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Pathophysiology and neuroprotection of global and focal perinatal brain injury: lessons from animal models

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Abstract

BACKGROUND—Arterial ischemic stroke occurs most frequently in term newborns than in the elderly, and brain immaturity affects mechanisms of ischemic injury and recovery. The susceptibility to injury of the brain was assumed to be lower in the perinatal period as compared to childhood. This concept was recently challenged by clinical studies showing marked motor disabilities after stroke in neonates, with the severity of motor and cortical sensory deficits similar in both perinatal and childhood ischemic stroke. The understanding of the triggers and the pathophysiological mechanisms of perinatal stroke has greatly improved in recent years, but many aspects remain still unclear.

METHODS—In this review, we will focus on the pathophysiology of perinatal stroke and on therapeutic strategies that can protect the immature brain from the consequences of stroke by targeting inflammation and brain microenvironment.

RESULTS—Studies in neonatal rodent models of cerebral ischemia have shown a potential role for soluble inflammatory molecules as important modulators of injury and recovery. A great effort has been made and is still in act to try neuroprotective molecules based on the new physiopatological acquisition.

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All authors disclose any potential conflict of interest

CONCLUSION—In this review we aim to give a comprehensive view of new insights concerning pathophysiological mechanism of focal and global perinatal brain injury and its new therapeutic approaches.

Keywords

stroke; newborn; neuroprotection; brain repair; inflammation

INTRODUCTION

Perinatal stroke is defined as "a group of heterogeneous conditions in which there is focal disruption of cerebral blood flow secondary to arterial or cerebral venous thrombosis or embolization, between 20 weeks of fetal life through the 28th postnatal day, confirmed by neuroimaging or neuropathologic studies" [1]. The term *"focal"* underlines the difference between this pathology and the more common neonatal hypoxic-ischemic encephalopathy (HIE), where injury is more often bilateral and may preferentially affect white or grey matter structures depending on regional and cell-type specific vulnerability at the time of the insult [2].

Perinatal arterial ischemic stroke (PAIS, Table 1) has an incidence of 1 in 2,300 to 5,000 births [3-7]. It is a subset of perinatal ischemic stroke and it is associated with mortality and significant long-term neurologic morbidity. As opposed to white matter injuries, that affect typically preterm infants, PAIS occurs more frequently in term neonates. The clinical presentation of PAIS depends on the age at diagnosis: in newborns the main symptom is seizures, but also lethargy, hypotonia, poor feeding, irritability or apnea [8]. Children who suffer PAIS typically develop long-term disabilities including motor deficits, epilepsy, cognitive and behavior disorders, deficits in language and vision. PAIS and HIE may share common risk factors and mechanisms, and can furthermore coexist in the same baby[9]. Most reported risk factors were derived from descriptive epidemiologic studies; thus, their causal relationship to perinatal stroke can only be assumed[10].

In this review, we will discuss data from rodent models of PAIS and hypoxic-ischemic (HI) injury to emphasize some of the common mechanisms of ischemic brain injury in the neonatal brain. We will describe potential therapeutic strategies considering that the sequence of key events in brain maturation is largely consistent between humans and rodents [11].

THE INTEREST OF RODENT MODELS

An elegant work [11] carefully marked the differences between rodents and humans in particular when comparing the maturational age of the CNS during normal and disrupted development. There is considerable cross-species alignment in terms of key developmental milestones, behavioural phenotypes and regional vulnerability to brain injury. It is now accepted that the maturation state of the brain, and in particular, specific processes of synaptogenesis and myelinisation, rather than chronological age, is the critical determinant of outcome after brain injury. Thus, comparisons can be made taking into consideration the timing of indices of neurobiological development, to gauge the impact of specific insults at

different developmental stages, and best model the process of interest to the investigator. For a more detailed explanation of how the postnatal days in the animal model correlate with humans please refer to Table 2. Developmentally related differences are of importance not only to our understanding of the healthy brain during maturation, but also to predict differential responses to injury and potential therapeutics.

In the literature there are sufficient evidences for selection of an age-appropriate rodent model that is predicated on biochemical and neuroanatomical changes during early postnatal development, as well as the emergence of age-specific behaviors. Ongoing in parallel research on cerebral development in both humans and rodents will provide a greater understanding of how all these factors interact, and how is the appropriate therapy for different injury and ages.

Another important aspect to be mentioned is the choice of the best model of hypoxia-ischemia. An ideal animal model, in addition to mimic the developmental stage of a human being, should also be reproducible and give the smallest variability. An important factor that influences variability in lesions obtained, and in related mortality and histopathological changes, is the cerebral blood flow supply. Across a wide range of species, two carotids and two vertebral arteries supply blood flow to the brain. However, the relative contribution of these large conductance vessels is highly variable. The cerebral circulation attempts to maintain constant cerebral perfusion despite changes in systemic conditions, due to its ability to autoregulate the blood flow. Occlusion of one carotid artery in rodents inconsistently results in brain ischemia unless combined with systemic hypotension. The combination of these insults in the P7 rat are needed to create a lesion. This is the principle used by the most important rodent models of systemic HI injury, which started with the Vannucci model in 1981 and that was further modified with the use of different ages and animal strands (Table 3). In contrast, a single permanent Middle Cerebral Artery (MCA) occlusion in the mouse appears sufficient to create an ischemic lesion. Heterogeneity within species observed in cerebral lesion size could be partly explained by collateral recruitment through the circle of Willis and/or through the cortical anastomoses between the vascular beds of the three terminal cerebral arteries. The lesion created by a single permanent artery occlusion is more similar to the lesion that we can have in PAIS, and for this reason is more and more used by scientists to investigate the effects of pure ischemia without hypoxia and the process of reperfusion injury [12].

TIMING OF ONSET OF INJURY

Patterns of brain injury depend on the gestational age at which they occur [13]: injury to the periventricular white matter in the preterm infant leads to permanent alterations in cerebral myelination, suggesting that oligodendrocytes are a target of injury. Oligodendrocytes progress through a phenotypic lineage comprised of successive stages of replication-capable progenitors that culminates with the postmitotic mature myelinating oligodendrocyte. Mature oligodendrocytes are relatively resistant to injury. During the window of vulnerability for periventricular white matter injury (gestational week, GW 23-32), before the onset of myelination, the subcortical white matter is populated predominantly by oligodendrocyte progenitors. Specifically, late oligodendrocyte progenitors

