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Preconditioning and Postinsult Therapies for Perinatal Hypoxic–Ischemic Injury at Term

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ABSTRACT

Perinatal hypoxic–ischemic encephalopathy can be a devastating complication of childbirth. Herein, the authors review the pathophysiology of hypoxic–ischemic encephalopathy and the current status of neuroprotective strategies to ameliorate the injury centering on four themes: (1) monitoring in the perinatal period, (2) rapid identification of affected neonates to allow timely institution of therapy, (3) preconditioning therapy (a therapeutic that reduces the brain vulnerability) before hypoxic–ischemic encephalopathy, and (4) prompt institution of postinsult therapies to ameliorate the evolving injury. Recent clinical trials have demonstrated the significant benefit for hypothermic therapy in the postnatal period; furthermore, there is accumulating preclinical evidence that adjunctive therapies can enhance hypothermic neuroprotection. Advances in the understanding of preconditioning may lead to the administration of neuroprotective agents earlier during childbirth. Although most of these neuroprotective strategies have not yet entered clinical practice, there is a significant hope that further developments will enhance hypothermic neuroprotection.

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MODERATE to severe hypoxic–ischemic encephalopathy (HIE) occurs at a rate of approximately 1–2 per 1,000 full-term live births,[1,2] with a total HIE incidence of three to five cases per 1,000 full-term live births.[3] The incidence is up to 10-fold higher in developing countries and globally, 23% of the 4 million annual neonatal deaths are attributed to birth asphyxia.[4] Perinatal asphyxia is believed to account for 10–20% of cases of cerebral palsy in term infants; however, certain subtypes of cerebral palsy such as dyskinetic cerebral palsy may have a higher incidence (up to 80%) of HIE etiology.[5] Neonates with a moderate HIE have a 10% risk of death and a 30% risk of disabilities with more subtle cognitive impairments potentially occurring with even greater frequency.[6]

Neuroprotective strategies to combat HIE are urgently required; these could include (1) improved monitoring in the perinatal period, (2) rapid identification of affected neonates to allow timely institution of therapy, (3) preconditioning therapy (a therapeutic that reduces the brain vulnerability) before HIE, and (4) prompt institution of postinsult therapies to ameliorate the evolving injury. This review will cover developments in these four themes of the neuroprotective strategy, focusing on potential interventions of the future following discussion of the pathogenesis of HIE.

Pathogenesis of Perinatal Hypoxic–Ischemic Neuronal Injury

In the nonpathogenic state, the central nervous system has a relatively high requirement for oxygen and glucose that is mostly metabolized by oxidative phosphorylation. In HIE, injury occurs to the areas of the human brain with a high metabolic rate and blood flow and a large number of excitatory glutamatergic neuronal synapses.[5] A rapid reduction in oxidative phosphorylation induces a primary energy failure in these neurons with subsequent neurotoxicity. The pattern of brain injury depends on both the gestational age of the infant and the intensity and duration of the hypoxia–ischemia (acute near total hypoxia–ischemia or chronic partial). With the help of magnetic resonance imaging studies in term infants with HIE, two main patterns have been described—basal ganglia/thalamus predominant (typically infants with an acute profound episode of hypoxia–ischemia who require significant resuscitation at birth) and watershed predominant pattern (typically infants with more prolonged partial hypoxia–ischemia who are less depressed at birth), although a mixed or atypical pattern may also occur.[7] A significant problem with developing generalized therapies is that the injury can vary significantly between afflicted individuals. Consistently, however, studies in term infants with perinatal asphyxia[8] and animals[9] employing magnetic resonance spectroscopy have defined a biphasic pattern of energy failure during and after a period of hypoxia–ischemia (fig. 1).

Primary Energy Failure and Excitotoxicity in Hypoxic–Ischemic Encephalopathy

Impaired neuronal energetics, secondary to hypoxia–ischemia, results in the dysregulation of ionic gradients in the brain. Energy depletion results in dysfunction of adenosine triphosphate-dependent ion channels and ion exchangers leading to cellular depolarization and the release of excitatory neurotransmitters such as glutamate (fig. 2).[10] Excess glutamatergic neurotransmission induces excitotoxic cell death (neuronal overexcitation leading to a cellular death); extracellular concentrations of glutamate can rise up to 10-fold. This excitotoxic injury is compounded by the failure of energy-dependent glutamate uptake mechanisms, which may even reverse, thereby exacerbating the excitatory load. Activation of postsynaptic glutamate receptors such as α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors and *N*-methyl-D-aspartate (NMDA) receptors mediates this injury, producing a transmembrane flux of sodium and calcium cations. NMDA receptors are abundant in early life (because of their role in the brain development, cell differentiation, axonal growth, and cell pruning)[11] meaning the immature brain is particularly vulnerable to excitotoxic injury.[12] In particular, the NR2B subunit containing NMDA receptors is prevalent,[11] and this may be important as NMDA receptors containing NR1/NR2B decay three to four times more slowly than NR1/NR2A receptors[13] and thus invoke greater cation movement.

