1 in these forms of programmed necrosis) [26–30]. These pathways to necrosis will not be emphasized since RIP-1-dependent pathways are the focus of this paper and have been most extensively studied.

2.1. The Many Faces of RIP-1: Making the Decision between Living or Dying. Maximal execution of RIP-1-mediated activation of programmed necrosis occurs in the setting of caspase inhibition [20, 31] which can occur as a consequence of pharmacologic inhibition or significant mitochondrial dysfunction and adenosine-5′-triphosphate (ATP) depletion [32–35]. Others and we have hypothesized that energy failure interrupts the neonatal brain's proclivity to apoptosis [6, 32, 33, 36] resulting in the hybrid, "continuum" cell death, or programmed necrosis morphology, possibly via activation of RIP-1 kinase [7]. Following activation of tumor necrosis factor (TNF) receptor (TNFR), RIP-1 signaling leads to a variety of cell fates and has been, for the most part, studied in cell culture [16]. In the setting of energy sufficiency, activation of members of TNFR superfamily (i.e; TNFR1, Fas death receptor (Fas-DR)) by their cognate ligands (TNF-α and FasL, resp.), produce a conformational change in the receptor and recruitment of RIP-1, TNFR- associated death domain (TRADD), and TNFR-associated factor (TRAF) 2 and 5 to the cell membrane. Together these components constitute complex I [32]. TRAF2 recruits the cellular inhibitor of apoptosis (cIAP) that allows polyubiquitylation of RIP-1 leading to activation of p38-mitogen-activated protein (MAP) kinase, nuclear factor- kappa B (NFκB) and cell survival [37–40] (Figure 1). In a rodent model of neonatal HI, preservation of cIAP, via blockade of Smac/DIABLO, decreases injury size and improve outcomes [41], suggesting a possible role of RIP-1 ubiquitylation in cellular survival in this model. Likewise, preservation of RIP-1 ubiquitylation by genetic deletion of cylindromatosis (CYLD, deubiquitinating enzyme) in cultured cells results in resistance to TNF-induced programmed necrosis [42, 43] which persists despite zVAD-fmk treatment (pan-caspase inhibitor) [44]. The roles of caspase 8 (known to cleave CYLD [44]), CYLD, and ubiquitylation of RIP-1 in determining activation of signaling pathways for programmed necrosis or survival are entirely unexplored territory in the investigation of neonatal brain injury following HI. Furthermore, RIP-1 ubiquitylation and complex I have been recently linked to cell death via Nox1 activation suggesting that many other modulators may play an important role in the elaborate intracellular signaling leading to cell survival or death [45] (Figure 1).

In the setting of energy insufficiency, activation of TNFR signals for cellular death via a variety of mechanisms is triggered by the degree of energy deficit. If cellular energy is only partially limited, RIP-1 polyubiquitylation declines

Table 1: Components of continuum-programmed necrosis pathway in neonatal HI models.

Component	Finding	(Year) Researchers
AIF	Translocation from mitochondria to nucleus produces DNA condensation. ↑ is correlated with ↑ infarct size (Rat model)	(2003) Zhu et al. [46]
	AIF effect on DNA is nitric oxide independent (Rat Model)	(2004) Zhu et al. [47]
	Hsp-70 ↓ translocation of AIF to the nucleus (Mouse model)	(2005) Matsumori et al. [48]
	TAT-Bcl-xL ↓ AIF translocation to nucleus and caspase activation providing neuroprotection post HI (Rat model)	(2006) Yin et al. [49]
	↑ nuclear translocation in males associated with ↑ injury Female mice show greater caspase 3 activity. (Mouse model)	(2006) Zhu et al. [50]
	Hypothermia ↓ AIF translocation. (Rat model)	(2011) Askalan et al. [51]
Calpains	m-calpain but not μ-calpain cleaves caspase-3 (Rat model)	(2001) Blomgren et al. [52]
	Calpain inhibition (using MDL28170) provides neuroprotection and ↓ necrosis (Rat model)	(2005) Kawamura et al. [53]
	Prolonged hypothermia ↓ calpain activation (Rat Model)	(2005) Ohmura et al. [54]
	Polyphenols (pomegranate) provide neuroprotection and decrease calpain activation (Mouse model)	(2007) West et al. [55]
	Inhibition produced by inhibition of JNK (using D-JNKI1) (Rat model)	(2009) Ginet et al. [56]
	TAT-mGluR1 blocks the calpain cleavage site of mGluR1α and provide neuroprotection (Rat model)	(2009) Zhou et al. [57]
	Inhibition of JNK (using TAT-JBD) prevents calpain-mediated brain injury after HI (Rat model)	(2010) Nijboer et al. [41]
	Calpain modulates the ↓ in Bcl-2 following HI (Rat model)	(2010) Zhu et al. [58]
	Ethyl pyruvate is neuroprotective via inhibition of calpain activation and Ca^{2+} dysregulation. (Rat model)	(2010) Shen et al. [59]
Cathepsins	Propidium ioidide + cells in cortex and hippocampus were + for cathepsin B after HI suggesting necrosis (Rat model)	(2007) Carloni et al. [60]
	Cathepsin D ↑ at 6 h and 24 h post-HI (Rat model)	(2009) Ginet et al. [56]
FADD	Expression is independent of gluthatione levels and hydrogen peroxide accumulation (Mouse model)	(2007) Payton et al. [61]
	Inhibition of RIP-1 kinase activity restores the RIP-3/FADD interaction (Mouse model)	(2011) Northington et al. [7]
Fas-DR	↑ in the thalamus following HI along with ↑ cleavage of caspase 8. (Rat model) ↑ after HI and genetic deletion provides neuroprotection to cortex (Mouse model)	(2001) Northington et al. [62] (2004) Graham et al. [63]
Hsp-90	—	No *in vivo* HI studies
Hsp-70	Hsp-70 overexpression provide protection against apoptosis (Mouse model)	(2005) Matsumori et al. [48]
	↑ FLIP levels, ↓ caspase-8 and 9 cleavage, and cytochrome C translocation to cytosol (Mouse model)	(2006) Matsumori et al. [64]
JNK	Activated after HI. Genetic deletion ↓ brain tissue loss. Activates c-JUN, ATF-2, Bim/PUMA (Mouse model)	(2007) Pirianov et al. [65]
	Inhibition (using D-JNKI1), ↓ caspase-3 activation. (Rat model)	(2009) Ginet et al. [56]
	Inhibition (using TAT-JBD) ↓ injury, improves outcomes, and preserves IAP (via inhibition of Smac/DIABLO). (Rat model)	(2010) Nijboer et al. [41]
p53	↑ in mitochondria → ↑ cytochrome C and Smac/DIABLO translocation. ↓ p53 → ↓ infarct (better outcomes). (Rat model)	(2011) Nijboer et al. [66]
PARP-1	Activation after HI but ↓ NAD^+ only in male mice and genetic deletion affords neuroprotection in males. (Mouse model)	(2004) Hagberg et al. [26]
	Simvastatin ↓ PARP-1 activation and IL-1β expression and provides neuroprotection (Rat model)	(2006) Carloni et al. [67]
	Immunoreactivity (IHC) peaks at 30 min and then again at 12 h post HI (Rat model)	(2005) Martin et al. [68]
RIP1/RIP3	↓ complex (necrosome) formation by necrostatin after HI affords neuroprotection, ↓ oxidation and FLIP (Mouse model)	(2011) Northington, et al. [7]
TNFR	NF-κB inhibition ↓ brain damage and switches the HI-induced TNF-R profile from ↑ TNF-R1 to ↑ TNF-R2. (Rat model)	(2009) Nijboer et al. [69]
TRADD	—	No *in vivo* HI studies

AIF: apoptosis inducing factor; FADD: Fas-associated protein; Fas-DR: Fas death receptor; FLIP: (Fas-associated death-domain-like IL-1β converting enzyme)-inhibitory protein; HI: Hypoxia-ischemia, Hsp: heat shock protein; IAP: inhibitor of apoptosis JNK, Jun N-terminal kinase; NFκB: nuclear factor-kappa B; PARP-1: Poly [ADP-ribose] polymerase-1; RIP: receptor interacting protein; TNFR: tumor necrosis factor receptor; TRADD: TNFR-associated death domain.

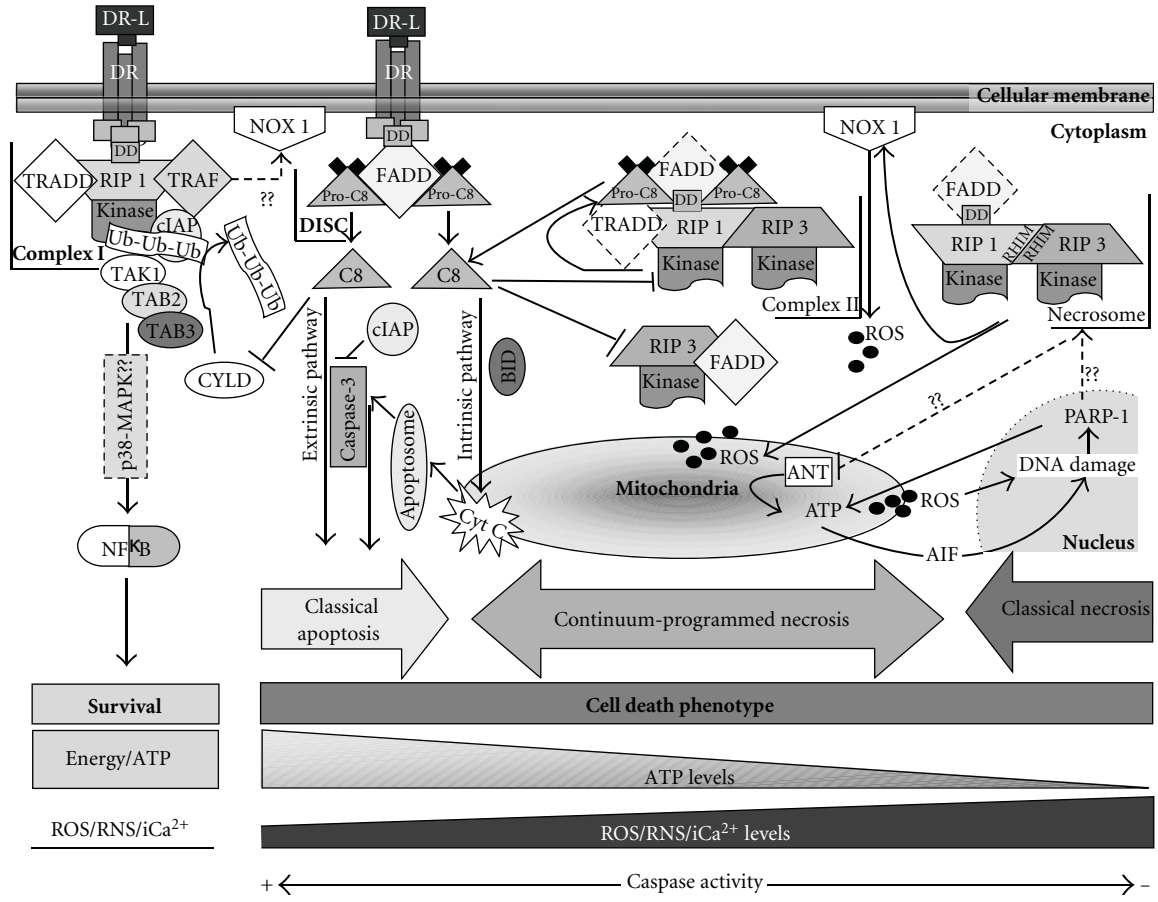

Figure 1: The role of RIP-1 in programmed necrosis. Following neonatal HI, members of the TNFR superfamily (also called death receptors, DR) are activated by their ligands (DR-L) (i.e., FasL, TNF-α). In the setting of energy sufficiency and upon TNFR activation, TNFR undergoes a conformational modification of its cytoplasmic portion allowing the interaction with receptor interacting protein (RIP)-1 with the death domain (DD), TNFR-associated death domain (TRADD), and TNFR-associated factor (TRAF)-2 and -5. They in turn recruit the cellular inhibitor of apoptosis (cIAP) forming the complex I. cIAP inhibits caspase-3 activation and allows ubiquitylation of RIP-1. Next, transforming growth factor-β-activated kinase (TAK)-1/TAK-1 binding protein (TAB)-2/TAB-3 form a complex that binds to ubiquitin residues on RIP-1 and activates nuclear factor-κB (NFκB). This may occur via a p38 mitogen-activated-protein-kinase-(p38-MAPK-) dependent pathway. Complex I may interact with NADPH oxidase (NOX 1) producing ROS, also possibly triggering programmed necrosis. Deubiquitylation of RIP-1 by the enzyme cylindromatosis (CYLD) favors the transformation of complex I to complex II binding to the internalized death-inducing-signaling-complex (DISC, formed by FAS-associated protein with death domain (FADD) and procaspase-8 (Pro-C8)) and RIP-3 (Complex II). If energy is only partially insufficient, RIP-1 activates caspase-8 (C8) signaling for classical apoptosis via intrinsic (where truncated BID binds to the outer mitochondrial membrane allowing the release of cytochrome C (Cyt C) and triggering apoptosome formation) or extrinsic pathway resulting in caspase-3 activation. In this setting, caspase-8 cleaves RIP-1 and RIP-3 preserving signal for apoptosis; however, if energy failure evolves, caspase activity declines favoring (i) preservation of the RIP-1 kinase activity, (ii) decrease in RIP-3/FADD constitutive interaction, and (iii) autophosphorylation between RIP-1 with RIP-3 at the RIP homotypic interaction motif (RHIM) forming the necrosome. Necrosome induces reactive oxygen species (ROS) production via activation of NOX 1 at the cellular membrane or direct effects in the mitochondria. ROS cause DNA alkylation increasing activation of calpain-dependent poly(ADP-ribose)polymerase-1 (PARP-1) which is normally required for DNA repair. Hyperactivation of PARP-1 induces ATP depletion and apoptosis-inducing factor (AIF) translocation from the mitochondria to the nucleus which in turn produces further DNA damage and PARP-1 activation. Necrosome formation is a a potential intermediate step that follows PARP-1 activation potential intermediate steps that follow PARP-1 activation. There is some evidence that it produces ATP depletion via inhibition of adenine nucleotide translocase (ANT) in the inner mitochondrial membrane. Mitochondrial dysfunction is likely at the core of the events resulting in programmed necrosis.

favoring the transition of complex I to cytosolic complex II via internalization of activated TNFR and formation of the death-inducing signaling complex (DISC) containing TRADD, Fas-associated protein (FADD) and procaspase 8 [32, 70, 71]. When RIP-1 kinase is active, caspase-8 is cleaved and activated, initiating the intrinsic and extrinsic apoptotic cascades [72]. Activated caspase 8 can then cleave RIP-1 and RIP-3 and consequently limit programmed necrosis [73, 74] (Figure 1). However, in the setting of more severe ATP depletion, caspase activity is inhibited allowing the

formation of the RIP-1/RIP-3 complex, the necrosome, and cell death proceeds via programmed necrosis [10, 11, 75]. Interaction between RIP-1 and RIP-3 occurs at the RIP homotypic interaction motif (RHIM) which is the site of mutual phosphorylation [76]. Other RIP-1-dependent pathways do not require kinase activity as suggested by the lack of modulation of NFκB following RIP-1 kinase blockade with necrostatin in cell culture [19]. Once again, no studies have addressed the formation of complex II *in vivo* following neonatal HI.

The interaction between FADD, RIP-1, and RIP-3 appears to be critical following TNFR activation [77]. RIP-1 is recruited to FADD in a TNF-dependent manner, while RIP-3 is more constitutively associated with FADD [78]. Following TNF exposure of cell cultures, FADD-deficient cells undergo RIP-3- and CYLD-dependent programmed necrosis with prominent inflammation, suggesting that FADD may prevent formation of the necrosome [79]. In addition to FADD, caspase 8 also seems to be necessary for survival of cultured cells due to its role in modulating CYLD activity and perhaps other functions [80]. In the developing mouse brain, there is abundant expression of caspase 8, TNFR, FAS death receptor, FADD, RIP-1, and RIP-3 [6, 7, 63]. In the normal developing brain, RIP-3 and FADD coimmunoprecipitate; following HI, RIP-1 is recruited to complex with RIP-3 disrupting RIP3's association with FADD [7]. These events are RIP-1 kinase dependent as proven by the partial restoration of RIP-3 and FADD association following treatment with necrostatin [7].

In the neonatal HI model, necrostatin not only provides neuroprotection but also partially shifts the death phenotype from necrosis to apoptosis validating the reality of the cell death continuum and providing insights into mechanisms that may drive the cell death continuum [6, 7]. A similar finding has been reported in cell culture; knockdown of RIP-1 prior to TNFα exposure switches cell death from necroptosis to apoptosis [42]. Some factors that may permit a switch from necrosis to apoptosis in mice treated with necrostatin early after HI are (i) preservation of the mitochondrial function and consequently ATP production, (ii) inhibition of FLIP ((Fas-associated death-domain-like IL-1β converting enzyme)-inhibitory protein) gene and protein expression [7, 81]; (iii) the fact that RIP-1 pathways leading to survival and apoptotic cell death are not kinase dependent [10, 19, 82]. We suspect that necrostatin-1, by blocking programmed necrosis, may allow a "cleaner" and less inflammatory form of cell death, similar to what is described for therapeutic hypothermia [83]. This possibility has not yet been explored.

2.2. Energy: The Driving Force. Mitochondrial dysfunction and energy failure is a hallmark in necrotic cell death following neonatal HI [6, 84–88]. RIP-1-dependent necroptosis evolves with increased reactive oxygen species (ROS) production, decreased ATP production, and decreased mitochondrial membrane potential [89]. In cultured cells, nitric oxide inhibits NADH dehydrogenase (mitochondrial complex I) causing depletion of intracellular ATP and promoting a switch from apoptosis to necrosis [33, 90, 91]. Nitric-oxide-(NO-) induced inhibition of mitochondrial complex I is reversible at low concentrations [91–93] but irreversible at high concentrations resulting in additional free radical production [94, 95]. After neonatal HI, inducible nitric oxide synthase (iNOS) expression and NO accumulation increase, events that are followed by a progressive decline in complex I activity in forebrain during the first 24 h (unpublished data, Pediatric Academic Society Meeting 2011 abstract 2170.2; Neuroscience 2012, submitted). This decline in complex I activity results in a significant impairment in ATP production at early stages following HI that is also prevented by blockade of RIP-1 kinase [96]. Blockade of RIP-1/RIP-3 complex formation in cell culture using necrostatin or RIP-1 siRNA prevents 3-nitrotyrosine accumulation and nitrosylation of complex I and attenuates NO-dependent necrosis [95] similar to findings in the neonatal *in vivo* HI model. These data are consistent with the hypothesis that an intact mitochondrion is initially required to produce physiological superoxide (O_2^-) that will react with NO to generate peroxynitrite ($ONOO^-$) resulting in mitochondrial membrane potential loss [97, 98].

The link between programmed necrosis and opening of the mitochondrial permeability transition pore (MPTP) complex is controversial [22, 99]. However, RIP-1 appears to have direct effects in cellular energy production by translocating to the mitochondria and suppressing ADP/ATP exchange [20, 100] in cell culture. In concert with these findings, necrostatin also prevents the reduction in mitochondrial membrane potential caused by excitotoxic stimuli [101].

2.3. Free Radicals Targeting the Mitochondria. RIP-1 kinase activity is essential for cell death to proceed via the most well-recognized form of programmed necrosis. RIP-1 kinase activity mediates the formation of the necrosome (RIP-1/RIP-3 complex) which induces ROS production via effects on (i) Nox 1 nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and (ii) the mitochondria [23, 45, 102]. Nevertheless, necrostatin is not a direct antioxidant and does not prevent cell death caused by hydrogen peroxide in culture [12, 103]. However, much like hypothermia, inhibition of RIP-1 kinase activity attenuates oxidative injury to proteins following neonatal HI in the mouse and piglet [7, 83]. Similarly, genetic deletion of RIP-3 gene or treatment with RIP-3 silencing RNA (siRNA) in cultured cells prevents increase in ROS and programmed cell necrosis [78]. Potential oxidative injury mechanisms targeted by the blockade of programmed necrosis include (i) blockade of nitric-oxide-mediated mitochondrial dysfunction caused by lipopolysaccharides (LPS) stimulation of macrophages [95], (ii) inhibition of glutamate excitotoxicity [103], (iii) increased glutathione levels [103], and (iv) decreased ROS production [103].

Glutathione (GSH) levels decrease following both excitotoxic and HI insults but blockade of RIP-1 kinase with necrostatin increases GSH production in HT-22 cells following glutamate exposure [45, 103]. In the neonatal HI mouse

model, treatment with necrostatin appears to prevent glutathione oxidation rather than increasing GSH production *per se* [96]. This finding may reflect an indirect effect of the prevention of early protein carbonyl formation afforded by necrostatin-1 after neonatal HI [7] or it may simply be an indirect consequence of neural cell protection.

Recently, a role for Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNip3) has been described in a programmed necrotic-like cell death [104]. This BH3-only protein subfamily includes two members: BNip3 (also called NIP3) and BNip3L (also called NIX or BNip3-like) each with different recognized functions [105, 106]. BNip3 (30 kDa monomer) binds loosely to the outer mitochondria membrane (OMM) [107]. Free radical accumulation induces BNip3 dimerization and insertion into the OMM triggering necrotic-like cell death [104, 108]. In models of neonatal HI, necrostatin prevents early iNOS expression and NO accumulation and blocks hypoxia-inducible factor (HIF)-1α expression (unpublished data), a transcription factor that binds to the hypoxia response element (HRE) at the BNip3 promoter [109, 110]. Because NO modulates HIF-1α expression via Ras modification and phosphorylated extracellular-signal-regulated kinase (ERK) nuclear accumulation [109], it is possible that by preventing NO accumulation, necrostatin could indirectly decrease HIF-1α and consequently BNip3 expression following neonatal HI, protecting the mitochondria and preventing the progress of programmed necrosis. The second member of the BNip3 subfamily, BNip3L, has dual, but distinct, actions depending on the targeted organelle, mitochondria, or endoplasmic reticulum [106]. Although BNip3L has not been studied in models of neonatal HI, there is data from cellular cultures. At the mitochondria, BNip3L induces Bax/Bak-dependent OMM permeabilization, cytochrome c release, caspase activation and apoptosis, while, at the endoplasmic reticulum, BNip3L induces acute release of luminal Ca^{2+} that triggers cyclophilin-D-dependent MPTP complex opening, mitochondria swelling, mitochondrial membrane potential loss, ATP depletion, release of free radicals, and cellular necrosis [106]. Conversely, Bax/Bak has been also associated with programmed necrosis via release of AIF and mitochondrial depolarization [89, 111]. Therefore, both members of the BNip3 subfamily can be classified as sensors of mitochondrial stress as suggested previously [112] and because its expression is modulated by stimuli that are very well-recognized in association with HI, it is possible that both, BNip3 and BNip3L, are linked with the mitochondrial dysfunction seen following HI.

The pathways linking RIP-1 activity and RNS production are mostly unknown. Increased NO accumulation and iNOS expression potentiates glutamate release, *N*-methyl *D*-aspartate receptor (NMDAR) activity, necrotic neuronal death, and progression of excitotoxic injury in cell cultures [33, 113, 114]. Allosteric inhibition of RIP-1 kinase prevents the RNS formation as evidence by the decreased nitration of the NDUFB8 subunit preventing mitochondrial complex I dysfunction and depolarization [95]. Unpublished experiments from our laboratory are in agreement with these finding suggesting that blockade of RIP-1 kinase activity following neonatal HI decreases NO accumulation by 70% coincidently with a decrease in iNOS expression (unpublished data, Pediatric Academic Society Meeting 2011 abstract 2170.2). It remains unknown which mechanisms are operative and if they are directly linked to the inhibition of programmed necrosis. Anti-iNOS/NO effects of necrostatin may involve modulation of inflammatory mediators since cytokines are primary activators of iNOS production by astrocytes and necrostatin decreases cytokine expression [7, 115].

Ultimately, overproduced ROS and RNS attack the mitochondria, depleting ATP production and allowing programmed necrosis to proceed. ROS induces DNA alkylation, an event that increases the levels of calpain-dependent PARP-1 required for DNA repair [27, 28] in the setting of caspase 8 inhibition. Hyperactivity of PARP-1 following glutamate excitotoxicity produces poly-ADP-ribose (PAR) accumulation and ATP depletion inducing translocation of AIF from the mitochondria to the nucleus via a c-Jun-N-terminal-kinase-(JNK)-1-mediated mechanism resulting in chromatin condensation and DNA fragmentation [29, 30]. The importance of PARP-1 activation and AIF translocation in the neonatal brain after HI appears to be gender specific [26, 50]. PARP-1 level peaks at 30 min and again at 12 h following neonatal HI [68] along with an early decrease in nicotinamide adenine dinucleotide (NAD^+) in male mice [26]. Furthermore, PARP-1 genetic deletion [26] or inhibition [67] provides neuroprotection following neonatal HI in male but not female mice. Blockade of calpains, required for PARP-1 activation, using MDL28170 [53] or hypothermia [54] or blocking JNK pathway [41] also decreases necrotic injury after HI. The degree of AIF translocation to the nucleus, also greater in male mice [50], correlates with the infarct size following neonatal HI [46] and its inhibition by heat shock protein (Hsp)-70 [48] TAT-Bcl-xL [49] or hypothermia [51] provides neuroprotection. Although still unclear, steps following PARP-1 activation may include RIP-1 activation as evidenced by the protection against DNA alkylation in RIP-1 knockdown mouse embryonic fibroblast [29]. Altogether, these data suggest an important role of a PARP-1-AIF feedback cycle in the events leading to brain injury following neonatal HI, direct evidence of interaction of AIF with RIP-1 (or the necrosome) has yet to be reported in the immature brain.

2.4. Inflammation and Programmed Necrosis. The importance of inflammation following HI has been extensively studied in the immature brain [116–118]. In normal physiology, a primary function of RIP-1 is to transduce the NFκB signal leading to survival, hence RIP-deficient mice fail to thrive and die within three days after birth with extensive lymphoid apoptosis associated with failure to activate NFκB due to unfavorable conditions to form complex I [32, 119]. Cell culture studies failed to show that RIP-1 kinase modulates NFκB activation [19]. However, *in vivo*, we have shown that blockade of RIP-1 kinase activity using necrostatin following neonatal HI is associated with prevention of early increase in nuclear translocation of NFκB [7]. This effect is

likely indirect but may be of significance given the toxicity associated with early increases in NFκB levels after neonatal HI [69, 120]. Additional confirmation of a possible indirect modulatory effect on NFκB is that transcription of FLIP is downregulated following RIP-1 kinase blockade [7]. Because FLIP is under transcriptional control by NFκB, the decline in early FLIP [121] expression following blockade of RIP-1 kinase with necrostatin may be a reporter for changes in NFκB activity.

NFκB is a transcription factor that also mediates important apoptotic and inflammatory pathways which are central to HI-mediated brain injury in the immature brain [69, 120, 122]. Innate immune responses are dependent on activation of toll-like receptors (TLRs), recruitment of myeloid differentiation primary response gene (MyD)88 and interleukin-1 receptor-associated kinase (IRAK), association of TRAF6 and MAP3K, phosphorylation of I kappa B kinase (IKK) and release and nuclear translocation of the transcriptional factor NFκB (p65/RelA/p50), resulting in change in cytokine expression [122]. Other proinflammatory receptors linked to NFκB include the nucleotide-binding oligomerization domain (NOD) which with the interleukin (IL)-1 converting enzyme protease-activation factor (IPAF) activates caspase 1 (IL-1β converting enzyme) and forms the inflammasome [123–125]. Further details about the inflammatory pathways triggered by NFκB activation may be reviewed elsewhere [122]. Current understanding of the "crosstalk" between programmed necrosis and inflammatory pathways is very limited; however certain interactions can be suspected based on current data. Blockade of programmed cell necrosis and cytokine expression in the neonatal HI model following treatment with necrostatin suggest that inhibition of RIP-1 kinase decreases the activation of the inflammasome, as shown by decreased caspase 1 activity and decreased transcription of IL-1β [7]. Furthermore, TNF-α and IL-6 are also downregulated in mice treated by necrostatin following neonatal HI, suggesting that RIP-1 kinase modulates neuroinflammation. However, it remains unclear if these anti-inflammatory changes are a direct effect of blockade of programmed necrosis pathway or whether they are secondary to the overall neuroprotection.

Although astrocytes provide support to neurons, they also release cytokines that instigate and perpetuate neuroinflammation [126]. TLR are constitutively expressed in astrocytes and modulation of these receptors following HI has been characterized [127]. Following induction of programmed necrosis, reactive astrocytes release cytokines and express iNOS [128], suggesting that changes in the cytokine profile associated with RIP-1 kinase blockade in HI may be related to an effect on astrocytes. Our preliminary results show that following neonatal HI, necrostatin decreases iNOS and cytokine expression while preserving astrocyte mitochondrial ultrastructure and attenuating glial fibrillary acidic protein (GFAP) expression at later stages. One possible hypothesis explaining the neuroprotective and anti-inflammatory effect associated with RIP-1 kinase inhibition is that *in vivo* astrocytes are a primary therapeutic target of necrostatin and by protecting and preserving astrocyte structure and function, it protects neurons and prevents neuroinflammation.

2.5. Gender Differences in Programmed Necrosis. Gender differences have been reported in neonatal rodent models of HI brain injury [7, 26, 50]. These differences may result from intrinsic differences in primary injury pathways. We found a more robust neuroprotection in males than females in response to programmed necrosis blockade [7]. Mechanisms explaining these gender differences are unresolved, but may involve an effect of necrostatin on the more significant decline in NAD^+ following PARP-1 activation [26] and the preferential nuclear translocation of AIF [50] found in male rodents following neonatal HI. Therefore, necrostatin's blockade of RIP-1/RIP-3 interaction, oxidative damage, and inflammation may reflect mechanisms of action upstream and downstream of AIF translocation in male rodents.

3. Conclusions

Neonatal HI brain injury remains a common cause of developmental disability despite ongoing advances in obstetrical and neonatal care. With the advent of hypothermia for treatment of some infants with HI, morbidity has begun to decrease [129]. However, hypothermia is only partially neuroprotective after neonatal HI and 45% of all treated infants still suffer severe neurodevelopmental disability or death despite treatment [130]. Development of adjuvant therapies for hypothermia treatment has been limited to date. Novel approaches to understanding neurodegeneration after neonatal HI are needed. The conceptualization of the apoptosis-necrosis "continuum" in neonatal brain injury in 1997 predicted important mechanistic interactions between apoptosis and necrosis pathways [131]. Evidence of programmed necrosis in neonatal HI is in complete agreement with this sentinel observation and provides an important new direction for future research [7]. Programmed necrosis has been well studied in cellular cultures with new findings published routinely but the recognition of its importance in neonatal HI is just beginning. Many components of the signaling pathway now known to also regulate programmed necrosis have been studied over the last decade in models of neonatal HI as part of the apoptotic pathways showing the clear overlap of these pathways (Table 1). As we now begin to understand the contribution of programmed necrosis to neural cell fate following HI injury, we should take a fresh look at previous findings from these earlier studies. However, many questions remain unanswered with respect to programmed necrosis and neonatal HI including (i) direct effect, if any, of RIP-1 (or the necrosome) in disruption of mitochondrial bioenergetics; (ii) role of calpain-mediated lysosomal destabilization in the progression of injury; (iii) link between RIP-1 and PARP-1-AIF feedback cycle; (iv) identification of neural cell types most vulnerable programmed necrosis and the role of individual neural cell types in propagation or resistance to programmed necrosis; (v) the cellular mechanisms activated following necrosome formation in the immature brain; (vi) whether specific inhibitors of programmed necrosis will be clinically

useful; (vii) what effect, if any, current therapies have on programmed necrosis following HI. Studies such as these will provide new perspectives on the mechanisms of neuronal cell death *in vivo* and may lay the foundation for new effective therapies for neonatal HI.

Abbreviations

ATP: Adenosine-5′-triphosphate
AIF: Apoptosis inducing factor
BNIP: BCL2/adenovirus E1B 19 kDa protein-interacting protein
ASC: Caspase recruitment domain
cIAP: Cellular inhibitor of apoptosis
CYLD: Cylindromatosis
DISC: Death-inducing signaling complex
DNA: Deoxyribonucleic acid
ERK: Extracellular-signal-regulated kinase
FADD: Fas-associated protein
FLIP: (Fas-associated death-domain-like IL-1β converting enzyme)-inhibitory protein
Fas-DR: Fas death receptor
GFAP: Glial fibrillary acidic protein
GSH: Glutathione
Hsp: Heat shock protein
HIF: Hypoxia-inducible factor
HI: Hypoxia-ischemia;
HIE: Hypoxic-ischemic encephalopathy
HRE: Hypoxia response element
IKK: I kappa B kinase
iNOS: Inducible nitric oxide synthase
IL: Interleukin
IPAF: Interleukin (IL)-1 converting enzyme protease-activation factor
IRAK: Interleukin-1 receptor-associated kinase
JNK: Jun N-terminal kinase
LPS: Lipopolysaccharides
MyD: Myeloid differentiation primary response gene
MPTP: Mitochondrial permeability transition pore
MAP: Mitogen activated protein
NAD^+: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NO: Nitric oxide
NMDAR: *N*-methyl *D*-aspartate receptor
NFκB: Nuclear factor-kappa B
NOD: Nucleotide-binding oligomerization domain
OMM: Outer mitochondria membrane
PARP-1: Poly (ADP-ribose) polymerase 1
RNS: Reactive nitrogen species
RIP: Receptor interacting protein
RHIM: RIP homotypic interaction motif
$ONOO^-$: Peroxynitrite
ROS: Reactive oxygen species
siRNA: Silencing ribonucleic acid
O_2^-: Superoxide
TLR: Toll-like receptors
TNF: Tumor necrosis factor
TNFR: Tumor necrosis factor receptor
TRADD: TNFR-associated death domain
TRAF: TNFR-associated factor.

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Review Article

Hypoxic-Ischemic Injury in the Developing Brain: The Role of Reactive Oxygen Species Originating in Mitochondria

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Mitochondrial dysfunction is the most fundamental mechanism of cell damage in cerebral hypoxia-ischemia and reperfusion. Mitochondrial respiratory chain (MRC) is increasingly recognized as a source for reactive oxygen species (ROS) in the postischemic tissue. Potentially, ROS originating in MRC can contribute to the reperfusion-driven oxidative stress, promoting mitochondrial membrane permeabilization. The loss of mitochondrial membranes integrity during reperfusion is considered as the major mechanism of secondary energy failure. This paper focuses on current data that support a pathogenic role of ROS originating from mitochondrial respiratory chain in the promotion of secondary energy failure and proposes potential therapeutic strategy against reperfusion-driven oxidative stress following hypoxia-ischemia-reperfusion injury of the developing brain.

1. Introduction

Perinatal hypoxic-ischemic (HI) brain injury is one of the most common causes of severe neurological handicap in children. Estimated life-time costs to support children with cerebral palsy, a common outcome of HI brain injury in neonates, reached 11.5 billion dollars in 2003 [1]. Unfortunately, our understanding the mechanisms of the HI brain injury is not deep enough for the development of mechanism-targeted therapeutic interventions in this disease. Even therapeutic mechanisms of post-HI cerebral hypothermia (the only clinically proven neuroprotective strategy) are still not well defined which precludes an optimal use of this potentially powerful strategy.

Physiologically, HI brain injury could be defined as an acute oxygen and nutrients deprivation to the brain caused by a collapse of cerebral circulation. Hypoxia-ischemia results in severe cellular bioenergetics failure, and if cerebral circulation is not restored, then the brain death is unpreventable. However, if the cerebral circulation is restored for example, as a result of successful resuscitation, then cerebral reperfusion ensures with a full or partial brain recovery. Unfortunately, the same reperfusion can also contribute to the propagation of brain injury initiated by the HI insult. This implies that HI brain injury as a disease, consists of two fundamental pathophysiological events: hypoxia-ischemia and reperfusion. During hypoxia-ischemia and reperfusion mitochondrial dysfunction plays a fundamental role in brain injury. It is now recognized that not only mitochondrial failure to generate ATP during ischemia, but the generation of oxidative radicals and the release of proapoptotic proteins during reperfusion contribute to the cellular damage. The leading molecular mechanisms responsible for the evolution of cell damage and repair during reperfusion change at different timepoints following HI insult (Figure 1). A critical upstream mechanisms to consider in the management of HI brain injury are those linked to an oxidative stress [2]. Therefore, already at the initiation of resuscitation/reperfusion an attempt should be made to limit the reoxygenation-driven burst in generation of reactive oxygen species (ROS) in order to alleviate the severity of oxidative damage to the HI brain.

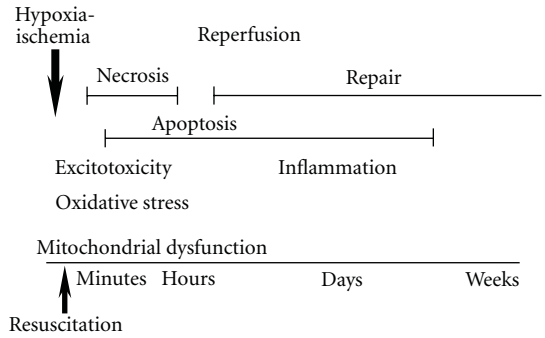

Figure 1: The evolution and major mechanisms of hypoxic-ischemic brain injury. Arrows indicate HI insult and resuscitation (reperfusion), two fundamental events that cause cerebral damage. Different mechanisms may take a lead in the evolution of brain injury: initiated by the bioenergetics mitochondrial dysfunction, cellular injury is driven by excitotoxicity and oxidative stress, followed by the neuroinflammation. The paper is focused on the proximal to the index event mechanism, an oxidative stress and the role of mitochondrial generation of ROS (see text), modified from [2].