(preoligodendrocytes) represent 90% of the total cells of the oligodendrocyte lineage present. Using a rodent model, it has been demonstrated that preoligodendrocytes manifest stage-specific vulnerability to one of the common insults occurring in early development, hypoxia-ischemia [14]. Taken together this data suggests that injury specifically to preoligodendrocytes accounts for subsequent myelination defects after periventricular white matter injury in the preterm infant. Two mechanisms have been proposed for the selective vulnerability of oligodendrocyte progenitors: oxidative stress and excitotoxicity. Preoligodendrocyte sensitivity to oxidative stress has been demonstrated in vitro by glutathione depletion and exposure to exogenous free radicals, and exogenous antioxidants protect preoligodendrocytes from glutathione depletion [15,16]. Mature oligodendrocytes, in comparison, are highly resistant to oxidative stress, in part because of differences in expression levels of antioxidant enzymes and proteins involved in programmed cell death [17]. Cognitive and sensory impairments are associated with periventricular white matter injury and are observed with increased frequency with decreasing gestation [18]. Cortical visual impairment is particularly common in infants with severe injury [19]. These observations suggest widespread abnormalities of cortical development after periventricular white matter injury, especially of the posterior visual pathways. It was determined that subplate neurons, cells that play a critical role in normal visual thalamocortical development [20], are selectively vulnerable to neonatal hypoxia-ischemia in the postnatal day 2 (P2) rodent model [21]. This rodent model is particularly relevant to the human preterm infant given the similarities in brain development [22], oligodendrocyte lineage progression [23], and the propensity to subcortical injury with HI insult [21] (Table 2). Subplate neurons are located beneath the developing neocortex [24], near areas of white matter signal abnormality observed on magnetic resonance imaging in preterm human infants [25] at risk for the diffuse type of periventricular white matter injury. Subplate neurons are a transient cell population that undergoes programmed cell death in the first postnatal week in mice [26]. In humans, the subplate zone peaks at the onset of the developmental window of vulnerability to periventricular white matter injury (GW 24), undergoes dissolution during the third trimester, and is largely absent after 6 months of postnatal age [27]. At its peak of development in human, the subplate zone is four times the width of the cortical plate [27]. Subplate neurons are involved in the formation of area-specific thalamocortical connections [26], and early subplate neuron ablation in cat prevents visual thalamo- cortical innervation [28]. However, periventricular white matter injury in human occurs after geniculocortical innervations [29]. Subplate neurons become incorporated into mature synaptic networks in the developing neocortex [30], and later subplate neuron ablation is linked to a disruption in the functional maturation of visual cortical columns, including ocular dominance [31] and orientation selectivity [32]. Late subplate neuron ablation leads to impaired synaptic transmission of visually driven activity from the lateral geniculate nucleus into cortical circuits [32], findings consistent with the cortical visual impairment observed in preterm humans with periventricular white matter injury [19]. In the rodent model, as in human, selective subplate neuron cell death after early hypoxia- ischemia occurs after the development of the geniculocortical projection, so that visual thalamocortical innervations is not disrupted. Rodents do not have anatomically segregated eye-specific thalamic input to visual cortex, thus the effects of subplate neuron cell death on patterned visual cortical innervation cannot be assessed in this model. However, the development of the patterned

somatosensory whisker representation is not affected [21]. The mechanism of selective vulnerability of subplate neurons to early hypoxia-ischemia is not known. Because neurons undergo programmed cell death during normal development to a much greater extent than other cortical neurons [33], there may be an enhanced susceptibility to programmed cell death after appropriate triggers,a finding observed in certain immature cortical neurons [34]. In addition, subplate neurons are known to mature earlier than other cortical neurons as assessed by the expression of a variety of markers [35], including a developmentally related increase in the NMDA-R1 glutamate receptor expression [36] and AMPA and kainite receptor [37].

Term human infants demonstrate a predilection for injury to thalamus and basal ganglia after hypoxia-ischemia in the neonatal rodent (P7), there are at least biphasic stages of neurodegeneration: early (1.5 to 3 hours) in forebrain and late (6 days) in striatum and thalamus [38]. Recent anatomic studies demonstrate injury evolution and ongoing cell death through 168 hours [39] in rat cortex and striatum, as well as in mouse hippocampus [40]. The nature of this cell death can be complex, and has recently been termed the “apoptotic-necrotic continuum “ [41]. However, late cell death in the thalamus is programmed cell death and is selective for sensory thalamic nuclei (lateral geniculate and ventral basal) [42] well established that programmed cell death plays a prominent role in normal development [43], and this may account for an enhanced susceptibility to programmed cell death after injury in different regions of the developing brain. A role for programmed thalamic cell death in neonatal hypoxia-ischemia is supported by the observation of Fas death receptor expression, cytochrome c release, and cleavage of procaspase 8 [44]. Fas, a member of the tumor necrosis family of receptors, plays a central role in the programmed elimination of lymphocytes in the immune system, and upregulation of Fas ligand (Fas-L) has increasingly been recognized in neuronal cell death resulting from trophic factor deprivation, injury, or stress [45]. Similar delayed programmed cell death in the thalamus is observed after mechanical injury to the developing visual cortex [46], suggesting that the cell death results from target deprivation. HIE in the term human infant is associated with selective injury to the deep gray nuclei, especially the basal ganglia. Within the basal ganglia, neuronal nitric oxide synthase (nNOS) expressing striatal neurons represent an example of selectively targeted cells, and these neurons are mechanistically involved in the selective vulnerability of nearby striatal projection neurons that do not express nNOS. nNOS-containing interneurons throughout the central nervous system produce nitric oxide (NO) dependent on the coupling and activation of the NMDA receptor and calcium entry [47]. The enzyme is maximally expressed in regions where the immature NMDA-R is expressed, especially the basal ganglia [48]. When NO is produced in excessive amounts during periods of oxidative stress in these regions of abundance, nitric oxide is converted to peroxynitrite, a potent mediator of free radical injury [49]. nNOS-expressing neurons are resistant to both hypoxia-ischemia an NMDA-mediated excitotoxicity [50,51], but this selective sparing of nNOS-expressing striatal neurons to NMDA agonists is limited to young ages (P7 in rodent) and as the brain matures this resistance is lost [51]. On the other hand, nNOS-expressing neurons are vulnerable to AMPA agonists [52]. Thus the selective vulnerability of striatal projection neurons to neonatal hypoxia-ischemia may result from a bystander effect attributable to their proximity to this enriched population of nNOS-expressing neurons. This hypothesis is

supported by the selective ablation of nNOS-expressing neurons with AMPA agonists before hypoxia-ischemia; a manipulation resulting in reduced injury from HI insult[53]. The susceptibility to injury of the immature brain was assumed to be lower in the perinatal period as compared to childhood [54,55]. This concept was recently challenged by clinical studies showing marked motor disabilities after stroke in neonates [54,56], with the severity of motor and sensory deficits similar in both perinatal and childhood ischemic stroke. Since PAIS results mostly from occlusion of the middle cerebral artery (MCA), the functions dependent on the brain regions supplied by this vessel (e.g. motor cortex) are more frequently affected [57-59]. Furthermore, in preterm infants, the structural immaturity of blood vessels, which appear thin walled, may predispose to bleeding [60]. Previously some studies have shown that the immature brain has a large potential to compensate for perinatal injury to the motor system [61, 62], but recently this concept has questioned by several authors[63,64]

PATHOPHYSIOLOGICAL MECHANISMS OF PERINATAL ISCHEMIC BRAIN INJURY

Cellular injury and neuronal cell death

Excitotoxicity, free radical formation and activation of the inflammatory cascades are the main mechanisms of ischemic brain injury at term (Figure 1). Each of these injury components elicits injury independently and they act in concert to aggravate injury.

Failure of ATP dependent calcium pumps results in increased intracellular calcium concentration, which is directly toxic to the mitochondria and activates several DNAses, proteases and lipases [65]. Failure of intracellular metabolism, particularly ATP depletion, results in neuronal depolarization and release of glutamate, which activates post-synaptic NMDA receptors, and other glutamate receptors, allowing for a greater influx of calcium and further cellular injury [65-68]. Higher levels of glutamate receptor expression [69], a different composition of individual NMDA receptor subunits [70,71], and intrinsic differences in the GABAergic system, which is immature and excitatory during early postnatal brain development, also contribute to excitotoxic injury [69,72]. Nitric oxide synthase (NOS), superoxide dismutase (SOD) and NADPH oxidase activated by ischemia form potent reactive oxygen species (ROS) [65,67,68].

