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Hypoxic Ischemic Encephalopathy in the Term Infant

Ali Fatemi, MD^a, Mary Ann Wilson, PhD^b, and Michael V. Johnston, MD^c

^a Assistant Professor of Neurology and Pediatrics, Kennedy Krieger Institute, Johns Hopkins Medical Institutions, Baltimore, MD

^b Associate Professor of Neurology and Neuroscience, Kennedy Krieger Institute, Johns Hopkins Medical Institutions, Baltimore, MD

^c Blum-Moser Chair for Pediatric Neurology at the Kennedy Krieger Institute, Professor of Neurology, Pediatrics, Physical Medicine and Rehabilitation, Johns Hopkins Medical Institutions, Baltimore, MD

Synopsis

Hypoxia-ischemia in the perinatal period is an important cause of cerebral palsy and associated disabilities in children. There has been significant research progress in hypoxic-ischemic encephalopathy over the last two decades and many new molecular mechanisms have been identified. Despite all these advances, therapeutic interventions are still limited. In this review paper, we discuss a number of molecular pathways involved in hypoxia-ischemia, and potential therapeutic targets.

Keywords

Hypoxia ischemia; neonatal encephalopathy; apoptosis; oxidative stress; hypothermia

Introduction

Hypoxia-ischemia in the perinatal period is an important cause of cerebral palsy and associated disabilities in children. Cerebral palsy is one of the most costly neurologic disabilities because of its frequency (2/1000 births) and persistence over the life span.¹ In the term infant, the most common mechanism of hypoxic injury is intrauterine asphyxia brought on by circulatory problems, such as clotting of placental arteries, placental abruption, or inflammatory processes.² These result in perinatal depression leading to diminished exchange of oxygen and carbon dioxide and severe lactic acidosis.² A recent study by Graham et al showed that the incidence of neonatal neurologic morbidity and mortality for term infants born with cord pH < 7.0 is approximately 25%.³ Reduced cardiac output in the setting of hypoxia is referred to as hypoxia-ischemia (HI).⁴ If an episode of HI is severe enough to damage the brain, it leads within 12 to 36 hours to a neonatal encephalopathy known as hypoxic-ischemic encephalopathy (HIE).⁵ This clinical syndrome includes seizures, epileptic activity on electroencephalogram (EEG),

Corresponding Author: Michael V. Johnston, MD, Kennedy Krieger Institute, Johns Hopkins Medical Institutions, 707 N. Broadway, Baltimore, MD, Johnston@kennedykrieger.org, phone 443 923 9315, fax 443 923 9317.

Co-authors:

Ali Fatemi, MD, Kennedy Krieger Institute, Johns Hopkins Medical Institutions, 707 N. Broadway, Baltimore, MD, Fatemi@kennedykrieger.org, phone 443 923 2684, fax 443 923 2775

Mary Ann Wilson, PhD, Kennedy Krieger Institute, Johns Hopkins Medical Institutions, 707 N. Broadway, Baltimore, MD, Wilson@kennedykrieger.org, phone 443 923 2694, fax 443 923 2695

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hypotonia, poor feeding, and a depressed level of consciousness that typically lasts from 7-14 days.⁶ Pathology studies of term neonates who sustained a profound hypoxic-ischemic event show relative cortical sparing and deep gray matter injury particularly involving hippocampi, lateral geniculate nuclei, putamen, ventrolateral thalami, and dorsal mesencephalon.⁷ There is no effective pharmacologic therapy, although hypothermia has shown promise in several clinical trials.^{8, 9} Magnetic resonance imaging (MRI) has markedly improved the understanding of the patterns of brain injury from perinatal asphyxia. The pattern produced by so-called “near total” asphyxia is easily recognized on MRI scans and includes relatively selective injury to the putamen, thalamus and peri-rolandic cerebral cortex, and often includes injury to the brainstem as well.¹⁰ This pattern is similar to the pathological pattern of diencephalic and brainstem injury described by Myers in his model of acute total asphyxia in nonhuman primates, developed in the early 1970s.¹¹ It can be distinguished from the injury produced by a partial prolonged insult that results in more extensive cortical injury. In most infants, white matter is relatively spared, although a transient increase in the T2-weighted MRI signal is often seen in the posterior internal capsule soon after injury.¹² Infants who demonstrate this pattern of insult may require vigorous resuscitation to survive and have severe metabolic acidosis in the umbilical cord blood.¹³ Metabolic derangements leading to oxidative stress, inflammatory factors, and excitotoxicity, and perhaps genetic factors, are thought to contribute to brain injury after HIE.

Delayed cell death in HIE

Both clinical and experimental observations demonstrate that HIE is not a single “event” but is rather an evolving process. The clinical signs of HIE reflect the evolution of a delayed cascade of molecular events triggered by the initial insult. MRI studies show progression of lesion size over the first few days after injury (Figure 1).¹⁴ The initial findings within the first few hours after near total asphyxia are subtle and often seen only on diffusion weighted imaging, which shows restricted diffusion typically starting as small lesions in the putamen and thalami and usually progressing over the next 3-4 days to involve more extensive areas of the brain.¹⁴ MR spectroscopy shows a similar pattern of progression, with an increase in lactic acid and reduction of N-acetyl-aspartate over the first few days after initial insult (Figure 2).¹⁵ Studies of animal models of HIE show that during the period after the insult, many neurons and other cells “commit” to die or survive over a period of days to weeks.¹⁶ Many of them might be rescued during this “window of opportunity.” Along with this notion, hypothermia has shown beneficial effect in HIE,⁹ suggesting that intervention after birth is still helpful possibly by preventing delayed cell death. Therefore, it is crucial to investigate molecular pathways involved in this event to identify potential therapeutic interventions.