2. HI and Resuscitation

It is known that reintroduction of the oxygen to ischemic tissue potentiates oxidative injury. An initial attempt to limit formation of ROS could be made by judicious use of oxygen during resuscitation. Not too long ago, in 2000 the use of 100% oxygen was indisputably recommended for the initiation of resuscitation in all depressed infants [3]. Now neonatologists have tempered their enthusiasm for the use of pure oxygen in neonatal resuscitation. Several clinical trials showed that in the majority of depressed infants the goal of resuscitation, an immediate survival, could be achieved with the use of room air, as effectively as with the use of 100% oxygen [4–6]. Oxygen is indispensable component of ROS. Therefore, regardless of the primary mechanisms of ROS generation during reperfusion, a switch from a routine use of 100% oxygen to the room air at the initiation of neonatal resuscitation, potentially, should limit the severity of an oxidative stress. Indeed, Vento et al. reported a significantly lower level of circulating markers of oxidative stress in neonates resuscitated with the room air (RA) compared to infants resuscitated with the 100% oxygen [7]. However, it remains to be determined to what extent the use of RA in the resuscitation of infants with HI brain injury attenuates an oxidative damage to the brain. Numerous animal studies clearly demonstrated that hyperoxic re-oxygenation maintained for 30–60 minutes of initial reperfusion was detrimental for neurological outcome in asphyxiated pigs and rodents [8–10]. The use of the 100% oxygen in these animals was strongly associated with exacerbation of an oxidative stress in the brain [8]. Of note, however, the hyperoxic resuscitation in these studies was used for 30–60 minutes. At these time-points of reperfusion a full restoration of systemic circulation was already achieved and this resulted in extreme hyperoxemia. Because the primary goal of resuscitation is the return of spontaneous circulation (ROSC), experiments in which the hyperoxic resuscitation is applied beyond the time-point of the ROSC have limited translational importance for the resuscitation science. However, the references cited above do provide an important translational message for the post-resuscitation medical care: All efforts should be made to avoid hyperoxemia in reperfusion.

Although, normoxic resuscitation has been shown to be effective in the majority of infants, it is still undetermined whether the use of RA in the resuscitation of severely (a complete circulatory arrest) asphyxiated infants is as effective as the use of 100% oxygen in achieving ROSC. After a prolonged (25 minutes) cardiopulmonary arrest in mature pigs, the resuscitation with the use of positive pressure ventilation significantly improved the rate of sustained ROSC and cardiac output only if the resuscitation was supplemented with hyperbaric (~400% O_2) re-oxygenation [11]. In contrast, following a brief (one minute) cardiac arrest a cardio-pulmonary resuscitation with the use of RA or 100% O_2 resulted in similar rates of ROSC in neonatal pigs [12, 13]. These data suggest that the duration of circulatory arrest may determine whether positive pressure ventilation needs supplementation with 100% O_2 to enhance the rate of ROSC. It is critical to understand that no attempts should be made to attenuate a reperfusion-driven oxidative stress at the expense of the efficacy of resuscitation.

Overall, current data suggest that the use of room air in resuscitation reduces the severity of oxidative stress in the majority of depressed infants at risk for HI brain injury. The simplicity of this approach (restriction of oxygen availability for the formation of ROS), however, underscores our incomplete understanding the mechanisms initiating an oxidative injury to the HI brain. Interestingly, Matsiukevich et al. showed that in neonatal mice subjected to a lethal HI insult evidenced by a complete circulatory collapse, hyperoxic resuscitation limited to the time (2 minutes) needed to achieve a sustained ROSC was not associated with exacerbation of reperfusion-driven acceleration in the rate of ROS emission from isolated brain mitochondria [14]. However, it is yet to be clarified whether ROS originating from mitochondria at the onset and during reperfusion cause an oxidative injury to the HI brain. To date, it is still unclear what are sources of pathogenic oxidative radicals in the HI brain, how to enhance antioxidative mechanisms and what are those mechanisms of injury which are initiated or exacerbated by the ROS.

3. Potential Sources of Reactive Oxygen Species in HI Injury to the Developing Brain

The evolution of ischemic brain injury following restoration of oxygen and nutrient delivery is a paradoxical biological phenomenon. Although, it is clear that without reperfusion/reoxygenation an ischemic tissue does not survive, maladaptive metabolic changes induced by ischemia predispose cell to dysfunction and death upon reperfusion/reoxygenation. The central role in this phenomenon was assigned to ROS, which can be formed only in the presence of O_2. Therefore, an oxidative damage occurs

mostly upon reintroduction of O_2 to the ischemic tissue. In the immature brain antioxidant system is underdeveloped which limits inactivation of some ROS and in particular, hydrogen peroxide (reviewed in [2]). The latter is perhaps the most important tissue-damaging ROS species due to its relative stability and the ability to cross lipid membranes. For example, upregulation of Cu/Zn superoxide dismutase (enzyme which converts superoxide into H_2O_2) increased, rather than decreased the extent of HI brain injury in neonatal rats [15]. This was associated with elevated level of H_2O_2 in the brain. In contrast, transgenic mice overexpressing glutathione peroxidase (enzyme which detoxifies H_2O_2 into H_2O) were markedly protected against HI insult [16]. What is the origin of this H_2O_2? What are the major sources of oxidative radicals responsible for an oxidative brain damage in HI? In an elegant study, Abramov and coauthors have identified three distinct ROS generating systems during simulated HI insult (oxygen-glucose deprivation (OGD)) and reperfusion in cultured neurons mitochondrial respiratory chain (MRC), xanthine oxidase and NADPH oxidase [17]. MRC responds to OGD with a burst of ROS emission, which declined by the end of HI insult secondary to a loss of mitochondrial membrane potential. At the end of HI insult a second elevation in cellular ROS generation was attributable to the activity of xanthine oxidase. A third peak in ROS production was due to activity of NADPH oxidase during reperfusion. Inhibition of either NADPH oxidase or xanthine oxidase resulted in a significant neuroprotection [17]. In immature animals and humans with HI brain injury, elevated level of hypoxanthine was proposed as the evidence for a pathogenic role of xanthine oxidase [18, 19]. However, an inhibition of xanthine oxidase with oxypurinol or allopurinol failed to reduce lipid peroxidation, and did not protect the brain in a rat model of HI injury [20] or in human neonates with perinatal HI insult [21]. Genetic or/and pharmacological inhibition of NADPH oxidase also did not exert neuroprotection in different models of perinatal HI brain injury [22]. Taken together these data challenge a pathogenic contribution of NADPH oxidase or xanthine oxidase to an oxidative brain damage following HI in neonates. Interestingly, Loor et al. using a model simulating HI reperfusion injury in cultured cardiomyocytes demonstrated that genetic overexpression of only intramitochondrial ROS-scavenging enzymes, Mn-superoxide dismutase or phospholipid hydroperoxide glutathione peroxidase protected cells against reperfusion-induced death [23]. In contrast, overexpression of Cu-Zn superoxide dismutase or catalase did not result in the protection [23].

Mitochondria are known as a major source for ROS production in the health and diseases, including brain ischemia-reperfusion injury (reviewed in [24]). In mature animal models of ischemia-reperfusion injury to the brain and heart, mitochondria have been increasingly recognized as an important source for the reperfusion-driven acceleration in ROS release [24–27]. However, rapidly emerging evidence supporting a deleterious role of ROS originating in mitochondria during reperfusion are partially counterbalanced by the reports suggesting a prosurvival signaling mediated by mitochondrial ROS in the heart preconditioning ([28], reviewed in [29]) and in postischemic reperfusion [30]. In the developing brain potential deleterious or prosurvival effects of mitochondrial ROS in HI reperfusion were not studied. In the following part of this paper we discuss the experimental data obtained in the mature animal models of the brain and heart ischemia-reperfusion injury which support the primary role of mitochondrial ROS in oxidative damage.

4. Mitochondrial ROS and HI Reperfusion Oxidative Stress

In mature animals several studies detected a reperfusion-driven acceleration in ROS generation from mitochondria associated with oxidative damage to the postischemic heart [25, 26] and brain [27]. A single study showed that in neonatal mice with genetically ablated C1q component of the classical complement activation pathway, the neuroprotection and attenuation of oxidative HI brain injury were associated with the ability of $C1q^{-/-}$ brain mitochondria to release significantly less ROS in response to HI reperfusion, rather then with altered activation of the terminal complement complex [31]. A pathogenic contribution of ROS originating from mitochondria is supported by the data demonstrating that extrinsic or genetic enhancement of mitochondria-targeted ROS scavengers reduces the extent of injury or/and oxidative stress in animal models of ischemia-reperfusion in several organs ([32–34], reviewed in [35]). Furthermore, pharmacological inhibition of ROS generation in the mitochondrial respiratory chain (MRC) limits the extent of ischemia-reperfusion damage and the expression of markers of oxidative injury [26, 36, 37]. These data highlight MRC as a potential target for an antioxidative therapeutic strategy against HI brain injury. In the MRC, complex I and complex III are two major sites for ROS generation during reperfusion [32, 38]. An inhibitory effect of ischemia on complex I has been suggested as a cause for an accelerated generation of ROS in MRC in hearts [26]. However, interpreting the data on postischemic mitochondrial ROS production might be difficult and requires an appropriate experience. The data on mitochondrial function in ischemia-reperfusion mostly were obtained in isolated mitochondria in vitro, when results depended on the choice of experimental conditions. For example, in mitochondria isolated from different organs, including neonatal mouse brain, the response to inhibition of complex I is either increase or dramatic decrease in ROS emission rates, depending upon a substrate used to donate electrons to MRC. NAD-linked substrates such as malate, glutamate, pyruvate, and so forth, invariably support an elevation in mitochondrial ROS emission following an inhibition of complex I with rotenone (Figure 2(a)). In contrast, the use of FAD-linked substrates such as for example, succinate results in robust decrease in mitochondrial ROS emission following an inhibition of complex I with rotenone (Figure 2(a)). These differences in ROS generation by MRC in response to the same complex I inhibitor are well understood and explained by the differences in the electron transport flows, supported by NAD- or FAD-linked substrates (reviewed in [39]). NAD-linked substrates

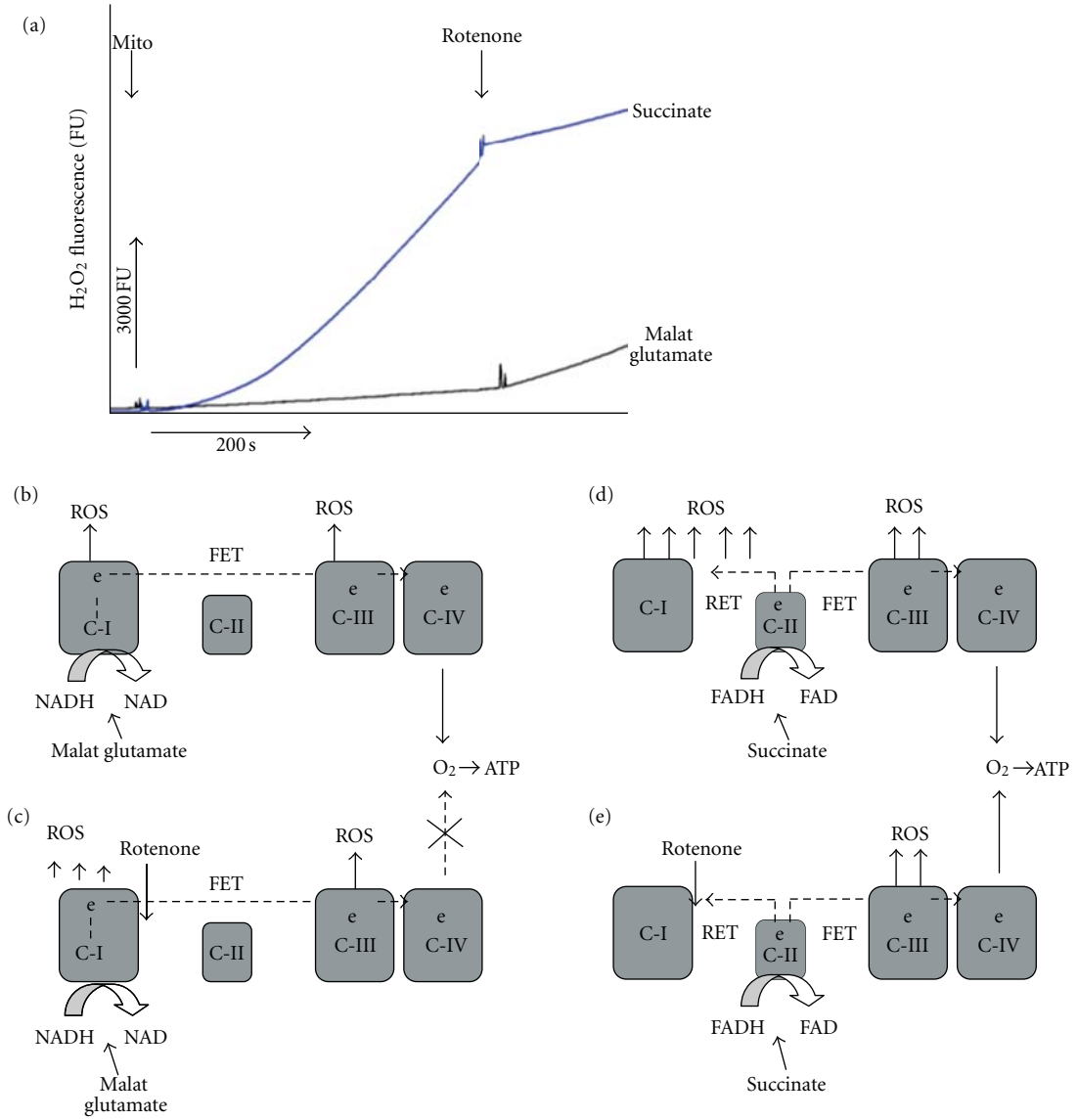

FIGURE 2: (a) H_2O_2 emission rate from brain mitochondria isolated from p10 naïve mouse and supported either with succinate (FAD-linked substrate) or malate-glutamate (NAD-linked substrates). Time-points when mitochondria (mito, 0.05 mg/mL) or rotenone (1 μM) were added are indicated. Cerebral nonsynaptic mitochondria were isolated and mitochondrial H_2O_2 fluorescence was measured using Amplex-ultra-red and horse radish peroxidase assay as described in [31]. (b–e), a schematic mechanism for ROS generation in MRC fueled with NAD-linked substrate, before (b) and after inhibition of complex I with rotenone (c), or FAD-linked substrate, before (d) and after rotenone supplementation (e). RET: reverse electron trasport, FET: forward electron transport.

support only forward electron transport flow (FET), from complex I—to membrane-dissolved ubiquinone—to Complex III—to cytochrome c and finally to oxygen through complex IV (cytochrome c oxidase). During this FET, low levels of superoxide can be generated at unspecified MRC sites (likely at complex I and complex III), because some electrons accidentally escape from MRC electron carriers onto O_2 (Figure 2(b)). Rotenone, pyridaben, thiobarbiturates and other complex I inhibitors interrupt FET between the complex I electron carriers and membrane-dissolved ubiquinone. This interruption of FET increases ROS emission from complex I (Figure 2(c)) secondary to over-reduction of electron carriers (flavin and/or FeS-center N2 and complex I-bound ubiquinone) within this complex (reviewed in [40]). It also stimulates ROS emission from other sources located in the mitochondrial matrix such as for example, dihydrolipoamide dehydrogenase [41, 42], a subcomponent of pyruvate dehydrogenase and ketoglutarate dehydrogenase. This stimulation in ROS production is caused by a decrease in mitochondrial NAD/NADH ratio (as a result of inability of compelx I to oxidize NADH). On the other hand, in the mitochondria fueled with FAD-linked substrates (e.g., succinate) the main electrons flow bypasses Complex I and proceeds from the succinate dehydrogenase (Complex II) to membrane-dissolved ubiquinone, Complex III, cytochrome c, and cytochrome c oxidase. Under specific

conditions, such as moderately elevated membrane potential and abundance of FAD-linked substrate, electron flux can—and does—proceed back from complex II, ubiquinone to complex I and further to the matrix-located NAD. This is called reverse electron transport (RET) flow (Figure 2(d)). It was found that RET is associated with very high rates of ROS emission, about 100 folds greater than that obtained with NAD-linked substrates (reviewed in [39]). The major sites of ROS emission in mitochondria fueled with FAD-linked substrate are thought to be complex I and matrix-located enzymes pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase. Inhibition of complex I with rotenone or similar inhibitors interrupts RET flow and, therefore, substantially diminishes the rate of ROS emission (5–8 folds) (Figures 2(a) and 2(d)) [39]. The RET flow represents the major mechanism for ROS production by mitochondria fueled with succinate, especially in the brain and the heart [43]. It should be noted, that both FET and RET generate proton-motive force and support oxidative phosphorylation of ADP; with RET being about 30% less efficient in terms of energy production but generating tremendously more ROS.

In vivo, under non-pathological conditions the primary electron donor for MRC in brain mitochondria are NAD-linked substrates for example, pyruvate generated in glycolysis. During ischemia-reperfusion, however, substrate availability significantly differs from that in normal cells. There are several lines of evidence to consider that at the onset of reperfusion postischemic mitochondria actively metabolize succinate. Complex I is the most sensitive among all five complexes to the reduction of the cerebral blood flow, and at the end of ischemia the activity of this complex is significantly reduced [44, 45]. In the immature brain HI resulted in slightly (9% on malate-glutamate) to moderately (21% on pyruvate-malate) greater inhibition of mitochondrial respiration tested on NAD-linked substrates compared to that tested on the FAD-oriented substrate, succinate [46]. In mature rats, forebrain ischemia and six hours of reperfusion resulted in a significant inhibition of mitochondrial respiration tested on NAD-linked substrates. However, no significant differences from the control values were detected when the same mitochondria respired on succinate [47]. This suggests, that after brain ischemia the activity of complex II— is better preserved compared to complex I. This favors a succinate-supported respiration upon reintroduction of O_2. Indeed, in the rat brain, ischemia resulted in a profound (8–10 fold) depletion of all NAD-linked substrates: pyruvate, citrate, alpha-ketoglutarate, oxaloacetate, fumarate, and malate. In contrast, the concentration of the succinate increased by ~300% [48] and remained elevated at 15 minutes of reperfusion [49]. Following an acute systemic hypoxemia an oxidation of succinate and glutamate by isolated rat brain mitochondria was significantly (>60%) increased [50, 51]. Furthermore, it is known that succinate oxidation inhibits an oxidation of pyruvate and other NAD-linked respiratory substrates, an event associated with over-reduction of mitochondrial pyridine nucleotides [52]. In the heart, the level of succinate also is markedly elevated during ischemia followed by normalization within 30–60 minutes of reperfusion [53, 54], the time-point associated with near-full restoration of mitochondrial metabolic activity in neonatal HI reperfusion [31]. Thus, if at the initial stage of reperfusion mitochondria actively utilize succinate, then interruption of RET flow by complex I inhibiting agents should reduce ROS generation without significant changing ATP-production rate. If the RET flow-dependent production of ROS causes an oxidative damage following HI, then inhibition of complex I recovery upon reperfusion should reduce an oxidative injury. Indeed, in rats with global cerebral ischemia an inhibition of complex I by rotenone or haloperidol significantly reduced tissue accumulation of hydroxyl radicals, resulting in near-complete abrogation of the reperfusion-driven surge in lipid peroxidation products [27]. Ambrosio et al. reported that inhibition of complex I with the thio-barbiturate amytal resulted in significant reduction in the level of free radicals associated with attenuation of lipid peroxidation in isolated rabbit hearts subjected to ischemia-reperfusion [25]. Our data demonstrated that inhibition of complex I with pyridaben significantly reduced cerebral infarct volume and signs of oxidative injury to the brain tissue and mitochondria following HI in neonatal mice [55]. In the model of cardiac arrest and reperfusion, complex I was proposed as a primary generator of ROS [56]. Taken together, these data suggest that ROS generated in complex I participate in oxidative damage to the postischemic brain and heart, making this complex a reasonable therapeutic target against oxidative stress in the early stages of reperfusion.

In addition to the complex I, complex III has been recognized as an important source for emission of ROS in ischemia and reperfusion [30, 57]. However, experiments with isolated nerve terminals revealed that only very high level of complex III inhibition (70–80%) resulted in detectable elevation in generation of H_2O_2 [58]. Given, that after brain ischemia mitochondrial respiration on succinate was shown to be markedly better preserved compared to that tested on complex I linked substrates [47], the rationale to consider complex III as a therapeutic target in reperfusion is weak. Indeed, in mitochondria respiring on succinate the RET flow (complex I) contribute the most to ROS production. Finally, it is unrealistic to inhibit complex III without robust reduction in production of ATP which could be detrimental for the tissue recovery.

5. The Pathogenic Mechanisms Targeted by Mitochondrial ROS in HI Reperfusion.

Traditionally, a detrimental effect of oxidative stress is supported by evidence of structural oxidative alterations to the post-HI brain. However, it is also important to determine what specific mechanism of injury could be targeted by ROS during reperfusion. In the design of neuroprotective strategies, it is not only a source of injurious ROS, but also a particular mechanism of damage triggered/exacerbated by these ROS is important to consider. Logistically, if an oxidative stress is one of the earliest reperfusion-driven damaging events, the mechanism targeted by ROS should be in close temporal proximity to the index event.

In the ischemic brain, cells experience glutamate-receptors over-stimulation and cellular Ca^{++} overload, which

occurs to a markedly greater extent in the neonatal brain than in the mature CNS [59, 60]. Mitochondria actively participate in preservation of cellular Ca^{++} homeostasis by up take of Ca^{++} from the cytosol into mitochondrial matrix space (reviewed in [61]). However, if mitochondrial Ca^{++} load exceeds mitochondrial capacity to hold Ca^{++}, then mitochondrial membranes loose their integrity via opening a channel in the inner membrane, termed the mitochondrial permeability transition pore (mPTP). Transient and permanent opening of mPTP has been strongly considered as one of the leading mechanisms of necrotic and apoptotic cell death in the brain and other organs following ischemia-reperfusion injury ([62, 63], reviewed in [64]). It has been shown, that mitochondrial ROS can initiate an opening of mPTP during ischemia [22] and reperfusion [65, 66] even in the absence of cyclophilin-D (the only known structural component of mPTP) or Ca^{++} overload [67, 68]. Mitochondria-targeted antioxidant, mitoTEMPO, partially prevented mPTP opening and attenuated necrosis and apoptosis following simulated ischemia-reperfusion injury in cultured renal tubular cells [69]. Taken together these data suggest, that regardless of the type of the organ, ROS originating from mitochondria upon reperfusion can trigger a loss of integrity in mitochondrial inner membrane, the event suggested as the "point of no return" in propagation of cell death following HI insult.

6. The Role of Mitochondrial Membrane Permeabilization in the HI Brain Injury

6.1. Inner Mitochondrial Membrane Permeability Transition Pore (mPTP) and HI Injury in the Developing Brain. Independent of the developmental stage, HI insult severely inhibits mitochondrial oxidative phosphorylation. It has been shown that in immature brain, at the end of HI insult mitochondrial phosphorylating respiration was significantly suppressed [31, 70, 71]. Reoxygenation/reperfusion restores mitochondrial ADP-phosphorylating capacity, normalizing ATP content in the post-HI brain. However, following several hours of reperfusion mitochondria exhibit a profound decline in their ADP-phosphorylating respiration rates [31, 46], the event known as a secondary energy failure. The molecular mechanism proposed to explain the pathogenesis of secondary energy failure is opening of mPTP. mPTP renders organelles incapable of ATP production due to a loss of proton-motive force and NAD. This bioenergetics failure results in mitochondrial swelling, leading to a permeabilization of the outer mitochondrial membrane and release of pro-apoptotic proteins which eventuates in necrotic and apoptotic cell death [72–74]. It has been shown that in neonatal rats inner mitochondrial membrane opens mPTP at 0–1.5 hours and at 6–8 hours after HI [75]. However, the pathogenic significance of mPTP in the reperfusion injury in the developing HI brain remains uncertain. For example, as opposite to adult mice, neonatal cyclophilin-D knock-out mice were found to be susceptible to HI injury [76]. Earlier the same group has reported that antagonist of cyclophilin-D, cyclosporin-A did not attenuate the extent of HI brain damage in neonatal rats [77]. In contrast, using the same model Hwang et al. reported that cyclosporin-A, injected immediately after HI insult significantly protected developing brain, attenuating both necrotic and apoptotic cell death in neonatal rats [78]. Similar results were obtained in neonatal rats subjected to a mild focal cerebral ischemia-reperfusion [79]. In neonatal rats and mice subjected to a global hypoxia-ischemia-reperfusion injury, a post-treatment with cyclosporine A markedly potentiated the neuroprotective effect of Ca^{++} channel antagonist, nimodipine [80]. Given, that in mature animal models of ischemia-reperfusion injury a pathogenic role for mPTP has been strongly suggested, more extensive research is needed to clarify the contribution of mPTP opening to cerebral HI reperfusion injury in the developing brain.

6.2. Outer Mitochondrial Membrane Pore (OMMP) and HI Injury to the Developing Brain. Following an ischemic insult mitochondrial membrane permeabilization can occur via opening of outer mitochondrial membrane pore (OMMP) induced by Bak/Bax translocation into mitochondria. This pore is thought to be primarily responsible for a release of pro-apoptotic proteins from the mitochondrial inter-membrane space, leading to an apoptotic cell death [81, 82], including that induced by an oxidative stress ([83], reviewed in [84]). Importantly, in HI reperfusion injury to the developing brain Bax dependent OMMP has been suggested as a primary mechanism of injury ([76], reviewed in [85]). Developmental shift toward a priority of the Bax-dependent OMMP over the cyclophylin-D dependent mPTP opening in the HI brain damage has been supported by the data obtained in cyclophilin D knock-out neonatal mice [76], as well as by neuroprotective effect of Bax-inhibiting peptide [86]. However, in contrast to a better understanding of events leading to secondary energy failure and necrotic cell death following an opening of mPTP, it is less clear how Bax/Bak mediated OMMP opening affects oxidative phosphorylation and results in secondary energy failure and necrosis. One possibility is that postischemic opening of OMMP results in a massive loss of cytochrome c from the inter-membrane mitochondrial space which results in secondary inhibition of oxidative phosphorylation. However, this loss of cytochrome c was not mediated by mPTP opening, and was not associated with changes in mitochondrial Bax, Bad, Bak or Bid [87]. Although, mitochondrial ROS appeared to be critical for the execution of Bax/Bak dependent apoptosis induced by anti-cancer drugs [88, 89], we have not found data that ROS originating in mitochondria are involved in the Bax/Bak-induced apoptosis in HI brain injury. Interestingly, oxidative stress-induced cell apoptosis clearly required the presence of ROS originating from MRC to signal mPTP opening, but this apoptosis was independent of Bax translocation [90]. The existence of two relatively independent mechanisms of mitochondrial membrane permeabilization does not exclude the contribution of each of these mechanisms in HI damage to the developing brain. Indeed, there is evidence for involvement of cyclophilin D dependent mPTP opening in the Bax-driven cytochrome c release in the isolated mitochondria [91].

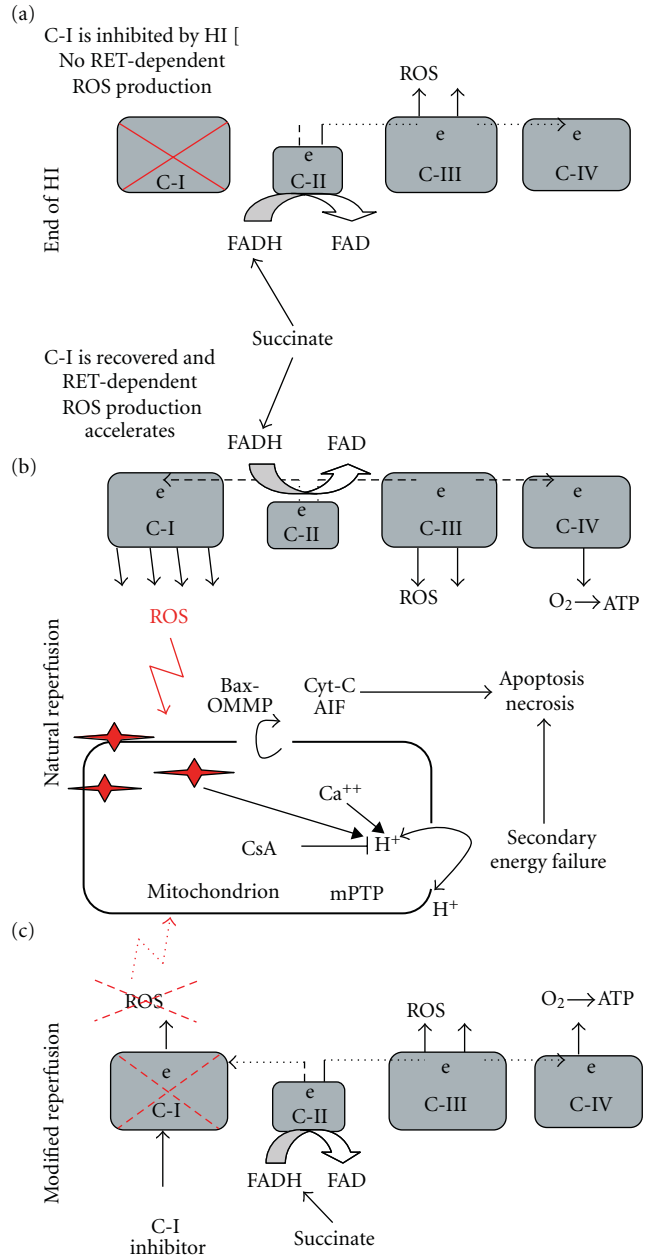

FIGURE 3: Proposed mechanisms of HI injury targeted by mitochondrial ROS and mechanisms of ROS generation in the MRC at the end of HI insult (a), at the initiation of a natural reperfusion (b), and the reperfusion therapeutically modified by an inhibition of the reperfusion-driven complex I recovery (c). Arrows indicate the leak of cytochrome c and apoptosis inducing factor (AIF), the loss of proton motive force and Ca^{++} and ROS contribution to the mPTP opening. CsA is a cyclosporine A which partially inhibits mPTP.

In conclusion, the analysis of current data supports the hypothesis that in the developing HI brain reoxygenation/reperfusion causes not only recovery of cell bioenergetics, but also accelerates ROS generation in mitochondrial respiratory chain (Figures 3(a) and 3(b)). These ROS can cause an oxidative damage to mitochondrial membranes. This damage occurs in the forms of mPTP and Bax/Bak dependent outer membrane pores, both of which are considered as a "point of no return" in the evolution of HI injury. With data that complex I contributes to accelerated generation of ROS during reperfusion, a novel neuroprotective strategy against reperfusion-driven mitochondrial membrane permeabilization may consist of reversible pharmacological inhibition of complex I recovery following HI insult (Figure 3(c)).

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Research Article

Regional Differences in Susceptibility to Hypoxic-Ischemic Injury in the Preterm Brain: Exploring the Spectrum from White Matter Loss to Selective Grey Matter Injury in a Rat Model

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Models of premature brain injury have largely focused on the white matter injury thought to underlie periventricular leukomalacia (PVL). However, with increased survival of very low birth weight infants, injury patterns involving grey matter are now recognized. We aimed to determine how grey matter lesions relate to hypoxic-ischemic- (HI) mediated white matter injury by modifying our rat model of PVL. Following HI, microglial infiltration, astrocytosis, and neuronal and axonal degeneration increased in a region-specific manner dependent on the severity of myelin loss in pericallosal white matter. The spectrum of injury ranged from mild, where diffuse white matter abnormalities were dominant and were associated with mild axonal injury and local microglial activation, to severe HI injury characterized by focal MBP loss, widespread neuronal degeneration, axonal damage, and gliosis throughout the neocortex, caudate putamen, and thalamus. In sum, selective regional white matter loss occurs in the preterm rat concomitantly with a clinically relevant spectrum of grey matter injury. These data demonstrate an interspecies similarity of brain injury patterns and further substantiates the reliable use of this model for the study of preterm brain injury.

1. Introduction

Preterm deliveries make up more than 500,000, or approximately 12.5 percent, of all infant births in the United States [1]. Although technological advances in neonatal care have dramatically improved the survival rates for the smallest and youngest infants, such advances have yet to fully protect the developing brain from injury and prevent the neurological morbidities associated with prematurity. Of those infants born less than 32 weeks gestational age and weighing less than 1500 g (very low birth weight, VLBW), approximately 10% have motor deficits and up to 60% have neurocognitive disabilities and/or behavioral issues [2, 3]. The most common predisposing factors to premature brain injury are hypoxia-ischemia (HI) and/or sepsis [4–7]. However, all premature newborns are at risk for brain injury and a specific ischemic episode is not required [8]. Specifically, *in utero* HI events (placental insufficiency, chronic fetal-to-maternal hemorrhage, stroke, infection, and inflammation), perinatal events (placental abruption, respiratory failure), and neonatal disorders (chronic lung disease, congenital cardiac abnormalities) are associated with acquired brain injuries that lead to cerebral palsy, intellectual disability, epilepsy, visual and hearing impairment, and issues with school readiness [8–12]. Further, the risk of brain injury and abnormal brain development in the premature newborn can be altered by systemic illness and by critical care therapies [10, 13]. In addition to the individual familial burdens of caring for infants and children with these disabilities, the socioeconomic impact of such care in the United States is estimated to cost in excess of $26.2 billion a year [1].

The neuropathology of premature brain injury is diverse and comprised of multiple lesions. The most commonly observed is periventricular leukomalacia (PVL), and it occurs in greater than 50% of VLBW infants [14, 15]. Historically, it was believed that white matter was exclusively injured following HI in preterm infants, as macroscopic focal necrotic lesions and cysts were easily identifiable on standard, acute cranial ultrasonography [16]. Over the past 10–15 years, cystic PVL has declined in incidence and currently occurs in less than 5% of VLBW infants [15]. However, the increasing application of MRI to the clinical assessment of brain injury in the preterm newborn has now revealed that diffuse noncystic white matter injury is the dominant pattern of white matter injury [13, 17], accounting for more than 90% of PVL and occurring in up to half of premature VLBW newborns [13, 15, 18, 19]. Diffuse PVL is a cell-specific lesion consisting of acute loss of early differentiating/premyelinating oligodendrocytes (preOLs) with accompanying astrogliosis and microgliosis, followed by a deficit in mature myelin producing OLs and subsequent cerebral hypomyelination [16]. The routine use of more advanced MRI methodologies also indicate that cerebral white matter abnormalities are accompanied by injury to grey matter structures in the cerebrum, diencephalon, brain stem, and cerebellum in preterm infants [8, 14]. Increasingly, the term "encephalopathy of prematurity" is used to describe PVL and the associated neuronal/axonal abnormalities and is believed to accurately represent the complex brain injury observed in this patient population [14, 20, 21]. However, it remains undefined how severity of insult relates to the pattern of injury observed.

Many studies conducted in animals and humans have investigated the etiology and pathophysiology of preterm brain injury and its developmental sequelae [22–28]. Following HI, with or without underlying infection, an intricate cascade of cellular injury comprised of excitotoxic, oxidative, and inflammatory events converge to produce cell death in an immature brain that is temporally and developmentally vulnerable [14, 16]. Although cerebral ischemia and systemic infection/inflammation are the two major upstream pathogenic mechanisms, preOLs are intrinsically vulnerable in the preterm brain and are immensely susceptible to excitotoxicity, microglial activation, and free radical attack [16, 28–31]. We have studied the pathophysiology of HI-injury *in vivo*, using a rat model of unilateral carotid artery ligation (UCL) followed by hypoxia [25, 31, 32]. Many variations and modifications to this model have been made over the years allowing for the study of preterm and term equivalent brain injury. While there is considerable data from human pathological studies of encephalopathy of prematurity [14], few rodent studies have addressed the relative susceptibilities of different brain regions exposed to HI injury at a preterm equivalent age. The goal of this study was to investigate the regional relationships, susceptibilities, and patterns of HI induced grey matter injury as it relates to a clearly defined spectrum of diffuse white matter injury in the preterm rodent brain. It was hypothesized that mild HI would result in white matter injury alone, and that an increase in the severity of HI-induced white injury would result in an increase in the severity and the regional diversity of cortical and subcortical grey matter injury.

2. Materials and Methods

2.1. Varying Hypoxic-Ischemic (HI) Injury with Carotid Artery Ligation and Hypoxia. All procedures were approved and in accordance with guidelines set forth by the Animal Care and Use Committee of Children's Hospital Boston (Boston, MA, USA). To perform carotid artery ligation, male P6 Long-Evans rat pups were anesthetized with ether. A midline incision at the base of the neck was made, and the left common carotid artery was exposed, isolated from the sympathetic chain and vagus nerve, and permanently ligated using a microelectrocauterizer. One-to-two midline sutures were placed and the neck wound closed. After surgery and recovery from sedation, but prior to hypoxia, pups were allowed to reside with dam for 1-2 hr to ensure full recovery and appropriate hydration. To induce hypoxia, pups were placed in a sealed, global hypoxic environment held at 6% O_2 balanced N_2. Normothermia was maintained throughout hypoxia with the aid of thermal blankets. Core body temperature was monitored by rectal probe prior to and after surgery. Surgical times and weights of the animals at P6 and P9 were obtained (Table 1).

To create a spectrum of HI-induced brain injury, pups were randomized to one of five groups of hypoxia exposure duration. These groups included: exposure to 6% O_2 for 1 hr 0 min ($n = 10$), 1 hr 5 min ($n = 9$), 1 hr 10 min ($n = 8$), 1 hr 15 min ($n = 11$), or 1 hr 20 min ($n = 11$). Litter-matched sham controls were neither subject to carotid ligation nor hypoxia (Table 1). Following hypoxia, pups were returned to their dams until euthanasia.