In rat, ionotropic glutamate receptors undergo rapid maturational changes: NMDAR density peaks late in the first postnatal week in many forebrain structures, including hippocampus and the neocortex [73], whereas AMPAR density peaks in the second postnatal week at around P10 [73]. Both NMDA receptors and AMPA receptors overshoot adult expression levels, resulting in heightened glutamate-mediated plasticity [74,75]. Maturational regulation of glutamate receptor subunit composition also enhances their ability to mediate activity-dependent synaptic plasticity in early postnatal life. The maturational regulation of AMPA receptor composition and function also enhances glutamate-mediated plasticity in early postnatal life. The ratio of GluR2 expression to that of other AMPA receptor subunits is significantly lower in immature neocortex and hippocampus compared to the adult [76,77], and AMPA receptors that lack a GluR2 subunit exhibit higher Ca2+ permeability than those that contain GluR2 [78-81]. The increased Ca2+ influx through AMPA receptors in immature

neurons may trigger mechanisms of plasticity that would not be triggered in the mature brain. In addition, the presence of such receptors may provide potential mechanisms for excitotoxicity. Glutamate receptors are also developmentally regulated on non-neuronal cells. In particular, olygodendrocytes express functional glutamate receptors in vitro, and these are exclusively of the non-NMDA subtype [82-84]. Glutamate has been shown to be toxic to oligodendroglia in vitro by receptor-independent [16,85-87], and receptor-mediated mechanisms [88-91].

Reperfusion and associated reoxygenation of the ischemic brain tissue following focal ischemic stroke causes a second wave of ROS formation that occurs in activated peripheral leukocytes and in multiple cell types in injured brain regions [65,67,68]. Inflammation associated with ROS triggers production and release of various toxic mediators, including cytokines, amplifying injury. As a result, necrotic neuronal death begins occurring almost immediately in the “core” of the ischemic region whereas neuronal apoptosis and several intermediate cell death states that exhibit features of both necrosis and apoptosis, occur within hours to days following ischemia, mostly affecting cells in the penumbral regions [92,93].

Zhou et al [94] showed that pyramidal neurons engaged in cortico-cortical connectivity in limbic cortex are vulnerable to denervation lesions. At least one trigger of this transsynaptic degenerative phenomenon is the activation of inhibitory interneurons in layer I, which are induced to upregulate neuronal nitric oxide synthase (nNOS) and release NO.

The improvement of transsynaptic degeneration with AMPA antagonist confirm that transsynaptic apoptosis and anoxic/ischemic neuronal necrosis are both glutamatergic events that involve the synthesis and release of NO [95]. Although the apoptotic or necrotic outcome is related to levels of superoxide anion generated with NO release (i.e., apoptosis involves a lesser degree of free radical burden [96]), the two mechanism have distinct neuropathology. In its classical formulation, anoxic-ischemic injury is a necrotic phenomenon that involves an early activation of NMDA receptors and the downstream induction of nNOS and intracellular toxic release of NO. In contrast, transsynaptic cell death is an apoptotic phenomenon that appears to involve the interaction of two neurons, one of which upregulates nNOS in response to AMPA signaling and releases NO to the toxic effects of which it is resistant because of a concomitant induction of the manganese isoform of SOD, a known superoxide anion scavenger in vivo [95,97,98].

On the other hand, the success of therapy with AMPA receptor antagonist in HIE white matter injury in P7 rat does not exclude that the transsynaptic mechanism may be involved in HI injury, too.

Both caspase -dependent [99,100] and –independent [100,101] pathways contribute to neuronal death after brain injury. The abundance of caspases in the neonatal brain, in particular caspase-3, may in part account for a more marked caspase-3-dependent cell death observed after neonatal brain injury than after similar injury in the adult. Neuronal death and injury are preferentially reduced following pharmacological inhibition or genetic deletion of PARP-1 in male pups [102], but following caspase-3 inhibition in female pups [100]. Hagberg’s

work suggests that the degree of PAR accumulation during early (1–4 h) post-HI reperfusion was similar in females and males, whereas the drop in NAD+ was only found in males and in consequence the degree of mitochondrial impairment may depend on gender. PARP-1-dependent neuronal injury in vitro has also been shown to rely on translocation of apoptosis inducing factor (AIF) from mitochondria to the nucleus[103]; furthermore, the genes for AIF, as well as for several other proteins involved in perinatal hypoxia- ischemia that may be related to PARP-1 (e.g.: X-linked inhibitor of apoptosis), are localized on the X-chromosome and may, in addition to NAD+, be differentially expressed in males and females. On the other hand, Renolleau et al. showed that concentration of cytochrome c release did not differ between males and females but it appeared as a sharp peak at 12 h post-ischaemia in males and then rapidly decreased at 24 h, whereas a regular increase from 0 h up to 16 h followed by a slight decrease at 24 h was observed in females. This might represent a mitochondrial dysfunction in males, but not in females. Recent data demonstrated that male neurons displayed a more pronounced translocation of apoptosis-inducing factor (AIF) and female neurons a stronger activation and cleavage of caspase 3 [104,105]. Accordingly, it is also interesting to note a gender-specific neuroprotection by iminobiotin, an inhibitor of nitric oxide synthases, via the cytochrome c and caspase 3 after HI insult in female P7 rats[104], suggesting that the intrinsic apoptotic pathway might predominate in females. Sex differences have also been reported for hypothermia, which provides more effective long-term neuroprotection in female than in male 7-day-old rats [106].

It also to be considered the importance of calcium in ischemic cell death, calcium antagonists came into the focus of ischemic neuroprotection [107]. Magnesium prevents cellular calcium influx and excitatory aminoacid release in neurons [108] by blockade of N-type and L-type calcium channels [109], prevents cellular calcium entry through NMDA-receptor channels [110], reduces calcium-induced mitochondrial dysfunction [111] and preserves cellular energy metabolism [112]. By these mechanisms, magnesium may inhibit or delay ischemic cell death during and after cerebral ischemic events [113]. However, despite promising results in animal models, magnesium was not found to reduce excitotoxicity following adult stroke. A multicenter phase III trial was conducted to determine the effect of magnesium therapy on outcome in aneurysmal subarachnoid hemorrhage. A total of 1,204 patients were administered intravenous magnesium sulfate (64 mmol/day) or placebo and evaluated for outcome on the modified Rankin Scale for up to 90 days. No improvement in outcome was seen with magnesium treatment versus controls. In addition, a retrospective analysis of 2,047 patients from previous trials was also performed and similarly concluded that magnesium has no benefit in the treatment of aneurysmal subarachnoid hemorrhage[114,115].

Blood-brain barrier (BBB) disruption and contribution of peripheral immune cells

BBB breakdown is a key injury factor in stroke. Both the structure and function of the BBB are influenced by a number of relatively independent pathophysiological processes, including oxidative/nitrosative endothelial cell damage, cytokine-dependent activation of the endothelium, altered phosphorylation and cellular localization of endothelial tight junction proteins, and degradation of individual BBB components by activated proteases [116-120]. Transmigration of peripheral immune cells and retraction of astrocyte endfeet further affect

functional integrity of the BBB [121]. Paradoxically, functional integrity of the BBB is better preserved after acute neonatal stroke than after adult stroke in the rat[122]. Compared to acute adult stroke, the expression of several tight junction proteins (occludin, ZO-1 and claudin-5) is better preserved after acute neonatal stroke, which would support the integrity of tight junctions in injured neonates [122]. However, it is unknown if phosphorylation or subcellular distribution of the tight junction proteins following stroke differs between the two ages. The higher resistance of the BBB to stroke in neonates is likely related to the maturation-dependent leukocyte-vascular interaction after injury. Compared to acute adult stroke, infiltration of neutrophils and monocytes is low after neonatal HI injury and focal stroke despite markedly increased leukocyte chemoattractant levels in injured neonatal brain [122,123].