Animal studies have led to new insights in HIE. Rodent models combine unilateral carotid artery ligation with exposure to a period of hypoxia to replicate the combination of hypoxemia and ischemia seen in human infants after asphyxia.¹⁷ Comparison of histology from the animal model with MRI of human term infants after near-total asphyxia reveals remarkably similar patterns of injury to the basal ganglia and cerebral cortex.^{10, 13, 16} Unilateral carotid ligation plus hypoxia results in predominant injury on one side with modest or no injury on the other.¹⁸ These studies show that during the initial phase of HI there is rapid depletion of adenosine-tri-phosphate (ATP)^{19, 20} leading to failure of Na/K-pump and depolarization of the cell, with severe cell swelling and cytoplasmic calcium accumulation leading to necrosis and activation of multiple cascades that eventually lead to more cell death.

The form of cell death depends on the severity of ischemic injury.²¹ Necrosis predominates in more severe cases, whereas apoptosis occurs in areas with milder ischemic injury, often days after the initial insult.²² We have shown that activation of the pro-apoptotic protein, caspase-3, in a neonatal rodent model of cerebral hypoxia-ischemia is prolonged and that moderate to

high levels of activated caspase-3 persist for at least 7 d after hypoxic ischemic injury.¹⁶ The regional and temporal patterns of caspase-3 activation correspond well with those for apoptosis,¹⁶ lending further support for a prolonged role of apoptosis in hypoxic-ischemic injury in the neonatal brain. The newborn brain is primed to respond to various insults with activation of apoptotic cascades due to the importance of programmed cell death in the normal development of the CNS.²³ Proapoptotic proteins are highly expressed in the developing brain,²⁴ and caspase-3 and -9 deficient mice present with severe brain-overgrowth malformations.^{25, 26}

In addition to necrosis and apoptosis, neurons in rodents subjected to neonatal HI display morphologic features along an apoptosis-necrosis continuum.^{16, 27} Cells showing a morphology intermediate between that of classic apoptosis and necrosis, referred to as “hybrid” cells, are observed.²⁷ The nuclei of such cells have large, irregularly shaped chromatin clumps, similar to apoptotic neurons, but the cytoplasm shows changes similar to necrotic neurons (Fig. 3A,A1). A study by Northington et al²⁸ showed that the evolution of this morphology coincides with mitochondrial bioenergetic and structural failure superimposed upon activation of apoptotic pathways after neonatal HI. Bioenergetic failure likely prevents execution of a full apoptotic phenotype. Evidence that apoptotic pathways are activated following neonatal HI coexists with evidence for incomplete execution of these pathways, energy failure, and biochemical evidence of necrosis.²⁸ It is presumed that mitochondrial failure may interrupt apoptotic cascades initiated by injury to the immature rodent brain and result in the hybrid phenotype of neuronal cell death.

Role of neurotransmitter receptors and excitotoxicity in hypoxia-ischemia

Excitotoxicity has emerged as an important mechanism of injury in the brain, and the concept is important for understanding perinatal brain pathology. Glutamate is the predominant excitatory amino acid neurotransmitter in the brain, and most neurons and many glia possess receptors for glutamate.²⁹ Neuronal pathways that utilize glutamate as their neurotransmitter are ubiquitous in the brain, mediating vision, hearing, somatosensory function, learning and memory, and other functions.³⁰ The development of excitatory neuronal circuits, as well as expression of specific glutamate receptor subtypes in excitatory synapses, are dynamic in the perinatal brain, and these changes can be related to changing patterns of pathology at different gestational ages.^{31, 32} There are three major groups of glutamate receptors within the post-synaptic membrane that operate ion channels, so called ionotropic receptors, and a group of G-protein linked metabotropic glutamate receptors. The three major types of ionotropic receptors are the N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainic acid (KA). Normally, glutamate is contained within the pre-synaptic nerve terminal until release is stimulated by neuronal depolarization;³³ when release into the synaptic cleft does occur, the neurotransmitter is quickly taken up by high-capacity glutamate transporters in astroglia that surround synapses and nerve terminals.³³ Glutamate taken up into the astroglia is converted to glutamine before being transported back into the nerve terminal to recycle glutamate neurotransmitter.³⁴ The glutamate transporter is dependent on a sodium gradient created by Na⁺/K⁺ ATPase that is powered by anaerobic glucose metabolism, and impaired delivery of glucose to the brain by ischemia and/or hypoglycemia impairs glutamate removal from the synapse.^{35, 36} Severe hypoxia associated with HI or ischemia also leads to reversal of glutamate transporters presumably via an NF- κ B mediated mechanism leading to additional accumulation of synaptic glutamate.³⁷ Elevations in extracellular glutamate have been measured in animal models of perinatal HI using intracerebral microdialysis.^{38, 39} While the accumulation of glutamate within synapses and in the brain's extracellular space is a generic phenomenon that occurs in most regions of the brain where glutamate-containing pathways are present, the toxic effect of this accumulation is determined by the local repertoire of postsynaptic glutamate receptors. The distribution and molecular characteristics of NMDA-type glutamate receptors appear to be an especially