2.2. Histological and Immunohistochemical Analysis of Brain Injury. All pups (control and UCL/hypoxia) were euthanized by terminal pentobarbital anesthesia followed by intracardiac perfusion of PBS and 4% paraformaldehyde (PFA) at 72 hr (P9). Brains were then removed and postfixed in 4% PFA, at 4°C, followed by cryoprotection in 30% sucrose. Serial, 16 μm, coronal sections were obtained via cryostat (Leica CM3050S) and collected from each animal at the level of the anterior hippocampus through to the posterior hippocampus. Hematoxylin and eosin (H&E) staining and Fluoro-Jade B (FJB) (Chemicon) staining were performed according to standard and manufacturer protocols. Immunohistochemistry was performed as previously published [29, 31, 33, 34] using the following primary antibodies: mouse monoclonal antibodies to myelin basic protein (MBP/SMI-99, 1 : 1000, Covance), CD68 (1 : 100, Serotec), and glial fibrillary acidic protein (GFAP/SMI-22, 1 : 1000, Covance); rabbit polyclonal antibody to fractin (1 : 1000, Chemicon). Briefly, sections were blocked with 5% normal goat serum and then incubated overnight at 4°C with the appropriate primary antibody. The following day, a species appropriate secondary antibody (goat anti-mouse Alexa Fluor 488 or 568, Invitrogen) was applied to the slides for 1 hr at room temperature. Slides were then rinsed and cover-slipped with

Table 1: Summary and comparison of study animal characteristics by hypoxia time.

Characteristics	Controls $n = 10$	1 hr 0 min $n = 10$	1 hr 5 min $n = 9$	1 hr 10 min $n = 8$	1 hr 15 min $n = 11$	1 hr 20 min $n = 11$
Postnatal day at study start/surgery	6	6	6	6	6	6
Postnatal day at sacrifice	9	9	9	9	9	9
Mean weight at PD 6, grams ± SEM	13.19 ± 0.45	13.61 ± 0.36	13.67 ± 0.32	14.08 ± 1.35	13.45 ± 0.67	13.19 ± 0.33
Mean weight at PD 9, grams ± SEM	19.53 ± 0.63	19.20 ± 0.46	19.70 ± 0.35	20.07 ± 0.34	19.78 ± 0.26	19.08 ± 0.40
Mean weight gain, grams ± SEM	6.34 ± 0.33	5.59 ± 0.27	6.03 ± 0.20	6.01 ± 0.21	6.33 ± 0.16	5.9 ± 0.20
Surgery time, min ± SEM	N/A	6.83 ± 1.38	9.33 ± 0.88	8.56 ± 0.73	10.18 ± 0.77	7.2 ± 0.64
Mean core temperature at surgery start, Celsius ± SEM	N/A	36.13 ± 0.40	35.2 ± 0.25	34.90 ± 0.18	34.90 ± 0.25	34.80 ± 0.40
Mean core temperature at surgery end, Celsius ± SEM	N/A	33.28 ± 0.62	31.9 ± 0.20	32.20 ± 0.32	32.20 ± 0.42	32.00 ± 0.28
Temperature decrease during surgery, Celsius ± SEM	N/A	2.9 ± 0.31	3.3 ± 0.25	3.0 ± 0.33	2.9 ± 0.56	2.8 ± 0.45

SEM: standard error of mean.
PD: postnatal day.

antifade medium (Fluoromount-G; Southern Biotechnology). Images were obtained on a Zeiss Axioscope, using a Spot Digital Camera and Advanced 4.5 software (Diagnostic Instruments).

2.3. Scoring and Image Analysis. An observer blinded to every aspect of the experimental protocol performed all scoring and image analyses. H&E sections were evaluated by light microscopy for cell loss, pyknotic nuclei, dense areas of eosinophilia, and macrocyst formation in periventricular white matter (WM) and overlying temporal-parietal cortex. Using our previously published semiquantitative scoring system [31], loss of WM was measured by Image J quantification of 2.4 mm^2 field of MBP in periventricular WM at the level of the middorsal hippocampus, 2.8–3.1 mm from bregma, 2.6–3.0 mm lateral to midline, and anatomically similar cross-sections [35]. The total area of MBP staining ipsilateral to UCL/hypoxia was compared to total area of MBP in the hemisphere contralateral to carotid ligation to determine percent WM change in the HI animals and percent WM in sham controls. Pups were then stratified into groups based on the percent of WM loss in the periventricular region ipsilateral to carotid ligation as compared to the contralateral hemisphere. Analysis groups were assigned as follows. Grade 0: *no discernable MBP loss* (0% reduction in MBP ipsilateral to carotid ligation as compared to contralateral WM), Grade 1: *mild MBP loss* (1–37% reduction in MBP ipsilateral to carotid ligation compared to contralateral WM), Grade 2: *moderate MBP loss* (38–69% reduction in WM ipsilateral carotid ligation compared to contralateral WM), and Grade 3: *severe MBP loss* (70–100% reduction in MBP ipsilateral to carotid ligation compared to contralateral WM) [31]. All sham control animals were scored using the same methodology.

Gliosis and evidence of neuroinflammation were defined by concurrent reactive astrocytosis and activated microglia and were identified by immunostaining for GFAP and CD68, respectively. Neuronal degeneration and axonal injury were identified using FJB and fractin immunostaining, respectively. Regions evaluated were the periventricular WM and overlying temporal-parietal cortex, hippocampus, thalamus, internal capsule, and caudate putamen as per the Stereotactic coordinates listed above. For each stain/immunostain, scoring was evaluated on a 0–3 point scale based on the density of FJB/immunopositive cells, where 0: no GFAP, CD68, fractin or FJB-positive cells; 1: diffuse areas of mild staining/immunoreactivity; 2: moderate staining/immunoreactivity occurring in dense, focal or columnar patches; 3: widespread severe staining/immunoreactivity distributed throughout the entire brain region (Figures 2 and 3).

2.4. Statistical Analysis. Data is expressed as mean ± standard error of the mean (SEM). Normally distributed data differences between two groups were compared using Student's t-test. Multiple groups were compared using one-way ANOVA with the Bonferroni multiple-comparison *post hoc* test. Nonparametric datasets were compared using the Mann-Whitney rank sum test. A P value of ≤ 0.05 was considered statistically significant. Data were analyzed with SigmaStat 3.11 software (Systat Software 2004).

3. Results

3.1. Graduated Increase of Periventricular White Matter Loss with Lengthening Periods of Hypoxia after Carotid Ligation. A total of 49 HI rat pups and 10 litter matched sham control pups (no surgery, no hypoxia) were evaluated in this study. There were no statistically significant differences in weight at P6 or P9, weight gain/growth over the 72 hr evaluation period, core body temperature at start or end of surgery, overall core body temperature decrease during surgery, and time for UCL surgical procedure between rats that had different durations of hypoxia (1 hr 0 min, 1 hr 5 min, 1 hr 10 min, 1 hr 15 min, and 1 hr 20 min) after UCL at P6 (Tables 1 and 2). However, the different durations of hypoxia resulted in a spectrum of WM loss from undetectable to mild, moderate, and severe as evidenced by a graduated reduction in MBP immunoreactivity at P9 (Figure 1). Coronal sections from all HI animals (HI) were evaluated and then classified in myelination groupings as no MBP loss (Grade 0; $n = 16$), mild MBP loss (Grade 1; $n = 13$), moderate MBP loss

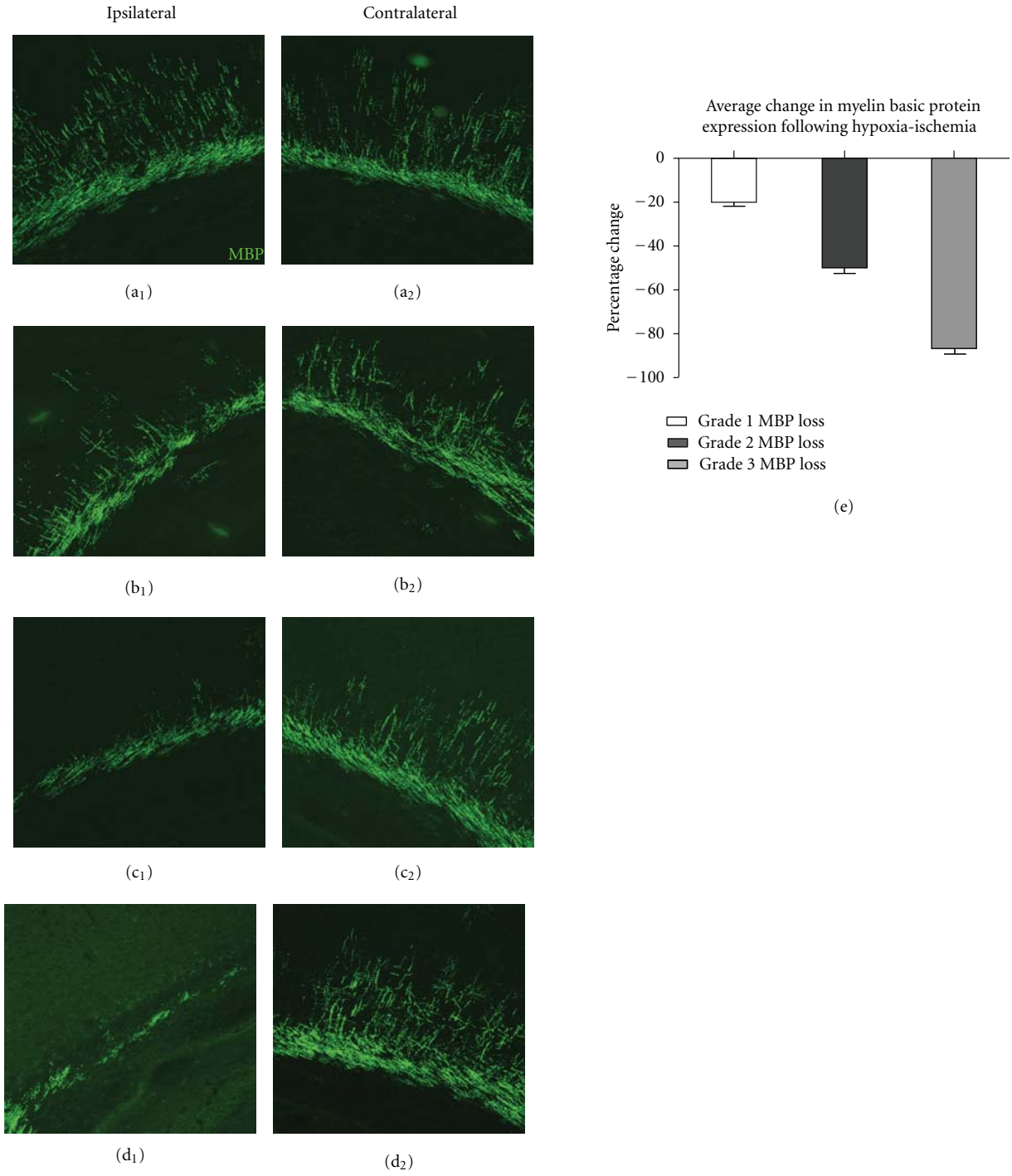

Figure 1: Periventricular white matter loss following hypoxia-ischemia (HI) in postnatal day 6 (P6) rats. Seventy-two hours following HI at P6, myelin basic protein (MBP) is significantly depleted in the hemisphere ipsilateral to carotid ligation. Representative photomicrographs show MBP in hemispheres both ipsilateral (a_1–d_1) and contralateral (a_2–d_2) to carotid ligation in animals with Grade 0 MBP loss (no discernable white matter injury, a_1-a_2), Grade 1 MBP loss (mild white matter injury, b_1-b_2), Grade 2 MBP loss (moderate white matter injury, c_1-c_2), and Grade 3 MBP loss (severe white matter injury, d_1-d_2). Histogram shows average percent change in MBP for each group (e).

Table 2: Summary and comparison of study animal characteristics: by analysis group.

Characteristics	Controls $n = 10$	Grade 0 MBP Loss $n = 16$	Grade 1 MBP Loss $n = 13$	Grade 2 MBP Loss $n = 9$	Grade 3 MBP Loss $n = 11$
Postnatal day at study start/surgery	6	6	6	6	6
Postnatal day at sacrifice	9	9	9	9	9
Mean weight at PD 6, grams ± SEM	13.19 ± 0.45	13.56 ± 0.23	13.12 ± 0.28	13.76 ± 0.34	13.95 ± 0.32
Mean weight at PD 9, grams ± SEM	19.53 ± 0.63	19.68 ± 0.28	19.03 ± 0.34	19.67 ± 0.36	20.11 ± 0.32
Mean weight gain, grams ± SEM	6.34 ± 0.33	6.12 ± 0.13	5.91 ± 0.20	5.91 ± 0.21	6.16 ± 0.20
Surgery time, min ± SEM	N/A	8.06 ± 0.74	8.08 ± 0.94	9.56 ± 0.60	9.45 ± 0.86
Mean core temperature at surgery start, Celsius ± SEM	N/A	35.22 ± 0.31	35.21 ± 0.25	35.29 ± 0.21	35.16 ± 0.35
Mean core temperature at surgery end, Celsius ± SEM	N/A	32.77 ± 0.36	31.82 ± 0.45	31.99 ± 0.17	32.05 ± 0.24
Temperature decrease during surgery, Celsius ± SEM	N/A	2.45 ± 0.27	3.39 ± 0.54	3.36 ± 0.23	3.11 ± 0.36

SEM: standard error of mean.
PD: postnatal day.

(Grade 2; $n = 9$), and severe MBP loss (Grade 3; $n = 11$) based on percent WM reduction (Table 2). Animals with mild MBP loss had a mean reduction in MBP ipsilateral to carotid ligation of 20.21 ± 2.09%, $P < 0.001$ (Figure 1(b)). Animals with moderate MBP loss had a mean reduction ipsilateral to carotid ligation of 50.11 ± 2.67%, $P < 0.001$ (Figure 1(c)), and animals with severe MBP loss animals had a mean reduction ipsilateral to carotid ligation of 86.02 ± 3.24%, $P < 0.001$ (Figure 1(d)).

3.2. Relationship of Inflammation and Gliosis to MBP Loss. Next, microglial activation was evaluated as a function of MBP loss (Figures 2(e)–2(h)). Of note, in uninjured sham controls activated microglia and reactive astrocytes were only observed in the white matter, hippocampus, and thalamus. All HI rat pups, including those with Grade 0 MBP loss, had significantly increased numbers of activated microglia within white matter as evidenced by increases in CD68 immunoreactivity compared to controls (mean score 1.88 ± 0.13 versus 1.22 ± 0.15, $P < 0.01$, Figure 2(h)). With increasing WM loss, there were further significant increases in the density of CD68 immunoreactivity within the white matter (mean score Grade 1: 2.08 ± 0.14; mean score Grade 2: 2.67 ± 0.17; mean score Grade 3: 3.0 ± 0.0, $P < 0.01$ for all, Figure 2(h)). In both Grade 1 and Grade 2 MBP loss groups, activated microglia were not only observed in WM but were present in a discrete, patchy columnar pattern in the overlying cortex (Figures 2(e)-2(f)). Accordingly, in brains displaying Grade 2 MBP loss, the density of activated microglia in the cortex was significantly increased compared to control (mean score 1.00 ± 0.29 versus 0.31 ± 0.18, $P = 0.001$). In brains exhibiting Grade 3 MBP loss, activated microglia were significantly more numerous and the columnar pattern to their distribution in the cortex was lost (mean score 2.22 ± 0.22). Specifically, the distribution of the CD68 positive cells was no longer detected in isolated focal patches and was increasingly widespread throughout the brain including the hippocampus (mean score 0.9 ± 0.25, $P = 0.007$), thalamus (mean score 1.72 ± 0.27, $P < 0.001$), and caudate putamen (mean score 1.36 ± 0.28, $P < 0.001$).

Regional patterns and severity of reactive astrocytosis, as evidenced by significant increases in GFAP immunoreactivity in HI pups compared to controls, were similar to the patterns of activated microglia described above (Figures 2(a)–2(d)). Specifically, HI rat pups with Grade 0 MBP loss had a significant increase in WM reactive astrocytosis compared to controls (2.67 ± 0.19 versus 1.80 ± 0.29, $P = 0.04$). In contrast to pups with Grade 0 MBP loss that had numerous reactive astrocytes in the WM but not the cortex, those pups with Grade 1 MBP loss had a statistically significant increase in cortical reactive astrocytosis (mean score 0.5 ± 0.17, $P = 0.03$). In addition, as WM became increasingly injured in the pups with Grades 2 and 3 MBP loss, GFAP immunoreactivity similarly became increasingly dense in the WM (mean score Grade 2: 2.88 ± 0.11; mean score Grade 3: 3.00 ± 0.00) and extended throughout other areas of the brain including the hippocampus, and thalamus ($P < 0.05$ for all regions, Figure 2(d)).

3.3. Regional Predilection and Severity of Cortical and Subcortical Neuronal Degeneration and Axonal Injury as a Function of Periventricular White Matter Injury. Neuronal degeneration occurred more often and with greater severity as WM loss increased (Figure 3). In brains with Grade 3 MBP loss, FJB degenerating cells were significantly increased in the cortex overlying the white matter (mean score 2.18 ± 0.30, $P = 0.001$), thalamus (mean score 1.45 ± 0.34, $P = 0.018$), and caudate putamen (mean score 1.72 ± 0.38, $P = 0.018$).

In addition to assessing neuronal cell body degeneration, axonal injury was assessed with immunostaining for fractin (Figures 3(c), 3(f), 3(i), 3(k)). Unlike FJB, fractin immunoreactivity was present in brains with the mildest white matter injury and regional axonal injury was evident prior to the appearance of FJB-positive cells (Figures 3(j) and 3(k)). Despite this, no statistically significant differences in fractin protein expression was observed in Grade 0 and Grade 1 MBP loss brains, although there was a trend for an increase in these mildly injured brains (Figure 3(k)). In contrast, HI pups with moderate and severe WM loss had statistically significant increases in fractin immunoreactivity

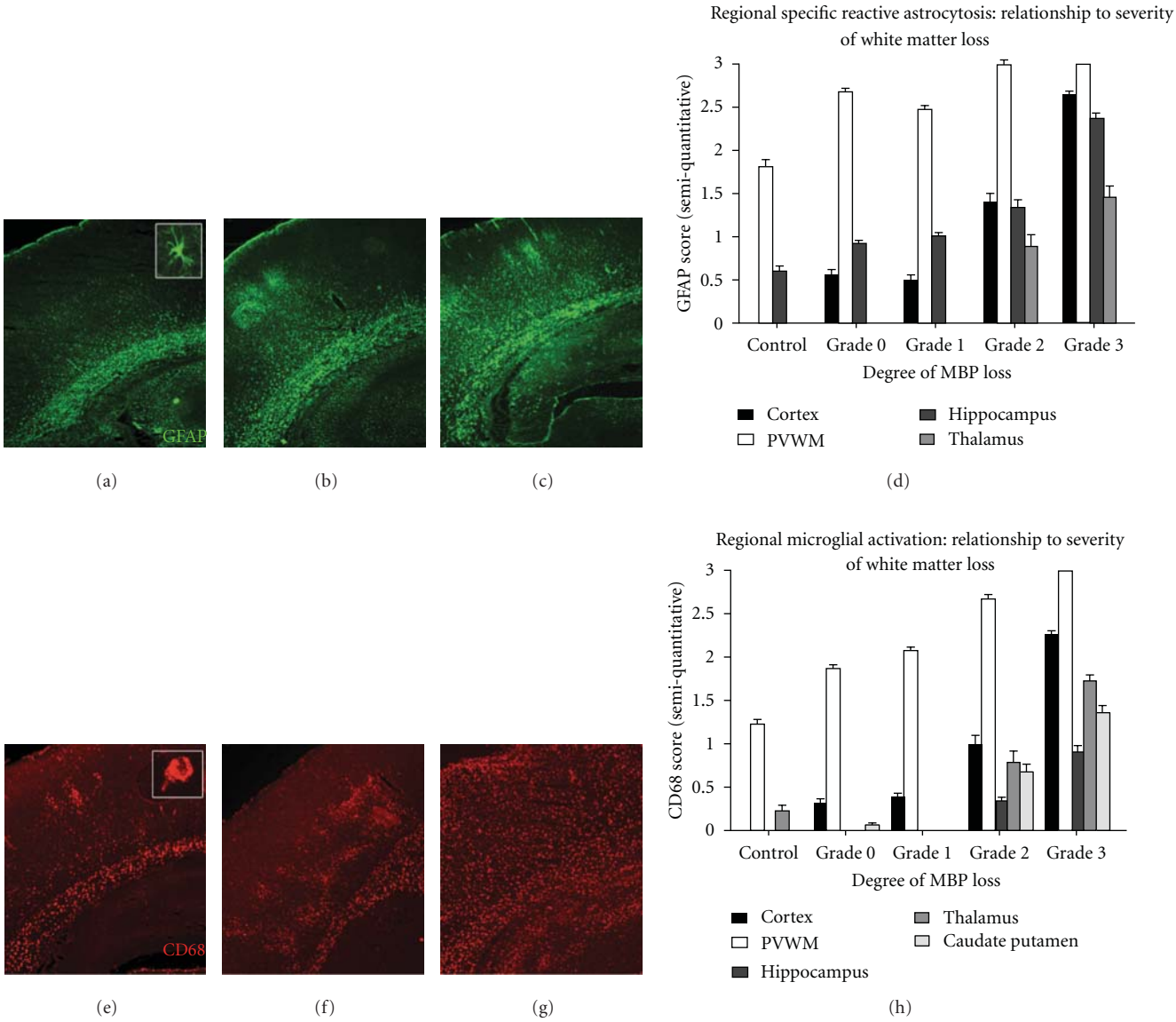

Figure 2: Astrogliosis and microgliosis in relation to severity of white matter injury in hypoxic-ischemic (HI) neonatal rats. Seventy-two hours following HI at postnatal day 6 (P6), numerous reactive astrocytes (a–d) and activated microglia (e–h) are present throughout the brains of neonatal rats with significant white matter injury. Representative photomicrographs depict GFAP-positive astrocytes and mild (a), moderate (b), and severe (c) astrogliosis following HI. Histogram in (d) displays the region-specific pattern and degree of reactive astrocytosis as a function of MBP loss. Lower panels depict CD68-positive microglia and mild (e), moderate (f), and severe (g) microgliosis following HI. Histogram in (h) shows the region-specific pattern and degree of microglial activation as a function of white matter injury severity. Magnification 40x.

(Figures 3(f), and 3(i)). HI animals with Grade 2 MBP loss were observed to have moderate axonal injury in the cortex (mean score 1.51 ± 0.26, $P = 0.015$) and caudate putamen (mean score 1.55 ± 0.39, $P = 0.043$) compared to controls. Axonal injury was greatest in brains with Grade 3 MBP loss, with significant increases in fractin immunoreactivity in the cortex (mean score 2.27 ± 0.27, $P < 0.001$), thalamus (mean score 1.72 ± 0.41, $P = 0.005$), caudate putamen (mean 2.45 ± 0.21, $P < 0.001$), and internal capsule (mean score 2.64 ± 0.28, $P < 0.001$) (Figure 3(k)).

4. Discussion

Clinically, neuroimaging and postmortem analyses have shown that preterm brain injury involves both white and grey matter injury [8, 20, 36–38], and it has been difficult to generate a clinically relevant spectrum of HI pathology in neonatal rodents that closely resembles that observed in humans [9]. Although rodent models are inherently limited due to simplicity of brain structure relative to the human, the data presented here suggest that there is a

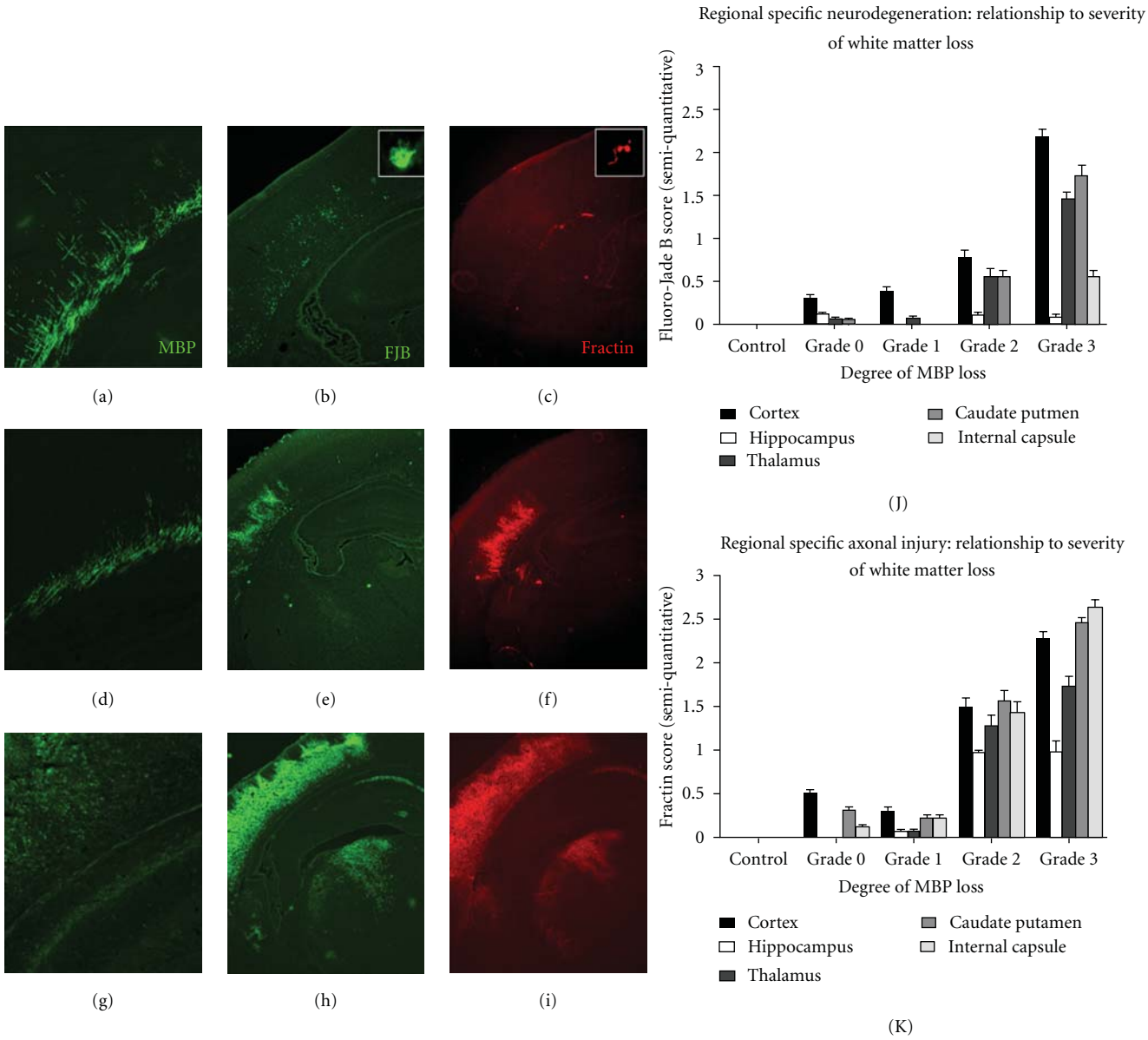

Figure 3: Significant grey matter injury accompanies myelin basic protein (MBP) loss in postnatal day 6 (P6) neonatal rats. Grey matter injury, as determined by neuronal and axonal degeneration, increases in hypoxic-ischemic neonatal rats with the severity of white matter injury. Representative photomicrographs show Grade 1 MBP loss (a), with mild cortical neuronal degeneration (FJB staining, (b)) and axonal injury (fractin immunoreactivity, (c)). With Grade 2 MBP loss (d), the density and distribution of FJB-positive neuronal cell bodies (e) and fractin-positive axons (f) increase in the cortex overlying the periventricular white matter. With Grade 3 MBP loss (g), the density of FJB-positive neuronal cell bodies (h) and fractin-positive spheroids (i) are further increased and encompasses the majority of the cortical mantle, as well as the hippocampus, thalamus, caudate putamen, and internal capsule. Histograms display region-specific neurodegeneration (j) and axonal injury (k) in relation to the severity of white matter loss. Magnification 100x for MBP and 25x for FJB and fractin.

spatiotemporal order of appearance of MBP loss, microglial and astrocytic infiltration, and neuronal somatic and axonal injury following HI. Specifically, we show that HI-mediated white matter injury, even when resulting in mild reductions of MBP, occurs in preterm equivalent rats concomitantly with significant white matter microglial activation and reactive astrocytosis. However, as the degree of periventricular/pericallosal white matter loss increases, the severity and frequency of cortical and subcortical grey matter injury also increase, with a widespread distribution of FJB-positive cells, fractin immunoreactivity, reactive astrocytes and activated microglia, and a regional predilection for the temporal-parietal cortex, internal capsule, caudate putamen, and thalamus. Further, as evidenced by the trend towards increased fractin-positive cells in the brains of animals with no discernable MBP loss, it is possible that mild grey matter injury may be present in this animal model even when periventricular WM loss is not. These findings are similar to injury patterns and

susceptibilities noted in prior human preterm brain injury studies.

Recent experimental evidence, in combination with advanced imaging in the newborn, has led to "a blurring of the grey-white (term-preterm) dichotomy" [8]. It is now common to recognize white matter injury in the term baby and appreciate injury to grey matter structures, such as the thalamus and cerebellum, in the preterm brain [8]. In this investigation, we found that MBP loss appears to be the most sensitive measure of injury, and severity of MBP loss can be used as a benchmark in order to stage other pathophysiological responses such as microglial and astrocytic reactivity and neuronal injury. Interestingly, the damage to grey matter in the preterm rodent occurs in cortical and subcortical structures with similar pattern and distribution to that observed in the human preterm infant [14, 24, 39]. Importantly, these data corroborate the most important cellular aspects of the encephalopathy of prematurity described in the human, and the neuropathology documenting that neuronal loss and/or gliosis is present in 13–30% of cases of noncystic PVL [22]. We show that the cortex, thalamus, internal capsule, and caudate putamen are injured with HI exposure at P6 and that the severity and presence of this injury evaluated at P9 closely correlates to an increasing spectrum of periventricular WM loss. This is especially relevant when it is considered that a Long-Evans rat brain at P6 and P9 is developmentally similar to a 30-week and 40-week gestational age human brain, respectively [33, 34].

Many sequences of events have been proposed to contribute to the major brain sequelae observed in premature infants with PVL, including injury to preOLs, axons, subplate neurons, migrating GABAergic neurons, and thalamus [16, 20, 38]. Primary injury in any one of these areas could lead to the OL cell death, hypomyelination and impaired cortical and thalamic development commonly observed in both the human brain following HI and our model of rodent brain injury [16]. It is well established, however, that developing neurons are highly dependent on trophic support for survival, and that target deprivation and failed tract formation also results in degeneration [8]. Delayed neurodegeneration in a systems-preferential manner is an important component to preterm brain injury, as it results in impairments in the human newborn that evolve into complex disabilities over time [8]. For example, cortical injury following HI will result in later thalamic damage due to loss of trophic support [8]. Detailed neuropathological examinations in human PVL have shown the thalamus to be similarly vulnerable, with neuronal loss, gliosis, and axonal degeneration present in 60% of cases [40]. Further, marked reductions in the density of layer V cortical neurons in human PVL cases have also been documented and may be reflective of injury secondary to necrosis in the underlying white matter [41]. These neuropathological findings in the human cortex corroborate our previous data indicating HI rat pups have significantly reduced cerebral mantle thickness [31]. Collectively, these data highlight the vulnerability of this region and are consistent with long-term MRI followup of older infants and children diagnosed with PVL as preterm babies that also demonstrate a reduction in the cerebral mantle, constituted by decreased cortical and white matter volume [23, 42]. Importantly in this investigation, we show that as injury to the periventricular white matter increases, the severity and frequency of cortical and subcortical grey matter neuronal degeneration increase with a regional predilection for the cortex, caudate putamen, and thalamus. We also document that all HI rat pups, even those without WM loss, exhibit a degree of axonal injury, as evidenced by presence of fractin immunoreactivity. During the peak period of vulnerability to PVL, cerebral white matter axons are rapidly growing. The occurrence of axonal injury in the necrotic foci of severe PVL has been known for years, but the widespread axonal degeneration in diffuse PVL, separate from focal necroses has only recently been documented [14, 38, 43–45]. Consistent with these observations, diffusion tensor imaging in noncystic PVL shows blunting of the normal maturational increase in fractional anisotropy in various axonal tracts [46–50]. Our data indicates that rats with moderate or severe loss of MBP have significantly increased axonal degeneration in the temporal-parietal cortex, caudate putamen, thalamus and internal capsule. As reported in prior animal studies, we also confirm that the hippocampus appears to be less susceptible to axonal injury and neuronal degeneration when exposed to HI at P6 and evaluated at P9 [51]. In our study hippocampal axonal injury occurs most notably, when WM injury is moderate to severe.

Subtle white matter and microstructural abnormalities in preterm infants are commonly associated with developmental impairment and abnormal visual, motor, and cognitive function [13, 18, 26]. Interestingly, we found that pups without evidence of gross periventricular white matter loss exhibited mild selective grey matter injury, as evidenced by mild axonal injury and neuronal degeneration, in the cortex, internal capsule, and caudate putamen; structures central to language processing and understanding, and motor and sensory function. Injury in these regions, even if mild, may be implicated in the neurocognitive disturbances noted in preterm survivors who do not demonstrate other clinical or radiological evidence of overt periventricular white matter injury [14]. Further, these findings demonstrate the increased necessity of combining traditional pathological techniques with high-resolution neuroimaging in animals. Just as diffuse white matter lesions were undetectable in preterm babies before the routine use of advance MRI sequences, subtle white matter abnormalities and HI changes to brain microstructure could go unrecognized in an animal model. Studies currently underway in our laboratory are addressing the connection between MBP loss, structural coherence of white matter, and the 3D course of axonal pathways following HI in the neonatal rodent.

vspace.21ptIn this investigation, all HI pups, including those without MBP loss, had significantly increased numbers of activated microglia and reactive astrocytes in white matter. Additionally, as white matter injury became increasingly severe, the numbers of activated microglia and reactive astrocytes increased in both white and grey matter, including the cortex, hippocampus, thalamus, and caudate putamen. Of note, when examining gliotic changes in the evaluated grey and white matter regions, a low to moderate level of baseline

microglial activation was noted in the periventricular white matter and thalamus of control animals. Similarly, a low to moderate level of reactive astrocytes were also noted in the periventricular WM and hippocampus of controls. In the normal brain, microglia are first prominent in the forebrain in the 16–22 weeks of gestation and reach peak abundance in the cerebral white matter later in gestation [52–54]. In a recent longitudinal study of human brain, microglia density in white matter peaked during the greatest vulnerability to PVL (early third trimester), and then declined in white matter after 37 weeks gestation [16, 54]. Interestingly, as microglia declined in the white matter, their density increased in the cortex [16]. The presence of these cells in the uninjured brain is likely due to their function in a rapidly developing and dynamic brain, and these normal features are consistent with the recognized roles for microglia in brain development, including apoptosis, vascularization, axonal development, and myelination [20, 54]. The role of neuroinflammation in preterm brain injury has similarly been studied and microglia have been suggested to be a convergence point in the potentiation of HI and infection/inflammatory insults [16]. Premature infants are subject to numerous inflammatory conditions and microglia have been recognized as a prominent component of diffuse PVL [16, 55, 56]. Our findings related to the regional distribution of microglial activation, and astrogliosis confirm prior published reports of expression of these cellular subtypes in rodent models of HI induced brain injury [30, 57, 58]. The increase in reactive astrocytes and activated microglia in cortex, caudate, thalamus, and hippocampus may be a consequence of and/or a secondary pathophysiologic response to injury of the cortical neuronal populations, the oligodendrocyte precursors cells, and the subplate neurons that reside in regions adjacent to and in the periventricular WM region and cortical grey matter structures. Confirmatory of these mechanisms are previously published studies showing marked neuroprotection by agents such as doxycycline and minocycline that attenuate microglial activation and neuroinflammation [30, 57, 59].

5. Conclusion

In summary, the data presented here is the first to evaluate the relationship between degree of periventricular WM injury and its associated regional grey matter injury *in vivo* in a Long-Evans rat model of preterm HI brain injury. We show that as WM loss increases, the severity and frequency of cortical and subcortical grey matter injury increase with a regional predilection for the temporal-parietal cortex, internal capsule, caudate putamen, and thalamus. These findings are similar to injury patterns and susceptibilities noted in prior human preterm brain injury studies. Collectively, these data indicate that numerous cellular and molecular questions can be addressed in this translational Long-Evans rat model. This will allow for rapid progress in understanding the pathophysiology and appropriate avenues for intervention after HI injury in the developing nervous system.

Authors' Contribution

D. B. Selip and L. L. Jantzie contributed equally to this work.

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Review Article

The Role of Cytokines and Inflammatory Cells in Perinatal Brain Injury

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Perinatal brain injury frequently complicates preterm birth and leads to significant long-term morbidity. Cytokines and inflammatory cells are mediators in the common pathways associated with perinatal brain injury induced by a variety of insults, such as hypoxic-ischemic injury, reperfusion injury, toxin-mediated injury, and infection. This paper examines our current knowledge regarding cytokine-related perinatal brain injury and specifically discusses strategies for attenuating cytokine-mediated brain damage.

1. Introduction

Preterm birth affects 12.5% of pregnancies in the United States [1, 2] and is the leading cause of neonatal morbidity and mortality, accounting for nearly half of the long-term neurologic morbidity in children [3]. The majority of premature infants in developed countries survive; however, 5–10% of survivors develop cerebral palsy (CP), and 40–50% develop cognitive and behavioral deficits [4, 5]. The prolonged vulnerability of the developing white and gray matter to excitotoxic, oxidative, and inflammatory forms of injury is a major factor in the pathogenesis of perinatal brain injury. While acute catastrophic brain injuries sometime occur (e.g., severe intraparenchymal hemorrhage), injury to white and gray matter regions is most often the cumulative result of metabolic, infectious and/or inflammatory, and hypoxic-ischemic insults over time [6]. For example, early respiratory compromise and systemic hypotension can precipitate glutamate, free radical, and cytokine toxicity to developing oligodendrocytes and neurons. The clinical course might be further complicated by late-onset or necrotizing enterocolitis (NEC). These sequential events result in different topographic patterns of injury based on developmental and genetic susceptibilities.