The active pathophysiological role of the systemic inflammation was demonstrated in experimental stroke in the adult [124]. In newborns with ischemic brain injury, several clinical studies showed an increase in cytokine and chemokine serum levels [125]. Hu et al [126] showed a hypoxia/ischemia-induced alteration of cortical development by proteome analysis of the cortex 2h after HI. Of the altered proteins, 14-3-3ε and TUC-2, both playing an important role in the development of the CNS, decrease after HI events, consistent with an early disturbance of cortical development. These observations suggested that changes in peripheral levels of individual inflammatory molecules may potentially serve as indicators of both cerebral damage and prognosis.

Glial cells and neuroinflammation

Endogenous brain macrophages—microglial cells—are the main cell type that provides immuno-surveillance in the brain, but these cells have been traditionally considered deleterious after stroke due to production of cytokines, chemokines, ROS, induction of proteases in response to injury and ultimate activation of cell death mechanisms and degradation of BBB components. However, microglial cells are the source of growth factors, and several known mediators produced in microglia/macrophages, such as MMPs, can exert dual effects: they can harm during the initial injury stages but enhance neural repair through remodeling of the extracellular matrix and the assembly of a neurogenic niche during the recovery phase [127].

The microglia/macrophage population is heterogeneous. Microglia is capable of acquiring diverse phenotype in response to injury: M1 (classically activated microglia with cytotoxic properties in response to infections), M2a (alternate activation and involvement in repair and regeneration), M2b (immunoregolatory phenotype), M2c (acquired-deactivating phenotype). Functions are known to overlap and be context dependent[128,129]. It was considered that cytotoxic phenotype develops due to insult/injury and the same cell shift to an M2-repair/regenerative phenotype over time[130] and depends on the combination of specific signals received from the local microenvironment [131]. However the precence of an early M2 phenotype (preceding M1) has recently been reported in a after hypoxic/ischemic insult in vivo[132]. Furthermore, brain macrophages that originate from invading monocytes may affect injury progression differently than activated microglia. It remains still unknown if the

phenotypical composition of the microglia/macrophage population is the same during early postnatal development and adulthood.

Interestingly, selective depletion of microglia (by intracranial administration of liposomes containing clodronate) in neonatal rats does not improve, but worsens brain injury during the sub-acute phase after MCAO [133]. A larger infarct size in these animals is associated with additional accumulation of several pro-inflammatory cytokines and chemokines, including TNFα, MCP-1 and MIP-1a [133] and expression of these mediators, which are typically produced by microglia, is overcompensated by production in other cells (astrocytes, neurons and endothelial cells) when microglia are absent [133]. These data indicate that at least a subpopulation of microglial cells can exert beneficial effects in injured neonatal brain, likely by acting as a "buffering" component in the brain inflammatory response.

Astrocytes actively participate in several pathophysiological stroke mechanisms, including excitotoxicity through regulation of the extracellular glutamate accumulation, oedema formation through increased aquaporin-4 expression, inflammation through cytokine production, and changes in BBB stability due to retraction of astrocyte endfeet from the vascular surface [134,135]. Compared to astrocyte functions in adult stroke, relatively little is known about the role of these cells in injury in the neonate. The finding that expression of several cytokines and chemokines in astrocytes is increased after focal ischemia in neonatal rats with pre-depleted microglia [133] suggests that astrocytes modulate the inflammatory response early after injury. However, genetic deletion of GAFP and attenuated reactive gliosis in the developing brain does not affect infarct volume after sub-chronic HI insult [136].

Receptor-mediated and intracellular signalling pathways

Death receptors—Death receptors, such as TNF-α receptor 1 (TNFR1), Fas, and TNF-related apoptosis inducing ligand (TRAIL) receptors, respond to inflammatory cytokines and initiate the extrinsic apoptotic pathway through the oligomerization of the adaptor molecule Fas-associated protein with death domain (FADD) with the initiator caspase, caspase-8 [137]. Activated caspase-8 then cleaves and activates effector caspases. Upregulation of Fas receptor after neonatal HI injury is concomitant with increased cleavage of Casp-8, -9 and -3 and neuronal apoptosis [42,138]. Genetic deletion of Fas protects [138], in part via increased expression of its counteracting protein c-FLIP [139].

Toll-like receptors (TLRs) and scavenger receptor CD36—TLRs are a receptor superfamiliy that mediate innate immune responses and activation of microglia in response to changed local microenvironment[140]. TLR intracellular signalling is complex and involves the activation of NF-kB and the subsequent expression of different gene sets [140]. TLR2 and TLR4 are the two most studied members of the TLR superfamily in the context of brain ischemia. While TLR4 seems to be purely injurious, TLR2 may elicit context- and tissue-dependent effects—injurious [141,142] or beneficial [143]—based on the types of heterodimers that it forms with other TLRs. Administration of selective TLR2 and TLR4 agonists to neonatal rats impairs normal postnatal myelination and induces hippocampal neuronal death even without stroke [144], indicating that activation of innate immunity is sufficient to interfere with normal postnatal development of the white matter. However, the role of TLR4

on progression of neonatal HI injury is unclear, since deletion of its intracellular effector protein, Myd88, does not result in improve neuroprotection [145]. At the same time, TLR2 deletion does reduce injury [146], suggesting that the particular contribution of each TLR subtype to injury differs between adults and neonates.

The scavenger class B receptor CD36 can signal independently as well as in cooperation with TLRs. Genetic deletion of CD36 protects against acute injury after MCAO in the adult [147,148] but worsens injury in the neonate [149]. While the exact mechanisms of such striking age-dependant differences are largely unknown, the pattern of changes for CD36 and its downstream effectors may be important injury modifiers. Genetic deletion of CD36 is associated with diminished engulfment and phagocytosis of apoptotic neurons that are abundant in injured neonatal brain and results in additional accumulation of cleaved caspase-3 [149]. CD36 toxicity depends on superoxide produced through NADPH oxidase (Nox) activation, but the relative contribution of individual Nox isoforms [150] and superoxide utilization [151] in injured neonates differs from that after focal stroke in adults. A markedly increased superoxide accumulation in microglia/macrophages in acutely injured adult brain [148], but not in neonatal brain [149], may activate distinct CD36-dependent intracellular signalling pathways.

Nuclear factor kappa B (NF-kB)—NF-kB is a ubiquitously expressed transcription factor that regulates expression of genes involved in inflammation, cell survival and apoptosis and plays a major role in regulation of neuronal death and injury in the ischemic brain [152]. Activation of several signalling pathways after neonatal brain injury converges in the activation of NF-kB. Upon stimulation, dissociation of NF-kB from its endogenous inhibitor protein IkB allows NF-kB to translocate from the cytoplasm to the nucleus and induce an array of genes in a cell-type and stimulus-specific way. NF-kB activation leads to the production of inflammatory mediators, but also induces the expression of pro-survival factors including antioxidants, growth factors and antiapoptotic molecules [153,154]. This dual role of NF-kB may underlie the time-dependent and sometimes opposite effects of NF-kB inhibition observed after neonatal HI insult. NF-kB inhibition during the early phase of activation (0-3 h) after HI injury limits neuroinflammation, prevents up-regulation and accumulation of NF-kB in the nucleus, reduces caspase-3 activation, and leads to marked long-term functional neuroprotection [155] and improved cognitive outcome [156]. In contrast, inhibition over a more extended time period after HI events abolishes the protective effect on neuronal apoptosis and exacerbates injury [155]. These opposing results suggest that the time window of NF-kB inhibition needs to be considered carefully in order to translate these results to the clinic.