important determinant of the pattern of neuronal injury in the perinatal brain.³¹ The NMDA receptor requires co-activation by both glutamate and glycine.^{40, 41} The NMDA receptor channel is blocked by magnesium at rest and requires depolarization of the post-synaptic membrane for the channel to release this block and allow calcium to flux inward.⁴² These special features allow the NMDA receptor to play a role in activity-dependent synaptic plasticity, including long-term potentiation (LTP) and refinement of synaptic connections.^{31, 43} However, failure of ATP-dependent Na⁺ transport during HI disrupts membrane potentials, which can overcome the magnesium block and allow Ca⁺⁺ influx through the NMDA channels.^{44, 45} Drugs that block NMDA receptors or channels, such as dizocilpine (MK-801),⁴⁶ dextromethorphan,⁴⁷ ketamine,⁴⁸ or magnesium,⁴² are strongly protective against hypoxic-ischemic injury if given before or shortly after HI or other insults in neonatal rodent models. At around 7 days of age the rodent brain is much more sensitive to direct intracerebral injections of NMDA, ibotenic acid, HI or trauma than the adult brain.¹⁷ Hypersensitivity to NMDA receptor activation during the neonatal period can be correlated with molecular features of the immature NMDA receptor channels that allow them to open more easily and flux more calcium than their adult counterparts.^{40, 49} NMDA receptors probably mediate much of the injury to neurons in structures such as cerebral cortex, basal ganglia, hippocampus and thalamus associated with hypoxic-ischemic injury in animal models.^{29, 50}

Activation of AMPA receptors, which primarily flux sodium and mediate most of the fast excitatory activity in the brain, also contribute to injury.⁵¹ From a developmental standpoint, NMDA receptors are the first glutamate receptors to appear at new synapses, followed by AMPA receptors associated with increasing neuronal activity.^{52, 53} Immature AMPA receptor channels flux calcium similar to NMDA receptors, but increasing expression of GluR2 receptor subunits and RNA editing over the first two postnatal weeks in rodents shift the balance towards calcium impermeable AMPA receptors in the mature brain.^{51, 54} Direct injection of AMPA agonists at various postnatal ages produces greater injury during the postnatal period than during adulthood,⁵⁵ with a peak several days after that for NMDA. Calcium flooding through open NMDA and calcium-permeable AMPA receptor channels triggers a cascade of intracellular events that mediate cell death, including generation of reactive oxygen species and activation of apoptotic pathways (see Figure 4).

AMPA antagonist drugs are not as protective against hypoxic-ischemic neuronal injury as NMDA antagonists in the perinatal period,⁵⁶ although the AMPA antagonist topiramate has been shown to be protective in combination with hypothermia in a model of hypoxic-ischemic injury in infant rats.⁵⁷ The molecular features of both NMDA and AMPA receptors during the perinatal period that enhance Ca⁺⁺ entry allow them to support activity-dependent neuronal plasticity and development. One indication of their importance for normal development is the observation that prolonged blockade of NMDA receptors causes apoptosis in cell culture and animal models.⁵⁸ However, their vital role and enhanced function in the perinatal period also make neurons more vulnerable to excitotoxicity, thus creating a paradox: the immature brain can withstand longer periods of energy deprivation than the adult brain because of its low energy requirement, yet when a critical threshold of energy deprivation is reached, excitotoxic injury is enhanced because of developmentally enhanced excitatory pathways.

In contrast to the probable role that NMDA receptors play in perinatal damage to the cerebral cortex, thalamus and basal ganglia, AMPA and kainate receptors are implicated most strongly in perinatal damage to the brainstem.²⁹ Autoradiographic studies in human postmortem tissue indicate that AMPA and/or kainate receptor binding is elevated in these vulnerable regions in the midgestation fetus and neonate^{59, 60} and then declines at later ages, while NMDA receptor binding is undetectable at midgestation and then matures in the postnatal period. Elevated levels of AMPA/kainate receptors in the griseum pontis at midgestation and early infancy may be relevant to pontosubicular necrosis from HI during the last trimester and early infancy.⁶⁰

AMPA receptors probably mediate the stimulus of breathing movements via the nucleus of the solitary tract during the fetal period, while NMDA receptors likely mediate stimulation of this nucleus in response to hypoxia and sustained ventilation in the newborn and infant,⁶⁰ suggesting that the vulnerability of these brainstem structures to injury is related to the adaptive roles that the different types of glutamate receptors play in normal neuronal development and plasticity.

The targeting and clustering of AMPA- and NMDA-type glutamate receptors to the synapses in the CNS are essential for efficient excitatory synaptic transmission. Members of the Long Pentraxins family of proteins, including neuronal pentraxin 1 (NP1) and neuronal activity-regulated pentraxin (Narp, also called NP2), have several structural and functional characteristics that might play a role in promoting excitatory synapse formation and remodeling.^{61, 62} NP1 and NP2 are linked to glutamate receptors at synaptic sites, and regulate AMPA receptor clustering.⁶² Hossain et al have shown that the neuronal pentraxin NP1 is induced in hypoxic-ischemic injury in neonatal brain, primarily in the cerebral cortex and hippocampal pyramidal layers of the CA3 and CA1 regions, and that antisense oligonucleotides directed against NP1 mRNA prevent hypoxia-induced neuronal death.⁶¹ Particularly interesting and important are the findings that NP1 is associated with AMPA receptors and that hypoxia induces a time-dependent increase in NP1-GluR1 interactions.⁶² Thus, hypoxia recruits NP1 protein to the GluR1 subunit of the AMPA receptor concurrent with the hypoxic excitotoxic cascade.⁶¹ These results suggest a novel mechanism by which NP1 induction during HI accentuates excitotoxicity and thereby contributes to HI-induced neuronal death.