Although there has been much focus on white matter injury (WMI) in premature infants, gray matter abnormalities in cortical and deep nuclear structures, and cerebellar injuries are also common and likely contribute to development of cognitive delay, psychomotor delay, and CP [7]. A variety of inciting events such as hypoxic-ischemia, infection, and/or inflammation, can stimulate a cascade of secondary responses, including fluid-electrolyte imbalance, regional blood flow alterations, calcium-mediated cellular injury, free-radical generation, oxidative and nitrosative stress, glutamate-induced excitotoxicity, disturbances in proinflammatory cytokine production, mitochondrion function, and apoptotic cell death [6, 8]. These disturbances result in activation of inflammatory cells involved in the innate immune response including neutrophils, macrophages, and resident microglia, which may propagate brain injury through mechanisms that directly and indirectly lead to neuronal and preoligodendrocyte (preOL) cell death or dysfunction.

Cytokines and inflammatory cells are mediators in the common pathways associated with perinatal brain injury induced by a variety of insults [9–12]. A better understanding of the role of cytokines in perinatal brain injury is needed to facilitate the development of strategies to prevent and/or treat cerebral white and gray matter damage.

2. Cytokines Affecting the Fetus and Neonate: What Are They and Where Do They Come From?

Cytokines are small, cell signaling nonstructural proteins involved in regulating hematopoiesis, inflammation, and immune cell proliferation and differentiation. They are grouped into different classes based on biological activity [13]. The term cytokine encompasses a variety of soluble proteins including monokines, interleukins (IL), colony-stimulating factors, interferons (IFNs), tumor necrosis factor (TNF), and chemokines [14]. These messenger molecules link the neural, endocrine, and immune systems [15]. Cytokines can be pro- or anti-inflammatory, neuroprotective or destructive, depending on their state and concentration [16]. Although nearly all nucleated cells produce cytokines, they are mainly produced by glial cells in the central nervous system (CNS) or by immune cells, such as helper T cells and macrophages [14]. Stimuli inducing cytokine production may originate remote to, or within the CNS. The origin of cytokines acting within the CNS may include blood-borne and native CNS sources, including immune cells, brain endothelial cells, astrocytes, microglia, and neurons [17–19]. Cytokines act by binding to specific cell surface receptors, which then induce intracellular signaling mechanisms that up- or downregulate transcription factors, leading to pro- or anti-inflammatory reactions. Cytokines with generally proinflammatory properties include TNF-α, INF-γ, IL-1, IL-6, and IL-18, while cytokines that antagonize the proinflammatory responses include IL-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13, and transforming growth factor (TGF)-β. Soluble receptors for proinflammatory cytokines can have similar function. Note that IL-6 appears in both categories.

3. Differences in Neonatal and Adult Immune Responses

The immune system of the fetus and newborn reflects the unique interaction between the developing individual and its host-mother. The developing fetus must avoid precipitating a maternal immune response that results in rejection or preterm delivery, but still must protect itself from intrauterine infection and prepare for the transition from the sterile intrauterine environment to the extrauterine environment that is rich with antigenic challenges. This combination of factors results in a neonatal immune system that differs significantly from its adult counterpart. In comparison to adults, the neonatal immune response is biased towards a Th2 response, with a muted Th1 response [20]. Stimulated neonatal mononuclear cells secrete markedly less of the pro-inflammatory Th1-polarizing cytokines, TNF-α and IFN-γ, whereas secretion of IL-6, a cytokine with anti-inflammatory and Th2-polarizing properties, is actually greater in neonates than adults. This response is mediated by adenosine, an endogenous purine metabolite with immune-modulatory properties [21–23].

4. Barriers to Accessing the Brain

There are three interfaces where molecular and cellular exchange between blood and neural tissues or the cerebral spinal fluid occurs. These are the blood brain barrier (BBB) formed by the cerebrovascular endothelial cells between blood and brain interstitial fluid, the choroid plexus epithelium between blood and ventricular CSF (blood-CSF barrier, BCSFB) and the arachnoid epithelium between blood and subarachnoid CSF [24, 25]. The two barriers that represent the largest interface between blood and brain extracellular fluids are the BBB, formed by brain endothelial cells, and the BCSFB, formed by choroid plexus epithelial cells (Figure 1) [26]. The BBB, also termed the "neurovascular unit," consists of highly specialized endothelial cells interconnected by an elaborate network of complex tight junctions surrounded by basal lamina in which pericytes and perivascular antigen-presenting cells are embedded, with an outer ensheathment of astrocytic perivascular endfeet. Mast cells, which synthesize and store neuroactive and vasoactive substances, are located at perivascular locations on the brain side of the BBB in apposition with astrocytic and neuronal processes [27]. In addition to tight junctions, adherens junctions hold the endothelial cells together providing structural support required for formation of tight junctions and are necessary to prevent disruption of the BBB [26]. The astrocytes that surround the microvasculature provide the cellular link to the neurons and play an active role in signal transduction pathways and regulating the BBB [24]. In adults, there are five known routes by which materials can pass between the circulation and the brain across these barriers (Figure 2) [25]. These are via a paracellular aqueous pathway (across tight junctions) and through transcellular pathways including the lipophilic pathway, via transport proteins, receptor-mediated transcytosis, or adsorptive transcytosis [25, 28]. Whether these same mechanisms are active in the fetus and neonate remains unknown.

From the earliest stages of brain development, the BBB excludes the passage of protein and small lipid insoluble markers between the circulating blood and the brain extracellular fluid [32, 33]. Similarly, paracellular diffusion of protein and small, lipid-insoluble molecules is limited at the BCSFB by apical tight junctions between the choroid plexus epithelial cells [34]. However, these substances may pass by transcellular mechanisms in choroid plexus epithelial cells, and their permeability is much higher in immature compared to adult brain [35]. Stolp et al. studied BBB permeability resulting from lipopolysaccharide-(LPS-) induced systemic inflammation (defined as increased blood concentrations of acute-phase proteins or IL-1β and TNF-α) in rats and opossums [33]. They demonstrated a restricted period in brain development when protein permeability of the BBB, but not the BCSFB, is altered following systemic inflammation. This increased BBB permeability was specific to white matter and was related to stage of development and not BBB immaturity.

The BBB is a dynamic structure which can be modified by circulating factors or by chemicals secreted by cells associated with the BBB [25]. Agents known to impair adult

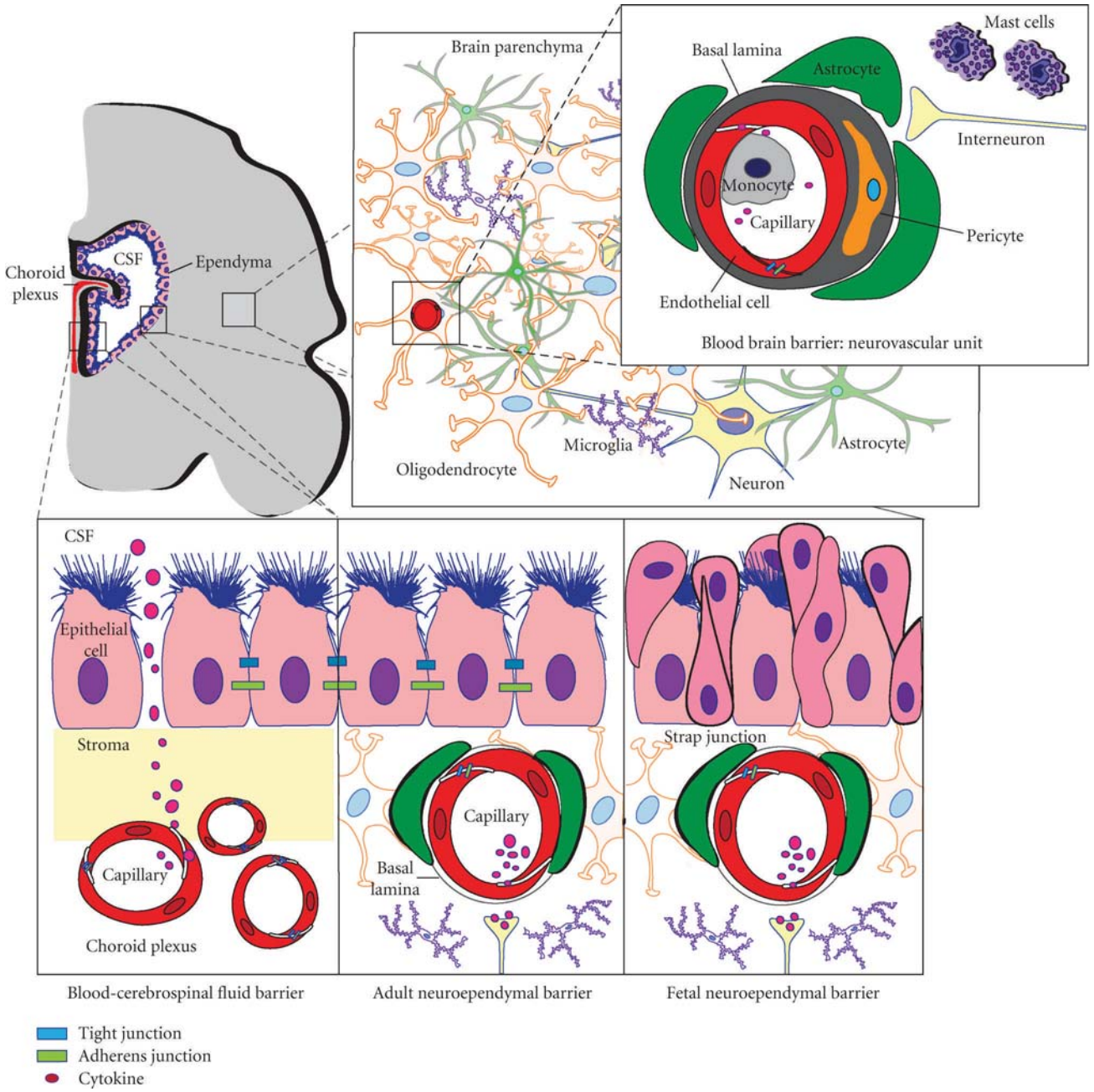

Figure 1: The blood-brain and the blood-cerebrospinal fluid barriers. A schematic diagram of the two barriers that represent the largest interface between blood and brain extracellular fluids: the brain endothelium forming the blood-brain barrier (BBB), also referred to as the neurovascular unit, and the choroid plexus epithelium forming the blood-cerebrospinal fluid (CSF) barrier. The neuroependymal surface lining of the ventricular system (inner CSF-brain barrier) is unique to the fetal brain and is not present in the adult. The neuroependymal cells are connected by "strap junctions" that prevent exchange of large molecules such as proteins between the CSF and brain [31]. Tight junctions and adherens junctions limit paracellular pathway endothelium and epithelium permeability. The neurovascular unit consists of specialized endothelial cells interconnected by tight junctions surrounded by basal lamina in which pericytes are embedded, with an outer ensheathment of astrocytic perivascular endfeet. Mast cells are located at perivascular locations in apposition with astrocytic and neuronal processes [27]. Inflammation may result in disruption of tight junctions and adherens junctions leading to paracellular passage of cytokines.

BBB function (increase leakiness) include bradykinin, histamine, serotonin, glutamate, purine nucleotides (ATP, ADP, AMP), adenosine, platelet-activating factor, phospholipase A2, arachidonic acid, prostaglandins, leukotrienes, interleukins (IL-1α, IL-1β, IL-6), TNFα, macrophage-inhibitory proteins MIP1 and MIP2, free radicals, and nitric oxide (NO) [25]. Many of these agents are upregulated after hypoxia or during infection.

It is not surprising then that localized or systemic inflammation/cytokinemia (e.g., chorioamnionitis and/or

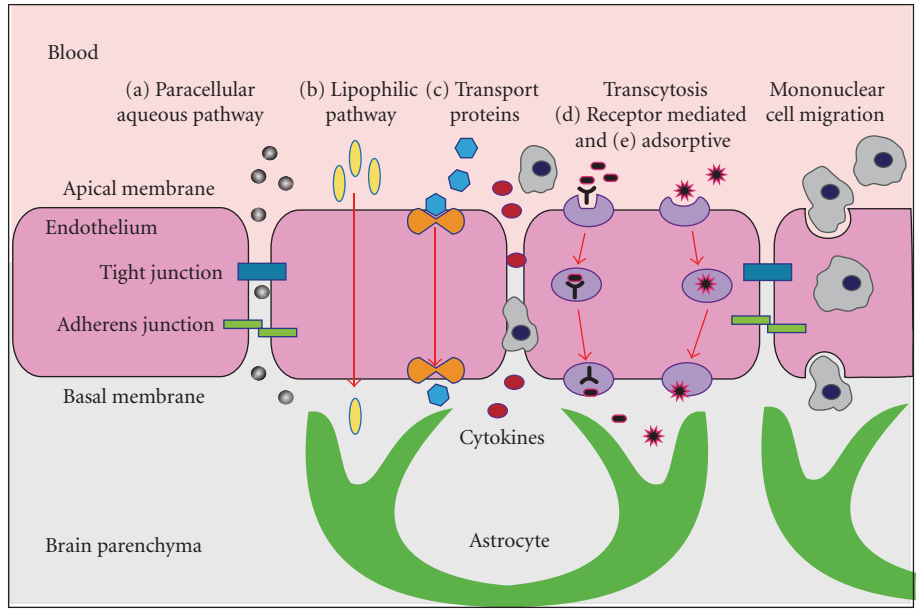

Figure 2: Access pathways across the cerebrovascular endothelial cells. An illustration depicting purposed access routes of materials across the endothelial cells of the blood-brain barrier (BBB). The pathways for cellular molecular movement from the circulation across the BBB may include (a) paracellular aqueous pathway across tight junctions, (b) transcellular pathways including the lipophilic pathway, (c) transport proteins, (d) receptor-mediated transcytosis, and (e) adsorptive transcytosis. Cytokine trafficking may occur via receptor-mediated transcytosis or possibly across disrupted tight junctions in the setting of inflammation. Cytokine movement is thought to occur mainly in the blood-to-brain direction; however, in the blood-cerebrospinal fluid barrier, bulk flow movement may lead to cytokine absorbtion into blood [19]. Mononuclear cells may penetrate the BBB by a process of transcellular diapedesis, directly through the cytoplasm of the endothelial cells without tight junction disruption [29]. During proinflammatory conditions, tight junctions between endothelial cells may be disrupted allowing mononuclear cells to gain access from the blood to the brain via paracellular routes, along with cytokines [30].

fetal inflammatory response) remote to the CNS may result in disruption of the BBB/BCSFB with increased cytokine access to the CNS [36, 37]. Activated CD4+ T lymphocytes, macrophages and dendritic cells must cross the endothelial and the parenchymal basement membranes and glia limitans before gaining direct access to the brain. Transmigration of these cytokine-producing immune cells appears to be influenced by ultrastructural alterations in the laminin isoform composition of the endothelial basement membrane, and by focal matrix metalloproteinase (MMP) activity of the parenchymal basement membrane [24]. To breach to BCSFB, circulating cytokines/immune cells must migrate across the fenestrated choroid plexus capillaries, enter the outer CNS parenchyma, and then penetrate the choroid plexus epithelial cell layer either by passing through the parallel tight junctions strands or transcellularly through the choroid plexus epithelial cells. However, evidence of inflammatory mediator access to the CNS across the BCSFB in the human fetus/neonate remains undefined.

The role of the neurovascular unit, which includes cellular (endothelial and epithelial cells, astrocytes, and pericytes) and acellular (e.g., the extracellular matrix networks) barriers in regulating cytokine access beyond the BBB and BCSFB to the CNS needs to be clarified in order to understand potential opportunities to mitigate the inflammatory cascade associated with perinatal brain injury. There is a paucity of information on *in vivo* human fetal/neonatal properties of barrier dysfunction and the available *in vitro* and adult animal models may not accurately reflect neurovascular unit functional permeability following injury/inflammation. For example, although experimental studies have demonstrated that LPS can induce WMI and neuroinflammation [38], evidence that LPS gains access to the fetal/neonatal brain causing human perinatal brain damage is lacking. However, since microglial cells possess LPS-binding toll-like receptor (TLR)4 receptors and seem to be necessary for LPS-induced oligodendrocyte death [39] this suggests that LPS can gain access to the brain. Additionally, how proinflammatory cytokines affect cellular inward and outward CNS barrier transfer mechanisms and alter CNS barrier function potentially influencing perinatal brain injury remains unknown. Identifying periods when the fetal/neonatal CNS is vulnerable to inflammatory mediator-induced barrier disruption and subsequent damage due to CNS penetration of peripheral toxic molecules is needed in order to define pharmacologic therapeutic windows to access injured brain regions.

The BBB can act as a regulatory conductor between the CNS and the peripheral circulation, establishing and maintaining CNS homeostasis, moderating the nutritional needs of the CNS, and governing influx and efflux of signaling molecules [19]. The BBB appears to have a dual role in regulating immune cell trafficking between the CNS and blood by controlling restrictive and selective permeability

[40]. Cytokines can disrupt the BBB [41, 42] and BCSFB, [43] and also can alter saturable neuropeptide transporter [44] and ATP-driven drug efflux pump activity [45] without affecting BBB integrity. The BBB can secrete cytokines [46–49] and may actively participate in inflammatory reactions of the CNS. Dysfunction of BBB and BCSFB mechanisms may be more than just a consequence of inflammation/injury, but also may constitute part of the disease process. Increased blood-spinal barrier permeability following spinal cord trauma involves an active upregulation in inflammatory cytokine transport systems in endothelial cells around the injured area [50]. Immune mediator traffic regulated by the BBB may also play a role in recovery following injury, as has been demonstrated in a murine model of hypothermic brain injury in which macrophages promote early posttraumatic reformation of the BBB [51]. The type and amount of cytokines transported across the BBB varies by CNS region, implying that there are different cytokine-specific regulatory mechanisms and effects [19]. Whether the human fetal/neonatal BBB also plays an active role (similar to animal models) not only in ongoing tissue damage, but also in the recovery process following CNS injury is not clear.

5. Infection

An *in utero* infection such as chorioamnionitis may trigger an innate immune system response, resulting in elevated cytokine levels. Microorganisms express conserved sequences known as pathogen-associated molecular patterns (PAMPs), such as LPS and double stranded RNA, on their surfaces. Recognition of these PAMPs by pattern recognition receptors on immune cells stimulate specific host cell TLRs [20]. For example, when stimulated by LPS, TLR4 signals through the adapter molecule myeloid differentiation factor 88, to activate the nuclear factor-κB (NF-κB) pathway that leads to an immune response characterized by the production of cytokines, antimicrobial products, and the regulation of costimulatory molecules [52]. The cytokine response may progress from the trophoblast, decidua, and amniotic epithelium [53, 54], to the amniotic fluid [55, 56] to the fetal lungs and then blood stream, or by direct hematogenous spread via the maternal-placental-fetal circulation. Initiation of a proinflammatory cytokine response following bacterial infection of placental tissues can lead to preterm labor [57]. Cytokines associated with preterm labor include IL-1β [58], IL-6 [59], IL-8 [60], and TNF-α [61]. Activated immune cells including circulating neutrophils, phagocytic macrophages, T cells, and NK cells, and resident CNS astrocytes and microglia produce biological mediators including cytokines, chemokines, adhesion molecules, and growth factors involved in complex intermolecular interactions that participate in the immunoinflammatory processes related to brain injury [62]. Cytokines in the fetal blood stream may contribute to a systemic fetal inflammatory response with eventual penetration across the BBB resulting in a chemical and or pathogen promoted inflammatory cascade in fetal brain [12].

6. Cytokines Expressed by Astrocytes and Microglia

Interaction between the CNS and the immune system relies on the expression of several cytokines and their receptors in both neurons and glial cells in the brain [63]. The two major reactive glial cell types that play significant roles during CNS injury and repair are microglia and astrocytes. These glial cells are involved in the intracerebral immune response where they act, in part, by secreting cytokines, chemokines, neurotrophic, or neurotoxic factors [64]. Cytokines and their receptors, like IL-1β and IL-1β receptor protein, are constitutively expressed in the CNS by astroglia, microglia, and oligodendrocyte progenitor cells (OPCs) [65].

Astrocytes are important players in neuroinflammatory processes and are capable of producing numerous cytokines including a variety of interleukins, TNF-α, and members of the interferon family [66]. The involvement of astrocytes in the pathogenesis of WMI is suggested by increased cytokine expression (IL-1β, IL-6, and TNF-α) in both the diffuse and focal components of periventricular leukomalacia (PVL) [67, 68]. Activated microglia produce cytokines, chemokines, free radical species, proteases, and other potential mediators of injury [69, 70]. Upon stimulation by LPS, microglia express IL-1β, which triggers astrocyte expression of tissue inhibitors of metalloproteinases (TIMPS) [71]. During CNS injury and repair, TIMPS play a critical role in regulating tissue proteolysis by neutralizing the effect of the MMP. TIMP-1 is involved in regulating the growth and morphology of cortical neurons in an MMP-dependent manner [72] and plays a role in oligodendrocyte generation and differentiation [73, 74]. Further studies are needed to determine the role of microglial IL-1β cytokine signaling and TIMP expression in perinatal brain inflammation and repair.

7. Brain Injury Associated with Prenatal Infection and/or Inflammatory Insults

Intrauterine infection might account for 25–40% of preterm births with up to 80% of preterm deliveries at <30 weeks of gestation having evidence of infection [75]. Clinical chorioamnionitis is significantly associated with cystic PVL and CP [76]. Neonates exposed to clinical chorioamnionitis or histological chorioamnionitis have increased risks of 140% and 80% for developing CP, respectively [77]. Bacterial infection of the decidua and placental membranes activates TLRs on the surface of inflammatory cells which results in release of proinflammatory cytokines, and initiates a local inflammatory reaction in the placenta [78, 79]. Elevated IL-6 concentrations measured in cord blood from neonates with white matter lesions associated with PVL supports the role of intrauterine inflammation and subsequent WMI [80]. Perinatal brain injury may not be contingent on pathogen penetration into the fetal CNS: intrauterine exposure to a systemic inflammatory stimulus alone can lead to brain damage in preterm neonates [10, 81].

Chorioamnionitis can be classified into acute and chronic chorioamnionitis [82]. Acute chorioamnionitis of

infectious origin is associated with elevated amniotic fluid IL-6 levels and results from microbial invasion of the amniotic cavity and intrauterine infection. Chronic chorioamnionitis of immunological origin is associated with elevated amniotic fluid CXCL10 levels and is a possible consequence of disrupted immune system hormones affecting CD8+ T-cell activity resulting in maternal antifetal rejection. Amniotic fluid proteomic analysis has demonstrated that acute chorioamnionitis and chronic chorioamnionitis are likely manifestations of different pathological processes [82]. Whether acute versus chronic chorioamnionitis also result in distinct alterations in perinatal brain injury patterns is not known.

8. Brain Injury Associated with Postnatal Infection and/or Inflammatory Insults

In preterm infants, known inflammatory conditions are associated with WMI. These include both early- [83] and late-onset sepsis [84], as well as NEC [85] and are generally associated with high plasma levels of IL-6, IL-8, and TNF-α [86]. Bronchopulmonary dysplasia, another comorbidity of prematurity, is associated with evidence of inflammation (neutrophils, macrophages, cytokines and toxic oxygen radicals) [87] and is also associated with increased risk of WMI [88].

9. Cytokines and Cerebral Palsy

CP, the most common cause of severe physical disability in childhood [89], is an umbrella term describing multiple diseases originating early in life characterized by variable motor impairments secondary to unspecified etiologies and cerebral pathologies. Preterm birth, perinatal infection, and neonatal encephalopathy are important risk factors for the development of CP [90].

9.1. Preterm Infants. Periventricular WMI is an important cause of disability in preterm low-birth-weight infants. Prior to 32 weeks of gestation, preOLs are particularly vulnerable to injury and developmental arrest [91]. Injury to these cells can result in a cystic necrosis of white matter tracts and/or diffuse noncystic lesions with hypomyelination [6]. Injury most commonly occurs in a watershed, periventricular distribution, which typically corresponds clinically with spastic diplegia, the most common form of CP diagnosed in preterm infants [92, 93]. Inflammation, mediated by proinflammatory cytokines, can contribute to the WMI that occurs in preterm infants [94]. In a study of 96 preterm babies with gestational age $\leq$32 weeks, elevated umbilical cord blood IL-8 concentrations were associated with CP (diagnosed by followup at 1 year of age) [95]. Another large multicenter study of infants with birth weights $\leq$1000 g (n = 1067) demonstrated that circulating IL-8 concentrations were higher on days 0–4 and subsequently in infants who developed CP compared with infants who did not develop CP in both unadjusted and adjusted analyses [96].

Macrophage infiltration and high levels of TNF-α and IL-1β have been demonstrated in brains of neonates with PVL compared to neonates with anoxic lesions who died shortly after birth [67]. These high cytokine concentrations may have direct cytotoxic effects on oligodendrocytes [97]. Neuronal cytotoxicity following exposure of preOLs to LPS is mediated by activated microglia via TLR-associated signaling pathways [98]. Both focal and diffuse forms of PVL are associated with activated microglia [8]. The release of proinflammatory cytokines from activated microglia has been implicated in neuronal and glia cell death [99]. Pang et al., using primary OPC cultures prepared from neonatal rat optic nerves, demonstrated that LPS-activated microglia mediate OPC death by two distinct mechanisms in a time-dependent manner [100]. An early phase of OPC damage occurs within 24 h after LPS treatment, mediated by NO-dependent oxidative damage, and a delayed phase of OPC death, evident at 48 h after LPS treatment, is mediated by cytokines and is prevented by blocking TNF-α activity. Whether these two distinct mechanisms of injury occur in human perinatal brain injury leading to PVL is not clear.

Inflammatory processes originating during vulnerable periods of neurodevelopment may result in perinatal programming. The effects of inflammation triggered by proinflammatory cytokines, prostaglandins, or LPS on the developing CNS of premature infants may have long-term consequences for the individual's ability to cope with environmental exposures during childhood and adulthood [36]. Lin et al. demonstrated that school-age preterm children with PVL-induced CP had significantly higher plasma concentrations of TNF-α, increased TNF-α released from LPS-stimulated peripheral blood mononuclear cells (PBMCs), and mRNA expression of inflammatory signaling molecules, including TLR4 and TNF-α, in PBMCs compared to normal control school-age preterm children [101]. Additionally, intracellular PBMC TNF-α levels were significantly higher in children with CP, but lower in controls following LPS stimulation. Whether or not children with CP who were born preterm with a history of PVL have long-term abnormalities of their immune responses remains unclear.

9.2. Cytokines, CP, and Neonatal Encephalopathy. Maternal inflammation contributes significantly to fetal susceptibility to hypoxia-ischemia [102–104] and the subsequent development of CP [105, 106]. Hypoxia-ischemia and infection can both induce a systemic inflammatory response associated with elevated cytokines [94, 107]. Higher concentrations of IL-1β, IL-6, TNF-α, and IL-8 in the blood of neonates who have encephalopathy have been associated with increased anaerobic brain metabolism, and with abnormal neurodevelopmental outcome [108]. Elevated concentrations of IL-6 and IL-8 have been demonstrated in the CSF of asphyxiated full-term infants, with intrathecal levels of these cytokines corresponding to the degree of hypoxic-ischemic encephalopathy [109].

Term neonates with encephalopathy have a risk for CP that is 100 times that of those infants who do not have encephalopathy [10]. Increased concentrations of IL-1β,

IL-6 and TNF-α in amniotic fluid [98], and IL-6 in cord blood [74, 110] secondary to maternal, placental, or fetal infections [97, 111] are associated with cerebral WMI and/or CP. Similarly, elevated neonatal blood concentrations of IL-6 and IL-8 were associated with the diagnosis of CP at 1 year of age in a study of 73 term babies (gestational age $\geq$36 weeks) [9].

Although infection and/or inflammation increase the risk for CP, they may not be sufficient causal factors to induce brain damage. In a 3-year follow-up study of high-risk infants, Yoon et al. reported that CP was diagnosed in only 18% (5/28) of infants born with documented microbial invasion of the amniotic cavity and 24% (11/45) of infants with evidence of intrauterine inflammation [112]. Another study compared early blood concentrations of inflammatory cytokines (IL-1, -6, and -8 and TNF-α) from 64 children later diagnosed with CP to 107 control children (all born at <32 weeks gestational age). Early cytokine concentrations were not predictive of later CP [104].

10. Dual-Role Cytokines

Inflammation in the CNS can result in significant brain damage, including injury to axons and myelin, the loss of preOLs, oligodendrocytes, and neurons [69]. However, neuroinflammation can be also be beneficial, promoting neuroprotection, the mobilization of neural precursors for repair, remyelination, and even axonal regeneration [69]. Some cytokines can have both pro- and anti-inflammatory effects. For example IL-4, IL-10, and IL-13 are potent activators of B lymphocytes, and also potent anti-inflammatory agents with the ability to suppress expression of proinflammatory cytokines IL-1 and TNF [13]. TNF-α and IL-1β can have both neuroprotective and damaging effects [113]. IL-6 and IL-8, typically associated with inflammation, have been associated with the release of nerve growth factor in the CSF of patients with traumatic brain injury suggesting their role in promoting repair of the CNS lesions as well as of axonal regeneration [16]. A dual role can also be seen in macrophages, which are key mediators of the immune response, particularly regarding their ability to produce cytokines. Macrophages can be subdivided into subtypes (M1 and M2) with M1 macrophages considered proinflammatory, producing molecules such as TNF-α, IL-1, IL-6, and NO, while the M2 subset is typically considered anti-inflammatory, producing molecules like IL-10, TGF-β, and IL-1 receptor antagonist [69]. The neuroimmune response appears to be dichotomous with the balance of pro- and anti-inflammatory cytokines likely influencing neurodevelopmental outcomes. Further research is needed to clarify what influences cytokines (e.g., timing, type, location, and duration of injury) to promote peace or wage war with regard to neuroprotection and neuroinflammation, respectively.

11. Cytokines and Genetic Susceptibility to Perinatal Brain Injury

Susceptibility to perinatal brain injury may be partially genetically determined by the balance of proinflammatory and anti-inflammatory cytokine expression. Single-nucleotide polymorphisms in genes encoding cytokines and their receptors might positively or negatively affect the risk of perinatal brain injury in infants. An increased risk for WMI has been associated with IL-8, IL-6, TNF-α, and TLR4 polymorphisms [10, 114].

A recent meta-analysis by Wu et al. demonstrated that CP is associated with IL-6 genetic polymorphisms [115]. Moderately preterm infant (32–36 weeks' gestational age) carriers of IL-6 gene -174 C allele, associated with upregulated IL-6 expression, may have an increased risk of developing quadriplegic CP [114]. Functional polymorphism in the IL-6 gene (-174 CC genotype) among term and near-term infants has been associated with an attributable risk percentage of 11.6% for developing CP [116]. The development of hemiplegic and quadriplegic CP has been demonstrated with IL-6 or IL-4 polymorphisms in the presence of viral exposure suggesting an association between candidate cytokine polymorphisms and a fetal inflammatory environment, which may be causally linked to the risk of CP development [114]. This proposed "double jeopardy" hypothesis linking neurotropic viral exposure and genetic susceptibility to infection needs further confirmation in susceptible neonatal population studies to establish causation of CP. In contrast, there may be protective gene polymorphisms. For example, preterm infants (<32 weeks gestation) homozygous for the high IL-10 producer -1082 G allele are significantly less likely to develop ultrasound defined PVL [117].

12. Cytokine Biomarkers of Perinatal Brain Injury

Accurate diagnostic, predictive, and prognostic biomarkers of brain injury are needed for optimizing the clinical treatment of at-risk neonates. Ideal biomarkers would accurately reflect the degree of brain injury, the timing and evolution of injury, and potential for response to therapy. These biomarkers would help to differentiate infants who do not require treatment from those at risk of permanent sequelae; infants that might benefit from intervention from those for whom treatment is futile and identify infants who are within a therapeutic window for a specific treatment. It is unlikely that a single biochemical or imaging biomarker measured at a single time point will achieve all these goals. Magnetic resonance imaging (MRI) and spectroscopy (MRS) have shown promise, but the most predictive protocols and the optimal timing of studies is still not fully established [118].

Measurement of inflammatory proteins in blood, including cytokines, shortly after birth in preterm infants may provide information about the risk of sonographic WMI (which correlates with neurodevelopmental outcome). Serial measurements of blood proteins during the first 2 postnatal weeks in extremely low gestational age newborns (born before the 28th week of gestation) in the ELGAN study demonstrated an increased risk of ventriculomegaly, sonographic indicator of diffuse cerebral WMI, in association with elevated concentrations of vascular endothelial growth

factor receptor 1, serum amyloid A, and macrophage inflammatory protein 1β on day 1 and IL-8 on day 7 [119]. An increased risk of an echolucent lesion, a sonographic indicator of focal cerebral white matter damage, was associated with elevated concentrations of macrophage inflammatory protein 1β on day 1 and intercellular adhesion molecule 1 on day 7 [119]. Interestingly, in this same study, elevated concentrations of the chemokine Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES, also known as CCL5) was associated with reduced risk of both ventriculomegaly and echolucent lesions. RANTES downregulates TLR4 ligation-induced IL-6 and TNF-α secretion by enhancing IL-10 production in PBMCs [120] and may play an anti-inflammatory role in perinatal brain injury.

Elevated cytokine levels have been associated with perinatal brain injury and show promise as diagnostic and/or prognostic biomarkers to be used in a multimodal approach along with MRI. Elevated levels of IL-1β, IL-6, IL-8, and lower levels of IL-12 following term delivery in infants with neonatal encephalopathy has been associated with impaired cerebral oxidative metabolism based on MRS and abnormal neurodevelopmental at 30 month of age, but not with detectable MRI changes in the neonatal period [108]. Procianoy and Silveira reported on the association between high cytokine concentrations with WMI in preterm infants and sepsis, looking at cohort of 84 very-low-birth-weight infants, 27 (32%) with WMI, and 57 (68%) control subjects (no WMI). WMI was increased in infants with clinical early-onset sepsis and higher plasma levels of IL-8, IL-6, and TNF-α. IL-8 levels $\geq$100 pg/mL had sensitivity 96%, specificity 83%, and negative predictive value 98% indicating that this chemokine may be a good predictor of WMI [86]. Although elevated levels of CSF cytokines have been associated with WMI, plasma cytokine concentrations may not reflect CSF cytokine levels or inflammatory events within the brain [94]. Therefore, relying on plasma cytokines as biomarkers of perinatal brain injury may prevent early recognition of localized brain inflammation. Additionally, measuring cytokines to assess perinatal brain injury has not been done routinely in the NICU setting and will likely require lowercost, automated, on-demand testing before these potential biomarkers are incorporated into standard diagnostic testing. Multiple assessments of these values over time may provide more accurate predictive values.

13. Prevention and Treatment of Perinatal Brain Injury

There are few interventions currently available to prevent or treat perinatal brain injury. Currently used strategies known to improve the outcome of prematurity include maternal prenatal treatments with magnesium sulfate and betamethasone, and postnatal neonatal use of caffeine. The only proven therapy available for term and near term infants with neonatal encephalopathy is therapeutic hypothermia. There are other promising therapies under active investigation for prevention and treatment of neonatal brain injury, including melatonin, erythropoietin (Epo), N-acetylcysteine, Epo mimetics, allopurinol, and xenon. Some of these approaches target anti-inflammatory mechanisms, and still others improve BBB function, thereby preventing the passage of cytokines and other potentially injurious factors into the brain. Examples of such approaches are explored below.

13.1. Erythropoietin. Epo is a hemopoietic growth factor produced by all vertebrates. Functional receptors for Epo are present on cell types other than erythrocyte progenitors, including neurons, and many glial cell types. Epo is a promising novel neuroprotective agent. It is widely available, affordable, and has been safe in over 25 years of neonatal studies of erythropoiesis. Epo triggers several different signaling pathways after binding to its receptor. Neuroprotective effects are associated with activation of Janus kinase/Stat5 and NFkB pathways [121], while Stat5 and Akt pathways are required for neurotrophic effects of Epo [122]. Epo also stimulates expression of several growth factors, including vascular endothelial growth factor secretion (VEGF) [123] and brain-derived neurotrophic factor (BDNF) [124], which may be beneficial in the injured brain. There are extensive data to support its neuroprotective effects *in vitro*, and in neonatal models of brain injury [125–131]. Epo has anti-apoptotic [128, 129] and anti-inflammatory effects (decreased Il-6 and IL-8) [132, 133], and it also increases neurogenesis, [134, 135] and protects oligodendrocytes from injury [136]. These combined effects might provide neuroprotective benefit for brain injury typical of preterm infants and term infants with hypoxic-ischemic injury. Phase I/II studies to determine safety and pharmacokinetics have been done [137, 138], and further phase II/III studies are underway or in the planning stages.

13.2. Melatonin. Melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring hormone which regulates circadian rhythms. Melatonin has antioxidant [139] and antiapoptotic effects [140, 141]. Prenatally administered low-dose melatonin can reduce cerebral inflammation and apoptosis following birth asphyxia in the spiny mouse [142]. In a fetal sheep model of perinatal asphyxia, melatonin attenuates the production of 8-isoprostanes and reduces activated microglia cells and TUNEL-positive cells in the brain [143]. In a neonatal rodent model of LPS-induced hypoxic-ischemic injury, multiple low-dose treatments with melatonin reduced injury by 45%, but higher dose treatment was not protective [144]. Clinically, melatonin has shown beneficial effects when given to both asphyxiated [145] and septic children [146].

13.3. Curcumin. Curcumin, the main active ingredient in turmeric, can prevent the onset of inflammation by inhibiting activation of NFκB, production of TNF-α, IFN-γ, and NO, expression of iNOS, and activation of nicotinamide adenine dinucleotide phosphate-oxidase (NOX) [147, 148]. Curcumin has been demonstrated to have a protective effect associated with suppression of iNOS and NOX activation injury in a neonatal rat model of LPS-induced WMI [149].

Table 1: Gaps in knowledge regarding human perinatal brain injury.