Individual mediators of injury

Reactive oxygen species (ROS)—Production of superoxide, hydrogen peroxide and other ROS is a part of normal brain function [157,158] but excessive accumulation of these species and the subsequent formation of hydroxyl radicals, lipid peroxides and peroxynitrate contribute to ischemic injury by altering cellular components and intracellular signaling, and by disrupting BBB integrity [159,160]. The neonatal brain is particularly susceptible to ROS accumulation due to a limited activity of endogenous antioxidants and anti-oxidative

enzymes [161,162]. Consistently, HI injury is diminished in transgenic mice over-expressing glutathione peroxidase-1 (GPx) [163,164], or by antioxidants [165,166]. However, in contrast to a functional neuroprotection in adult stroke, overexpression of SOD-1 exacerbates injury following neonatal HI insult [151]. These opposite effects are due to a further increase in H_2O_2 in injured neonatal brain because of insufficient catalase and GPx activity and the consequent metabolism of accumulated H_2O_2 produced via SOD-1 activation.

NO has dual effects on ischemic injury: it mediates vasodilation and neuroprotection when produced by endothelial NOS (eNOS), but is a major mediator of oxidative/nitrosative damage when produced by neuronal (nNOS) and inducible NOS (iNOS) [159,167]. Genetic deletion and pharmacological inhibition of iNOS and nNOS reduce injury associated with HI insult [159,168-172]. NO produced by iNOS also forms toxic peroxynitrate and increases activity of inducible cyclooxygenase (COX-2) [173,174], further propagating neonatal brain injury.

Cytokines

Increased cytokine production has been traditionally related to inflammatory states and injury progression following brain ischemia in adults. Several cytokines have been shown to modulate injury after neonatal HI events and focal stroke, as shown in Table 4. Expression of IL-1β is rapidly and locally upregulated after HI injury and transient MCAO in neonatal rodents [175,176]. Increased CSF levels of IL-1β have been identified as a marker of severe injury during the first 24 hours after asphyxia in babies [177,178], but the effects of this cytokine in injured neonatal brain are complex. While IL-1β contributes to injury after neonatal HI insult [179], genetic deletion of IL-1α or IL-1β, alone or in combination (IL-1αβ knockout), does not protect one week after HI events [180], whereas administration of IL-1 receptor antagonist (IL-1Ra) protects [175,181]. Furthermore, the data that brain levels of IL-1β remain elevated when pharmacological neuroprotective effects are achieved [182,183], suggests that a decrease in IL-1β levels, induced by injury, is not necessarily required for protecting brain from focal/global injury. TNF-α and IL-6 mRNA and protein expression are also up-regulated after neonatal HI injury [175,184]. TNF-α induces biological activity via stimulation of the tumor necrosis factor receptor (TNFR) [185,186].

Beneficial effects of therapeutic agents against HI damge are often associated with reduced brain levels of TNF-α, but the outcomes of inhibition of this mediator and the relative contribution of signaling via TNFR1 and TNFR2 remain largely undefined. TNF-α damages oligodendrocyte progenitors *in vitro* [187,188], but genetic deletion of TNF-α leads to impaired differentiation of myelinating oligodendrocytes during the postnatal period [189]. Recently R-7050, a novel cell-permeable triazoloquinoxaline, has been shown to attenuate neurovascular injury and improve neurobehavior sequels,by selectively inhibiting TNF-α induced cellular signaling [190].

Chemokines

Several studies have suggested an important role for chemokines in cerebral damage in models of ischemic stroke, HI and excitotoxic injury [191]. In P7 rats, secretion of a macrophage inflammatory protein-1α and MIP-1β mediates inflammatory cell recruitment

and activation after HI insult [192]. Another chemokine, the macrophage chemo-attractant protein (MCP)-1/CCL2, is a potent mediator of ischemic cerebral injury in adults [193,194] and neonates [195], likely by attracting circulating monocytes. MCP-1 also mediates excitotoxic injury in the neonatal rat brain [196], and the genetic [197] or pharmacological [198] inhibition of MCP-1-dependent pathways reduces cerebral damage, supporting a role for this chemokine in the early phases of the ischemic insult.

The CXC chemokine CINC-1/KC has been recently shown to paradoxically contribute to both BBB integrity and reduction of brain injury after MCAO in neonatal rats [122]. Changing the balance between the CINC-1 levels in plasma and in the brain increases transmigration of neutrophils, disrupts vascular function and extends injury [122].

CXCL12 (SDF-1), a chemokine that is upregulated in astrocytes and brain vessels in the peri-infarct area following adult stroke, has been shown to mediate the recruitment of circulating leukocytes into the brain [199,200]. In the neonatal brain, reduced activation of the CXCL12 receptor, CXCR4, by dexamethasone, leads to decreased lesion size following HI injury [201]. Importantly, the sustained induction of SDF-1 after stroke in adult mice but not in neonatal mice [202] may indicate a shorter temporal window for SDF-1 -mediated repair in the neonatal brain.

MEDIATORS OF CELL SURVIVAL AND RECOVERY

Growth factors and neurotrophins

The pharmacological and histologial neuroprotective effects of Brain Derived Neurtrophic Factor have been firmly established in adult experimental stroke [203-205] and at least several underlying mechanisms have been identified, including activation of pro-survival mechanisms [206], modulation of local inflammation [207], and reduction of excitotoxic injury [204,208]. In neonates, it protects against HI injury via ERK activation and blockade of caspase-3 activation [209,210]. BDNF is known to promote endothelial cell survival and mediate neurogenesis in ischemic tissue in the adult [211] and is likely to contribute angiogenesis in injured neonates.

Angiogenesis is essential for long-term repair, but recent findings show that in contrast to a relatively rapid induction of angiogenesis in the adult brain after MCAO, within days, in the neonate, increase in angiogenesis does not seem to occur for at least 10-14 days after MCAO [212]. Experimental studies from the adult brain suggest that the “early” up-regulation (between 1 h and 3 h post-MCAO) of VEGF could be associated with alterations in BBB permeability and contribute to exacerbating injury [213]. In both neonatal and adult models following hypoxia, intracerebroventricularly injection of VEGF 48h after Ischemia resulted in reduced brain injury, decreased infarct volume and decreased apoptotic cells without increasing BBB permeability. Both studies suggest this is related to the activation of the Akt/ERK pathway [214, 215]. Inhibition of VEGFR-2 has also been shown to decrease endothelial cell proliferation, increase cell death and worsen injury following neonatal stroke in rodents [216]. Taken together, these results indicate that VEGF-dependent angiogenesis is important for recovery of a neonatal stroke.

Another key hypoxia inducible gene is erythropoietin (EPO) that increases the capacity of red blood cells to supply oxygen following hypoxia [217,218]. Hypoxic up-regulation of EPO is regulated by HIF-1α and HIF-2α in vivo and in vitro [217,219,]. EPO is widely expressed in the brain by astrocytes, neurons, microglia, and endothelial cells [218,220]. Following cerebral ischemia, endothelial cells are the first to increase EPO expression, which could implicate that EPO mediates angiogenesis, probably by stimulating the expression of VEGF and its receptors on endothelial cells [221,222]. In vitro, EPO can also modulate angiogenesis by stimulating endothelial cell migration and proliferation [223]. Suggested to be neuroprotective following hypoxia, increased EPO expression has also been shown to increase anti-apoptotic gene expression and promote survival in oligodendrocytes, neurons, astrocytes, and microglia [217,218]. Treatment with recombinant human EPO following focal hypoxia-ischemia in neonatal rats results in enhanced revascularization, neurogenesis, endothelial cell and neuronal survival and increased Glut-1, Tie-1, and angpt-2 expression which resulted in enhanced neurovascular unit repair [221,224]. EPO treatment following neonatal stroke in rats has also shown significant neuroprotection ,[225-227], after neonatal HI injury [228] and MCAO [229,230].