Seizures are extremely common in infants with HIE. Despite dramatically lower synaptic connectivity,⁶³ the immature brain is much more susceptible to seizures than the adult brain.⁶⁴ One likely proconvulsant factor is the paradoxical action of the neurotransmitter GABA. GABAA receptor (GABAA-R) activation is inhibitory in the adult brain, both by virtue of the membrane hyperpolarization induced by chloride (Cl) influx through the GABAA ionophore and by virtue of shunting of dendritic excitatory inputs.⁶⁵ In the fetal and neonatal periods, however, the transmembrane chloride gradient is reversed.⁶⁵ As a result, GABAA-R activation depolarizes the neuronal membrane. GABAA receptor-mediated synaptic currents with depolarizing reversal potentials are common in the embryonic and neonatal brain⁶⁶ and are most likely explained by a high intracellular chloride concentration.⁶⁷ GABAergic synapses are established first and exert an excitatory action, as measured by the capacity for GABAA receptor activation to trigger action potentials in the postsynaptic cells.⁶⁸ In addition to triggering action potentials, the depolarizing action of GABA in the neonatal brain may induce Ca²⁺ entry through voltage-dependent Ca²⁺ channels,⁶⁹ contribute to removal of the Mg²⁺ block from NMDA channels, and further enhance glutamate excitotoxicity.

Inflammatory mechanisms involved in hypoxia-ischemia

Inflammatory cytokines have been associated with neonatal hypoxic-ischemic encephalopathy and are significantly elevated in term infants who later develop cerebral palsy.⁷⁰ Elevated levels of IL-6 and IL-8 in the cerebrospinal fluid of term newborns have been correlated with an increased degree of encephalopathy and poor neurodevelopmental outcome.⁷¹ A study employing MR Spectroscopy demonstrated a correlation between elevated lactic acid level in the basal ganglia and serum IL-1, IL-6, IL-8 and TNF α levels in infants with HIE.⁷² Studies in newborn mice subjected to unilateral carotid artery ligation demonstrate up-regulation of many inflammatory genes associated with cellular activation in the injured hemisphere.⁷³ Inflammatory gene expression is evident at 8 h and increases further at 24-72 h post HI, and the set of genes that is expressed suggests activation of microglia and other inflammatory cells.⁷³ There is also increased chemokine expression and infiltration of inflammatory cells around the lesion.⁷⁴ Microglial aggregation in the dentate gyrus has been observed in human infants

after HI insults.⁷⁵ Microglia may contribute to secondary brain injury through the production of proinflammatory cytokines, proteases, reactive oxygen species, NO, complement factors, and excitotoxic neurotransmitters such as quinolinic acid.

There is now substantial experimental evidence that pre-existing intrauterine inflammation can exacerbate HIE.^{76, 77} Lipopolysaccharide (LPS), also referred to as endotoxin, has been used extensively to induce an inflammatory response in animal models for HIE.⁷⁸⁻⁸¹ LPS binds to toll-like receptor 4⁷⁶ and myeloid differentiation factor 88⁸² to activate downstream signaling, including activation and nuclear translocation of the nuclear transcription factor NF- κ B,⁸³ which promotes transcription of proinflammatory cytokines such as interleukin-1 β , interleukin-6, and tumor necrosis factor- α , prostaglandins, and a variety of adhesion molecules and acute-phase proteins.⁸⁴ NF- κ B has been found in various cell populations in the brain including microglia, astrocytes, and neurons.⁸⁵ Hagberg et al demonstrated an upregulation of NF- κ B in the fetal brain at an early stage after LPS administration.⁸⁰ The timing of NF- κ B elevation in this study corresponded with the cytokine changes that occur after intrauterine LPS administration. NF- κ B has also been found to be involved in preconditioning-induced neuroprotection.⁸⁶ LPS treatment leads to activation of microglia with increased production of reactive oxygen species and NO.⁸⁷ Astrocytes also play a significant role in inflammation following HI.^{88, 89} Astrocytes are the major source of interleukin-6 (IL-6) in CNS injury and inflammation.⁸⁹ Reactive astrocytes also release TNF- α and IL-6 through a carrier-dependent mechanism, which can result in sustained modulatory action on neighboring neurons.^{88, 90}

Interestingly, low-dose treatment with intrauterine LPS, applied between the chorionic and amniotic membranes, dramatically increased the severity of injury after HI in neonatal mice but conferred protection against HI in adult rodents.⁸⁰ It appears, therefore, that inflammatory processes may either potentiate HI-induced injury or exert a neuroprotective effect in a time-dependent manner.