Barriers to Accessing the Brain
(i) Do pathogens, inflammatory mediators and inflammatory cells access the fetal and neonatal brain using the same mechanisms as in animal and adult models?
Infection
(i) Which leukocyte populations and which specific proinflammatory cytokines are the primary triggers for brain damage of premature infants?
(ii) What is the origin and the role of proteins differentially expressed in amniotic fluid associated with chronic chorioamnionitis cases compared to acute chorioamnionitis in the amniotic fluid detected by proteomic analysis?
(iii) What is the role of microglial IL-1β signaling and TIMP expression in perinatal brain inflammation and repair?
(iv) What are the mechanisms of brain injury from LPS-activated microglia leading to PVL?
Cerebral Palsy
(i) What are the roles of inflammatory cytokines in preterm infants that develop CP?
(ii) To what extent does an altered inflammatory response and persistent neuroinflammation originating in the perinatal period play a long-term role in preterm children with PVL-induced CP?
Dual Role of Cytokines
(i) What variables determine neuroprotective and neuroinflammatory properties of cytokines (e.g., timing, type, location, and duration of injury)?
Cytokines and Genetic Susceptibility to Perinatal Brain Injury
(i) Which cytokine gene polymorphisms predispose to CP?
(ii) How do cytokine gene polymorphisms interact with perinatal infections to cause CP?
Cytokine Biomarkers of Perinatal Brain Injury
(i) Are there accurate diagnostic, predictive, and prognostic cord blood and neonatal plasma cytokines biomarkers that reflect CSF cytokine levels or inflammatory events within the brain?
(ii) Are there biomarkers specific for precise inflammatory conditions associated with white matter injury (e.g., differentiating between septicemia and necrotizing enterocolitis) that will provide time-sensitive, pathogen and treatment specific information?
Prevention and Treatment of Perinatal Brain Injury
(i) Which anti-inflammatory cytokines and treatments will safely and effectively alter cytokine profiles promoting neuroprotection and repair?
(ii) What is the optimal timing of such treatments?

Abbreviations: TIMP: tissue inhibitors of metalloproteinases, LPS: lipopolysaccharide, PVL: periventricular leukomalacia, CP: cerebral palsy, CSF: cerebrospinal fluid.

14. Targeting the BBB to Fight Disease

Another approach to preventing or treating neonatal brain injury might be to target the BBB. Several neonatal pathologies involve increased leakiness or dysfunction of the BBB. Therefore, using agents that improve BBB function might improve outcomes. Steroids, hypothermia, intracellular cyclic AMP, adrenomedullin, and noradrenergic agents all stimulate an increase in BBB function. These approaches are under investigation or used therapeutically to treat some adult brain disorders. For example, dexamethasone treatment is currently used to decrease the brain edema associated with brain tumors [150], and Ca^{2+} channel blockers are under investigation as treatment for hypoxia-induced brain injury [151, 152]. Hypothermia, which also improves BBB function, is one of the few proven therapies available to treat neonates with hypoxic-ischemic brain injury and has the lowest number needed to treat to see benefit [153]. Stabilizing activated mast cells with disodium cromoglycate (Cromolyn) may decrease BBB leakiness by inhibiting release of potentially toxic factors including histamine, serotonin, neutral proteases, cytokines, chemokines, and free radicals [154, 155].

Another approach under investigation in adult models of disease is to improve the health of the endothelial cells involved in maintenance of the BBB. The use of exercise, fish oils, and specific fruits, soy, vitamins C an E, and red wine may all be of benefit (NNT = 7–9) [25]. The application of a select group of these strategies might be applicable to neonatal brain injury; however, each one must be studied with regard to safety, efficacy, and developmental implications.

15. Conclusion

Large knowledge gaps exist regarding the detailed roles of cytokines in brain injury, repair, and protection in the human fetus/neonate. Although animal studies have demonstrated an important role of cytokines in brain injury, many questions on the underlying cytokine-related mechanisms influencing brain injury remain unanswered. In humans, the fetal/neonatal brain injury knowledge gap is even wider (Table 1), with developmental differences in immune response and in the complex neurovascular barrier mechanisms that play a critical role in regulating inflammatory mediator traffic at the interface between

the systemic circulation and the brain. Understanding the balance between pro- and anti-inflammatory mediators and their roles in normal brain development and in the setting of inflammation is needed to tailor treatments that promote neuroprotection.

Future large animal studies aimed at developing diagnostic cytokine profiles of perinatal brain injury biomarkers must be designed to allow evaluation in the context that is clinically useful. While neonatal rodents models of brain injury provide vital information about mechanisms of brain injury and also neuroprotection, it is essential that information learned in these models be verified in larger animal models (fetal sheep, piglet, and nonhuman primate) that more closely reflect human brain development.

For example, for early-hospital diagnosis, a test that is reasonably specific and very sensitive to early perinatal brain injury secondary to infection or cytokines/inflammation would be necessary to facilitate time-sensitive anti-inflammatory strategies. Such a study should be specifically designed to address the incremental benefits of biomarker-based information beyond traditional means of assessment, such as standardized clinical examination, maternal history, risk factor assessment, and radiographic studies. For purposes of identifying risk of early deterioration, additional data might be obtained by serial measurements in the early hospital setting. Similarly, for functional prognosis, serial testing in the subacute setting might provide useful information. Patient heterogeneity (e.g., genetic factors), and the timing, type, degree, and duration of perinatal brain exposure to inflammatory mediators/cytokines likely influence long-term neurodevelopmental outcomes. The need for accurate biomarkers is well illustrated by infants affected by neonatal encephalopathy secondary to hypoxic ischemic encephalopathy. Over 1500 neonates have now been enrolled in randomized controlled trials of therapeutic hypothermia using the *best available entry criteria*: a combination of clinical assessments (Apgar scores, Sarnat or Thompson scores), laboratory assessment (lactic acid, pH, base deficit) and electrophysiologic function [153]. While these criteria identify a group of high risk neonates, their predictive value is poor: untreated, one-third of these infants do well with no long-term neurodevelopmental sequelae, while two thirds die or have significant long-term neurodevelopmental impairment. Treatment improves outcomes by approximately 15%, but the infants who will benefit cannot currently be differentiated from those who will not, nor from those who will do well without treatment.

Similarly, it is unlikely that one single biomarker, such as a cytokine, will be robust enough to have clinical utility for guiding treatment of infants with perinatal brain injury. A panel of biomarkers will therefore likely be more useful. Ideally, future biomarker biomarkers, which incorporate serum cytokine levels and imaging modalities will allow for early tailored individualized treatment strategies that will promote the proper treatment for the proper patient at the proper time. Similarly, in the subacute setting, a biomarker panel might be useful adjunctive tool combined with clinical information and radiographic imaging to determine risk stratification to direct aggressiveness of care for primary or secondary prevention of perinatal brain injury in patients with known risk factors.

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Review Article

Perinatal Cerebellar Injury in Human and Animal Models

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Cerebellar injury is increasingly recognized through advanced neonatal brain imaging as a complication of premature birth. Survivors of preterm birth demonstrate a constellation of long-term neurodevelopmental deficits, many of which are potentially referable to cerebellar injury, including impaired motor functions such as fine motor incoordination, impaired motor sequencing and also cognitive, behavioral dysfunction among older patients. This paper reviews the morphogenesis and histogenesis of the human and rodent developing cerebellum, and its more frequent injuries in preterm. Most cerebellar lesions are cerebellar hemorrhage and infarction usually leading to cerebellar abnormalities and/or atrophy, but the exact pathogenesis of lesions of the cerebellum is unknown. The different mechanisms involved have been investigated with animal models and are primarily hypoxia, ischemia, infection, and inflammation Exposure to drugs and undernutrition can also induce cerebellar abnormalities. Different models are detailed to analyze these various disturbances of cerebellar development around birth.

1. Introduction

Premature birth is a significant risk factor for adverse motor, coordination, cognitive, and behavioral outcomes in survivors [1]. The prevailing brain pathology in very preterm infants is diffuse white matter injury in the cerebral hemispheres [2]. In addition, a consistent pattern of regionally specific long-term volume reduction and abnormalities in cortical and deep grey matter structures in ex-preterm infants is now recognized [3, 4]. Injury and impaired development of the cerebellum, involving both white matter and grey matter components as a complication of premature birth, are also becoming increasingly recognized with advanced neonatal brain imaging [5–11].

Survivors of preterm birth demonstrate a constellation of long-term neurodevelopmental deficits, many of which are potentially related to cerebellar injury, including impaired motor functions such as hypotonia, fine motor incoordination, ataxia, and impaired motor sequencing [12, 13]. Cerebellar injury has also been implicated in cognitive, social, and behavioral dysfunction among older patients [14, 15] and may contribute to the long-term cognitive, language, and behavioral dysfunction seen among 25% to 50% formerly preterm infants [16–19].

The cerebellum is considered particularly vulnerable in the newborn human because of its very rapid growth at that time, a period comparable in the developing animal. The concept of a particular vulnerability of the cerebellum during its phase of rapid growth is documented in experimental models of undernutrition, glucocorticoid exposure, and X-irradiation [20–22].

This article reviews the morphogenesis and histogenesis of the human and rodent developing cerebellum, and its more frequent injuries in preterm. Most cerebellar lesions are cerebellar hemorrhage and infarction usually leading to cerebellar abnormalities and/or atrophy but the exact pathogenesis of lesions of the cerebellum is unknown. The different mechanisms involved, infection, inflammation,

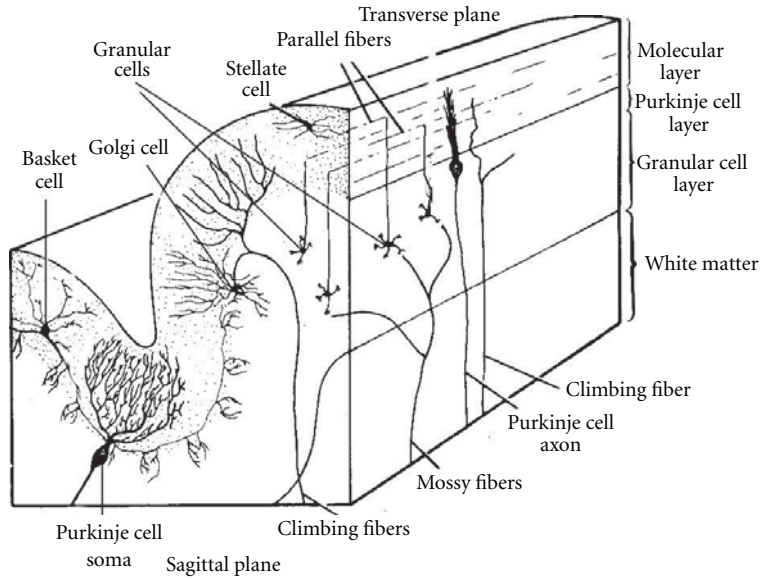

Figure 1: Organization of the mammalian cerebellar cortex in transverse and sagittal planes. Adapted from Brain Res 1981 [79].

hypoxia, ischemia, exposure to drugs, and undernutrition, have been investigated with animal models. These models will be detailed to analyze the disturbance of cerebellar development around birth.

2. Review of Cerebellar Histology and Development

2.1. Cytological Layering and Specific Cellular Organization of the Cerebellar Cortex. The cerebellum is composed of three major histological subdivisions: the cortex, the underlying white matter, and the deep cerebellar nuclei. The basic histological layering of the cerebellar cortex is similar in rodents and primates: the deep granular cell layer, the Purkinje cell layer, and the superficial molecular layer are shown in the simplified schema in coronal and sagittal planes (Figure 1). From the eight classes of cells found in the cerebellar cortex, only the Purkinje cell axons project outside the cortex [23]. The others are local circuit neurons: the granular cells and unipolar brush cells are glutamatergic whereas the others, in particular the stellate, the Golgi, and the basket cells, are GABAergic. The Purkinje cells give rise to the sole output pathway of the cerebellar cortex. The two main afferent pathways conveying information to the cerebellar cortex are the climbing and mossy fibers systems that direct their impulses differently to the Purkinje cells. The climbing fibers originate from the inferior olivary nucleus and established directed synaptic contacts with dendrites of the Purkinje cells. The afferent mossy fibers originate from neuronal populations from various nuclei of the spinal cord, the brain stem, and even the deep cerebellar nuclei. They reach the Purkinje cell indirectly through relay cells, the granular cells via their axonal field, and the parallel fibers [23]. The Purkinje cells are therefore the pivotal elements around which all the cerebellar circuits are organized by receiving information, processing it, and channeling towards efferent pathways.

2.2. Connectivity of the Cerebellum. The characteristic neuronal arrangement consists of a strict positioning of neurons and afferent fibers conferring to the cortex a stereotyped three-dimensional geometry [24], which is very helpful to analyze any changes which may occur in the properties of neurons and their connectivity. In addition, the organization of connectivity shows differences in primate versus rodents. The cerebral cortical areas of the forebrain make several axonal connections with the cerebellum via the pallidum, the thalamus, and the pons in mammals. Whereas in humans unilateral and crossed afferents connections running along the superior peduncle in the cerebellum are the most predominant, these corticopontocerebellar projections are bilateral in the rat brain.

2.3. Prenatal Development. Contrary to other regions of the central nervous system (CNS), cerebellar neurons are generated in two germinative neuroepithelia in two waves of proliferation and migration processes. This development occurs in similar order but at different rates in rodents and primates (Figure 2). During the embryonic period in mammals, the cerebellar primordium arises from both mesencephalic and rhombencephalic vesicles in the isthmic area under the control of the isthmic organizer [25]. The first neurons to be generated are the deep nuclear neurons and all the Purkinje cells that migrate immediately after to the cerebellar plate (Figure 3). In parallel, the first granular cell precursors are generated in the rostral rhombic lip (with other neuronal cell populations), and they migrate as precursor granular cells tangentially to cover the superficial zone of the cerebellar plate following a lateromedial and anteroposterior direction (see [23]). They form the extragranular layer (EGL).

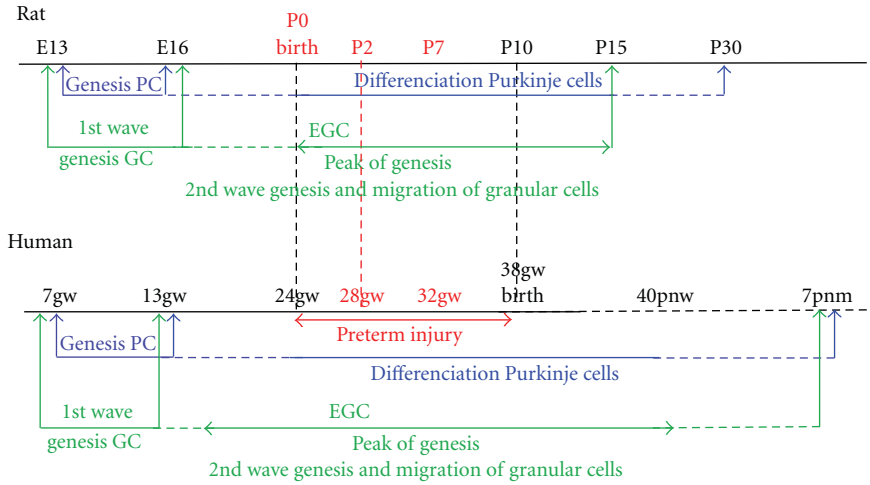

Figure 2: Comparison of timing of development of the Purkinje cells (PC) and granular cells (GC) in the cerebellar cortex in rat and human. EGL: external granular layer. Embryonic day (E), postnatal day 0 (P0), gestational weeks (gw), postnatal weeks (pnw), postnatal months (pnm).

2.4. Postnatal Development. During postnatal life, the second wave of proliferation occurs in the EGL, the secondary germinal zone giving rise to the granular cells which migrate radially inward to their final destination in the internal granular layer (IGL). The proliferation of granular cells is regulated by Purkinje cells (PC) secreting the Sonic hedgehog signaling factor [23]. In the molecular layer, the onset of synaptic inputs of the axons of the granular cells (parallel fibers) is concomitant with the onset of the final postsynaptic dendritogenesis of the Purkinje cells. The synaptic inputs, essentially from the parallel fibers but also from the climbing fibers, are essential for the achievement of the espalier arrangement of the dendritic trees of the Purkinje cells. In the rat, although the extension of the lateral domain of the dendritic tree of the PC is achieved at postnatal day 15 (P15), its final extension, that is, adult size, is reached at P30. Altman and Bayer [26] described in the rat a caudorostral gradient of development of the cerebellar cortex. In human, the adult number of folia is achieved around two months postnatally [27] and the EGL disappears around the 7th postnatal month [28]. Interestingly, in vivo 3-dimensional volumetric imaging techniques shows, an increase in the cerebellar volume of about 5-fold from 24 to 40 gestational weeks (gw) [29, 30].

3. Cerebellar Lesions of the Premature and Term Infants

Lesions such as cerebellar hemorrhage (CBH), infarction, and cerebellar atrophy have been increasingly diagnosed in preterm and term infants using improved neuroimaging techniques [4, 9, 10, 17, 31, 32]. The incidence of these lesions is strikingly dependent on the degree of prematurity. Thus, in the study of Limperopoulos et al. [17], the incidence of lesions in infants <750 g birth weight was 15%, and 2% were seen in those >750 g to 1499 g. The topography of the CBH is primarily focal, unilateral, and within the peripheral parenchyma of the cerebellar hemisphere. Subpial germinal matrix bleeding within the external granular layer may account for some intrahemispheric CBH. The vermis is involved in slightly less than one-third of patients [17]. Cases of vermian hemorrhage represent hemorrhage within the germinal matrix located in the subependymal layer of the roof of the fourth ventricle [33, 34].

CBH may occur concomitantly with cerebral lesions such as hemorrhagic parenchymal infarction, intraventricular hemorrhage with dilatation, and periventricular leukomalacia. In these last cases, premature infants at term-equivalent age have reduced cerebellar volume. This reduction may be due to a primary cerebellar injury that is not detectable by MR imaging at term-equivalent age or due to Wallerian degeneration secondary to cerebral lesions. Cerebellar atrophy is usually focal in the unilateral supratentorial lesions and often generalized in the bilateral cerebral lesions [3]. These data suggest important insights into the highly integrated anatomic and functional integrations between the cerebrum and the cerebellum during development, such as trophic transsynaptic effects along the corticopontocerebellar pathway.

The neuropathological basis of the decreased cerebellar volume remains to be elucidated. In preterm of 32 gestational week (gw), neuronal loss and gliosis were detected in dentate nucleus, cerebellar cortex, or the brain stem cerebellar relay nuclei, basis pontis, and inferior olive, in only 5% to 15% of infants, in particularly in presence of leukomalacia [6].

The possibility that cerebellum atrophy in premature infants may be related to adverse blood product as hemosiderin deposit following hemorrhage has been suggested

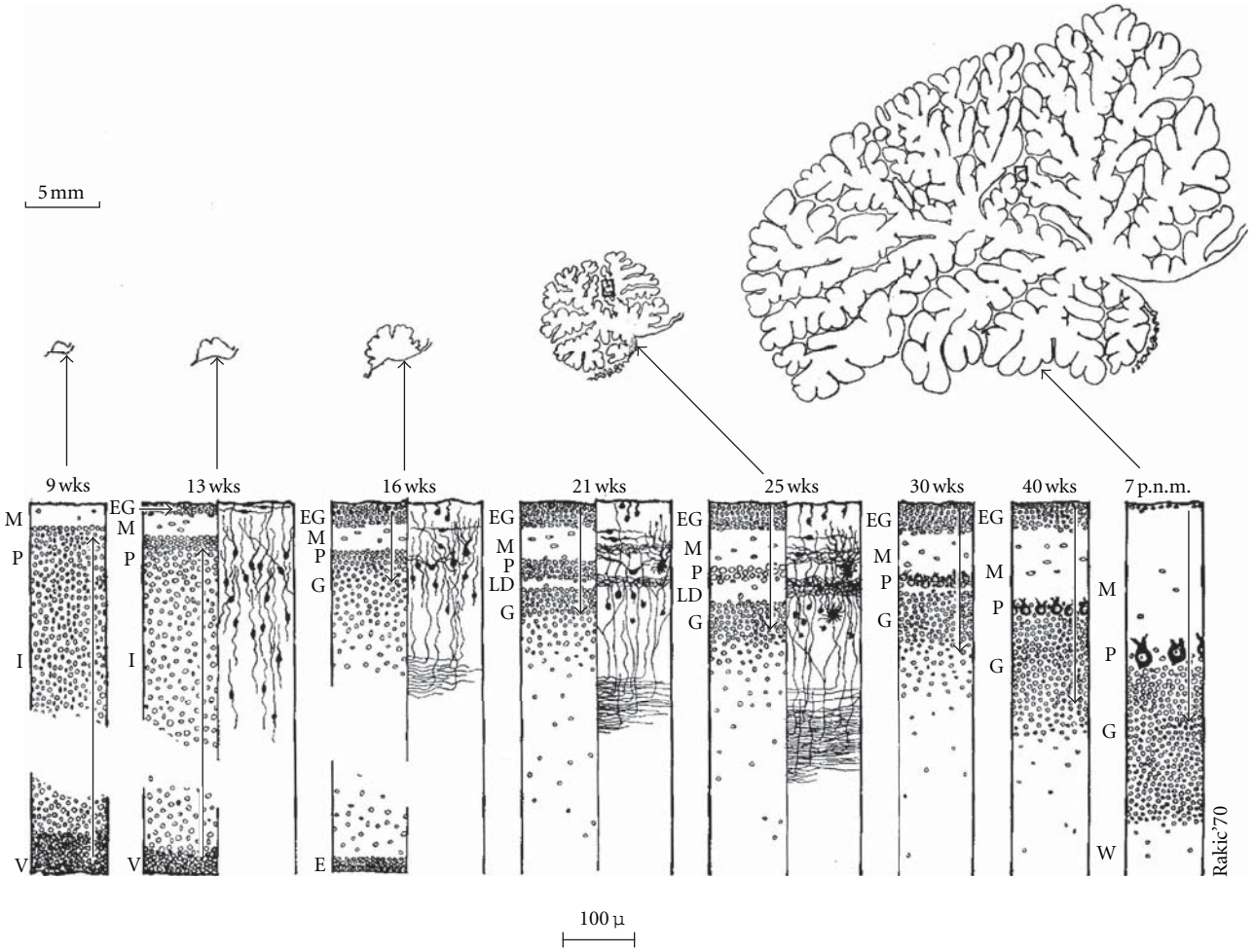

Figure 3: Summary of the main morphogenetic and histogenetic events during development of the human cerebellum from the ninth gestational week (wks) to the seventh postnatal month (p.n.m.) shown in sagittal plane at the level of the primary fissure. E: ependyma, EG: external granular layer, G: Granular layer, I: intermediate layer, L: laminar dissecans, M: molecular layer, P: Purkinje cell layer, V: ventricular zone. W: white matter. The 5 mm scale in the upper corner of the figure show the dramatic increase of the cerebellum primordium especially from the beginning of foliation to 16 wks to 7 pnm. Source: from Brain Res 1973 [28].

by Messerschmidt and colleagues [5, 19, 33]. Tam et al. [4] found that more severe supratentorial intraventricular hemorrhage (IVH) was associated with slower growth of cerebellar volumes. No changes in volumes were found with IVH at 30 weeks postmenstrual age (95% CI 26–33 weeks), but volumes by 40 weeks were 1.4 cm^3 lower in premature infants with grade 1-2 IVH and 5.4 cm^3 lower with grade 3-4 IVH. The same magnitude of decreased volume was found whether the IVH was ipsilateral or contralateral. No association was found with severity of white matter injury ($P = 0.3$).

Whether these blood products are crucial or not in the onset of the cerebellar lesion remain unclear (see [33]). Early effects of decreased cerebellar volume associated with supratentorial IVH in either hemisphere may be a result of concurrent cerebellar injury or direct effects of subarachnoid blood on cerebellar development.

Preterm delivery associated to other adverse insults could disrupt the developmental program of the cerebellum. A recent postmortem study on premature infants who had survived in an exutero environment reports cerebellar abnormalities in the development of granular cells which parallel a decrease of Sonic hedgehog in the Purkinje cell layer [35].

In fact the pathogenesis of lesions of the cerebellum is multifactorial. Univariate analyses identified maternal, intrapartum, and early postnatal hemodynamic risk factors; multivariate regressions indicate that emergent caesarian section, patent ductus arteriosus, and lower 5-day minimum pH independently increased the odds of cerebellar hemorrhage [17]. Different mechanisms appear plausible to explain the disturbance of cerebellar development after premature birth. The correlation of lesions of the cerebellum in preterm with animal models can highlight the precise pathophysiology of these lesions.

4. Mechanisms of Cerebellar Lesions in Preterm: Correlations with Animal Models

4.1. Hypoxia-Ischemia

4.1.1. Magnetic Resonance Imaging Studies. The damaging influence of hypoxia or hypoxia-ischemia to the cerebellar underdevelopment is suggested by the strong correlation of the cerebellar abnormality with MRI-demonstrated supratentorial injury [3, 4, 9, 11, 32]. In the largest reported MRI series of very preterm and preterm, a decrease in cerebellar volume at term equivalent age correlated with decreasing gestational age [30]. In the pathology of preterm infants, neuronal loss detected in the cerebellum and related brain stem nuclei was essentially confined to the infants with periventricular leukomalacia (25% to 30% of infants) [6]. Primary impaired cerebellar development of different origins, such as hypoxia-ischemia, has most often consisted of bilateral, generally symmetric deficits in the cerebellar hemispheric volumes [33]. On the other hand, a recent MRI study suggested that unilateral injury confined to the preterm cerebral hemisphere was associated with a significantly decreased volume of the contralateral cerebellar hemisphere [3]. These data suggest that two main mechanisms might induce the impaired cerebellar development of the premature brain: either a direct effect on the development of the cerebellar cortex or remote effects operating via trophic transsynaptic interaction between the telencephalic leukomalacia and the developing cerebellum via the corticopontocerebellar pathway. On the other hand a recent study by Tam et al. showed that cerebral white matter injury did not correlate with reductions in cerebellar volume [4].

4.2. Rodent Models at Postnatal Day 2. To address this question a model of the preterm human in neonatal rat pups was developed on postnatal day 2 (P2) which is comparable to 28 weeks of gestation in the human (Figure 2), when the cerebellar cortex is the most vulnerable to insult (see Section 2.4). As mentioned previously, the second wave of neuronal cerebellar proliferation plays a key role in the organization of the cerebellar cortex. In a previous study we demonstrated that global hypoxic injury or forebrain hypoxia-ischemia at P2 in rat pups produce dramatic cellular damages in the cerebellar cortex [36]. Interestingly, the addition of forebrain ischemia does not increase the huge cellular damage obtained following hypoxia which contradict the afore mentioned hypothesis about a possible correlation between cerebral and cerebellum [3]. Our results showing neuronal and white matter damage in both cerebellar hemispheres following hypoxia alone suggest that systemic hypoxia could adversely affect the developing cerebellum independent of its connections at this developmental stage. The defect in myelination detected following hypoxia alone is even more severe than that following hypoxia-ischemia. The lack of volume loss detected at P21 indicates that there can be significant cellular injury followed by gliosis and postlesional plasticity with axonal and dendritic growth. The presence of increased density of GFAP-positive cells and microglial activation in the white matter and cerebellar cortex of both hypoxic and hypoxic-ischemic injured rats supports a pathological event directly affecting the survival and/or maturation of neurons and preoligodendrocytes. These findings may explain some neurodevelopmental abnormalities seen in preterm babies even in the absence of gross cerebellar volume reduction.

4.3. Rodent Models at Postnatal Day 7. Following hypoxia-ischemia, selective vulnerability of different regions of the brain depends on its maturity and on the severity of the insult [37]. In the P7 hypoxic-ischemic model (Vannucci model) equivalent of human injury at 32–36 weeks of gestation (Figure 2) the areas with higher metabolism such as the cerebral cortex, hippocampi, and deep gray nuclei suffer the most after initial ischemic injury. Histological brain damage is generally confined to the cerebral hemisphere ipsilateral to the arterial occlusion, and consists of selective cell death or infarction and delayed neurodegeneration depending on the duration of the systemic hypoxia [38–40].

Other studies using perinatal hypoxia-ischemia have shown that cell death occurs in brain regions that are not directly affected by the ischemia, such as cerebellum [39, 41, 42] suggesting that neuronal connectivity may play a role in neurodegeneration following hypoxia-ischemia to the immature brain (P7 age). Taken together, these findings may reveal the connection networks which could exist between the damaged forebrain and cerebellum in the developing mammal brain. In rodent models, forebrain hypoxia-ischemia may affect differently the corticopontocerebellar connections according to the age of the insult. As aforementioned, these lesions may not occur at P2 but could be present at P7. In human, the activity in the ipsilateral pons, and also the contralateral cerebellar cortex, is a phenomenon known as crossed cerebellar diaschisis [43]. Limperopoulos et al. [3] showed that unilateral injury confined to the preterm cerebral hemisphere was associated with a significantly decreased volume of the contralateral cerebellar hemisphere, and that these effects were evident as early as term gestational age equivalent. Limperopoulos et al. [3] hypothesized that the corticopontocerebellar connections are involved in cerebellar damage. More studies are necessary to confirm this hypothesis.

4.4. Infection and Inflammation. A strong relation of maternal intrauterine infection with systemic fetal inflammation or of postnatal neonatal infection with systemic inflammation and the occurrence of periventricular leukomalacia is well documented [44, 45]. White matter damage, astrocytosis, and cytokine activation have been demonstrated in experimental model of intrauterine infection, all of which are capable of leading to delays in brain development [46, 47]. The cerebellum is particularly vulnerable to infections insults since it is not fully developed until after birth in both humans and rodents [22, 33]. Due to the nearly 5-fold increase in growth in the cerebellum in the last trimester of pregnancy, intrauterine infection, or activation of the fetal immune system could cause irreparable damage to this structure [29]. Experimental studies of E. *coli* injection

administered at gestational day 17 in rats decreased Purkinje cell density and volume [48]. The decrease in calbindin in Purkinje cells is also accompanied by impairment in motor coordination and balance in rats from the early postnatal period through adulthood [49]. In fetal preterm sheep, exposure to bacterial endotoxin (lipopolysaccharide; LPS) cause a diffuse pattern of cerebellar white matter damage [50, 51]. Injury to the cerebellar white matter involves diffuse loss of oligodendrocytes, associated with apoptotic and/or inflammatory processes, which is similar to the white matter injury observed in the forebrain of preterm infants [2] and in experimental immature animal models [52].

Human cytomegalovirus infection of the developing central nervous system (CNS) is also a major cause of neurological damage in newborn. To investigate the pathogenesis of this human infection, animal models of virus infection of the CNS are associated with a delay of the morphogenesis of the cerebellum [53, 54]. The defects in cerebellar development in infected animals located in the cerebellar cortex are correlated temporally with virus replication and CNS inflammation, spatially unrelated to foci of virus-infected cells. CMV-infected cells are more prevalent in the Purkinje cell layer than in the mitotic granule cell layer [55]. In an animal model of lymphocytic choriomeningitis virus [56], there is selective infection of several neuronal populations and in focal pathological changes. Astrocytes and Bergmann glia cells are the first cells of the brain parenchyma infected with LCMV and the virus spreads across the brain principally via contiguous glial cells. The virus then spreads from glial cells into neurons. LCMV infects neurons in only four specific brain regions: the cerebellum, olfactory bulb, dentate gyrus, and periventricular region. The cerebellum undergoes an acute and permanent destruction while the olfactory bulb is acutely hypoplastic but recovers fully with age. Neurons of the dentate gyrus are unaffected in the acute phase but undergo a delayed-onset mortality. In contrast, the periventricular region has neither acute nor late-onset cell loss.

Currently, there are no direct data on the role of infection/inflammation in the genesis of cerebellar abnormality of the human premature infant.

4.5. Drug Exposure. Maternal exposure to nicotine, cocaine, and ethanol during pregnancy is known to be a significant contributor to neurobehavioral deficits in the offspring [57], and specific studies of the cerebellum in this context are of particular interest.

In animal studies, nicotine treatment via injection during gestation has been shown to produce episodic hypoxia in the developing fetus. Abdel-Rahman et al. [58] evaluated the neurotoxicity in the offspring at pubertal stage of the development following continuous maternal exposure to nicotine via infusion during the gestation period. Histopathological findings showed a significant decrease in the surviving Purkinje neuronal cells in the cerebellum and CA1 subfield of hippocampus in the offspring on postnatal day 30 and 60. These pathological findings suggest that the deficits in the cerebellum could persist longer than other brain regions [59]. Furthermore, there was a significant increase in GFAP immunostaining in both cerebellar white matter and granular cell layer as well as the CA1 subfield of the hippocampus.

The mechanisms which alcohol induces their effects on development are unknown. A study by Bonthius et al. showed that gestational exposure to ethanol in a nonhuman primate species induced permanent doserelated deficits in the number of cerebellar Purkinje cells. The number of Purkinje cells and their linear frequencies were significantly reduced in the alcohol-treated subjects, and the deficits were dose-dependent. The findings suggest that alcohol-induced reduction in neuronal number may be an important factor underlying the CNS dysfunction in fetal alcohol syndrome [60].

4.6. Glucocorticoid Exposure. The developmental effects of glucocorticoids on the cerebellum are an important area of study as the cerebellum has the highest levels of glucocorticoid receptors in the brain, localized in the external granular layer [61, 62]. Studies in human preterm newborns reveal adverse effects of postnatal dexamethasone therapy on brain development, including decreased cerebral and cerebellar tissue volumes [63]. In a recent study by Tam et al. [11], preterm newborns were prospectively studied with serial MRI examinations near birth and again near term-equivalent age. Adjusting for relevant clinical factors, antenatal betamethasone was not associated with changes in cerebellar volume but postnatal exposure to clinically routine doses of hydrocortisone or dexamethasone was associated with impaired cerebellar growth. Cerebral growth was not affected [11, 64].

Animal models demonstrate reduced preterm cerebellar growth after exposure to all glucocorticoids including hydrocortisone, dexamethasone, and corticosterone [62, 65, 66]. In the developing cerebellum, glucocorticoids cause neuronal apoptosis and inhibit proliferation of immature granule neuron precursors. However, although 11-β-hydroxysteroid dehydrogenase type 2 is capable of degrading hydrocortisone but not dexamethasone, both glucocorticoids result in injury of the external granular layer in wild-type animals. This is suggested by rodent models showing similar effects of corticosterone (a known substrate of 11-β-hydroxysteroid dehydrogenase type 2) and dexamethasone on granule cell apoptosis with acute glucocorticoid exposure and inhibition of cell proliferation with chronic exposure [67]. Heine et al. [68] recently showed that systemic administration of a small-molecule agonist of the Sonic hedgehog-Smoothened pathway (SAG) prevents neurotoxic effects of GCs susceptible to metabolism by the enzyme 11β-hydroxysteroid dehydrogenase type 2, but that it does not interfere with beneficial effects of glucocorticoids on lung maturation. These findings suggest the potential of a small molecule agonist of Smoothened as a neuroprotective agent in the setting of glucocorticoid-induced neonatal cerebellar injury.

4.7. Undernutrition. In the study of Limperopoulos et al. [30], cerebellar volumes were significantly associated with head circumference and weight at term-equivalent age MRI. Insufficient postnatal catch-up growth in preterm infants

has been significantly associated with adverse neurodevelopmental outcome [69, 70]. These data suggest that impaired postnatal growth may be an important marker of impaired central nervous system integrity and, in particular, deficient cerebellar growth at term. However, prospective studies in preterm (less than 30 weeks' gestation age) suggest that suboptimal early nutrition in preterm infants can have a permanent effect on their cognitive function, emphasising the potential importance of dietary management decisions in this population [71, 72].

Many experimental data show that during its phase of rapid growth, the cerebellum is especially vulnerable to undernutrition [21, 22, 73]. Rees et al. [74] showed no overt signs of damage in sheep brains and cerebellum from intrauterine growth restricted (IUGR) fetuses; however, morphological analysis demonstrated subtle but important alterations in connectivity and function. In the cerebellum, the most important finding was a 20% reduction in the area of arborization of Purkinje cell dendrites and a 26% decrease in the total number of dendritic spines. As spines are the sites of synaptic apposition, synaptic input to Purkinje cells are reduced with a possible alteration in cerebellar function [74–76]. Restricted cerebellar growth and differentiation is also shown in studies of placental insufficiency in fetal sheep and guinea pigs [77, 78].

5. Conclusion

Cerebellar injury and disorders of development are now a recognized problem in preterm infants; these data suggest a potential pathological role in the long-term cognitive, behavioral and motor deficits associated or not with brain injury. The precise pathophysiology of cerebellar injury remains unknown in preterm infants, and it is necessary to interrogate animal models to unravel the main mechanisms. In parallel, sophisticated pathological data on premature cerebellum are necessary to analyze specific human features. In addition, pathological investigations associated with MRI studies on the same cerebellum are an essential step to define biomarkers necessary to improve the prognosis of cerebellar damage in preterm infants.

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Review Article

Sex Differences in Mechanisms and Outcome of Neonatal Hypoxia-Ischemia in Rodent Models: Implications for Sex-Specific Neuroprotection in Clinical Neonatal Practice

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Clinical findings show that male infants with hypoxic-ischemic injury (HI) fare more poorly than matched females on cognitive outcomes. Rodent models of neonatal hypoxia-ischemia support this difference, with data showing that perinatal brain injury leads to long-term behavioral deficits primarily in male rodents and in female rodents treated with early androgens. Results support the idea that sex-specific gonadal hormones may modulate developmental response to injury and dovetail with overwhelming evidence of developmental androgen effects on typical brain morphology and behavior. However, mechanisms underlying sex differences in response to early brain injury may be more complicated. Specifically, activation of cell death pathways in response to HI may also differ by sex. In females, the preferential activation of the caspase-dependent apoptotic pathway may actually afford greater protection, potentially due to the actions of X-linked inhibitor of apoptosis (XIAP) within this pathway. This contrasts the pattern of preferential activation of the caspase-independent pathway in males. While an integrated model of sex-specific hormonal and genetic modulation of response to early injury remains to be fully elucidated, these findings suggest that infants might benefit from sex-specific neuroprotection following HI injury.