Mediators with dual effects

Some of the mediators that are upregulated and are injurious during the acute injury phase, such as NO, MMPs, MIP-1a, MCP-1, and complement, may be beneficial and mediate the repair. Activation of MMPs, MMP-9 in particular, after stroke is thought to contribute to acute brain injury [231], but MMPs are critically involved in the remodelling of extracellular matrix and migration of immature neurons from the SVZ into the striatum [232]. Galectin-3 (Gal-3), a multifunctional carbohydrate-binding protein that controls numerous cell functions, has been identified as an angiogenic factor in adult stroke [233]. Like MMP-9, Gal-3 may have dual roles after stroke—harm initially but promote long-term repair. Anti-inflammatory agents may protect in part by reducing a subpopulation of microglia/macrophages with upregulated Gal-3. Genetic deletion of Gal-3 exacerbates injury and increases apoptosis after MCAO in the adult [234] but reduces hippocampal injury after HI insult [235]. Microglia-mediated IGF-1 production [234] and increased endothelial proliferation [233] were suggested as mechanisms of Gal-3-mediated remodeling after adult stroke. Gal-3 expression is rapidly increased after HI injury in the neonate [235], but its role in brain repair has not yet been explored in neonates.

TRANSLATIONAL ASPECTS AND TREATMENTS

In the past two decades, a broad range of therapeutic agents were used in neonatal ischemic brain injury models to target the excitotoxic, oxidative and inflammatory injury components, but, like in adult stroke, the results of the protective efforts have been mixed. Both broad-spectra and relatively selective anti-inflammatory treatments, including allopurinol [166], deferoxamine [236], N-acetylcysteine [237], melatonin [238-241], and minocycline [242], demonstrated beneficial effects but many studies revealed several limits in neuroprotection. As an example, the neuroprotective effect by minocycline is either short-lived or contingent on genetic background [183,243]. Targeting NF-kB signalling with peptide-based inhibitors showed that while sustainable neuroprotection can be achieved, the timing of administration

and the length of inhibition are crucial and that prolonged treatment can exacerbate injury [155,244]. Non-psychotropic cannabinoids have been considered as therapeutics based on numerous reports that demonstrated neuroprotection against neurodegenerative conditions [245,246], including not only neonatal rodent models [182] but HIE models in larger animals, newborn piglets and fetal lambs [247-250].

The HI white matter injury at P7 rats could be significantly attenuated by post-insult treatment with the AMPA receptor antagonist 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX)[251]. Likewise, intracerebral injections of AMPA (in combination with the *N*-methyl-d-aspartate receptor antagonist MK-801) demonstrated greater susceptibility of olygodendrocytes to injury at P7 than in younger or older pups and this injury was attenuated by systemic pretreatment with the AMPA antagonist NBQX. The AMPA receptor antagonist NBQX was effective at attenuating immature white matter injury in vivo, due either to direct receptor activation or hypoxia/ischemia. NBQX blocked the injury at P7 due to AMPA injections, consistent with the results of others in adult brain [88,91,252], and suggesting a receptor-mediated cause of injury. Notably, the acute seizures and the long-term enhanced seizure susceptibility are blocked by systemic administration of the AMPA receptor antagonist NBQX, whereas there is no effect of NMDA receptor antagonists (MK-801), GABA agonists (lorazepam and phenobarbital), or the conventional AED phenytoin [253].

The overactivation of NMDA receptor in immature rats are used as target of therapy of white matter injury [254]: the uncompetitive NMDA antagonist memantine attenuates in *vivo* acute loss of the developing oligodendrocytes cell surface marker O1 and the mature oligodendrocyte marker MBP(myelin basic protein), and also prevents the long-term reduction in cerebral mantle thickness seen at postnatal day 21. These protective doses of memantine do not affect normal myelination or cortical growth as previously observed for uncompetitive MK-801.

Attempts to protect the neonatal brain by inhibiting caspase-3 dependent apoptosis also showed mixed results. Pancaspase inhibitors and casp-3-selective inhibitors showed pharmacological neuroprotective effects in several [255-257] but not all [258,259] rodent studies of neonatal brain injury. While the age at the time of insult and the limited ability of individual inhibitors to distribute within the brain might affect injury outcome [259,260], caspase inhibition may activate caspase-independent cell death pathways, undermining neuroprotective efforts. Yet, targeting individual caspases upstream of Casp-3 may be promising [261], as was demonstrated by the potent neuroprotective role of a Casp2 inhibitor against perinatal ischemic brain damage in rodents [262].

Recently, the pluripotent capacity of stem cells from the human umbilical cord blood provides simultaneous targeting of multiple neuropathologic events initiated by a HI insult. Umbilical cord blood contains a mixture of mononuclear cells and other blood components, including red blood cells and platelets. The mononuclear cell fraction contains white blood cells, as well as progenitor and stem cells at an amount comparable to or exceeding that in bone marrow. Progenitor cells are defined as cells that can divide, producing more than one type of cell. Stem cells are defined as dividing cells that can differentiate into more mature

cell types. Studies on human blood samples showed that, compared with bone marrow-derived stem cells, umbilical cord blood cells are reported to display lower immunogenicity and risk of rejection [263] and an eightfold greater proliferative potential [264,265]. These features are advantageous for transplantation, and furthermore their acquisition does not require painful donor extraction procedures.

HIE neonatal models that have received systemic injections of umbilical cord blood cells commonly display human cells in the lesioned side of the brain and variable improvement in morphologic or functional outcome [266]. Pimentel-Coelho et al. [267] described a region-specific effect of mononuclear umbilical cord blood cells in a neonatal rat model of HIE. Is not clear if neurofunctional improvements are related to an anti-inflammatory effect of cord blood cell transplantation, as suggested by decreased microglial activation in the ischemic cortex at 7 days after injury, or they are due to cytokine or growth factor release from human cord blood cells. Further improvement in functional outcomes after brain injury has been achieved via adjunctive therapies[268-272]. EPO administration enhances neurogenesis and promotes functional recovery after neonatal HI injury [228] and MCAO [229,230] in rodents. While a single EPO dose has been found to be beneficial over a short time period, multi-dose EPO treatment markedly improves structural and functional outcomes over several months [230]. Its safety profile and beneficial effects via activation of multiple pathways makes EPO a good candidate as a treatment of newborns with brain injury. However, the combination of EPO with hypothermia showed no benefit over EPO alone after HI insult in neonatal rats [270], reinforcing the importance of proper timing for adjunctive therapies. BDNF and IGF-1 seem to be promising therapeutic approaches, but few data are available to date.

More recently, cell based therapies, including mesenchymal stem/progenitor cells (MSCs), have been shown to improve, in rats, functional outcomes after stroke [273-278] and traumatic brain injury [279] in the adult and after HI event in the neonate [280-284]. Intravenously administered MSC reduce apoptosis, promote endogenous cell proliferation [273], and reduce the expression of inhibitory factors in astrocytes, including a broad array of glycoproteins [285], but they increase production of VEGF and BDNF [274]. MSC may stimulate angiopoietin1 and VEGF signaling and amplify angiogenesis, a process necessary for vessel remodeling and neuroblast migration [274]. Intranasal MSC administration in mice was shown to markedly reduce infarct size, facilitate formation of new neurons and oligodendrocytes, and improve sensorimotor outcome following HI injury [281,282]. Although initially long-lasting neuroprotection by MSC against HI damage was attributed to replacement of dead neurons, the survival of MSC appeared to sharply decline to only 1% by 18 days after delivery whereas the beneficial effects continued [283]. This data, along with marked effects of MSC on gene expression of growth factors and inflammatory molecules [283], has suggested that engrafted cells stimulate the microenvironment, which, in turn, permits remodelling and improvement of neurological function.