Role of oxidative stress in hypoxia-ischemia

Fetal life elapses in a low oxygen environment with a mean intrauterine arterial oxygen saturation (SpO₂) under physiologic conditions of 40-45%.⁹¹ In the first minutes of life, an abrupt rise of SpO₂ to 80-90% occurs, which creates a pro-oxidant condition.⁹² This condition facilitates activation of specific metabolic pathways.⁹³ Under pathological conditions, such as birth asphyxia, a series of pathophysiologic events, such as excess calcium influx via glutamate receptors, leads to severe oxidative stress.⁹⁴ There is accumulation of hydrogen peroxide (H₂O₂) after HI in neonatal mice but not in adult mice.⁹⁵ H₂O₂ may be the critical mediator in determining whether downstream signaling will favor cell death or repair. Repeated episodes of hypoxia cause purine derivatives, such as adenosine or hypoxanthine, to accumulate and promote specific changes that predispose cells to enhanced damage on reoxygenation.⁹⁶ Activation of oxidases and nitrogen oxide synthase (NOS), and up-regulation of hypoxia inducible factor-1 alpha (HIF-1 α), as well as downregulation of antioxidant enzymes, such as superoxide dismutases, catalases, and glutathione peroxidases, generate a burst of reactive oxygen (ROS) species on reoxygenation.⁹⁷ There is a marked increase in nNOS immunoreactivity in nerve fibers for more than a week after HI in regions such as the thalamus.⁹⁸

Nitric oxide synthase comprises a family of enzymes that produces nitric oxide (NO). In addition, •OH can react with NO to form a powerful oxidant and nitrosylating agent in the CNS, peroxynitrite.^{99, 100} Mitochondria appear to be a major target of ROS attack, and the immature brain is particularly susceptible to free radical injury because of its poorly developed scavenging systems and high availability of iron for the catalytic formation of hydroxyl radicals.¹⁰¹ Formation of ROS in the brain after various insults is respiration dependent,

mitochondria in vitro are sensitive to ROS and peroxynitrite, and most data suggest that oxidative stress contributes to the post-ischemic impairment of mitochondrial respiration.^{102, 103} When the ROS levels exceed the capacity of the cell in general and the mitochondria in particular to scavenge and render them harmless, the resulting oxidative stress may initiate mitochondrial permeability transition (mtPT),¹⁰⁴ which in turn potentiates the oxidative stress.¹⁰¹ The mitochondrial permeability transition dissipates the proton motive force, uncoupling oxidative phosphorylation, and causes mitochondrial swelling.¹⁰⁵ Rupture of the outer membrane allows the release of mitochondrial intermembrane proteins with the potential to activate the initial steps of apoptosis (Figure 4).^{101, 105}

Consistent with the notion that excessive NO in the neonate may be detrimental, Ferriero et al have shown that neuronal NOS (nNOS) knock-out mice are protected from neonatal HI-induced histopathological brain damage.¹⁰⁶ Continuing production of nitric oxide during the period after injury is probably important in the evolution of injury, and it was shown that doses of the nNOS inhibitor 7-nitroindazole (7-NI) that inhibited nNOS by more than 50% over 9-12 hours were more effective in reducing brain injury than transient inhibition.¹⁰⁷ After maternal administration in a rabbit model of intrauterine HI, selective nNOS inhibitors were found to distribute to fetal brain and inhibit nNOS activity in vivo.¹⁰⁸ There was a reduction of NO concentration in fetal brain, and dramatic reduction of deaths and the number of newborn kits exhibiting signs of CP.¹⁰⁸ One has to note, however, that NO generated by endothelial NOS (eNOS) plays an important role in maintaining blood flow and blood pressure.¹⁰⁹ Animals that lack the eNOS gene have enlarged cerebral infarcts after HI.¹¹⁰ Therefore, NO may play a dual role in HIE.

The free radical scavenging agent N-acetylcysteine (NAC) is able to cross the placenta;¹¹¹ it is considered safe during pregnancy and, therefore, of potential therapeutic value in humans, and has been shown to reduce oxidative stress and inflammation.¹¹² NAC has been shown to provide marked neuroprotection in a clinically relevant model of combined LPS/HI in neonatal rats.¹¹³ The protective effect of NAC was much more pronounced than that produced by another free radical scavenger, melatonin, when administered before and after LPS/HI.¹¹³ NAC was also effective when administered directly after HI¹¹³ (three days after LPS). In addition to reducing total tissue loss, NAC reduced white matter injury. The mechanism of NAC neuroprotection appears to be related to reduced oxidative stress, as indicated by lower levels of isoprostane and nitrotyrosine, preservation of the scavengers GSH and Trx2, attenuated activation of apoptotic proteases (caspase-3, calpain), and reduced inflammation as indicated by attenuated activation of microglia and caspase-1.¹¹³

Apoptotic mechanisms involved in hypoxia-ischemia

Multiple apoptotic pathways have been shown to be involved in neonatal hypoxic-ischemic cell death. As stated above, excitotoxicity, oxidative stress and other factors lead to injury of the mitochondrial membrane. Mitochondrial permeability transition plays an important role as an event that marks the point of no return in multiple pathways to cell death.¹⁰¹ The opening of the permeability transition pore (PTP) in the inner mitochondrial membrane, a process enhanced by cyclophilin D (CypD), is believed to be responsible for MPT in the adult brain.¹¹⁴ However, Wang et al recently demonstrated that in the developing brain, the proapoptotic Bcl-2 associated X protein (Bax) plays a more prominent role in MPT.¹¹⁴ It is thought that Bax plays a central role in regulating apoptosis in early development.¹¹⁵ MPT leads to release of a number of proapoptotic factors into the cytoplasm including cytochrome c, apoptosis inducing factor (AIF), caspase-9, and endonuclease G.¹¹⁶ Release of cytochrome c and procaspase-9 into the cytoplasm leads to activation of caspase-9 between 3 and 24 hours after the insult and is followed conversion of procaspase-3 to active caspase-3 between 6-48 h after injury.^{16, 117} Caspase-3 activation results in proteolysis of essential cellular proteins, including

cytoskeletal proteins and kinases, and can commit the cell to the morphological changes characteristic of apoptosis, including nuclear fragmentation.¹¹⁸ This cytochrome c mediated pathway is also referred to as the intrinsic pathway. Activated caspase-3 has been shown in human post-mortem brain tissue of full term neonates with severe perinatal asphyxia.¹¹⁹