Water passively follows the transmembrane flux of sodium and chloride, causing neuronal swelling and cerebral edema. The rise in intracellular calcium initiates a series of cytoplasmic and nuclear events that promote tissue damage. For example, overactivation of enzyme systems, such as proteases, lipases, and endonucleases, degrades cytoskeletal proteins and generates free radicals that damage the membranes, mitochondria, and DNA with ensuing cell death.[14] Understanding the pathogenesis of excitotoxicity has stimulated interest in NMDA antagonists as postinsult neuroprotectants. An alternate approach is to increase the inhibitory tone in the brain by activation of α_2 adrenoceptors. In adults, the γ-aminobutyric acid type A channels are powerful inhibitory receptors that may have a role in neuroprotection; however, in the immature brain, these channels are excitatory rather than inhibitory.[15] This occurs because the neuronal chloride importer NKCC1 is overexpressed on immature neurons, resulting in high intracellular chloride ion concentrations leading to chloride efflux with receptor activation. Therefore, it is unclear whether γ-aminobutyric acid type A channels agonists will prove useful neuroprotective agents for HIE, although NKCC1 inhibitors are being examined for this purpose (see Postinsult Therapies, Pharmacological Neuroprotection, and Antiepileptic Therapies).

Oxidative Injury in Hypoxic–Ischemic Encephalopathy

The neonatal brain seems particularly vulnerable to oxidative injury[14] because of immature scavenging mechanisms and a relative abundance of iron that acts as a catalyst for the formation of free radicals. Reactive oxygen and nitrogen species are produced that damage proteins, initiate mitochondrial stress, opening of the mitochondrial transition pore, and activate apoptotic pathways *via* release of mitochondrial proteins. Indeed, mitochondrial swelling and excess calcium are common after reperfusion in HIE models[16]; these changes are improved by the administration of NMDA antagonists.[17] NMDA receptor activation drives the production of nitric oxide through neuronal nitric oxide synthase (although inducible nitric oxide synthase is also constitutively expressed in the postnatal period).[14] Another free radical, superoxide, is produced predominantly by mitochondrial stress. Excess nitric oxide may also play a role in the production of superoxide by inhibiting electron transport chain function. Sub-

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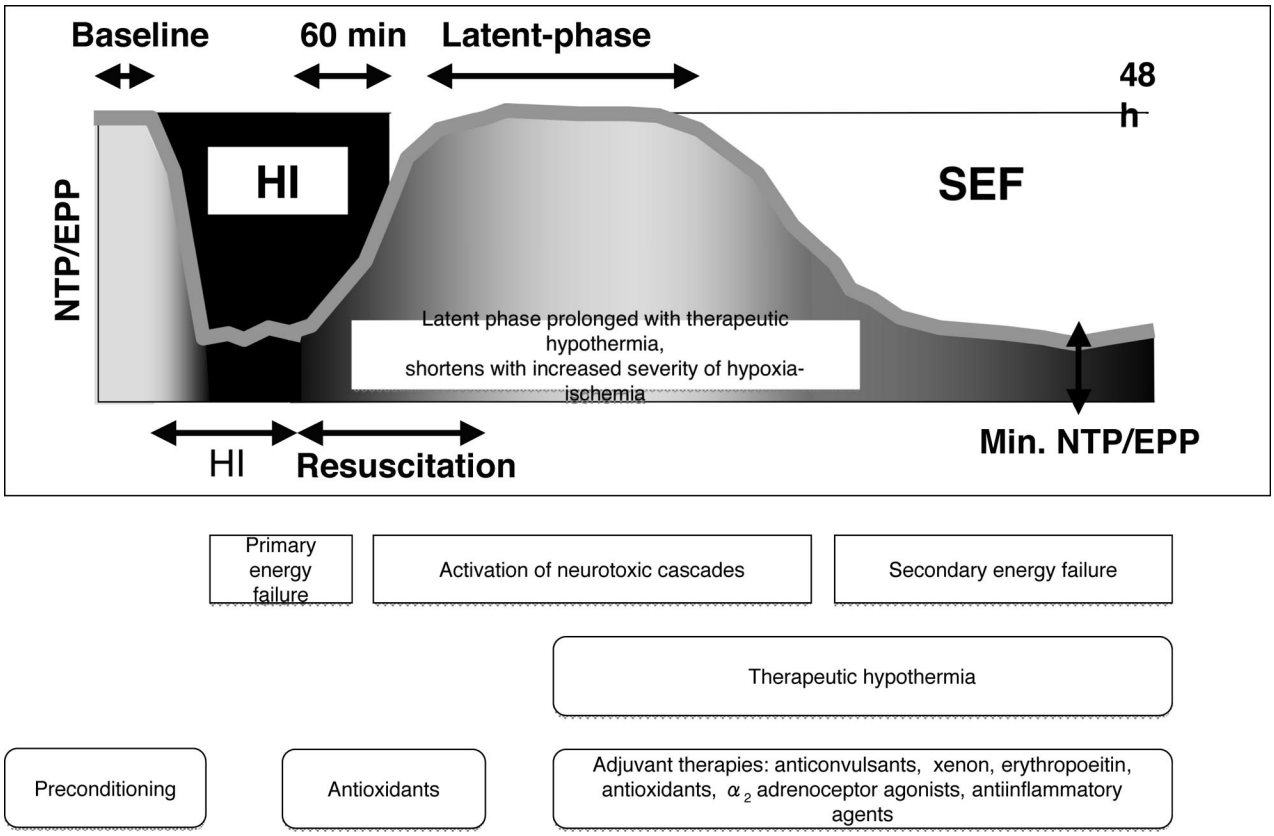