While further proof of the latter mechanism is needed, data that MSC that overexpress BDNF are even more potent in repair than MSC themselves supports the notion of a critical role of the changing microenvironment by MSC. One recent work [286] showed that Intranasal delivery of MSC- and MSC-BDNF significantly reduces infarct size and gray matter loss in

comparison with vehicle-treated rats without any significant difference between MSC- and MSC-BDNF– treatments. Treatment with MSC-BDNF significantly reduced white matter loss with no significant difference between MSC- and MSC-BDNF–treatment. Motor deficits were also improved by MSC treatment when compared with vehicle-treated rats. MSCBDNF–treatment resulted in an additional significant improvement of motor deficits 14 days after MCA occlusion, but there was no significant difference between MSC or MSC-BDNF 28 days after occlusion.

To date, all these translational treatments have been tested only in animal models.

As of today, hypothermia is the only neuroprotective treatments of proven efficacy in humans for injury resulting from perinatal HIE. Recent multicenter clinical trials demonstrated the effectiveness of hypothermia, when initiated within the first 6 hours in neonates with moderate HIE, eventually reducing the risk of major neurological disabilities [287,288]. However, approximately 40% of cooled infants died or survived with significant impairments. Selective brain cooling was shown to potentially induce anti-inflammatory effects [289]. The beneficial effects of hypothermia seen in experimental models of ischemia are the result of a wide range of biological effects, as outlined in Table 5. Hypothermia can protect by preserving energy metabolism [290], reducing proteolysis [291,292] and ROS production [292,293], as well as by affecting vascular integrity and neuroinflammation [294,295]. It has the potential to minimize secondary injury to vulnerable areas [296,297]. Although clinical and experimental studies show functional improvement after hypothermia in injured neonates, there is still the need to better understand the optimal depth, timing and duration of hypothermia in order to maximize beneficial effects and reduce long-term neurologic morbidity.

CONCLUSIONS

Global/Focal perinatal brain injury represents a complex disease, frequently occurring in the perinatal period and resulting in neurological sequelae. The various clinical and pathological outcomes in neonates could be associated with either post-ischemic processes or other insults occurring during neural development. The data is emerging that neuroinflammation plays a role not only in short-term injury outcomes, but in modulating the long-term repair after perinatal stroke and enhancing the effects of adjunctive therapies. Pharmacological inhibitors, in particular glutamate-receptor antagonists and caspase inhibitors that target neuro-inflammatory mediators, and cell based therapies, in association with hypothermia represent the most promising therapeutic options for trials in humans in the near future.

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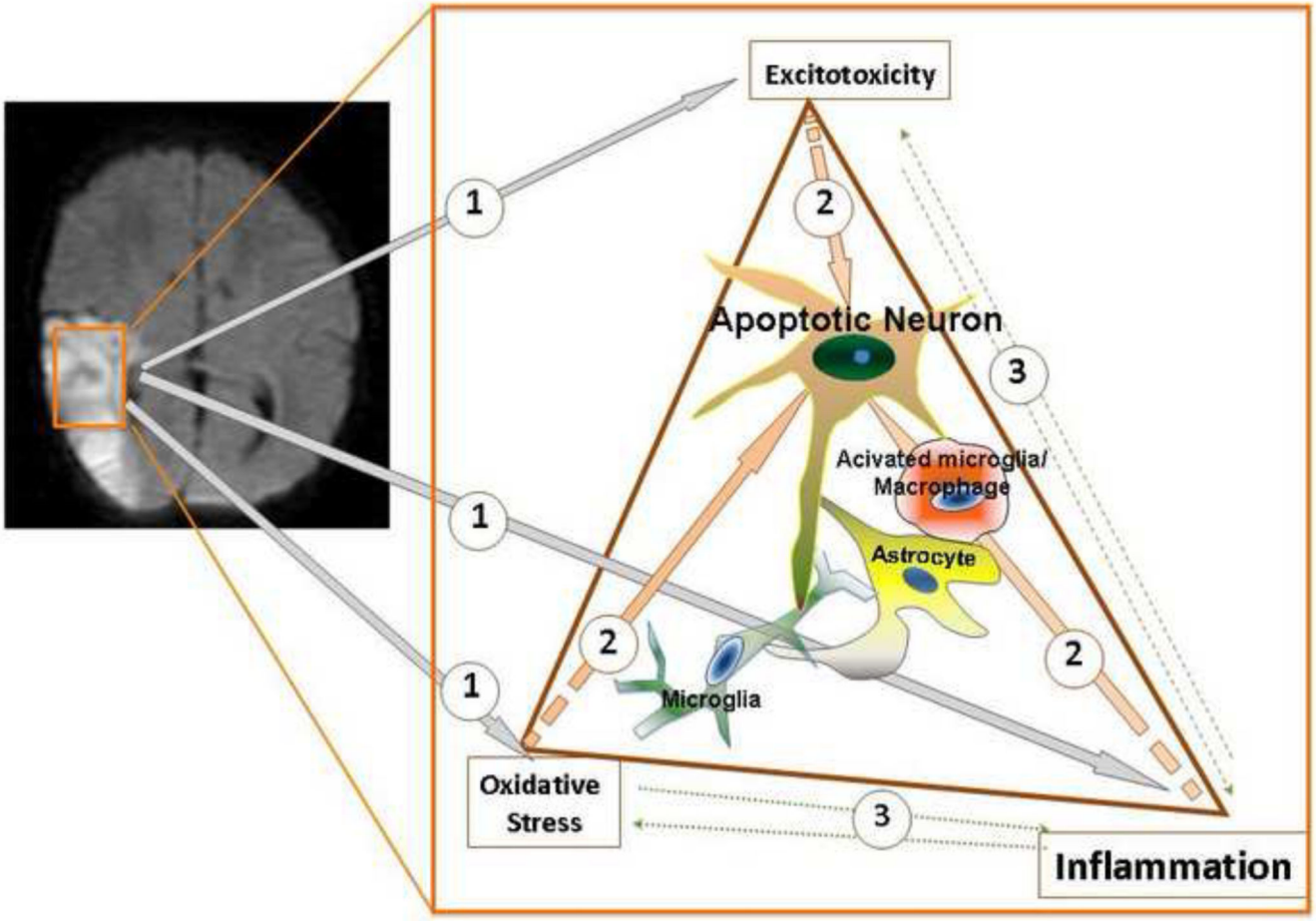

Figure 1. The mechanisms of injury in perinatal stroke and HIE

A. A representative MRI demonstrates a focal ischemic lesion in perinatal stroke. ***B***. Schematic representation of the major mechanisms contributing to neuronal death in post-ischemic neonatal brain. Excitotoxicity, oxidative damage and neuroinflammatory processes are the main mechanisms of injury (shown as (1)). These mechanisms of injury can independently lead to spectra of neuronal death mechanisms (2), but also feed and potentiate each others effects (3), further exacerbating brain injury.

Table 1

Risk factors for PAIS.

Maternal Factors	***Fetal/neonatal factors***	***Others***
Blood, homocysteine and lipid disorders	Inherited thrombophilias	Catheter-related complications
Thrombotic state during pregnancy	Twin tot twin transfusion	Male gender
Previous pregnancy related disorders	Systemic infections	Race and ethnicity
Primiparity, twin-twin pregnancy	Meningitis	Dehydration
Pre-eclampsia	Perinatal asphyxia	Trauma
Gestational diabetes	Congenital heart diseases	ECMO
Chorioamnionitis	Hypoglycemia (in preterm)	Emergency cesarean section
Labor and delivery complications	ELBW	
Autoimmune disorders	Polycythemia	
Drug abuse (cocaine)		
Infertility and its treatment		

Table 2

Summary of key developmental processes across comparable ages in humans and rodents.