A number of cell surface receptors respond to cytokine (inflammatory) stimulation, resulting in activation of cell death signaling programs.²³ The Tumor Necrosis Factor Receptor Superfamily (TNFRSF) belongs to this group of cytokine-responsive receptors.¹²⁰ Fas death receptor is one of the most extensively studied TNFRSF members.¹²¹ The apoptotic pathway that is regulated by Fas receptor involves caspase-8 and is referred to as the extrinsic pathway.¹²¹ Caspase-8 leads then to caspase-3 activation.¹²² Lack of a functional Fas death receptor is neuroprotective in adult models of HI.¹²³ Hypoxia-ischemia also activates Fas death receptor signaling in the neonatal brain.¹²⁴ Depending on the type of stimulus applied, a cell may undergo apoptosis, necrosis, or survival and proliferation in response to activation of the Fas death receptor.¹²¹ In addition to activation of the extrinsic caspase-directed apoptosis cascade in the presence of increased Fas ligand,¹²⁴ there is abundant evidence that the intrinsic apoptosis cascade is also activated following Fas death receptor signaling and functions to amplify Fas-mediated cell death.¹²¹ Inhibitors of both caspase-9 and caspase-8, given immediately after the hypoxic period in the neonatal rat HI model, result in long-term neuroprotection.^{125, 126}

A caspase-independent apoptotic pathway has also been extensively studied. Poly(ADP-ribose) polymerase (PARP-1) is a nuclear enzyme that transfers ADP ribose groups from NAD⁺ to nuclear proteins and facilitates DNA repair.¹²⁷ Mandir et al reported that PARP mediates neuronal cell death caused by NMDA but not non-NMDA excitotoxicity.¹²⁸ Ducrocq et al. found that the PARP inhibitor 3-aminobenzamide reduced infarct size in a neonatal model of focal ischemia.¹²⁹ DNA damage caused by hydrogen peroxide in PC12 cells also stimulated PARP-1, and 3-aminobenzamide decreased both apoptosis and necrosis in this model.¹³⁰ PARP activation consumes NAD⁺ needed for mitochondrial energy production, which in turn triggers release of cytochrome C and activation of caspases.¹²⁷ However, PARP's primary effect is to activate movement of apoptosis inducing factor (AIF) from mitochondria into the nucleus, a caspase-independent mechanism of cell death.¹²⁷ The translocation of AIF to the nucleus is preceded by increasing translocation of the pro-apoptotic bcl-2 family member Bid (BH3-interacting domain death agonist) to mitochondria, perinuclear accumulation of Bid-loaded mitochondria, and loss of mitochondrial membrane integrity.¹³¹ AIF translocation leads to release from the nucleus of molecular signals that impair mitochondrial function and ATP production.^{132, 133} Movement of AIF into the nucleus is greater in the immature brain than in the adult.¹³⁴ Recent reports also indicate that PARP-1 has important molecular roles beyond DNA repair, including regulation of chromatin structure and transcription.¹³⁵ PARP-1 represses transcription by DNA polymerase II at specific loci, but is released from chromatin when activated by NAD⁺, allowing de-repression.¹³⁵ These new data supplement older results indicating that PARP-1 can alter gene transcription by modifying histones and provide an explanation for its recently reported role in learning and memory.^{135, 136}

We made the unexpected observation that knocking out the *Parp1* gene in mice reduced brain damage from HI in 7-day-old male but not female mice.¹³⁷ We also found that NAD levels were significantly lower in males but not in female wildtype neonatal mice after HI.¹³⁷ We planned this experiment because PARP-1 is downstream in the NMDA activated excitotoxicity pathway, and it is activated by DNA strand breaks caused by nitric oxide (NO•) production.¹²⁸ Knocking out this gene in adult mice had been shown to protect the brain from damage from middle cerebral artery stroke,¹²⁷ and we wanted to determine if the protective effect was also present in neonates subjected to HI. McCullough et al confirmed our results on sexual dimorphism in adult mice.¹³⁸ The effect of sex had been missed in the initial report on *Parp1*

knockout on stroke in mice because only males had been used in those experiments.¹³⁸ McCullough et al found a more striking difference between males and females in adults than we found in neonates: males were protected, but injury in females was even greater in the Parp1 KO animals. They also showed that the effect of pharmacologic inhibition of nNOS was sexually dimorphic.¹³⁹ These gender differences are consistent with data from Li et al, who reported that inhibition of nNOS with 7-nitroindazole reduced injury from OGD in male but not female neurons in culture.¹⁴⁰ Male neurons also produced more nitrite and nitrate than female neurons in this study.