1. Introduction

Perinatal hypoxic-ischemic injury (HI; concurrent oxygen/blood deprivation in the brain) represents a major cause of mortality and long-term neurologic morbidity in premature/very low-birth-weight (VLBW) infants (<1500 g) and in term infants suffering birth trauma [1]. In premature infants, the vulnerability of the underdeveloped neural vascular system, coupled with poor cerebral autoregulation [2], can often result in intraventricular or periventricular hemorrhagic injury (IVH-PVH; bleeding within or surrounding the ventricles [3]). These bleeds, primarily located in the subependymal germinal matrix, lead to some immediate cell necrosis as well as a progressive apoptotic cell death cascade of germinal matrix and glial precursor cells [3]. In addition, poor cerebral autoregulation (including reperfusion failure) in preterm infants can lead to periventricular leukomalacia (PVL), a nonhemorrhagic ischemic injury associated with loss of white matter surrounding the ventricles [4]. Moreover, underdevelopment of the lungs can lead to reduced oxygenation of the blood, thus resulting in hypoxic conditions within the brain of premature infants. Animal models of acute preterm HI injury include the Rice-Vannucci model of unilateral carotid artery ligation followed by a period of exposure to 8% oxygen prior to postnatal day 7 (P7), typically performed in rodents [5–7]. Additional models are also used in which fetal blood supply is diminished by clamping of the placental blood supply [8, 9], and/or by raising dams in a low-oxygen environment for a period of days [10, 11].

In term infants, HI injury typically results from complications of birth (e.g., cord compression, placental disruption/failure, or cord asphyxia [1, 7, 12–14]) and can result in cerebral white matter injury typical of cerebral palsy [15] or in gray matter injury [1, 16, 17]. Thus, term-born children experiencing asphyxia exhibit injuries

more typical of hypoxic-ischemic encephalopathy (HIE), in contrast to injuries exhibited by preterm children (i.e., IVH-PVH, PVL). Animal models of term injury include the Rice-Vannucci method performed on P7-P10 [5–7] or methods of middle cerebral artery occlusion (MCAO, [18]). Although the mechanisms of neural damage in preterm versus term populations differ, these varied forms of injury result in similar activation of acute necrotic and delayed apoptotic cell death mechanisms that impact on cell populations most vulnerable during the time of injury [5–7].

Not surprisingly, the long-term consequences of neonatal HI injury in both populations can be severe. Nearly 50% of term-born infants suffering severe HIE die within weeks of birth, while up to 25% of those surviving exhibit permanent neuropsychological dysfunction [7]. Similarly, a 50% mortality rate exists for preterm infants experiencing severe HI, with 80% of survivors experiencing long-term complications [19], including reductions in cerebellar [20], cortical, and hippocampal volumes [21] associated in turn with cognitive and behavioral deficits, deficits in verbal and language domains [22, 23], reduced IQ measures [24], cerebral palsy, and mental retardation [25]. What is surprising, however, is the disproportionate incidence of, and increased severity of effects following, neonatal HI injury in males. Not only are male infants more vulnerable to perinatal insult (showing higher incidence of IVH and increased rates of mortality from prematurity or stillbirth), they also suffer more long-term cognitive deficits as compared to females with comparable injury [23, 25–31]. In fact, males in general show increased risk for brain-based developmental disorders, including speech and language disorders, stutter, dyslexia, autism, learning disabilities, attention-deficit-hyperactivity disorder, and cerebral palsy as compared to females [26, 27, 30, 32]. Importantly, males suffering intracranial bleeds at birth also display significantly lower full-scale, verbal, and performance IQ at early school age as compared to females matched for degree of prematurity and severity of intracranial bleed [33]. Overall, evidence suggests that among infants at risk for HI, females may be at a quite significant advantage as compared to their male counterparts who are two times more likely to experience prenatal anoxia, hemorrhage, and infection, and 1.8 times more likely to suffer cerebral birth trauma [26–28, 30].

Despite the overwhelming evidence of sex differences in outcome following neonatal HI injury, many researchers appear to remain naïve to the importance of sex in perinatal injury models and continue to utilize only male animals in research studies. However, recent work concerning hormones present during the perinatal period, as well as sex differences in mechanisms of cell death, have begun to illustrate dynamic and differing processes occurring in the neonatal brain following injury and emphasize the need for studies to include both sexes. This work suggests, first, that the substantially elevated level of testosterone present in human male fetuses during gestation through the first year of life [34–36] may enhance neuronal excitotoxicity following hypoxic-ischemic insult [37, 38] and may contribute to exacerbated deficits in males [39, 40]. Second, evidence suggests that following such injury, male and female cells diverge in the proportional activation of caspase-dependent and caspase-independent pathways leading to apoptotic death [41–43]. In fact, this difference may contribute to outcomes that show males to be more vulnerable to early brain damage [44–46]. Finally, data indicate that females may possess a gene-linked advantage through a family of inhibitors of apoptosis (IAPs [47]), the most potent being X-linked IAP (XIAP [48]). XIAP is known to act on the caspase-dependent apoptotic pathway [48–51], and it is possible that increased expression of XIAP [52] in females may contribute to a female advantage following neonatal HI. Taken together, this evidence suggests an interplay of hormonal modulation and genetically determined apoptotic mechanisms, through which perinatal females may be afforded a level of protection against HI injury that is greater than for perinatal males. The current paper will focus on factors that may play key roles in the outcome of hypoxic-ischemic events experienced by males and females, including perinatal exposure to sex-specific gonadal hormones and sex-specific cell death mechanisms. Research in this area could lead to the discovery and clinical implementation of sex-specific neuroprotectants for infants suffering from HI injury.

2. Early Hormonal Factors

Sex differences in androgen levels represent one principal difference in the male versus female neonatal brain and lead to substantial effects on brain morphology and subsequent behavior [34–36, 53–59]. Human testes develop around gestational week (GW) 6, with testosterone from the testes—as well as from the fetal adrenals (as a by-product of corticosteroid production)—circulating at detectable plasma levels in males by GW 8 [34, 35]. Testosterone secretion, however, is highest from GW 10 to 20, falling to lower levels by GW 24, followed by a second transient testosterone surge on the day of birth (in response to the drop in placental estrogen). In humans, testosterone levels gradually increase during the first week of life and remain high for the first year, peaking during the 3rd-4th month at levels similar to the second stage of puberty (200–300 ng/dL; [35]). Through aromatization, testosterone can be converted to 17-B estradiol, thus allowing it to bind to estrogen receptors within the brain [53, 54, 60]. In fetal male rats, where plasma testosterone is significantly higher than female littermates beginning at embryonic day 18 (E18) through P5 [61], the conversion of circulating testosterone to estradiol results in neural and behavioral masculinizing effects [36, 53]. However, no studies of which we are aware directly support this mechanism in humans. Difficulties in ascertaining the role of aromatization in human sexual differentiation reflect experimental constraints [53], but some evidence does support a role for aromatization in human development [62].

Human female fetuses are also exposed to androgens from the fetal adrenal glands, as well as the maternal adrenals, ovaries, and fat—though the amount is insufficient for masculinization. In humans, it is believed that a negative feedback loop between the fetal adrenal cortex and the

anterior pituitary corticotrophins minimizes female adrenal androgen secretion, acting as a transient mechanism that safeguards early human female development from virilization [63]. The fetal ovaries also develop at approximately GW 7, though no circulatory estrogen of fetal ovarian origin is present until very late in gestation [34, 35]. Likewise, studies of ovarian secretion of estrogen in the rat reveal detectable levels 5 days after birth (corresponding to the late third trimester human; [56]), although some central steroidogenesis may occur [64]. In rodents, females circumvent masculine development via maternal estrogen (consistent with masculinization via intracellular conversion of androgens as discussed above) through alpha-fetoprotein, a binding globulin found in late-gestation fetuses. This protein binds to estrogen within the bloodstream thereby rendering it inactive and preventing virilization of the female rodent brain [36, 54].

2.1. Testosterone and Brain Injury. As noted above, the presence of testosterone during development represents one of the foremost differences between neonatal male and female brains. However, despite a large and dynamic literature concerning modulatory effects of gonadal hormones on pathologic and behavioral response to stroke injury in adults, research data concerning hormonal modulation of injury in neonates remains scant (but see [65]). A brief review of this adult literature illustrates that studies using an induced injury model simulating adult stroke—middle cerebral artery occlusion (MCAO)—show consistent evidence that males benefit after injury from acute testosterone depletion, while the presence of testosterone increases glutamate toxicity following injury [37]. Furthermore, testosterone and its metabolite dihydrotestosterone (DHT) have been shown to increase stroke damage in young adult rats [37, 66, 67], while testosterone concentration was found to be inversely associated with stroke severity and 6-month mortality [68, 69]. Interestingly, young male rats were also found to incur larger strokes than their older counterparts, an effect hypothesized to be due to the ability of testosterone to alter the susceptibility of the brain to ischemic damage in an age-dependent manner. Alternately, though aromatase levels are stable over age (and thus the protection afforded to aged males is not likely due to increased capability for aromatization of testosterone to estrogen [70]), the declining effects of stroke damage in older males may reflect higher levels of testosterone in young rats [70].

In neonatal animals, baseline sex differences have been seen with an early hypoxia model [10, 71] as well as an HI model [40, 72, 73] of brain injury—both of which have shown that males exhibit increased brain volume loss [10, 40, 71, 74], disrupted myelination [10], and increased behavioral deficits [40, 71–73] following injury as compared to like-treated females. As with adult injury models, there is also some evidence that the presence of androgens can exacerbate induced brain damage—for example, following GABA-A mediated excitotoxicity [65]. Other studies of a rat model of focal ischemic injury leading to developmental cortical malformation (microgyria) found that androgenizing female rat pups via testosterone propionate (TP) prior to and following induction of microgyria via focal cortical freezing lesion on P1 led to a developmental shift in medial geniculate nucleus (MGN) neuronal size distribution in adulthood—similar to that seen in male microgyric rats—while vehicle-treated microgyric females were found to be identical to sham females (and showed no disruption of the MGN [39]). With regard to the specific long-term effects of testosterone as a modulator of neonatal HI injury, we are aware of only one study performed to date [40]. In this study, vehicle-treated male and female rat pups, as well as female rat pups that had been treated on postnatal days 1–5 (P1–5) with superphysiologic levels of testosterone propionate (TP), received the Rice-Vannucci HI procedure [5] on P7. Results showed subsequent deficits in auditory processing ability in males and TP-treated females with induced neonatal HI, while no effect of HI was found in vehicle-treated females [40], thus demonstrating the apparent deleterious effects of androgen exposure in modulating behavioral deficits associated with HI.

From these cumulative studies, it is evident that testosterone acts in some manner to exacerbate the response to early hypoxic-ischemic injury in rats, though the specific mechanism(s) of action remain to be defined. Moreover, since an aromatizable form of testosterone propionate was used in the above study [40], it is not possible to determine whether these effects were modulated directly by androgens or via intracellular conversion to estrogen. Future studies looking at neonatal HI while manipulating testosterone receptors, estrogen receptors, and/or aromatase blockers could potentially dissociate or clarify this issue. Nonetheless, further research in the area of neonatal testosterone exposure may help to better characterize the protection afforded to females, which potentially could be adapted to males through some form of neuroprotective treatment. Although the generalized use of androgen-blocking manipulations in male infants would be a clinically untenable intervention, a viable option could be to identify the delineated mechanisms of androgenic exacerbation of injury and block those specific effects only.

2.2. Estrogen and Brain Injury. It must also be noted that evidence from animal models of adult stroke shows substantial beneficial and protective effects of estrogen modulation [75–77]. In fact, adult female animals have a lower incidence of naturally occurring stroke [78] and show less sensitivity than male animals to the damaging effects of focal or global ischemic injury [79], and—in strains displaying conditions known to be stroke risk factors in humans (i.e., hypertension)—female animals display less tissue damage than males following induced stroke [80]. This female advantage has been attributed at least in part to protective effects of ovarian steroid hormones, since interventions that reduce estrogens (i.e., ovariectomy, estrogen receptor blockade, and natural aging) have all reduced differences in stroke outcome between the sexes [75]. Further, induced stroke during metestrus (when estrogen is lowest) increases tissue damage in comparison to strokes occurring during proestrus (when estrogen is highest; [77]). It should be noted that the effects of estrogen on adult human stroke

are less clear, and many of the successful studies of estrogen replacement in animal models have failed to translate to human clinical populations [75, 76]. Nonetheless, whatever the protective mechanism(s) of estrogen may be, they are less likely to fully account for the neonatal effects described here, since female protection has been shown in animal models of neonatal brain injury, when minimal circulating estrogen from the quiescent neonatal ovaries is present [40, 68, 72, 73, 81, 82] although central steroidogenesis may occur [64]. Still, it is certainly possible that some late developmental beneficial effects of ovarian estrogen on neural reorganization after injury could occur.

These hormonal data, taken together, suggest that early androgen exposure in males may be a primary contributor to the modulation of sex differences in response to HI injury (although whether these effects occur through aromatization cannot be determined, based on data collected to date). However, another promising line of research aimed at exploring sex differences in response to brain injury has begun to examine possible sex differences in the mechanisms of cell death following injury. These findings have led researchers to believe that hormonal differences are not the only key factor modulating sex differences in response to injury, and that the apoptotic cascade may be differentially activated in the male and female brain following HI.

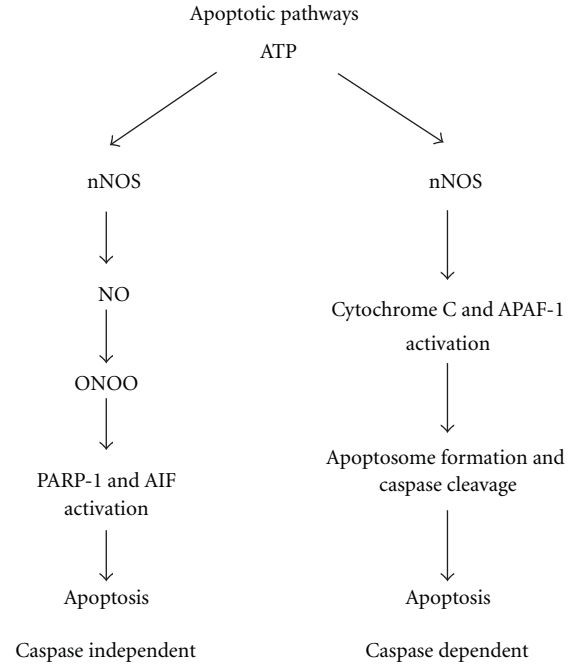

Figure 1: A diagram of the progression of caspase-independent and -dependent apoptotic mechanisms.

3. The Apoptotic Cascade

Apoptosis, or programmed cell death, can be triggered by various events including DNA damage, cytotoxic drugs, a lack of survival signals, and developmental death signals—among a variety of other mechanisms [83]. With regard to neuronal death in response to hypoxia-ischemia, events are initially triggered by a deprivation of oxygen and glucose supply to the cell(s), which depresses both adenosine triphosphate (ATP) synthesis as well as the cellular uptake of glutamate. The accumulation of excess extracellular glutamate triggers an increase in glutamate receptor (NMDA, AMPA, and kainate) activation and prolonged depolarization, leading to increased calcium and sodium influx. Sodium influx through AMPA and kainate receptors leads to cell swelling and rapid necrotic cell death, while calcium influx through NMDA and AMPA receptors lacking the GluR2 subunit (rendering the channel open to calcium) activates neuronal nitric oxide synthase (nNos). nNos, in turn, leads to the production of the free radicals, nitric oxide (NO), and peroxynitrate (ONOO). In caspase-*independent*-mediated cell death, a reduction of nicotinamide adenine dinucleotide (NAD^+, a high energy molecule) is caused by activation of poly(ADP-ribose) polymerase-1 (Parp-1, a DNA repair enzyme), leading to release of apoptosis-inducing factor (AIF) and endonuclease G from the mitochondria to the nucleus of the cell, and ultimately cell death [49–51]. Through a second caspase-*dependent* pathway, the increase in nNos ultimately leads to mitochondrial dysfunction and the translocation of cytochrome-c from the mitochondria to the nucleus, signaling apoptotic protease-activating factor-1 (APAF-1) and the formation of the apoptosome. The apoptosome binds with caspase-9 (the initiator caspase), which in turn cleaves downstream caspases 3, 6, and 7 (effector caspases) causing chromatin condensation, DNA fragmentation, and ultimately cell death [42, 50, 83–85], (see Figure 1).

Interestingly, research has revealed the sexes to differentially favor one of these two pathways (though not exclusively), with females relying more heavily on the caspase-dependent pathway and males largely utilizing the caspase-independent pathway of cell death following HI insult. These data derive from injury models in both adult stroke (MCAO) and neonatal HI models [41–46, 80, 86, 87].

3.1. Apoptotic Cascades and Brain Injury. In adult stroke models, male and female Parp-1-deficient mice both display a reduction in Parp-1 and AIF production, suggesting the activation of the caspase-independent apoptotic pathway by both sexes. However, only male Parp-1-deficient animals exhibited a reduction in stroke-induced brain damage following MCAO [86]. Likewise, female (but not male) mice were largely resistant to endotoxin-induced mortality, and Parp-1 inhibition decreased endotoxin-induced vascular and inflammatory response in male (but not female) mice [88]. Interestingly, ovariectomy partially reversed the protection normally seen in females, suggesting a modulating role of estrogen [88]. Similarly, inhibition of Parp-1 and nNOS was found to protect male animals from the damaging effects of MCAO (but not females, [89]). In fact, Parp-1 inhibition *increased* stroke damage in intact females and estrogen-replaced ovariectomized females, again suggesting a mediating role of estrogen [89]. Though the results of these two studies suggest that ovarian hormones may play a role in

modulating caspase-independent cell death in adult models, it is important to note that sex differences in neonatal HI are found when minimal circulating estrogen is present (though evidence indicates central steroidogenesis may be occurring [64]). These neonatal studies also report evidence of predominant male use of the caspase-independent pathway, suggesting such sex differences may not be exclusively mediated by exogenous hormones (see below). Likewise, preferential activation of the caspase-dependent apoptotic pathway has been seen in adult female animals following MCAO, as increased cytochrome-c release and caspase-3 cleavage was found relative to males, while inhibition of caspase activation was found to be neuroprotective in female animals only. This benefit was extended to ovariectomized and estrogen-replaced female animals, indicating these effects to be independent of hormones [80].

Recent work exploring cell death mechanisms in neonatal models also indicates sex differences in the preferred apoptotic pathway following early HI [41, 42]. With regard to the caspase-independent apoptotic pathway, both Parp-1 and AIF have been found in higher concentration in the brains of male mice following P9 HI injury, as compared to the brains of HI-injured females [43]. Likewise, significant protection from P7 HI injury has been shown in Parp-1 knockout male mice, though no comparable protection was seen in females [44]. Other studies report that cytochrome-c and various caspases—which are active in the caspase-dependent pathway—have been found in higher concentration in female as compared to male mice following P9 HI injury [43]. Likewise, inhibition of caspase cleavage has been shown to be neuroprotective in female (but not male) animals following P3 or P7 HI [45, 46], where translocation of cytochrome-c was prevented. Finally, neuronal cultures (absent of circulating hormones) subjected to cytotoxic challenge showed differing pathways of cell death—with XY neurons predominantly utilizing the AIF-mediated caspase-independent apoptotic pathway and XX neurons activating the cytochrome-c, caspase-dependent apoptotic pathway [38]. It should be noted that no studies (of which we are aware) have quantified the exact proportional activation of caspase-dependent and -independent apoptosis for either sex, but instead have measured the activation of elements specific to each pathway or the relative protection afforded to one sex over the other following knockout/inhibition of elements specific to one pathway as stated above. These sex differences are found to be significant in magnitude.

From these studies, it is evident that male and female neurons undergoing apoptosis capitalize on pathways that show sex differences unlikely to be exclusively related to hormones (though these apoptotic pathways are not exclusive to sex). However, detailed assessment of the potential interaction between the early (or concurrent) presence of gonadal hormones, and the activation of sex-specific apoptotic cascades, remains to be defined. Nonetheless, apoptosis is a major contributor to neuronal cell death and tissue loss following neonatal HI, and the development of neuroprotectants aimed at targeting the mechanisms most utilized by each sex represents a valuable venue of investigation for therapeutic interventions.

4. A Gene-Linked Female Advantage

An alternative or additional explanation for sex differences seen in neonatal HI outcome involves endogenous inhibitors of apoptosis. During development, the apoptotic cascade is a highly regulated process critical for healthy development and maintenance of tissue. This process of programmed cell death is counterbalanced by antiapoptotic signals that promote the survival of cells. A family of proteins, known as inhibitors of apoptosis (IAPs), serve as endogenous inhibitors of cell death [47, 90] and have been found to regulate apoptosis by blocking both the intrinsic and extrinsic mechanisms. Specifically, IAPs directly bind to and inhibit initiator and effector caspases [47, 49, 51]. The function of IAPs has recently been extended beyond its initial role in development and is now thought to play a role in processes such as cancer, tumor formation, autoimmune diseases, neurodegenerative disorders, and most recently, cell death following brain injury [51, 91].

Of the known IAPs, X-linked IAP (XIAP) is recognized to be the most potent [48]. XIAP effectively binds to the initiator caspase (caspase-9) and halts further cleavage of downstream caspases (caspases 3 and 7), thus preventing cell death [48–51]. XIAP has also been shown to bind and inhibit caspases 3 and 7 directly [48], and *in vitro* studies have revealed XIAP to severely inhibit nuclear destruction and cytochrome-c-induced caspase activation [48]. XIAP expression has been confirmed in both rodent and human brains following ischemic injury [92]. Moreover, it is understood that genetic balancing in females occurs via random inactivation of the second X chromosome; however, 15% of genes located on the second X chromosome always escape inactivation, and an additional 10% sometimes escape inactivation [52]. Therefore, it is possible that females present with an increased expression of XIAP relative to males. And since XIAP acts specifically on the caspase-dependent pathway of cell death preferentially activated in females, XIAP may play a role in the selective protection afforded to females following early HI injury.

4.1. X-Linked IAPs and Brain Injury. Currently very little is known about the regulation of IAPs and XIAP in neonatal HI injury, though surprisingly, results from studies of XIAP knockout [93] and overexpression [94] have largely failed to report sex differences in degree of tissue damage following early HI injury. Further investigation revealed that these results may be due to compensatory changes in other IAP family members (i.e., upregulation of c-IAP1 and c-IAP2 [95, 96]), with XIAP remaining a probable source of protection for females. One specific study examined the long-term behavioral effects of neonatal HI following inhibition of XIAP in male and female rats [73]. Based on cumulative evidence of sex differences in apoptotic mechanisms, coupled with evidence of potential female protection via XIAP, this study utilized embelin—a small molecule inhibitor of XIAP. Embelin binds to the BIR3 domain (the biding site of caspase-9) on the XIAP protein molecule [91], thus preventing endogenous inhibition of apoptosis by XIAP. Treatment

with embelin increased neuropathological damage and life-long behavioral deficits in HI females relative to vehicle-treated HI females, while no comparable effects were seen in males [73]. Thus, results demonstrate both the reliance on specific pathways of cell death between the sexes, as well as the importance of XIAP in the protection afforded to females following injury.

Clearly more research is needed on the role of IAPs in hypoxic-ischemic neuronal death, but the studies presented here emphasize the need for an improved understanding of innate mechanisms of protection in male and female neonates. Future studies will be needed to assess the potential interaction of hormonal exposure and genetic differences in sex chromosome gene expression within brain cells, since sex differences in response to early injury are almost certainly influenced by a combination of these factors.

5. Conclusions

Neonatal HI is a major cause of infant mortality and long-term neurologic morbidity in both preterm and term-injured populations. It is evident that the consequences of neonatal HI injury are severe, yet the difference in outcome experienced between the sexes is surprising. Male infants not only exhibit increased risk for HI, but also display greater behavioral and cognitive disruption following HI injury as compared to matched female counterparts. Animal studies utilizing induced neonatal HI suggest that this sex discrepancy may be modulated by (1) the presence of sex-specific hormones (e.g., testosterone), (2) sex differences in the preferred mechanisms of apoptosis, and/or (3) the protective effect of IAPs (which may be in greater quantity in female brain) on the caspase-dependent apoptotic pathway. Indeed, all three of these mechanisms may interact with each other, and sex differences in the effects of neonatal HI outcome likely reflect an interplay of both genetic and hormonal factors. One possible study to dissociate these interactive mechanisms could entail the use of a four-core genotype (FCG) mouse model (described in [97]), in which the Sry (testis determining) gene is deleted from the Y chromosome and inserted onto an autosome. A cross between this type of male and an XX female can then produce genetic females with insertion of the Y chromosome Sry gene modulating testicular development (thus leading to androgen exposure absent of all other Y chromosome genes), and XY males with knockout of the Sry gene (who develop as phenotypic females). Exploration of the consequences of neonatal HI in mice with Y genes but no testosterone, and testosterone but no other Y genes, could allow a more in-depth study of whether sex-based preference for apoptotic pathways may somehow be set by early androgen exposure, other genetic factors, or both.

In closing, further studies of the influence of both genetic and hormonal factors relevant to neonatal HI could have important clinical implications. For example, the modulation of hormonal mechanisms leading to increased damage in males, modulation of apoptotic cascades, or modulation of IAPs may all represent target candidates for therapeutic intervention in neonates suffering HI brain injury. Further, studies looking at neonatal HI while manipulating testosterone receptors, estrogen receptors, and/or aromatase blockers could potentially dissociate or clarify the mechanism of action promoting injury. Moreover, it seems plausible that a lack of exposure to placental hormones due to premature birth could also be detrimental to the neurological development of premature infants (though no studies, of which we are aware, have determined such effects). Given the tremendous amount of research focusing on sex differences in adult stroke, we suggest that future research should similarly focus on sex differences in the consequences of neonatal HI. In fact, research in this area could yield beneficial sex-specific neuroprotectants, with far reaching implications for improved clinical practice and treatment.

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Review Article

Disruption of the Serotonergic System after Neonatal Hypoxia-Ischemia in a Rodent Model

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Identifying which specific neuronal phenotypes are vulnerable to neonatal hypoxia-ischemia, where in the brain they are damaged, and the mechanisms that produce neuronal losses are critical to determine the anatomical substrates responsible for neurological impairments in hypoxic-ischemic brain-injured neonates. Here we describe our current work investigating how the serotonergic network in the brain is disrupted in a rodent model of preterm hypoxia-ischemia. One week after postnatal day 3 hypoxia-ischemia, losses of serotonergic raphé neurons, reductions in serotonin levels in the brain, and reduced serotonin transporter expression are evident. These changes can be prevented using two anti-inflammatory interventions; the postinsult administration of minocycline or ibuprofen. However, each drug has its own limitations and benefits for use in neonates to stem damage to the serotonergic network after hypoxia-ischemia. By understanding the fundamental mechanisms underpinning hypoxia-ischemia-induced serotonergic damage we will hopefully move closer to developing a successful clinical intervention to treat neonatal brain injury.

1. General Characteristics of Neonatal Brain Injury

Approximately 4 in 1000 babies are born each year with brain damage. Being born premature (<37 weeks gestation) and exposure to a hypoxic-ischemic insult (HI; reduced oxygen and blood flow to the brain) are the major risk factors that contribute to this statistic [1, 2]. An HI insult can ensue after many possible factors including placental dysfunction, haemorrhage, hypotension, umbilical cord occlusion, and stroke [1]. A considerable number of these preterm neonates estimate as high as 50% [3], develop neurological and functional impairments such as cerebral palsy, motor deficits, sleep disorders, hyperactivity, anxiety, depression, and cognitive and autonomic disabilities [4–8]. These lifelong disabilities place enormous burdens on the individual as well as family, healthcare, educational, and community resources.

Although significant advances in neonatal care have increased survival rates of preterm infants, particularly those less than 28 weeks gestation, a concomitant decrease in morbidity has not been achieved. In addition, aside from the recent development of early cooling of the neonatal brain [9, 10], there is no therapeutic intervention available to treat neonatal brain injury. Thus the substantial associated lifelong burdens are growing and there is an urgent need to identify neuroprotective drugs that target neuronal networks to prevent, slow, or abate the deleterious effects of HI in the neonatal brain.

White matter damage is a hallmark feature of brain injury after HI in the preterm neonate. Enlarged ventricles (ventriculomegaly), loss of vulnerable oligodendrocyte progenitor cells, periventricular leukomalacia (PVL), hypomyelination, thinning of the corpus callosum, astrogliosis, and microgliosis are typical features of white matter damage [11–16]. Characterising white matter injury and searching

for the mechanisms contributing to this injury have been major avenues of investigation in the area of preterm HI brain injury. However, neuronal loss is also a critical neuropathological feature of HI and the pattern of brain injury in preterm neonates is described as a combination of white and grey matter damage [11–13]. Moreover, it is plausible that disrupted neuronal function and neural circuit connectivity are a consequence of white matter loss and axonal disruption.

2. Neuronal Damage in the Preterm HI Brain

With the advent of more sophisticated and higher resolution imaging techniques scientists are beginning to discriminate white and gray matter, delineate neural connectivity, and identify biochemical markers so that brain injury in the neonate is increasingly being characterized in much finer detail. It is well established that there are volumetric reductions in certain brain areas of HI-affected preterm infants including the thalamus, basal ganglia, and cerebral cortex and that these effects are manifested in association with PVL and other white matter features [17–21]. Axonal pathology and neuronal injury have been reported in these regions as well as in the brainstem, cerebellum, striatum, hippocampus, and hypothalamus after HI in the human preterm brain [8, 22–24] and animal models [25–28]. Furthermore, long-term changes in neuronal neurotransmitter content and release can also occur after neonatal HI [29–32]. Disruption of neuropeptides and neurotransmitters, critical for the development of synapses and formation of neuronal networks, has been postulated to underlie behavioural deficits and neuroendocrine disorders in the growing child and adult human with a history of preterm HI [33]. It is pertinent that some types of neurons (e.g., dopaminergic, noradrenergic, and cholinergic neurons) may be more vulnerable to perinatal injury than others (e.g., magnocellular neurons in the hypothalamus) [28, 34–36].

Identifying which specific neuronal phenotypes are vulnerable to HI, where in the brain they are damaged, the timing and mechanisms underlying neuronal losses are necessary directions to establish the anatomical substrates underpinning functional impairments in HI-affected neonates. These are important issues to determine because if particular neuronal phenotypes or brain regions are injured at different times or differ in their vulnerability to HI then selective neuroprotective interventions may also be temporally and spatially distinct. One neural network that we have a particular focus on is the serotonergic system in the brain.

3. The Serotonergic System: A Candidate Network Disrupted after Neonatal HI

Virtually all brain regions reportedly injured after neonatal HI receive substantial serotonergic fibre projections from the brainstem. In addition, the rostral brainstem, where serotonergic cell bodies reside, is damaged after neonatal HI [8]. It is well established that interruption of the central serotonergic system can lead to numerous functional deficits and many outcomes are similar to those observed in preterm neonates exposed to HI. These observations prompted us to hypothesise the serotonergic network in the brain is a major system that is disrupted after preterm HI and that this system is a pivotal neural candidate to target with neuroprotective interventions after preterm HI.

Serotonin (5-hydroxytryptamine, 5-HT) is pivotal in fetal and postnatal brain development [37]. The serotonergic network in the brain develops very early during gestation and is one of the first transmitter systems to appear in the developing brain. Indicative of its pervasive innervation of the central nervous system in the postnatal and mature brain, 5-HT is a neurochemical that is involved in a vast array of functions. In addition, dysfunction of serotonergic neurotransmission has been implicated in a host of physiological, metabolic, and behavioural changes in disease states such as epilepsy, depression, movement disorders, autism, anxiety and sudden infant death syndrome (SIDS) [38–43]. In the context of neonatal brain injury, it is pertinent that many of these deficits match those observed in HI-affected neonates [4, 5, 7, 44]. In addition, decreased serotonergic function is a hallmark feature of depression and depressed patients show 31% loss of dorsal raphé neurons [45]. Cerebral palsy is a notable disability in some HI-affected neonates and these patients have been reported to suffer depression [46, 47]. Although, whether altered serotonergic function accounts for certain HI-induced neurological deficits is not known. It is important to first characterise the effects of neonatal HI on major elements of the serotonergic system in the brain and begin to decipher whether these specific nuclei constitute primary candidate networks that underpin neonatal HI-induced neurological deficits.

Utilising a postnatal day 3 (P3) HI model of preterm HI we have recently investigated how P3 HI affects the serotonergic system in the brain. The P3 rat pup is subjected to HI by right common carotid artery ligation followed by 6% oxygen for 30 min. In the rat, the P3 brain development stage is analogous to the preterm human neonate brain at approximately 24–28 weeks gestation in terms of cellular development, number of synapses, neurochemical development, and cortical organization [48]. This preclinical model produces typical behavioural and pathological features including encephalopathy and hypomyelination observed in human preterm neonates affected by HI [4, 28, 48–51].

4. The Synthesis and Release of 5-HT in the Central Nervous System

Serotonin is synthesised in the brain in serotonergic neurons from the amino acid L-tryptophan and its metabolite 5-hydroxytryptophan (5-HTP). Synthesis occurs via tryptophan hydroxylase (TpH), 5-HT's rate-limiting enzyme and a second enzyme amino acid decarboxylase. Two isoforms of TpH are known to exist (TpH_1 and TpH_2) but only TpH_2 is found in the brain [52]. The major regulator of 5-HT levels in the brain is the serotonin transporter (SERT). The transporter consists of 12 transmembrane domains that span the presynaptic membrane of 5-HT-releasing

cells [53]. Localised on the presynaptic membrane of serotonergic neurons, SERT terminates serotonergic signalling by the efficient reuptake of extracellular serotonin back into the presynaptic neuron (Figure 1) thereby controlling the duration of action and post-synaptic signalling of 5-HT in the brain. Consequently, SERT is a major target for drugs such as selective serotonin reuptake inhibitors (SSRIs) that can increase 5-HT availability in the brain and are useful drugs in the treatment of depression. Serotonin is also broken down by monoamine oxidase (MAO) enzymes, preferentially MAO-A, into 5-hydroxyindoleacetic acid (5-HIAA); serotonin's major metabolite.

Nine groups of 5-HT-containing cell bodies represented in raphé subdivisions in the pons and upper brainstem were first described using histochemical techniques and designated B_{1-9} [54]. The bilateral raphé subdivisions are predominantly populated with serotonergic neuronal cell bodies and provide an extensive serotonergic network throughout the central nervous system. Based on their cytoarchitecture, neurochemistry, and neural projections, nomenclature for the clusters of 5-HT neurons describes their location in the dorsal, lateral, midline, or caudal portion of the pons and medulla oblongata [55, 56].

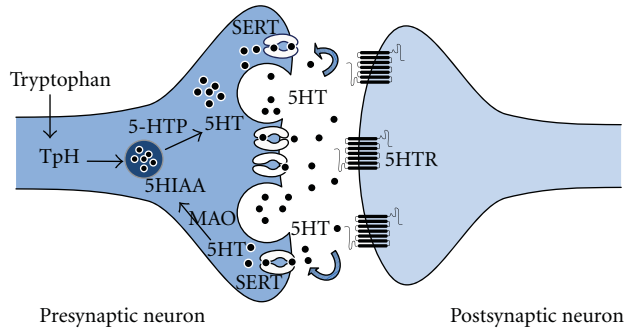

Figure 1: Schematic diagram depicting the major pathways involved in the synthesis, release, re-uptake and metabolism of serotonin in serotonergic neurons. Components of the figure have been modified from Motifolio. TpH: tryptophan hydroxylase; 5-HTP: 5-hydroxy-L-tryptophan; 5-HT: serotonin; SERT: serotonin transporter; MAO: monoamine oxidase; 5-HIAA: 5-hydroxyindoleacetic acid; 5-HTR: serotonergic receptor.

5. Serotonergic Damage in the Immature Brain after HI

In human neonates with HI encephalopathy tryptophan hydroxylase, the 5-HT rate-limiting enzyme, is reduced in the brainstem [41, 57]. Damage to human dorsal brainstem nuclei, where serotonergic cell bodies are located, is also apparent [8]. However, until our initial study in 2010 in a rodent P3 HI model [58], information about the effects of HI on specific raphé nuclei was scarce. We found an overall significant loss of 5-HT-positive raphé neurons after P3 HI, consistent with previous animal studies [59, 60] and reports from human neonates [8]. However it is interesting that certain serotonergic raphé nuclei appear to be more vulnerable to P3 HI-induced injury than others. One week after P3 HI, 5-HT-positive neuronal losses occur in the dorsal raphé caudal, dorsal raphé ventrolateral, and dorsal raphé dorsal nuclei. In contrast, the dorsal raphé interfascicular and the raphé magnus nuclei showed no reduction in number of 5-HT-positive neurons on P10 and P45. Six weeks after P3 HI, on P45, only the dorsal raphé ventrolateral and the dorsal raphé dorsal demonstrated a maintained and significant decrease in numbers of 5-HT-positive neurons [58].

The rostrocaudal distribution of the raphé serotonergic neurons may determine their vulnerability to HI injury. It is evident that the anterior raphé subdivisions are more affected by P3 HI than the more posterior and caudally located raphé nuclei such as the raphé magnus and the dorsal raphé interfascicular nuclei [58]. The topographical clustering of different raphé subdivisions in the midbrain and brainstem also represents differential connectivity patterns in the brain. As such the dorsal raphé caudal, dorsal raphé ventrolateral, and dorsal raphé dorsal nuclei primarily innervate the cerebral cortex, basal ganglia, thalamus, hypothalamus, hippocampus, and amygdala [55, 61, 62]. In contrast, the more caudal nuclei predominantly send neural projections to the spinal cord and other parts of the brainstem [63]. The afferent and efferent connections of each raphé subdivision are integral to producing characteristic serotonergic-dependent functions. Thus selective losses of serotonergic raphé nuclei may underpin particular deficits reported in HI-affected preterm infants. On the other hand, it appears that the more caudal raphé magnus and dorsal raphé interfascicular nuclei are not susceptible to P3 HI injury and therefore the serotonergic innervation of the spinal cord remains relatively intact and functional after P3 HI [58]. Indeed previous reports suggest that spinal cord injury only occurs after severe neonatal HI insults [64, 65].

Functional disruption of the serotonergic system after neonatal HI is clearly reflected in the reduction in 5-HT levels in the brain. We and others have demonstrated reduced 5-HT levels in cortical, thalamic, and brainstem regions after HI produced in the immature rodent brain [66, 67]. The losses of brainstem dorsal raphé neurons and their neural projections after HI are most likely responsible for the reduced 5-HT levels in the forebrain. Although regional differences are apparent, the association between direct serotonergic neural inputs to forebrain regions from specific raphé nuclei in the brainstem is not known. Thus determining whether specific ascending and descending neural connections are disrupted after HI injury may predict raphé nuclei vulnerability to P3 HI injury and the effects they have on brain regions innervated by serotonergic afferents and efferents.