Human	Rodent	Developmental milestones	Reference (s)
23–32 wk gestation (pre-term infant)	pnd 1–3	Oligodendrocyte maturation state changes—pre-dominance of mitotically active pre-OLs[a].	Craig et al. (2003), Lodygensky et al. (2010), Dean et al., 2011a and Dean et al., 2011b
		Immune system development.	Holsapple et al. (2003)
		Establishment of the blood-brain barrier.	Engelhardt (2003), Daneman et al. (2010)
36–40 wk gestation (term infant)	pnd 7–10	Peak brain growth spurt.	Dobbing and Sands (1979), Bockhorst et al. (2008)
		Peak in gliogenesis.	Catalani et al. (2002), Kriegstein and Alvarez-Buylla (2009)
		Increasing axonal and dendritic density.	Cowan (1979), Bockhorst et al. (2008), Baloch et al. (2009)
		Oligodendrocyte maturation state changes–switch to a pre-dominance of immature OLs.	Craig et al. (2003), Dean et al., 2011a and Dean et al., 2011b
		Consolidation of the immune system.	Holsapple et al. (2003)
2–3 year old	pnd 20–21	Brain reaches 90–95% of adult weight.	Dobbing and Sands, 1973 and Dobbing and Sands, 1979, Dekaban et al. (1987), Giedd et al. (1999)
		Peak in synaptic density at 50% > adult levels.	Huttenlocher (1979), Micheva and Beaulieu (1996)
		Peak in myelination rate.	Keshavan et al. (2002)
		Neurotransmitter and receptor changes.	Hedner et al. (1986), Romijn et al. (1991)
4–11 year old	pnd 25–35	Fractionation/specialization of prefrontal cortex neural networks (structural maturation).	Tsujimoto (2008)
		Maximum volume of grey matter and cortical thickness.	Sowell et al. (1999), Bansal et al. (2008)

Table 3

Systemic hypoxia-ischemia (H-I) and ischemia-reperfusion rodent models

HUMAN PATHOLOGIC CONDITION	ANIMAL USED AND AGE OF THE ANIMAL	TYPE OF DOMMAGE	REFERENCE
H-I models	P7 Wistar rats	Permanent occlusion of the Middle Cerebral Artery (MCA), pMCAo + O2 8%	Rice et al. 1981
	Rice's model adapted to other rat's ages	"	Derugin et al 1998, Oshima 2012
			Derugin 2005
	Mouse P7	Blockade of the past External Carotid Artery- Internal Carotid Artery (ECA-ICA) bifurcation, external-internal carotid artery	Oshima 2012
	Mouse P8	Permanent occlusion of the Middle Cerebral Artery (MCA), pMCAo + O2 8%	Hagberg 2004
	Mouse P10	"	Tsuji 2013
	Mouse P12	"	
Ischemia-Reperfusion Models (=Stroke-Like lesions)	Wistar Rat P7	pMCAo +bilateral transient Common Carotid Artery (tCCAo) occlusion	Renolleau 1998
	Wistar Rat P10	"	Mitsufujii 1996
	CB17 Mouse P12	pMCAO	Tsuji 2013

Table 4

Pro- and anti-inflammatory mediators involved in the ischemic cascades.

Mediators	Post ischemia	Neuroprotection	Treatment	CK target	Effect
IL-1	upregulated in H-I in mice	IL1Ra ↓ brain injury			
IL-18	upregulated in H-I in mice	IL18 KO mice ↓ brain injury in neonate			
TNFα	• upregulated in mice, • ↓ survival/ maturation olygodendrocyte progenitor	**?**	**R-7050**	TNFα	Attenuate neurovascolar injury
IL-2	**?**	**?**			
IL-6	upregulated in H-I in mice	**?**			
IL-8	**?**	**?**			
IL-9	induce histamine release→cell injury	inhibit post-mitotic neuronal apoptosis in the newborn mouse cortex			
IL-10	**?**	exogenous IL10 therapy			
IL-4	**?**	in adult stroke			
INFγ	**?**	IL4/ INFγ ↓ apoptosis in oligodendrocyte progenitors and astrocytes			
NFkB	dual role pro-anti apoptotic	Precoce (0-3 h) inibhition ↓ neuroinflammation and Casp 3 activation	**Minocycline**	NF-kB	Attenuation of the postischaemic inflammatory response and ↓ in white matter damage in the immature rodent brain (HI model)
MIP	upregulated in H-I in mice		**Estradiol**	MIP-2; CCR7	MIP-2; CCR7
MCP/CCL2	upregulated in H-I in mice	Genetic deletion/ pharmacological inhibition	**vMIP-II** chemokine analogue peptide acting as antagonist against several chemokines and chemokine receptors.	Various chemokines (CCL2, CCL3) and chemokine receptors (CCR1,2,3,4,5,8; CXCR3,4).	Attenuation of brain infarction in mice

Mediators	Post ischemia	Neuroprotection	Treatment	CK target	Effect
			Dimemorfan sigma-1 receptor agonist.	CCL2	↓ of infarct size and glutamate-mediated excitotoxicity in rats
			Rosiglitazone agonists of ligand-activated transcription factors (PPAR)-γ.	CCL2; CXCL8	↓ of infarct size and improved functional outcomes in mice
RANTES	upregulated in H-I in mice	**?**			
H_2O_2	injure neonate mice/rats after H-I	**?**	**N-acetylcysteine**	Oxygen radical scavenger	↓ in cerebral oxidative stress and cerebral injury; improvement in myelin expression and neurological outcome in neonatal rats (combined with hypothermia) (HI model)
			Melatonin	Oxygen radical scavenger	↓ in white matter damage and promotion of repair by induction of axonal regrowth or sprouting in newborn mice (ibotenate-mediated excitotoxic brain injury). ↓ in oxidative damage mediators in 7-day-old rats (HI model)
NO	nNOS, iNOS	eNOS inhibition of nNOS, i-NOS	**2-iminobiotin**	Selective nNOS and iNOS inhibitor	Improvement in cerebral energy state, ↓ in vasogenic oedema and neuronal death (HI model)
			7-nitroindazole	Selective nNOS inhibitor	Suppression of both two peaks of NO metabolites (in hypoxia and re-oxygenation period) (HI model)
			Aminoguanidine	Selective iNOS inhibitor	Suppression only theNOmetabolites peak in the re-oxygenation period. ↓ in cerebral injury in a neonatal rats (HI model)

Mediators	Post ischemia	Neuroprotection	Treatment	CK target	Effect
COX-2	upregulated in H-I	COX2 inhibition **?**			
CXC	increased in periphery and in the brain in neonatal MCAO; protect BBB early after injury	CXC12L→improvement of neurogenesis, neuroblast migration, vasculogenesis	**JWH-133** Synthetic cannabinoid 2 receptor agonist	CXCL2	Inhibition neutrophil migration CXCL2 - mediated in ischaemic brain in mice
			Candesartan angiotensin AT1 receptor blockers. **Pioglitazone** thiazolidinediones	CXCL1	Down regulation of CXCL1 and TNF-α expression and reduction of cerebral infarct size in rats
Galectin-3	upregulated in H-I in mice	Gal3 KO mice ↓ injury in neonate			
Caspases	markedly upregulated; cross-talk between inflammatory mediators and apoptosis pathways	• inhibition casp2 • inhibition casp3 – mixed effects			

Table 5

Mechanisms of action by which hypothermia can limit ischemic damage.

Reduced metabolic demand
Reduced proteolysis
Cell membrane stabilization
Inhibited spreading depolarization
Decreased excitotoxic damage
Lower lactate and tissue acidosis
Reduced free radical and reactive oxygen species formation
Altered proapoptotic signals
Reduced neuronal calcium influx and toxicity
Reduced ischemia-associated gene expression
Inhibited inflammation and cytokine production
Reduced disruption of the blood brain barrier
Preserved cerebral autoregulation