Du et al reported that the cell death in response to cytotoxic challenge proceeds by different cell death pathways in male and female rat neurons cultured separately.¹⁴¹ They reported that XY neurons are more sensitive to nitrosative stress and glutamate excitotoxicity, while XX neurons are more sensitive to etoposide and staurosporine, agents that activate caspase-dependent apoptosis. Their results showed that male neurons die predominantly through activation of an AIF-dependent pathway while female neurons preferentially release cytochrome c from mitochondria and die as a result of subsequent activation of caspase 3. Male neurons also had lower levels of glutathione following nitrosative stress than neurons from females. Our finding that Parp1 KO protects males, but not females, is consistent with the preferential activation of the NMDA→NO→PARP-1→AIF release in males.¹³⁷ Two recent papers confirmed that these sex-related differences in cell death pathways are present in neonatal mice and rats in vivo.^{142, 143} The first paper by Zhu studied 9-day-old mice subjected to HI.¹⁴² This paper reported that there was no difference in injury between males and females at 9 days, but there was greater translocation of AIF from mitochondria to nuclei in males and greater activation of caspase-3 in females. The second paper by Nijboer et al reported that HI in 7-day-old rat pups caused translocation of AIF only in males, and 2-iminobiotin treatment was neuroprotective and reduced cytochrome c release and caspase activation only in females.¹⁴³ These papers support the hypothesis that gender-specific cell death pathways are conserved across species in the perinatal brain. We have also shown that the glutamate antagonist dextromethorphan is protective against stroke in male but not female mice at 12 days of age.¹⁴⁴ This finding also supports the greater influence of the excitotoxic pathway in immature males.

This new information about gender differences in neuronal death pathways in experimental models is probably directly relevant to gender differences reported in the response of infants and children to brain injuries. The 2005 Surveillance of Cerebral Palsy in Europe (SCPE) study reported that male babies are at higher risk for cerebral palsy than females.¹⁴⁵ This finding agrees with the observation that arterial stroke and cerebral sinovenous thrombosis are more commonly diagnosed in boys than in girls in the neonatal period.^{146, 147} This observation is consistent with earlier data showing that the cognitive and motor outcome of brain injury is worse in male than in female low birth weight infants.¹⁴⁶ Quantitative imaging showed that male premature infants are more vulnerable than girls to white matter injury from intraventricular hemorrhage, but girls are more vulnerable to gray matter injury.¹⁴⁸ It follows directly from this information that an infant's sex could influence the efficacy of neuroprotective agents and the cell types most at risk. A striking example of this effect was reported from the prospective Indomethacin Intraventricular Hemorrhage (IVH) Prevention Trial.¹⁴⁹ In this study, indomethacin eliminated parenchymal hemorrhage and improved verbal scores in boys at ages 3 to 8 years, but had no effect on girls. As stated in the editorial that accompanied publication of this paper, "it is becoming increasingly clear that gender differences are not simply a result of hormonal influence but are profound properties of individual cells."

Mechanisms of hypothermic protection in hypoxia-ischemia

While a number of different agents have shown a neuroprotective effect in animal models of HI, hypothermia is the only therapeutic intervention that has been extensively investigated in the newborn patient population.^{8, 150-153} This clinical investigation was preceded by a large number of preclinical studies in multiple animal models that demonstrated a neuroprotective effect.^{90, 154-159} Clinical studies have shown an overall reduction in mortality accompanied by a reduction in disability among newborns with HIE that were enrolled in hypothermia protocols within the first day of life.⁹ Further supportive information comes from MRI studies that suggested both head cooling and total body cooling were associated with a reduced incidence of basal ganglia/thalamic brain lesions. To date, two large randomized controlled trials and one large pilot study have been completed evaluating hypothermia in infants with HIE. The multicenter Cool Cap Study involved 243 infants with moderate or severe encephalopathy and an abnormal amplitude-integrated EEG (aEEG), who were either cooled to a temperature of 34–35°C for 72 h or treated with temperature maintenance in normothermia range with conventional care.¹⁶⁰ The effect of head cooling for infants with the most severe aEEG changes was not protective; on the other hand, the effect of head cooling for infants with less severe aEEG changes was protective. The National Institute of Child Health and Human Development (NICHD) Neonatal Research Network trial evaluated 102 infants randomized to hypothermia with whole body cooling to 33.5°C for 72 h compared with 106 control infants randomized to conventional care.¹⁶¹ Death or moderate/severe disability at 18 months of age was noted in 44% of infants in the hypothermia group compared with 62% of infants in the control group. The mode of cooling used in each trial was different, and it is unknown if one cooling regimen (head cooling versus whole body hypothermia) is superior to the other. Current meta-analyses suggest that the only consistent adverse effects of hypothermia are clinically benign physiological sinus bradycardia and increased thrombocytopenia, and there seems to be a borderline increase in inotropic support but no increase in the incidence or degree of hypotension or other major adverse events.⁹ Eicher et al¹⁶² demonstrated an increase in intraventricular hemorrhage, but this finding was not confirmed in two other larger randomized trials. It is reassuring that in piglet studies where the cortex was cooled significantly (to <30°C), no cerebral hemorrhages were seen, and a recent retrospective case series suggests that hypothermia may be safe even down to rectal temperatures as low as 30°C.^{163, 164}