In concert with the loss of raphé neurons and reductions in 5-HT levels in the brain, SERT expression is significantly reduced in the brain [58, 67, 68]. We have characterised SERT losses after P3 HI using both Western blot and immunolabelling techniques. The distribution of SERT in fibres, dendrites, cell bodies, and axon terminals [69] makes it an excellent marker of the serotonergic network in the brain [70, 71]. As such the distribution of SERT in the brain closely reflects that of serotonergic neuronal cell bodies

and innervating fibres [72, 73]. Serotonergic fibre losses and damage are observed after P3 HI in several key forebrain regions such as the motor and somatosensory cortex, lateral hypothalamus, ventrolateral thalamus, and horizontal limb of the diagonal band [67]. The parallel and concomitant reductions in 5-HT levels and SERT indicate that there was reduced availability of 5-HT for release as well as limited reuptake of 5-HT. This is analogous to findings after ischemia in P7 rat pups whereby there is concurrent attenuation of 5-HT and its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), suggesting that injury to the serotonergic neuronal network ensues rather than direct modulation of SERT itself or of serotonergic metabolism [66]. We therefore speculate that P3 HI induces disruption to the serotonergic system as a result of loss or damage to serotonergic neurons.

The efficient reuptake of 5-HT is primarily dependent on the localisation of SERT on cell bodies, dendrites, and fibres of serotonergic neurons in the central nervous system [72]. However there is evidence that the reuptake of 5-HT can occur by glial cells whereby SERT may be also localised on astrocytes [74–77] and/or microglial cells [78]. Thus glial SERT could potentially assist in the clearance of 5-HT from the serotonergic synapse [74]. However of the few studies that have specifically examined this possibility, most have only reported localisation of SERT in glial cell lines and primary cultures. Through our own *in vivo* investigations, we have found no evidence of SERT localisation on microglia or astrocytes in the normal or P3 HI-injured neonatal rodent brain (unpublished).

From our studies it is interesting to note that, in general, proportionately greater serotonergic changes occur in the forebrain regions compared to the brainstem raphé nuclei [58, 67, 68]. This observation has led us to speculate that damage to the serotonergic fibres in the forebrain core/penumbral areas of the HI-injured brain may occur before injury to the brainstem raphé nuclei. In our P3 HI model in the rodent, ligation of the common carotid artery affects a vascular field encompassing primarily forebrain regions, whereas the brainstem lies outside this vascular field and is seemingly spared of immediate hypoxic and ischemic conditions. It has been shown that blood flow to the brainstem tends to increase during HI [79]. In addition we have consistently found that, unlike the forebrain, there is no change in brainstem hemisphere area after P3 HI [28, 58, 67, 68]. The dorsal raphé nuclei can be considered remote from the damaged forebrain sites and therefore serotonergic neuronal injury in the brainstem might develop as a result of secondary mechanisms. One such secondary injury mechanism that we have had a particular focus on is P3 HI-induced neuroinflammation.

6. Role of Neuroinflammation in Producing Neuronal Injury

Two phases of injury can be defined after a neonatal HI insult; an early primary phase within 24–48 h causing mainly irreversible injury in the brain and a later secondary injury phase then ensues. Early neuronal injury after HI is thought to evolve primarily via necrosis resulting from excitotoxic damage produced by excessive release of glutamate from presynaptic nerve terminals and astrocytes, causing calcium overload and cell death [80]. Brain injury during the primary phase can also result from high levels of free radicals including reactive nitrogen species and reactive oxygen species accumulating in the brain tissue [81, 82]. Both caspase-dependent and caspase-independent mediators of cell death are also initiated after neonatal HI [83, 84].

The subsequent secondary phase can continue for weeks, months, or longer after HI. A vast array of mechanisms may contribute to neuronal injury during this phase and the majority of these have been identified as features of neuroinflammation. Key features of this phase include increased numbers of activated microglia, astrogliosis, increased levels of proinflammatory cytokines (e.g., interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), decreased levels of anti-inflammatory cytokines, increased cyclooxygenase (COX-1 and COX-2) expression, prostaglandins (PGE_2 and PGI_2), nuclear factor kappa-light-chain-enhancer of activated B cells, increased expression of chemokines and chemokine receptors, cell adhesion molecule expression, and matrix metalloproteinases [85–89]. Proinflammatory cytokines, particularly IL-1β and TNF-α, are synthesized and released by activated microglia although IL-1β is also expressed by astrocytes and developing oligodendrocytes [90–92]. Astrocytes are important sources of lactate, neurotrophic factors, and pyruvate for neurons and contribute to maintaining neurotransmitter and metabolic homeostasis in the brain [93, 94]. However, although inhibition of astrogliosis after neonatal HI improves the survival of newborn neurons it does not alter infarct volume [95]. The infiltration of peripheral cells such as lymphocytes, neutrophils, and mast cells can also ensue if there is sufficient breakdown and leakage across the blood-brain barrier [89, 96, 97]. The hallmark feature of neuroinflammation in the HI-affected brain that we have focused on, in terms of a potential mechanism underpinning serotonergic neurodegeneration, is the elevated number of activated microglia.

Numbers of activated microglia peak within the first week after HI although can remain elevated for weeks or months after the initial ischemic episode as observed in human and preclinical studies [68, 84, 89, 91, 98–100]. Microglia are the resident immune cells of the CNS that, in the normal brain, survey the extracellular environment and scavenge and clear the brain of debris and dying cells [101–103]. However microglia can also respond quickly to changes induced by HI in the brain and within 48 h can switch from a resting to an active state, multiply and migrate to sites of ischemic injury [104, 105]. Activated microglia produce and release excessive levels of IL-1β and TNF-α [50, 84, 89, 100, 106], that are toxic to neurons, can cause neurodegeneration, and negatively affect the neurodevelopment of neonates [107, 108]. We have demonstrated that numbers of activated microglia and levels of TNF-α and IL-1β in the brain are elevated over the critical first week after P3 HI, particularly in the cortex, thalamic nuclei, and white matter, and closely parallel injury to the serotonergic system [67, 109]. Thus an association between neuroinflammation and serotonergic injury is evident and the period after the P3 HI insult

is a critical window of opportunity for interventions that target neuroinflammation. Two anti-inflammatory drugs are proving to be potential interventions to ameliorate HI-induced damage to the serotonergic system. These are minocycline and ibuprofen.

(a) *Effects of Minocycline on the Serotonergic System after Neonatal HI.* The role of activated microglia and raised levels of proinflammatory cytokines in contributing to serotonergic neuronal disruption can be addressed by blocking microglial function with anti-inflammatory drugs. Minocycline is a broad-spectrum antibiotic that also has anti-inflammatory properties in the brain primarily because it is a potent inhibitor of activated microglia [110, 111]. Minocycline does not appear to affect astrocytes after neonatal HI [84, 111, 112]. Minocycline readily crosses the blood-brain barrier after systemic delivery [113, 114] and is an effective neuroprotective intervention when delivered after-insult [50, 67, 83, 115, 116]. The opportunity to alter HI-induced brain injury *after* the insult is an important prospect because clinical diagnosis of HI in the preterm neonate is often not made until 3 days after birth, well into the secondary injury phase. Furthermore it is difficult to predict if an HI insult is imminent and therefore prophylactic treatments during pregnancy or labour are difficult to administer.

Recent studies in the adult rat demonstrate that minocycline reverses 3-nitropropionic acid neurotoxicity-induced changes in 5-HT levels [117] and reduces the 3,4-methylenedioxymethamphetamine-induced reduction in SERT expression [118]. We have now shown in our neonatal model that minocycline, initiated 2 h after P3 HI and administered daily for 1 week, inhibits P3 HI-induced microglial activation and TNF-α and IL-1β levels and also results in fewer raphé neurons being lost, maintenance of normal 5-HT levels, and increases SERT expression [67]. However not all effects of minocycline on the serotonergic system damage are completely prevented. Furthermore, using the same 1-week-long minocycline regimen, HI-induced neuroinflammation is still inhibited 6 weeks later [68] but minocycline's long-term neuroprotection of the serotonergic system is less effective than at P10. At 6 weeks after-HI SERT expression and serotonergic fibre content appear to be close to control levels but 5-HT levels remain reduced [68]. Nonetheless minocycline, a robust inhibitor of P3 HI-induced neuroinflammatory mediators, significantly improves serotonergic outcomes; however, HI induced damage to the serotonergic network.

Although minocycline treatment could be a novel therapy to minimise serotonergic changes after neonatal HI and preserve the integrity of 5-HT neurocircuitry in the brain, the use of minocycline in neonates is controversial. Minocycline is an excellent tool to block microglial activation and has considerable neuroprotective effects, not only in neonatal HI animal models. Moreover minocycline has proven to be highly beneficial in numerous adult human trials to treat a variety of neurodegenerative conditions [119–122]. Nevertheless its use in human neonates must be undertaken with caution because of the adverse effects associated with chronic tetracycline that historically have tarnished their administration to neonates [123]. Minocycline can produce bone stunting, staining, and pitting of teeth [123–125]. Tetracyclines may also prevent the binding of bilirubin to albumin and possibly lead to bilirubin-induced brain damage in neonates. In contrast, recent studies have demonstrated that minocycline does not produce some of the side effects historically associated with tetracycline use in neonates [126–129]. The development of new derivatives of minocycline, with fewer adverse side effects, could be promising interventions to develop for clinical translation. Alternatively, given the potential of anti-inflammatory interventions to prevent serotonergic injury, testing other anti-inflammatory drugs that may be more clinically acceptable for use in neonates is a rational approach.

(b) *Effects of Ibuprofen on the Serotonergic System after Neonatal HI.* Nonsteroidal anti-inflammatory drugs (NSAIDs) constitute an alternative anti-inflammatory treatment to stem brain injury after neonatal HI. In this class, drugs such as ibuprofen and indomethacin are commonly used to treat patent ductus arteriosus in preterm neonates [130, 131]. Ibuprofen is a lipophilic compound and after systemic delivery easily crosses the blood-brain barrier [132]. A canonical mechanism of action of NSAIDs is to inhibit cyclooxygenase 1 and 2 enzymes (COX-1, COX-2) and the conversion and synthesis of arachidonic acid to downstream inflammatory effectors such as cytokines and prostaglandins.

Systemic delivery of ibuprofen can inhibit central neuroinflammation and elicit neuroprotective effects although these outcomes have primarily been demonstrated in adult models of cerebral ischemia [133–136]. In human preterm neonates (<28 weeks gestation) indomethacin reduces white matter loss [137] although ibuprofen combined with ascorbic acid treatment in neonates reportedly has little effect on brain injury after severe HI [138]. Consistent with previous studies [86, 139–142], we have shown that COX-2 is elevated in the brain after P3 HI and that ibuprofen significantly prevents this effect as well as P3 HI-induced increases in numbers of activated microglia, IL-1β, and TNF-α levels [143]. In association with these anti-inflammatory effects ibuprofen ameliorated reductions in cerebral hemisphere size, O4-positive pre-myelinating, O1-positive immature oligodendrocyte cell counts, and myelin content [143].

The potential of ibuprofen to be a neuroprotective agent in neonates to stem HI brain injury is further supported by findings that systemic indomethacin or COX-2 inhibitors (NS398) attenuate inflammatory changes as well as functional impairments after neonatal HI in the rodent [142, 144, 145]. In contrast, the effects of NSAIDs on serotonergic neuronal injury after HI are not known. Preliminary evidence in our preclinical HI model indicates that ibuprofen prevents reductions in SERT expression, 5-HT levels (in the frontal cortex and thalamic nuclei), and serotonergic raphé neuronal counts (unpublished). Our findings suggest that ibuprofen is as effective at preventing serotonergic injury however, like minocycline, it does not appear to completely ameliorate damage to this neuronal network. Thus it is plausible that other mechanisms of injury

also contribute to HI-induced serotonergic damage in the neonatal brain.

7. Lack of P3 HI-Induced Neuroinflammatory Mediators in the Brainstem

From our studies, it is interesting to note that a pattern of neuroinflammation is beginning to emerge. The brainstem dorsal raphé and frontal cortex, for example, represent two areas where neuroinflammatory mediator profiles differ markedly. In the frontal cortex substantial and significant increases in activated microglia and proinflammatory cytokines occur after P3 HI. In contrast, in the brainstem, we have observed that the brainstem does not elicit any major signs of neuroinflammation after P3 HI. Numbers of activated microglia are relatively small, there are no apparent changes in proinflammatory cytokines [67] and more recent data from our laboratory indicates there are no changes in COX-2 expression in raphé serotonergic subdivisions (unpublished). It therefore appears that serotonergic raphé cell bodies are not lost because of local neuroinflammation in the brainstem. Thus, even though inhibition of neuroinflammation has a significant beneficial effect on P3 HI-induced losses of raphé neurons [67], we speculate that the effects of anti-inflammatory drugs such as minocycline are not directly effective at the level of the raphé nuclei. We postulate that the losses of 5-HT-positive neurons in the brainstem after P3 HI, and the neuroprotective effects of minocycline are therefore likely to occur via other, indirect secondary mechanisms.

As stated earlier, at least in our model, the brainstem is located outside the vascular field of the common carotid artery and should not be directly affected by immediate changes in perfusion after HI. Instead neuroinflammation could contribute to brainstem injury via remote actions originating from primary injury sites. Inflammatory mediators may damage afferent and efferent fibres of dorsal raphé nuclei in the forebrain and subsequently compromise the survival of brainstem nuclei by retrograde degeneration and/or target deprivation. The thalamus, substantia nigra, hippocampus, and amygdala have substantial neural connections with primary injury sites (e.g., the cerebral cortex) and can undergo prolonged periods of apoptosis and degeneration in the neonatal brain after HI [25–27, 146]. It has been shown that after ischemic conditions, disrupted somatosensory transmission in the thalamus is associated with increased numbers of thalamic neurons degenerating in the secondary phase [26, 147, 148]. Progressive loss of serotonergic neural connections with damaged areas could lead to the disruption and loss of raphé serotonergic neurons in the brainstem. Indeed the regional differences in vulnerability of 5-HT-positive neurons in the dorsal raphé nuclei after P3 HI [58] might be attributed to the serotonergic innervation pattern to damaged and undamaged forebrain regions.

The two mechanisms of HI-induced neuroinflammation and neural disruption may not be mutually exclusive. Activated microglia have also been shown to be present in brain regions as a consequence of a loss of connectivity with a target region or axonal interruption [149–151]. It remains to be investigated whether forebrain neuroinflammation after neonatal HI initiates subsequent serotonergic neuronal damage in the remote brainstem via retrograde degeneration and/or target deprivation mechanisms.

8. Conclusions and Future Directions

We have identified the serotonergic system as a pervasive network that is disrupted after neonatal HI in a rodent model. The concomitant reductions in SERT, 5-HT levels and 5-HT-positive raphé neurons suggest that serotonergic network injury is a consequence of degenerating serotonergic neurons that project to the HI-damaged forebrain. A change in the levels of 5-HT in the brain gives a "readout" of the functional integrity of the serotonergic system. However determining how the synthesis of 5-HT is affected, the storage, release mechanisms, postsynaptic signaling and the breakdown of 5-HT would further our understanding of how HI-injury affects the serotonergic network and possibly reveal new targets for selective interventions. Moreover key components of the serotonergic system have been a critical focus of our recent work, but whether serotonergic changes manifest as specific impairments of neurological performance is not known. It is plausible that disruption of the serotonergic system may underpin impairments such as hyperactivity, cardiorespiratory, cognitive, and attention deficits observed in preterm children who have experienced neonatal HI [4–7, 152]. Also, current theories implicate a disrupted 5-HT neurocircuitry in the brainstem raphé nuclei as the putative underlying mechanism of cardiorespiratory dysfunction in neonates and increased susceptibility to SIDS [41, 153–155]. Being born preterm is a significant risk factor for SIDS [156] and exposure to a HI insult may be sufficient to alter raphé serotonergic function and increase a neonate's susceptibility to later cardiorespiratory complications and possibly SIDS [41, 155].

The serotonergic system does show some degree of recovery weeks after the initial P3 HI insult [58, 68]. Greater density of serotonergic innervation, increased arborization and axonal length, and higher expression of the SERT occur in the postnatal brain; indicating plasticity and temporal differences depending on the region examined [157–159]. It is also remarkable that serotonergic neurons have an ability to sprout and potentially reinnervate after injury [160–162]. In the HI-injured neonatal brain this avenue of investigation remains to be explored, and possibly exploited, to test new therapeutic strategies.

To date, evidence suggests that both minocycline and ibuprofen are successful postinsult interventions to ameliorate neuroinflammation and reducing neuronal loss. Both of these potential anti-inflammatory treatments could be beneficial for HI-induced injury to other neurons in the brain [163–165]. However neither intervention appears to be sufficient to completely reverse the HI-induced decrease in brain 5-HT levels. The dose, timing, and specificity of anti-inflammatory interventions are likely to be key parameters that dictate their success. Alternatively, selectively targeting the serotonergic system to improve its function, in concert with changes produced by anti-inflammatory

drugs, could be an ideal combination treatment to achieve long-term improvement of the serotonergic system after neonatal HI. By understanding the fundamental mechanisms of serotonergic damage after neonatal HI we will hopefully move closer to providing a clinical intervention.

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Review Article

Molecular Mechanisms of Neonatal Brain Injury

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Fetal/neonatal brain injury is an important cause of neurological disability. Hypoxia-ischemia and excitotoxicity are considered important insults, and, in spite of their acute nature, brain injury develops over a protracted time period during the primary, secondary, and tertiary phases. The concept that most of the injury develops with a delay after the insult makes it possible to provide effective neuroprotective treatment after the insult. Indeed, hypothermia applied within 6 hours after birth in neonatal encephalopathy reduces neurological disability in clinical trials. In order to develop the next generation of treatment, we need to know more about the pathophysiological mechanism during the secondary and tertiary phases of injury. We review some of the critical molecular events related to mitochondrial dysfunction and apoptosis during the secondary phase and report some recent evidence that intervention may be feasible also days-weeks after the insult.

1. Introduction

Brain injury occurring during the perinatal period is a common cause of life-long neurological disability. The etiology is complex and multifactorial, but hypoxia-ischemia (HI), infection/inflammation, and excitotoxicity are considered important causes or precipitating insults of preventable/treatable forms of perinatal brain injury. Genetic background, maturational age, sex, and degree of brain development of particular regions affect vulnerability and the mechanisms of brain injury [1, 2]. Furthermore, antecedents like infection/inflammation, intrauterine growth restriction, or preexposure to hypoxia can modulate brain vulnerability [3–5]. Brain injury evolves over time, and different mechanisms are critical during the primary, secondary, and tertiary phases. Indeed, recent experimental data suggests that interventions can be effective if administered hours, days, or even weeks after the primary insult [6, 7].

The aim of the present paper is to describe the critical mechanisms of brain injury during the different stages after an acute insult with particular emphasis on mitochondrial impairment, apoptotic events and the tertiary phase of injury.

2. Secondary Brain Injury

Cerebral HI that is sufficiently severe to cause depletion of tissue energy reserves (primary insult) is often followed by transient but complete restoration of glucose utilization, ATP and phosphocreatine upon reoxygenation [8–10]. Thereafter a secondary decrease of high energy phosphates occurs in experimental studies that parallel a decrease in tissue glucose metabolism and development of cell injury [8–10]. In a similar way, infants with neonatal encephalopathy exhibit characteristic abnormalities in cerebral energy metabolism,

which is frequently normal soon after birth, but shows a progressive decline in [PCr]/[Pi] some hours later [11]. Infants displaying this phenomenon develop neurodevelopmental impairment or die, and there is a close relationship between the magnitude of the late decline in [PCr]/[Pi] and the severity of long-term neurodevelopmental impairment [12].

These findings suggest that most of the injury after HI evolves with delayed onset *after* rather than during the insult. There are many examples of successful posttreatment after HI in animals suggesting a therapeutic window following HI prior to the secondary phase of tissue impairment [13]. Hypothermia following HI reduces secondary energy failure and brain injury in newborns with neonatal encephalopathy [14]. However, the mechanisms involved in secondary brain injury are largely unknown and such knowledge is critical for development of future therapies for preterm infants or to be combined with hypothermia in severely asphyxiated infants at term, hopefully, to further reduce serious disability in children and adults.

3. Mitochondrial Functional Impairment

Mitochondria are small membrane-enclosed organelles, remarkably mobile and plastic, constantly changing their shape and undergoing fusion and fission [15]. Many factors can challenge mitochondrial balance and good functioning: DNA mutations, increase of intracellular calcium, reactive oxygen species, inflammation, decrease in trophic factors, and mitochondrial dysfunction plays a crucial role in brain injury [16]. Because of the heterogeneity of mitochondria existing in the brain, to understand variations in mitochondria functioning and consequent selective vulnerability to injury, the organelle must be placed within the context of its cellular, functional, developmental, and neuroanatomical environment [17, 18]. The location of mitochondria in the cell varies between cell types, but they are most often localized near sites of high ATP utilization as their major role is to produce and supply energy, ATP, to the cells through the enzyme complexes forming the respiratory chain. Mitochondrial function is critically important during development and throughout life in metabolic tasks like cellular proliferation, regulation of the cellular red-ox state, apoptosis, and excitotoxic injury.

Interest is growing in mitochondrial diseases or mitochondria-related injury where the respiratory chain/oxidative phosphorylation system starts to malfunction. Mitochondrial diseases are principally due to mutations in either nuclear or mitochondrial DNA, provoking impairment of transcription, translation and assembly of the enzyme complexes, leading to the malformation and/or malfunction of the mitochondria [19, 20]. Impairment of the respiratory chain is associated with ageing, neurodegenerative disorders [21], and mitochondrial diseases [19]. During ageing, inefficiency of the respiratory chain has been linked to the decreased activity of AMP-activated protein kinase (AMPK) leading to decreased mitochondrial biogenesis and function [22, 23]. In neurodegenerative disorders, like Parkinson's and ALS, an increase of oxidative stress is shown to be a crucial initiator affecting respiratory chains, leading ultimately to cell death [21, 24]. As well, recent discoveries of mutation associated with hereditary form of those diseases render the story even more complex [25].

Very little is known of what happens to the respiratory chain in injuries like stroke or during perinatal brain damage. After neonatal hypoxia-ischemia (HI), there is a significant energy failure in the brain, followed by a recovery period before a second energy failure [2, 26–29]. Those primary and secondary energy failures are associated with the primary and secondary injury [30]. Currently, most of the research on perinatal brain damage is focusing on the secondary insult leading to cell death and tissue injury [31]. However, what is happening during the primary energy failure, what is happening during the short recovery, and what mechanisms lead to the second energy failure and injury remain unknown.

4. The Role of AMPK in Mitochondrial Energy Crisis

Challenges to mitochondrial biogenesis and integrity are most likely to happen quite early in the cascade of events leading ultimately to injury. Before being involved in the apoptotic process after HI ([31–33] and see paragraph below) and considering the role of mitochondria as a major ATP supplier, it is most likely that mitochondria are involved from the first steps of the injury process after the insult. For instance, our group recently identified a peak of AMPK activity as early as 20 min after an HI insult in the brain of neonatal mice (Rousset et al., unpublished data). AMPK is well known as the energy sensor of the cell and is activated when there is an imbalance in the AMP : ATP ratio such as that which occurs in heat shock, anoxia, and so forth [34]. Once activated, AMPK will inhibit energy-consuming pathways (fatty acid/cholesterol synthesis) and promote energy-producing pathways (glycolysis, e.g., or through PGC-1α increasing mitochondria biogenesis, [35, 36]) in an attempt to restore energy balance which is critical to cell survival. AMPK is activated through two upstream kinases: LKB1 and CaMKKβ [37–41]. The latter is activated by a surge of intracellular calcium within the cell [40], which happens during excitotoxicity, a well-described feature of HI injury mechanism [42]. Furthermore, AMPK has recently been shown to mediate apoptosis through expression of the proapoptotic protein Bim after an excitotoxic challenge *in vitro* [43].

Hypothetically, as a first step, the calcium surge provoked by excitotoxicity and ROS signalling [44, 45] could not only activate CaMKKβ and then AMPK but could also simultaneously challenge the mitochondrial respiratory chain leading to an imbalance in the AMP/ATP ratio, reinforcing AMPK activation through the second upstream kinase LKB1. The activation of downstream pathways of AMPK to restore energy balance, could logically explain the return to basal level of ATP in the brain after the primary energy failure. Subsequently, events in the mechanistic cascade responsible for HI injury, like inflammation [32], could theoretically once again impede mitochondrial function, causing the

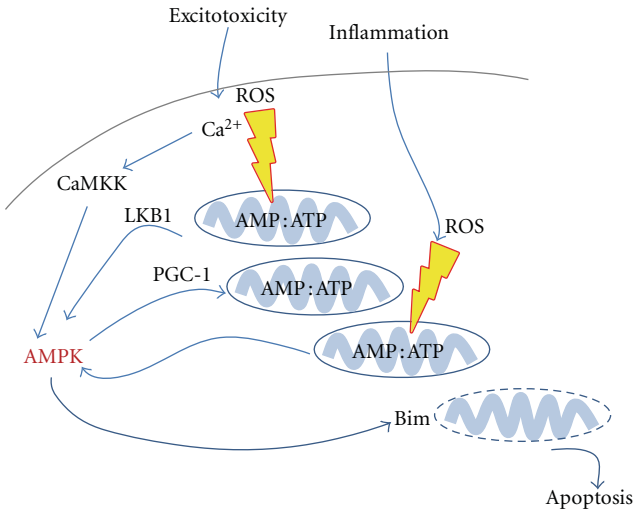

Figure 1: A potential role for AMPK in neonatal brain injury. AMPK is activated in response to stresses which change either intracellular calcium levels (e.g., excitotoxicity) or deplete intracellular ATP concentrations (e.g., inflammation, reactive oxygen species). Although AMPK works to return energy levels to baseline, prolonged activation results in upregulation of the proapoptotic protein, Bim.

secondary energy failure (Figure 1). This, cumulating with an overactivation of AMPK, which has been reported to exacerbate injury after stroke [46, 47], and still ongoing deleterious consequences from previous events, could provoke in the most vulnerable cells a final mitochondrial challenge, leading to its membrane permeabilisation and ultimately cell death through apoptotic pathways.

5. Mitochondrial Fusion and Fission

Mitochondria constantly fuse and divide, and the mechanisms governing this aspect of mitochondrial behaviour are currently the focus of many investigations. This property to fuse and divide appears to be crucial for a number of functions, the maintenance of organelle fidelity, mediating DNA or protein quality control, and, finally, it may be an important feature during apoptosis [48]. Mitochondrial fusion proteins attenuate apoptosis by inhibiting the release of proapoptotic agents like cytochrome c, while mitochondrial fission protein DRP-1 promotes apoptosis through Bax, leading to mitochondrial outer membrane permeabilization and cell death [49]. However, it is of note that fusion and fission have not yet been investigated in the immature brain, but this is surely something of great interest to push forward.

6. Intrinsic Pathway of Apoptosis and Secondary Brain Injury

Apoptosis (programmed cell death) is essential for the normal development of tissues and is especially key in neuronal development. The balance between cell survival and cell death is therefore required to be highly regulated; as such it is unsurprising that aberrant activation of apoptotic pathways occurs in a number of pathological conditions including stroke and a variety of neurodegenerative diseases [50].

Cellular apoptosis can be achieved through two routes, the extrinsic pathway (discussed later) activated in response to extracellular signals such as Fas and TNFα and mediated by death receptors [51] and the intrinsic pathway activated in response to DNA damage or cellular stress. Although each pathway has unique members, both mechanisms converge downstream at the level of the mitochondrion, where if the insult is severe enough, there is catastrophic permeabilisation from which the cell cannot recover. Mitochondrial permeabilisation results in the release of mitochondrial apoptogenic factors into the cytosol including apoptosis-inducing factor (AIF), endonuclease g (endo G) cytochrome c (cyt c), and Smac/Diablo. These proteins have a number of pro-apoptotic functions; cyt c interacts with Apaf-1 to form an active apoptosome, providing a platform for procaspase-9 cleavage; Smac/Diablo interacts with inhibitors of apoptosis (IAP) reducing their negative influence on the activity of caspases [50]. In contrast with cyt c and Smac/Diablo, AIF and endo G operate through a caspase-independent pathway. Both are translocated to the nucleus from the mitochondria in response to death—inducing stimuli where they induce fragmentation of nuclear DNA [52, 53].

7. The Role of Caspases in Neonatal Brain Injury

Caspases play a key role in apoptosis and inflammation. Caspases can be divided into three groups: initiator caspases (caspase-2, -8, -9, -10), effector caspases (caspase-3, -6, -7), and inflammatory caspases (caspase-1, -4, -5, -11, -12). Whereas effector caspases are activated by the initiator caspases, initiator caspases are activated by different, more complex mechanisms [54].

In the extrinsic pathway, binding ligands to death receptor leads to recruitment of adaptor protein, which recruits caspase-8, forming DISC (death-inducing signaling complex) leading to dimerization and activation of caspase-8. Caspase-8 then cleaves and activates effector caspases. In the intrinsic pathway, after cyt c is released from mitochondria into cytosol, it interacts with Apaf-1. This complex binds to procaspase-9 in the presence of dATP/ATP and forms the apoptosome which cleaves and activates initiator caspase, caspase-9 which, in turn, activates effector caspases (in particular, caspase-3) by cleaving between their large and small subunits [55]. Activated effector caspases cleave cellular substrates, such as PARP (poly(ADP-ribose) polymerase), lamin, fodrin, ROCK1 (Rho-associated kinase 1), and ICAD (inhibitor of CAD), leading to DNA fragmentation, cell shrinkage, and membrane blebbing [56–58]. Among the effector caspases, caspase-3 cleaves a broad range of substrates and the main effector caspase in the brain.

During brain development, a large number of neurons are eliminated by apoptosis to optimize neural networks. The activation of caspase-3 appears in the execution of neuronal apoptosis in the brain during development and after acute injury like HI. The extent of caspase-3 activation

following brain injury is greater in immature brain than adults [59, 60]. Caspases are important for apoptosis in developing brain. Nevertheless, there is the implication that caspase-independent death pathways may also influence nervous system development and may provide an alternative mechanism for regulating neuronal death.

The initial report characterising caspase-3-deficient mice showed defects of apoptosis in the nervous system; these mice die during embryonic development or in the perinatal period, in a manner similar to the phenotype of caspase-9 and Apaf1-deficient mice. Subsequently, it was reported that caspase-3 deficiency on C57/BL/6J background produced only minor neuropathological changes and caspase-3-deficient C57/BL/6J mice survived into adulthood [61]. Moreover, neonatal HI brain injury in caspase-3-deficient mice is worse compared with the previous model [62]. In rats subjected to neonatal HI, there is a peak of caspase-3 activity observed 24 h after the insult which remains elevated for a significant number of days [63]. These data suggest that the apoptotic pathway is likely to be strain dependent and caspase-independent death pathways may also influence nervous system development and may provide an alternative mechanism for regulating neuronal death. Recent studies have also revealed the nonapoptotic function of caspases. In particular, caspase-3 is suggested to function in neurogenesis and synaptic activity [64].

Caspase-6 is an effector caspase, and, in apoptotic pathways, lamin, a structural protein of nuclear envelope, is thought to be the only substrate cleaved exclusively by caspase-6. In other pathways, caspase-6 is also known to cleave cytoskeletal and structural proteins, such as the microtubule-associated protein tau and amyloid precursor protein (APP), and caspase-6 is detected in neurodegenerative diseases, such as Alzheimer's disease and Huntington's disease. Recently, Nikolaev and colleagues identified APP/death receptor-6 (DR6)/caspase-6 pathway as the mechanism specific for axonal pruning and degeneration by trophic factor withdrawal in developing neurons [65]. As a result, the involvement of caspase-6 in axonal degeneration has come under a high degree of scrutiny [66, 67]. Recently, it was demonstrated that *caspase-6* gene deficiency conferred protection in a mouse model of adult stroke with a reduction of axonal degeneration and improvement of functional outcome [66]. We have recently found that caspase-6 is activated (cleaved) also in neurites in the immature brain after HI (Miyakuni et al., personal communication), but its pathophysiological importance remains unknown.

8. A Role for Mitochondrial Permeabilisation in Secondary Brain Injury in Neonatal HI

Mitochondrial permeabilisation (MP) therefore represents the "point of no return" in the life cycle of the cell. Two forms of permeability have been identified. Mitochondrial outer membrane permeability (MOMP) is the result of Bcl-2 family members such as Bax relocating from the cytosol to the mitochondria. Once there, Bax interacts with another Bcl-2 family member Bak to form pores in the outer membrane enabling proteins located between the inner and outer membranes to leak into the cytosol [68]. In contrast, a permeability transition pore (PTP) is formed at points where both the inner and outer leaflets of the mitochondrion are at their closest points. In contrast with MOMP, the inner mitochondrial membrane is permeabilised resulting in leakage of solutes, depolarisation due to proton gradient equilibration, and generation of reaction oxygen species. ATP production ceases and the mitochondrion swells ultimately disrupting the outer membrane. PTP-mediated cell death is predominantly necrotic (through calcium imbalance and bioenergetic failure), although in extreme cases, if sufficient ATP is present, apoptosis can occur through activation of caspases [69]. Induction of the PTP is enhanced by cyclophilin D, a mitochondrial matrix protein which has previously been implicated in adult ischaemic injury [70]. However, our recent studies demonstrated that Bax-mediated MOMP rather than cyclophilin-D-mediated PTP is critical in mouse models of neonatal HI [71]. Indeed, previous work from our group and others suggests that, in neonatal brain, Bax-dependent mitochondrial outer membrane permeabilisation is implicated (Figure 2).

9. Involvement of Bax and Other Proapoptotic Bcl-2 Family Members in Neonatal HI

A study examining Bax-deficient mice found that these animals were protected in immature brain injury paradigms [72]. Furthermore, studies which ablate the effects of Bax-mediated mitochondrial membrane permeabilisation (e.g., knockout models of Bim and Bad [73], Tat-Bcl-xL-mediated neuroprotection [74], *Bcl-xL* transgenic mice [75]) all exhibit reduced brain injury after neonatal HI. Pharmacologically, intracerebroventricular injection of Bax inhibitory peptide prior to induction of HI in a neonatal mouse model conferred neuroprotection in both grey and white matters [76]. Finally, both caspase-dependent and AIF pathways are activated to a much greater extent in the immature brain compared with the adult brain [60]. Taken together, these data suggest that Bax-dependent mitochondrial permeabilisation is a critical event in delayed brain injury because it leads to both activation of caspase-dependent and caspase-independent cell death and mitochondrial functional impairment.

10. Upstream Regulators of Proapoptotic Bcl-2 Family Members

10.1. p53. It is a tumour suppressor that triggers apoptosis via multiple pathways including cell cycle arrest and the regulation of autophagy through transactivating proapoptotic and repressing antiapoptotic genes [77]. It is highly conserved and regulates cell death resulting from a wide variety of both physiological and pathological stimuli [78]. p53 also has transcription-independent, cytoplasmic actions at the mitochondrial level and can promote Bax-dependent mitochondrial permeabilisation [79]. In unstressed neurons, p53 expression is generally low, limited by its association with its negative regulator MDM2 which functions as a ubiquitin

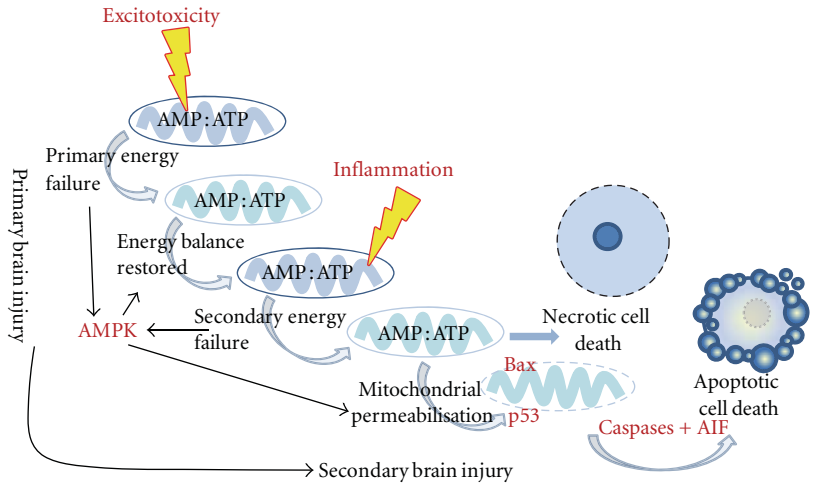

Figure 2: The development of secondary brain injury. Energy depletion culminating in Bax-dependent mitochondrial permeabilisation represents an irreversible commitment to cell death in neonatal brain injury.

ligase, targeting polyubiquitinated p53 for degradation [80]. Cellular stress displaces p53 from MDM2, and subsequently p53 expression is stabilised through substantial posttranslational modification [77]. The classical role for p53 is as an activator of transcription, and, on stabilisation, it accumulates in the nucleus where it upregulates the transcription of proapoptotic genes such as *PUMA, BAX,* and *Noxa* [81]. More recently a cytosolic, transcription-independent role was described in which activated p53 accumulates in the cytosol where it is sequestered by the antiapoptotic Bcl2 proteins for example, Bcl-xL [79]. However, increased PUMA expression mediated by nuclear p53 displaces Bcl-xL allowing p53 to activate Bax, promoting its oligomerisation, mitochondrial outer membrane permeabilisation, and inducing apoptosis [79, 82].