The remarkable neuroprotective effect of mild hypothermia against ischemic brain injury is likely attributed to its broad inhibitory actions on a variety of harmful cellular processes induced by HI. Among the proposed key mechanisms underlying hypothermic neuroprotection is the inhibition of intracellular signaling events that initiate the cell death cascade. Under injury conditions, hypothermia decreases loss of high-energy organic phosphates, slows the rates of metabolite consumption and lactic acid accumulation, and reduces oxygen consumption.^{163, 165} Hypothermia was originally thought to protect the brain by reducing cerebral metabolism during conditions of reduced substrate availability and increased anaerobic glycolysis. However, hypothermia slows but does not completely prevent the eventual depletion of ATP, and several other studies suggest that metabolism is not significantly altered despite remarkable neuroprotection.¹⁶⁶⁻¹⁶⁸ For example, rodents subjected to 20 minutes of forebrain ischemia showed marked protection, but brain levels of various metabolites were no different from normothermic ischemic controls. Thus, the influence of hypothermia on cerebral metabolism probably does not fully explain its protective effect. Mild hypothermia during ischemia diminishes oxidative DNA damage in the brain after severe focal ischemia and reperfusion in adult rats.⁵⁰ Furthermore, hypothermia inhibits the activation of apoptotic signaling pathways in the ischemic brain.¹⁵⁹ Hypothermia has also been shown to reduce inflammation triggered by ischemia.¹⁶⁹ Post-ischemic hypothermia reduces IL-18 expression and suppresses microglial activation accompanied by decreased loss of MAP-2 immunoreactivity (a marker of neuronal integrity).¹⁶⁹ In adult rats treated with hypothermia after HI, a reduction in volume

of infarct and neuronal loss was also associated with a marked reduction of astrocytosis and of TNF- α and IL-6 mRNA and protein levels in the ipsilateral hippocampus.⁹⁰ In summary, it is likely that hypothermia influences multiple pathways ultimately leading to neuroprotection.

Future directions

There has been significant research progress in hypoxic-ischemic encephalopathy over the last 2 decades, and many new molecular mechanisms have been identified. Despite all these advances, therapeutic interventions are still limited. As mentioned above, hypothermia is slowly becoming a promising approach to reduce the degree of injury and perhaps allow a longer window of opportunity to intervene. One of the difficulties with evaluation of therapeutics is the limited availability of surrogate markers for outcome as well as ethical dilemmas associated with clinical trials in newborns. But we are hopeful that there will be significant progress in this field soon. First, the addition of advanced magnetic resonance imaging methods has not only contributed to a better understanding of the pathological events but also allows quantitative monitoring of disease progression that may guide decision regarding interventions. In addition, recent advances in stem cell engineering may soon lead to cell-based interventions in HIE. Our group and others have demonstrated a protective effect of neural stem cell transplantation in rodents with neonatal HI and very recent reports have been published on similar protective effect by systemic injection of cord blood cells. There is currently an ongoing trial to assess the role of cord blood transplantation in infants with HI.

Finally, given the number of different pathways involved in HIE, it is likely that the best outcome will be achieved by a multi-modal therapeutic approach such as a combination of glutamate receptor antagonists with anti-oxidants and hypothermia.

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Figure 1.

Diffusion-Weighted Imaging (DWI) and T2-weighted imaging in a neonate with HIE 24 hours (A) and 3 days (B) after birth. DWI at 24h shows hyperintensities in the bilateral thalami and posterior limbs of the internal capsule (left greater than right), which are not recognized on T2-weighted images at the same levels. Repeat DWI at 3 days of life demonstrates diffuse hyperintensities in the bilateral basal ganglia and thalami as well as the internal capsule. Gyral enhancement consistent with cortical injury is observed in multiple areas, and is most prominent in the bilateral medial temporal and occipital lobes and less evident in the parasagittal frontal and parietal lobes. These changes are present but much less prominent on T2-weighted images at the same levels. (Modified from Takeoka M. et al, *Pediatr Neurol* 2002;26:274-281.)

Figure 2.

Proton Magnetic Resonance Spectroscopy in newborn infants after HI showing Choline (Cho), Creatine (Cr), N-Acetyl-Aspartate (NAA), Lactate (Lac). A) Spectra of a healthy newborn showing normal NAA to Creatine ratio, and minimum lactate double-peak. B) Newborn with moderate HIE, showing normal NAA, but elevated lactate double-peak. C) Newborn with severe HIE showing low NAA and markedly increased lactate. (Modified from Cheong JLY et al. *AJNR* 2006;27:1546-1554.)

Figure 3.

Necrosis-Apoptosis spectrum in neurons after HI in the neonatal rat. Nuclear changes in degenerating cells in cortex 48 hr after HI. Light microscopic photographs of 1 mm, Nissl-stained sections (A–C) and electron micrographs (A1–C1) are shown. An intermediate type of degenerating neurons was found, hybrid cells (arrow, A, A1) with large, chromatin clumps in the nucleus that were similar in size to those found in cells undergoing apoptosis but that were more irregular in shape. Typical necrotic neurons had smaller clumps of irregularly shaped, condensed chromatin (asterisk in A; arrows in B, B1). Necrotic neurons, which had a relatively homogeneous nucleus with a few irregular chromatin clumps and condensed granular cytoplasm (C, C1) and were typically found in adult ischemic models, were rarely identified in this model. Scale bars: A–C, 10 mm; A1, C1, 2 mm; B1, 1 mm. (Reprinted from *J Neuroscience*, November 1, 2000, 20(21):7994–8004)

Figure 4.
Cell death pathways involved in hypoxic-ischemic brain injury