A previous study found that p53 was upregulated and accumulated in the nucleus and mitochondria in an *in vivo* rat model of neonatal HI. In consequence, there was an upregulation of apoptotic pathways leading to activation of caspase-3. The authors identified a pathway involving NFκB upstream of p53 and were able to decrease p53 accumulation (thus increasing neuronal survival), in response to neonatal HI by treating with the NFκB inhibitor NBD peptide [83, 84]. Subsequently, this has translated into improved long-term function in behavioural tests [85]. More recently, the same group confirmed the importance of p53 activation in neonatal HI by use of a small molecule inhibitor of p53, pifithrin-μ. Injection of this peptide into mice which have previously been subjected to an HI paradigm results in a high degree of protection in both white and grey matters which translates into long-lasting behavioural benefits compared with sham-injected animals [86]. As pifithrin-μ is widely believed to inhibit the mitochondrial but not nuclear functions of p53 [87], this strengthens the case for critical involvement of a p53-Bax pathway in neonatal HI.

10.2. C-Jun N-Terminal Kinases (JNKs). These are members of the mitogen-activated protein kinase (MAPK) family and, as such, are activated in response to stress. There are three mammalian *jnk* genes and 10 expressed isoforms as the result of alternative splicing; however, it is JNK3 that is predominantly active in the brain [88]. In a mouse model in which JNK3 expression is ablated (JNK3 KO), both adult and neonatal animals were partially protected against HI insult, and, in newborn animals, levels of c-jun were reduced compared with wild-type animals [89, 90]. This correlates with an earlier study suggesting that expression of *c-Jun* and its subsequent phosphorylation was increased on ischaemic injury [91]. JNK3 is hypothesised to act upstream of the proapoptotic Bcl-2 family as JNK3-mediated increases in Bim and PUMA expression were absent in the JNK3 KO animal [90]. In addition, activation of caspase-3 was also decreased suggesting that activation of JNK3 in response to hypoxic-ischaemic insult results in caspase-dependent apoptosis.

10.3. Caspase-2. It is a member of the initiator subgroup of caspases and is developmentally regulated [92]. Activation of caspase-2 is dependent on its dimerisation and subsequent cleavage which is facilitated through interaction with PIDD (p53-induced death domain-containing protein) and RAIDD (RIP-associated ICH-1/CED3 homologous protein with a death domain) [93–95]. Once activated, caspase-2 promotes Bid cleavage resulting in Bax translocation and release of cyt c [96]. In a very recent study, caspase-2 null newborn mice were found to be partially protected in both excitotoxic and HI paradigms [97] in contrast with the adult caspase knockout mouse model [98]. As the study also showed high expression of caspase-2 in neonatal mice and rats which decreased postnatally, it is probably unsurprising that there are age-dependent differences in caspase-2 function. Interestingly, a group II caspase inhibitor, TRP601, has recently been developed which targets caspase-2 and caspase-3 functions. Neonatal animals subjected to excitotoxicity, arterial stroke, or HI insult were significantly protected against white and grey matter loss [99].

11. Death Receptors and the Extrinsic Pathway of Apoptosis

During inflammation such as that which has been reported in perinatal brain injury [32], activation of mast cells [100] and microglia will produce reactive oxygen species, release excitatory amino acid agonists, proinflammatory cytokines (e.g., IL-1γ, IL-18, TNF-α), chemokines [101, 102], and tumour necrosis factors (e.g., TNF-α, TNF-β, FasL, TRAIL, TWEAK) [101, 103–105] that will contribute to cell death most often characterized by a mixed apoptotic-necrotic phenotype [59, 106].

From the time TNF was cloned and characterized in 1984 [107], roughly 20 ligand-receptor pairings are now included in the TNF superfamily. These TNF and TNF-receptor-like molecules are similar in structure to TNF and are functioning as trimers (both ligands and receptors). The receptors are largely membrane-bound signalling molecules with exception of some soluble decoy receptors (e.g., Osteoprotegerin). The ligands instead can be either membrane or soluble forms and both forms can have physiological activity. Because of the similarity of their structure, multiple ligands are able to bind and induce signalling through one receptor, or a single ligand is able to bind multiple receptors. Some of the receptors contain the so-called death domain in their intracellular domain (e.g., TNF-R1, DR4, DR5, Fas) and are able to trigger apoptosis when activated from the binding of the corresponding ligand (e.g., TNF- α, TRAIL, FasL). This extrinsic pathway of apoptosis continues with the activation of a death-inducing signalling complex (DISC) adjacent to the death domain of the receptor. Activated DISC catalyzes the proteolytic cleavage and transactivation of procaspase-8 [108]. Activated caspase-8 either directly activates caspase-3 or mediates cleavage of Bcl-2 interacting domain (Bid) to truncated Bid (tBid), which integrates different death pathways at the mitochondria ([109]; Figure 3). tBid translocates to mitochondria where it interacts with other proapoptotic proteins and triggers the release of apoptogenic factors like cyt c and apoptosis-inducing factor (AIF) from the mitochondria. Apoptosis then proceeds in the same way as for the intrinsic pathway with caspase-dependent and caspase-independent cell death.

12. Necroptotic Cell Death

Activation of death receptors in the presence of broad-spectrum caspase inhibitors induces a newly described cell death process called necroptosis. Necroptotic cell death initiated by TNF-α, Fas, or TRAIL is mediated by formation of a complex of two kinases, RIP1 and RIP3. This complex promotes mitochondrial reactive oxygen species (ROS) production and eventual collapse of cellular energy production [110].

13. Involvement of Death Receptors in Neonatal Brain Injury

TNF-α activity is mediated through activation of two receptors: low, affinity TNFR1 (p55) and the high-affinity TNFR2 (p75) [111], found on both neuronal [112, 113] and glial cell populations [114]. Although the extracellular domains of both receptors have a high degree of homology, their intracellular domains differ significantly [115]. This leads to complex signal transduction pathways that can be triggered and may result in activation of the antagonistic functions of these two receptors [111, 116]. When activated, the intracellular part of TNFR1 containing the death domain triggers apoptosis [117], whereas TNFR2 lacks that domain—its activation triggers neuroprotection through activation of NFκB [118]. There are several pieces of evidence that suggest the involvement of the TNF pathway in the development of white matter damage (WMD). Children who develop cerebral palsy show increased blood levels of TNF-α [119], and TNF receptor 1 is critical for LPS-mediated sensitization to oxygen glucose deprivation *in vitro* [120]. Moreover, deletion of the TNF gene cluster abolishes LPS-mediated sensitization of the neonatal brain to HI insult [121]. TNF-α treatment appears to be toxic for the oligodendroprecursor (OPC) cell [122] and potentiates the IFN-γ toxicity on those cells *in vitro* [123]. TNF-α has also been shown to stimulate astrocyte [124] and microglial [114] activation and proliferation. TNF-α-mediated cell destruction may be mediated directly, via activation of its TNFR and subsequent cell death signalling pathways, or indirectly by enhancing glutamate excitotoxicity [125]. TNF is also implicated in brain neuroprotection. It is shown that neuronal damage by focal cerebral ischemia and excitotoxic insults are enhanced in TNFR KO mice [126]. The neuroprotective role for TNF in cerebral ischemia is mainly attributed to TNFR2 activity [127].

FasL is able to bind with Fas death receptor triggering apoptosis and with Decoy receptor 3 (DcR3) [128]. Fas death receptor is one of the most extensively studied of this group of receptors. Lack of functional Fas receptor is neuroprotective in adult models of HI [129, 130]. HI also activates Fas death receptor signalling in the neonatal brain especially in areas where apoptosis is a prominent feature [131–133]. Although the Fas/FasL system is primarily linked to apoptosis, Fas activation can also induce caspase-independent cell death [134], initiate cell necrosis [135], or induce proliferation and differentiation signals [136]. It is shown that Fas expression in primary OPC is higher than in mature oligodendrocytes [123], implying higher susceptibility to FasL at earlier developmental stages. Fas expression can be upregulated in OPCs exposed to an inflammatory stimulus [123] which may imply that in an inflammatory environment these cells would have increased vulnerability to Fas-induced apoptosis.

In humans, four membrane-bound and one soluble receptor for TRAIL have been identified. Of these, two contain cytoplasmic death domain (DR4 and DR5) and have the capacity to induce apoptotic cell death [137, 138], whereas DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) lack functional death domains and thus are considered to act as decoy receptors [139, 140]. Osteoprotegerin (OPG) is a secreted TNF receptor family member that besides receptor activator of nuclear factor kappa-B ligand (RANKL) can bind TRAIL as well [141, 142]. In mice, two membrane decoy receptors mDcTRAILR1 and mDcTRAILR2 have been reported [143],

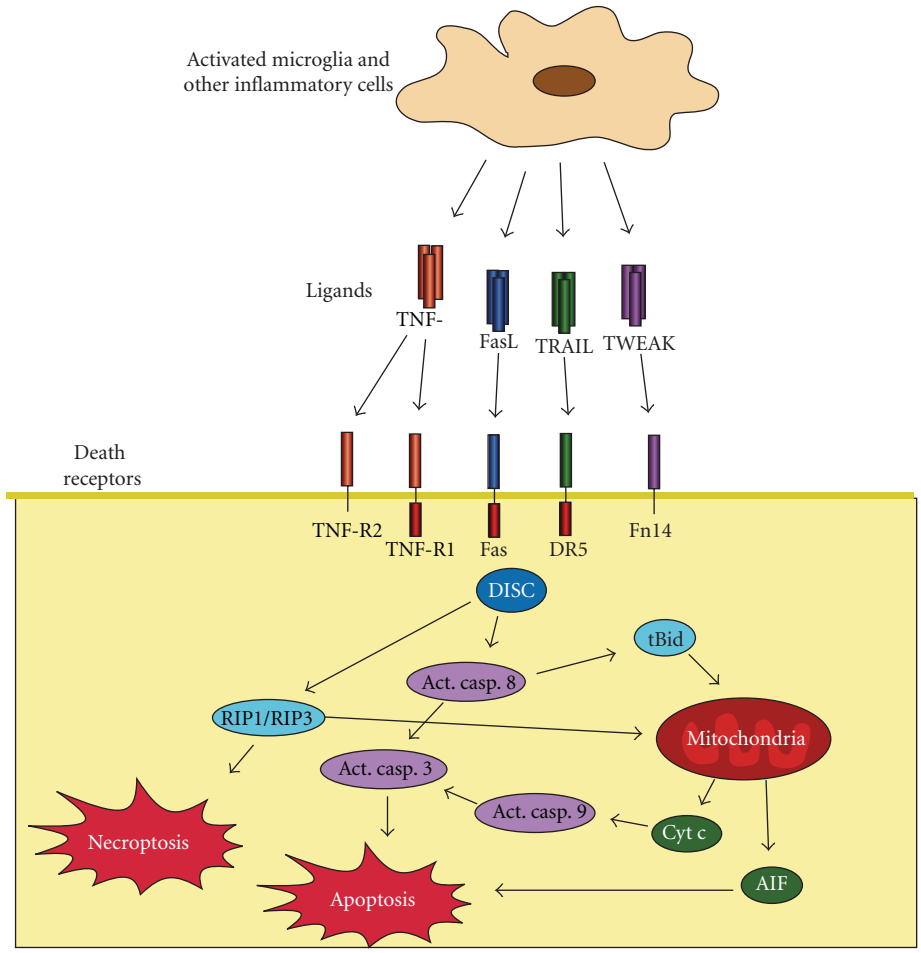

Figure 3: The extrinsic pathway of apoptosis. Inflammatory cells secrete death receptor ligands which bind to receptors on neurons, oligodendroglial and other receptor-expressing cells, recruiting the death-inducing signalling complex (DISC) and triggering both apoptotic and necroptotic pathways.

one soluble OPG [142], and only one death-mediating TRAIL receptor which has the highest homology with the human TRAIL receptor DR5 [144].

Only one receptor for TWEAK has been identified so far in both humans and rodents, fibroblast growth factor-inducible 14 (Fn14) [145]. Binding of TWEAK to this receptor can trigger proliferation, differentiation, migration, and cell death [146]. The Fn14 cytoplasmic tail does not contain a canonical death domain, and TWEAK binding to Fn14 can induce multiple cell death pathways in different cellular contexts [147, 148].

Although many studies have been conducted in the cancer- or inflammation-related systems, the role of TRAIL and TWEAK in the development of WMD after HI is still unclear. The studies that implicate TRAIL and TWEAK signalling in the pathogenesis of ischemic cerebral damage are performed in adult models of stroke or multiple sclerosis and concern mainly neurons [105, 148–150]. To date very few studies relate these pathways to OPC death [61]. However, intracerebroventricular injection of soluble DR5 receptor [150] or Fn14 [105] is able to reduce significantly the infarct volume after HI in adult rodent models, strongly implicating TRAIL and TWEAK signalling in neuronal cell death after HI.

14. Tertiary Brain Injury

Tertiary brain injury will be defined as that occurring following the commonly defined events of primary and secondary cell death. As outlined previously, perinatal brain injury is predominantly caused by inflammation/infection and hypoxic-ischemic events that cause metabolic dysfunction and cell death. Even after secondary cell death has subsided, effects on the brain persist including sensitisation to inflammation or injury, increased seizure susceptibility, impaired oligodendrocyte maturation and myelination, and persistent inflammation and gliosis [151–156]. More speculatively, perinatal inflammation is suggested to play a critical role in the pathogenesis of autism and schizophrenia [157–159].

When considering treatments for tertiary brain injuries, we could distinguish between strategies aiming at extending the window of therapeutic intervention from the acute phase to the subacute phase and strategies targeting more

long-term events such as chronic inflammation or postlesional plasticity.

15. Extending the Window

One key issue for protecting the perinatal brain is the available window for intervention in the processes leading to cell death. From a clinical point of view, the longer this window, better the chance to implement viable interventions. For example, hypothermia has to be initiated within the first 6 hours of life to be protective in term infants with neonatal encephalopathy [160]. Such a short window does not allow applying this treatment to all neonates who might benefit from it. As a strategy to enhance the efficacy of hypothermia, some groups have been trying to extend the window of intervention of hypothermia by giving first an antiepileptic drug prior to delayed hypothermia. Using the classical Rice-Vannucci P7 rat model, Liu and colleagues have shown that a combination of low-dose topiramate administered 15 minutes after the HI insult and 3-hour hypothermia initiated 3 hours after the insult was neuroprotective while topiramate alone or hypothermia alone had no significant effect [161]. More recently, the same group showed that early administration of Phenobarbital also enhanced the efficacy of delayed hypothermia [162]. It remains to be seen if drugs used successfully in parallel with hypothermia, such as melatonin and xenon, might also be able to extend the therapeutic window of this treatment [163, 164].

An alternative strategy would be to use early but short-term hypothermia to enhance the window of opportunity for a protective drug. This strategy could allow reducing the duration of hypothermia. Accordingly, it was shown that fructose-1,6-biphosphate (FBP) was neuroprotective against neonatal excitotoxic cortical damage [165]. However, the drug had to be given within the first 8 hours to be neuroprotective. Interestingly, a moderate but transient (4 hours) cooling immediately after the insult extended the therapeutic window for FBP, as FBP administered 24 h after the excitotoxic insult was still significantly neuroprotective in these pups.

16. Targeting the Long-Lasting Inflammation

A recent and intriguing study performed in preterm infants with cerebral palsy [155] suggests that, at least in some patients with perinatal brain damage, there could be a long-lasting inflammation as measured by increased TNF-α levels in the plasma and the supernatants of peripheral blood mononuclear cells after lipopolysaccharide stimulation. This long-lasting altered inflammatory response could have deleterious effects on the progression of disease and/or on the clinical symptoms. If such a pathophysiological event was confirmed, recognizing and blocking such a persistent inflammation could be of therapeutic value.

Additional studies are necessary to confirm these new hypotheses and to determine whether or not there is a long-lasting CNS inflammatory process. Techniques such as PET with markers of microglia or MRI using ferromagnetic particles taken up by activated microglia could be instrumental in this perspective. Indeed, a study using this approach has revealed that for many years after traumatic brain injury in human adults microglia remain activated [166]. Although these studies have not yet been reproduced in children/young adults following perinatal injury, a similar activation might be ongoing and therefore a target for reducing tertiary phase injury.

17. Targeting Epigenetic Marks

The term epigenetics refers to the enzymatic (e.g., acetylation, methylation) and nonenzymatic mechanisms (microRNA) by which gene expression/cell phenotype is modified without altering the sequence of genomic DNA. Inflammation, growth restriction, and maternal stress are known to alter the epigenome [167–170], and although in the perinatal period these effects alone may not lead to classic brain injury, they may cause long-lasting cognitive, motor, and/or behavioural impairments [151, 167, 171].

The underlying mechanisms by which modifying the epigenome could have lasting effects includes myelin deficit linked to blockade of oligodendrocyte maturation, impaired neuronal migration, increased neuronal cell death, impaired axonal growth, or altered synaptogenesis [172–175]. Of particular interest, microRNAs with suggested roles in regeneration and repair are upregulated from 3 days after MCAO [176], and microRNAs are capable of enhancing the beneficial microglial M2 phenotype [177]. If microRNAs do indeed represent an endogenous repair and immunomodulatory mechanism, they may be a novel strategy to treat brain injury in the tertiary phase.

Drugs specifically targeting acetylation have shown great efficacy in treating acute-phase adult cerebral injuries (see, [178]), and evidence is mounting to suggest efficacy in neonatal models ([179]; Fleiss and Mallard, unpublished). We do not yet know if modulating the epigenome after the secondary phase will have any efficacy after inflammation or HI. However, adult changes in behaviour stemming from perinatal maternal stress and linked to increased methylation can be abolished in adulthood by increasing acetylation [180]. This raises hope for the future design of innovative treatments that could be implemented way beyond the perinatal insult.

18. Promoting Positive Post-Lesional Brain Regeneration with M2 Microglia

Activated microglia have been shown to be detrimental for the production of hippocampal neurons, but microglia and macrophages can also be beneficial and support neurogenesis, progenitor proliferation, survival, migration, and differentiation in other brain regions. Recent studies suggest that the phenotypic expression of macrophages can vary depending on the situation and pro-inflammatory macrophages (M1) can undergo transition into an anti-inflammatory-reparative (M2) phenotype. More recently, three activation states of microglia in CNS have been proposed: classical acti-

vation (tissue defence, pro-inflammatory), alternative activation (repair, anti-inflammatory, fibrosis, extracellular matrix reconstruction), and acquired deactivation (immunosuppression, phagocytosis of apoptotic cells [181, 182]).

Strategies aiming at activating microglia when it has reached the M2 phase could be beneficial for facilitating repair and plasticity. Of note, the early phases of microglial activation (M1 type of activation) have typically been described as deleterious for the brain. More recently, preventing early microglial activation has been shown to be detrimental in focal ischaemia [183, 184]. This suggests caution in timing of any intervention to modify microglial activity.

Alternatively, or in parallel, strategies aiming at accelerating the M1-M2 switch could also be of major interest. At this point, it is not known if modulation of the activation state of microglia/macrophages can be used for development of novel therapeutic strategies in the developing brain, but a recent report suggests that M2 (alternative activation/acquired deactivation) macrophage cell therapy indeed can provide protective effects in an animal model of multiple sclerosis [185].

19. Promoting Positive Post-Lesional Brain Regeneration with Exogenous Stem Cells

The development of an adequate protocol for stem cell culturing and application has envisaged the use of these cells for the reparation of perinatal cerebral lesions. Some studies have shown a positive effect of neural or mesenchymal stem cell therapy on the lesion extent and/or cognitive or motor outcome following perinatal brain lesions [7, 186]. Interestingly, in some of these studies, positive effects were observed when stem cells were injected several days (up to 10 days) after the insult. Furthermore, in an adult MCAO model, stem cells given even 30 d post-insult improved neurobehavioural scoring assessed 50 d later suggesting efficacy may be possible even in the tertiary phase of perinatal brain injury [187].

The therapeutic potential of neural stem cells in acute neonatal brain injuries has been evaluated in a rodent excitotoxic model [186]. Early (4-hour) and late (72-hour) neural stem cells implantation significantly reduced brain lesion size in this neonatal model. The implanted cells, modified *in vitro* prior to transplantation toward the oligodendrocytic lineage, were capable of migrating toward the lesion site even when implanted contralaterally to the lesion. At the lesion site, the neural stem cells underwent transient differentiation into neurons and oligodendrocytes but not astrocytes, suggesting that fate specification was achieved by the culture conditions. Pre-implantation cell fate determination may offer some ability to specifically target white matter injury, such as predominates in the injured immature brain [188–191]. In parallel with the reduction in lesion size, the injured mice displayed a persistent and marked improvement in temporal and spatial memory at 3 and 6 weeks of age compared to littermates given intracerebroventricular injections of saline or fibroblasts.

Similarly, it was recently shown that two administrations of bone marrow-derived mesenchymal stem cells to neonatal mice 3 and 10 days after unilateral right carotid artery occlusion on P9 produced a 46% improvement in sensorimotor function as observed in the cylinder rearing test and a 60% decrease in neuronal loss, compared with vehicle-treated animals [7]. Moreover, cellular proliferation and differentiation of the proliferated cells into cells expressing neuronal, oligodendroglial and astrocyte markers was observed. Interestingly, remodeling of the corticospinal tract correlated with sensorimotor improvement.

It is not clear yet whether the stem cells themselves or factors secreted by stem cells mediate the positive effect. Increased neurotrophin production with eventual loss of injected cells is linked to improvements [186], while in some studies cells become functionally integrated [192]. The ethical problem associated with the use of human stem cells is less evident in mesenchymal stem cells or stem cells derived from cord blood. Such cells permit an autologous transplant and do not entail the problem of immune tolerance of the transplanted cells. A clinical study is currently being performed using stem cells in children with neonatal encephalopathy at the Duke University [193].

A further intriguing alternative to treatment with stem cells is to stimulate the production of endogenous neuronal stem cells. It has already been shown that stem cells accumulate in the subventricular zone following an acute brain lesion. These results open a new perspective: the stimulation of this stem cell population to support the physiological reparation processes of a lesion. A variant of this strategy would be to redirect new cell production from astroglia to oligodendrocytes and neurons [194]. Critically, stem cell therapies and stimulating endogenous proliferation bears the theoretical risk of cancer induction [193].

20. Promoting Positive Post-Lesional Brain Regeneration with Pharmacological Agents

Fostering positive post-lesional plasticity appears a very promising strategy for delayed interventions aiming at improving long-term neurological and cognitive function. However, there is still limited knowledge about the cellular and molecular mechanisms underlying post-lesional brain plasticity.

Different growth factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), erythropoietin (EPO), or vasoactive intestinal peptide (VIP), have been shown to reduce delayed neuronal death in various animal models of perinatal brain damage [195–199]. As for hypothermia, the window for intervention, when tested, was rather restricted to the first hours after the insult. However, beyond their potential capability to prevent neuronal cell death, growth factors appear as good candidates to target mechanisms involved in plasticity such as proliferation of neuronal precursors, axonal growth and sprouting, or synaptogenesis and synaptic stabilization.

Accordingly, BDNF and VIP have been shown to promote axonal sprouting following excitotoxic injury of the periventricular white matter in newborn mice [198, 199]. Although growth factors like BDNF are big molecules unlikely to cross easily through the intact blood-brain barrier,

ampakines, allosteric positive modulators of glutamatergic AMPA receptors, are small and diffusible molecules able to induce BDNF production in the brain when administered systemically. Interestingly, ampakines have been shown to mimic BDNF effects on axonal sprouting in the mouse model of excitotoxic white matter injury [200].

Similarly, melatonin was shown to promote plasticity using the same model of neonatal excitotoxic white matter damage [42]. Although melatonin did not prevent the initial appearance of white matter damage, it promoted repair of secondary lesion with axonal regrowth and/or sprouting. Recent data have shown that the window for intervention is at least 24 hours after the insult (Gressens P, personal communication). Behavioural studies support the hypothesis that melatonin-induced white matter histological repair is accompanied by improved learning capabilities. Neuroprotective properties of melatonin have been confirmed in several animal models of perinatal brain damage, including fetal sheep [201]. Melatonin is a safe compound, including newborns [202], and it crosses the blood-brain barrier as well as the placenta. Based on these data, a clinical trial testing the neuroprotective effects of melatonin has been initiated in preterm infants at high risk of developing brain damage and neurological handicap [203].

Although this study needs to be replicated, an intriguing clinical study has recently shown that EPO, when given on an average of 24 hours after birth, had very significant neuroprotective effects in human term infants with neonatal encephalopathy [204]. Evidently, the precise mechanism for this neuroprotection is unknown, but the timing of intervention argues on favour of an effect of EPO on post-lesional plasticity although a direct effect on delayed neuronal cell death cannot be excluded.

Authors Contribution

C. Thornton and C. I. Rousset contributed equally to this work. P. Gressens and H. Hagberg shared senior authorship.

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Review Article

Infection-Induced Vulnerability of Perinatal Brain Injury

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A growing body of evidence demonstrates that susceptibility and progression of both acute and chronic central nervous system disease in the newborn is closely associated with an innate immune response that can manifest from either direct infection and/or infection-triggered damage. A common feature of many of these diseases is the systemic exposure of the neonate to bacterial infections that elicit brain inflammation. In recent years, the importance of innate immune receptors in newborn brain injury, the so-called Toll-like receptors, has been demonstrated. In this paper we will discuss how neonatal sepsis, with particular emphasis on *Escherichia coli*, coagulase-negative staphylococci, and group B streptococcal infections in preterm infants, and Toll-like receptor-mediated inflammation can increase the vulnerability of the newborn brain to injury.

1. Introduction

Perinatal brain injury represents a significant clinical problem [1]. A growing body of evidence demonstrates that susceptibility and progression of both acute and chronic central nervous system (CNS) disease is closely associated with an innate immune response that can manifest from either direct infection and/or infection-triggered damage [2]. A common feature of these diseases is the systemic activation of inflammatory mediators, which via the blood can disrupt the blood-brain barrier, affect the circumventricular organs in the brain (which lack a blood-brain barrier), or interact with the brain endothelium, thereby eliciting brain inflammation [3]. Furthermore, the presence of activated inflammatory cells derived from systemic circulation or from dormant brain resident populations is a key feature of many CNS diseases. More recently, the importance of innate immune receptors in CNS injury, the so-called Toll-like receptors (TLRs), has also been emphasized. In this paper we will focus on how neonatal sepsis and TLR-mediated inflammation increase the vulnerability of the newborn brain.

2. Neonatal Sepsis and Brain Injury

Infants with sepsis have an increased incidence of cerebral palsy [4] and white matter abnormalities [5–11]. In a large study of 6093 extremely low birth weight (<1000 g) infants, those who were infected (including early-onset sepsis, suspected sepsis (culture negative), and had necrotizing enterocolitis (NEC)) were more likely to have cerebral palsy than children who did not have a neonatal infection [12]. In another recent large sample-size study involving 1155 infants born at 23 to 27 weeks gestation, it was found that children who had both late bacteremia (positive blood culture result after the first postnatal week) and surgical NEC were at increased risk of diparetic cerebral palsy compared with children who had neither [13]. Moreover, by comparing outcomes of 150 infants with periventricular leukomalacia (PVL) with controls matched for gestational age, it was found that infants with bacterial sepsis were twice as likely to develop PVL, and those with meningitis were almost four times as likely to develop white matter disease [14]. Similar findings were noted in a smaller case-control study, where

associations between cerebral palsy, clinical chorioamnionitis and sepsis were demonstrated [15]. Moreover, there was an increased incidence of Gram-negative bacterial and fungal infections in a very low birth weight population, and these infants were at significantly increased risk for moderate to severe cerebral palsy and neurodevelopmental impairment at 18 months of age [16].

2.1. Bacterial Pathogens in Neonatal Sepsis. *Escherichia coli* is one of the main pathogens causing early-onset infections in preterm neonates, accounting for up to 40% of the cases of bacteremia among very low birth weight preterm infants (<1,500 g) [17]. Cerebral white matter injury has been found by MRI following *Escherichia coli* meningitis in human newborn infants [18]. Furthermore, *Escherichia coli* induce brain damage in a number of antenatal rabbit and rodent models [19–26]. Also, in a recent study, white matter injury was demonstrated in an animal model of neonatal *Escherichia coli* sepsis in 5-day-old rat pups [27]. Experimental studies show that early-life *Escherichia coli* exposure can also have long-term effects, influencing the vulnerability to other factors in adulthood, for example, age-related cognitive decline [28] as well as attenuated glial and cytokine responses to amphetamine challenge [29].

In recent years, coagulase-negative staphylococci (CONS) have emerged as the most prevalent and important neonatal pathogens, responsible for approximately 50% of all episodes of late-onset neonatal sepsis in neonatal intensive care units around the world [30–33]. CONS cause significant morbidity, mortality, and healthcare costs worldwide in preterm newborns, especially in very low birth weight infants [34–38]. The vulnerability of preterm infants to CONS infection has been suggested to be due to the special characteristics of the premature infant's innate immunity [39]. Although there is no direct evidence of CONS causing perinatal brain injury, the presence of CONS in the chorioamnion space at delivery is associated with increased risk for the development of cerebral palsy in preterm infants [40, 41]. Further, in children with an established diagnosis of cerebral palsy, who are admitted to pediatric intensive care, there is a high rate of carriage of abnormal bacteria, including CONS [42].

In very low birth weight preterm infants with early onset neonatal sepsis, the rate of group B streptococcal (GBS) infections is relatively low in comparison with *E. coli* infections [17]. There is no direct evidence of GBS sepsis playing a role in cerebral palsy; however, nearly half of all infants who survive an episode of GBS meningitis suffer from long-term neurodevelopmental sequelae [43]. Further, extensive cortical neuronal injury was found in GBS-infected neonatal rats, which was mediated through reactive oxygen intermediates [44, 45].

3. Toll-Like Receptor-Mediated Vulnerability of the Immature Brain

3.1. Toll-Like Receptors. Toll-like receptors (TLRs) play a central role in primary recognition of infectious and viral pathogens. The presence of all 13 known TLRs has been demonstrated in the brain [46–48]. TLR4 mediates cellular activation in response to LPS derived from *Escherichia coli* [49], while CONS [39] and GBS infections [50] are, at least partly, believed to be mediated by TLR2. Interestingly, the role of TLRs in nonbacterial-induced brain injury has also recently been highlighted [51]. TLRs signal through the recruitment of intracellular adaptor proteins, followed by activation of protein kinases and transcription factors that induce the production of inflammatory mediators (Figure 1). The adaptor protein MyD88 is used by most TLRs, except TLR3, while the TRIF adaptor protein is used only by TLR3 and TLR4. LPS-induced activation of TLR4 elicits, via both MyD88 and TRIF, a broad inflammatory response in tissues, including the immature brain [52].

3.2. TLR Expression during Brain Development. There is relatively little information regarding the expression of TLRs in the developing brain. During embryonic life, protein expression of both TLR-3 and -8 has been identified [53, 54], while TLR-2 expression is relatively low before birth and increases during the first two weeks of life [55]. We have shown that mRNA for TLR1-9 is expressed in the neonatal mouse brain [56]. It appears that some of the TLRs may play important roles during normal brain development, as TLR2 inhibits neural progenitor cell proliferation during the embryonic period, and TLR3 deficiency increases proliferation of neural progenitor cells, while TLR8 stimulation inhibits neurite outgrowth [53–55]. In support, TLR2 and TLR4 have been shown to regulate hippocampal neurogenesis in the adult brain [57].

3.3. LPS-Induced Brain Injury. We, and others, have shown that systemic administration of LPS results in brain injury in both fetal and newborn animals [58–60]. These injuries appear, both histologically and by MRI analysis, to be very similar to those found in preterm infants [61]. Furthermore, it is now well established that pre-exposure to LPS can increase the vulnerability of the immature brain to hypoxia-ischemia (HI), in both rats [62, 63] and mice [64]. These effects are TLR4 [65] and MyD88 dependent [64, 66]. In a recent study, it was also shown that a very low dose of LPS, specifically increased the vulnerability of the immature white matter [67]. Low-dose LPS (0.05 mg/kg) sensitized HI injury in P2 rat pups by selectively reducing myelin basic protein expression and the number of oligodendrocytes while increasing neuroinflammation and blood-brain barrier damage in the white matter. The neuroinflammatory responses to LPS/HI appears to be age dependent [68]. Rat pups subjected to LPS/HI at P1 responded with weak cytokine response, while there was a prominent upregulation of cytokines in P12 pups subjected to the same insult. Interestingly, IL-1β was upregulated at both ages; IL-1β injections sensitize the newborn brain to excitotoxicity [69] and repeated IL-1β exposure during the neonatal period induces preterm like brain injury in mice [70].

Although it has clearly been demonstrated that LPS can increase the vulnerability to HI, under certain circumstances LPS can also induce tolerance to brain injury. We have

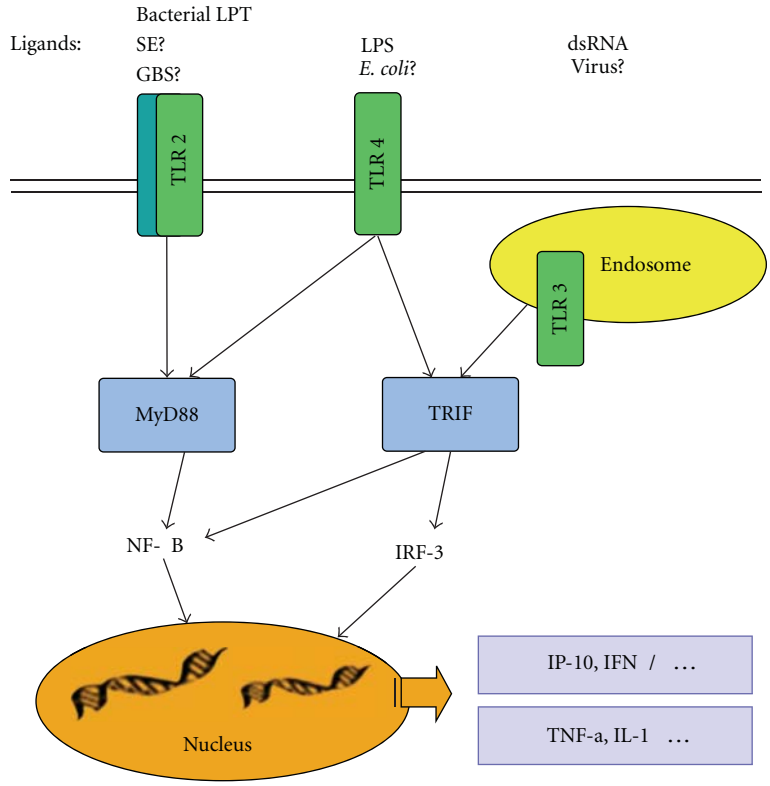

Figure 1: Diagram outlining infectious agents, TLRs, and major signaling pathways. Abbreviations: SE: S. epidermidis; GBS: group B streptococcus; LPT: lipopeptides. LPS: lipopolysaccharide; MyD88: myeloid differentiation primary response gene (88); TRIF: TIR domain-containing adaptor inducing interferon-β-mediated transcription factor; NF-κB: nuclear factor-KappaB; IRF: interferon regulatory factor; IP-10: interferon gamma-induced protein 10; IFN: interferon; TNF: tumor necrosis factor; IL-1: Interleukin -1.

shown that the time interval between LPS exposure and the subsequent HI is imperative to the outcome [71, 72], where a 24 h interval seems to induce a tolerant state that makes the brain less vulnerable. This has been confirmed by others who have implicated several possible mechanisms, including upregulation of corticosterone [73], which is further supported by the fact that administration of dexamethasone prevents learning impairment following LPS/HI in neonatal rats [74]. Furthermore, Akt-mediated eNOS upregulation in neurons and vascular endothelial cells have been implicated in LPS-induced preconditioning [75].

The importance of the time interval between LPS and other insults seems to be a generalized phenomenon. We have recently demonstrated in an in vitro model that conditioned medium from LPS-activated microglia affects the antioxidant Nrf2 system and cell survival in astrocytes in a time-dependent manner. LPS-induced inflammation had dual, time-dependent, effects on the Nrf2 system in that sustained activation (72 h) of GSK3beta and p38 downregulated the Nrf2 system, possibly via the activation of histone deacetylases, changes that were not observed with a 24 h (tolerance) interval [76, 77]. These studies support our previous report demonstrating that reductions in antioxidants were more pronounced when HI was preceded by LPS injection in 8-day rats 3 days prior to the HI insult [78].

3.4. Other TLRs in Perinatal Brain Injury. Compared to TLR4, much less is known about other TLRs in perinatal brain injury. As mentioned above, TLR2, TLR3, and TLR8 can affect normal brain development [53–55]. Activation of TLR2 in neonatal mice decreases volume of cerebral gray matter, white matter in the forebrain, and cerebellar molecular layer [79]. Further, we have recently demonstrated the expression of both TLR1 and TLR2 in the neonatal mouse brain following HI. In these studies, TLR2 deficiency resulted in reduced infarct volume after HI, while TLR-1-deficient mice were not protected [56].

Maternal viral immune activation is believed to increase the risk of psychiatric disorders such as schizophrenia in offspring, and in order to examine this relationship, several authors have investigated the vulnerability of the fetal brain to synthetic double-stranded RNA, polyriboinosinic-polyribocytidilic acid (poly I:C), a TLR3 agonist. Maternal injection with poly I:C towards the end of gestation ($\geq$G15) causes sensorimotor gating deficits in the adult offspring in mice [80] and increased sensitivity to the locomotor-stimulating effects of MK-801 [81]. The effects of Poly I:C appear to be gestational age dependent [82]. Maternal Poly I:C injection on GD9, but not GD17, significantly impaired sensorimotor gating and reduced prefrontal dopamine D1 receptors in adulthood, whereas prenatal immune activation

in late gestation impaired working memory, potentiated the locomotor reaction to a NMDA-receptor antagonist, and reduced hippocampal NMDA-receptor subunit 1 expression. In particular, Poly I:C injections early during rodent pregnancy affect structural brain development, such as a transient decrease of myelin basic protein in the neonatal offspring [83] and cerebellar pathology [84].

4. Conclusion

E. coli infections are common in preterm neonates, and considerable evidence suggests that *E. coli*-induced inflammation play a role in the development of white matter damage in preterm infants. There is much less data available concerning the importance of two other common neonatal pathogens, CONS and GBS, in perinatal brain injury. Furthermore, it is becoming clear that TLRs have important roles during development and may be involved in both pathogen-induced damage as well as so called "sterile" HI-induced inflammation. In order to better understand the underlying causes of perinatal brain injury, the interaction between common neonatal pathogens and TLRs in the newborn brain deserves further investigation.

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