Fig. 1. Schematic diagram illustrating the biphasic pattern of energy failure associated with a transient hypoxic–ischemic (HI) insult visualized using phosphorus 31 magnetic resonance spectroscopy in the piglet model. The nucleotide triphosphate (NTP) concentration relative to the total high-energy exchangeable phosphate pool (EPP; EPP = Pi + PCr + NTP) is shown on the y-axis. The change in NTP/EPP during transient HI, resuscitation, the latent phase (period between the recovery from acute HI and the evolution of secondary energy failure [SEF]) and SEF itself is shown. During the acute energy depletion, some cells undergo primary cell death, the magnitude of which will depend on the severity and duration of HI. After perfusion, the initial hypoxia-induced cytotoxic edema and accumulation of excitatory amino acids typically resolve over 30–60 min with apparent recovery of cerebral oxidative metabolism (latent phase). It is believed that the neurotoxic cascade is largely inhibited during the latent phase and that this period provides a "therapeutic window" for therapies such as hypothermia and other agents. Cerebral oxidative metabolism may then secondarily deteriorate 6–15 h later (as SEF). This phase is marked by the onset of seizures, secondary cytotoxic edema, accumulation of cytokines, and mitochondrial failure. Mitochondrial failure is a key step leading to delayed cell death.

sequent reaction of nitric oxide with superoxide produces peroxynitrite that is particularly damaging to lipid membranes and proteins. A dearth of scavenging mechanisms, including glutathione peroxidase, means there is little to oppose the generation of further free radicals.[14] During reperfusion, hyperoxia may then compound the formation of free radicals, further increasing oxidative stress.[14,18]

Apoptotic Injury in Hypoxic–Ischemic Encephalopathy

The initial excitotoxic and oxidative injury accompanying primary energy failure is followed by a wave of programmed cell death or apoptosis (fig. 2).[19,20] During normal brain development, redundant neurons are deleted *via* apoptosis; this is an important physiologic process to ensure the formation of appropriate neuronal networks. However, after hypoxia–ischemia, this apoptotic component is pathologic, leading to excessive neuronal loss. Apoptosis may occur secondary to a loss of synaptic connectivity (because of the first wave of cell death killing an innervating cell), loss of trophic factor support, inflammatory activation of death receptors, and mitochondrial impairment related to excitotoxic/oxidative stress. This process involves the mitochondrial translocation of the proapoptotic protein Bax in the immature brain (rather than opening of the mitochondrial permeability transition pore as in the adult brain[21]) with subsequent mitochondrial release of cytochrome *C* and activation of aspartate-specific cysteine proteases or caspases. Apoptotic neuronal injury is particularly important in the very young[18,19] and evolves over time, taking hours to develop. It is likely that this form of injury could be a target for novel neuroprotective regimens.

A limitation of the use of some therapeutics for HIE, particularly anesthetics and antiepileptic agents, is that they

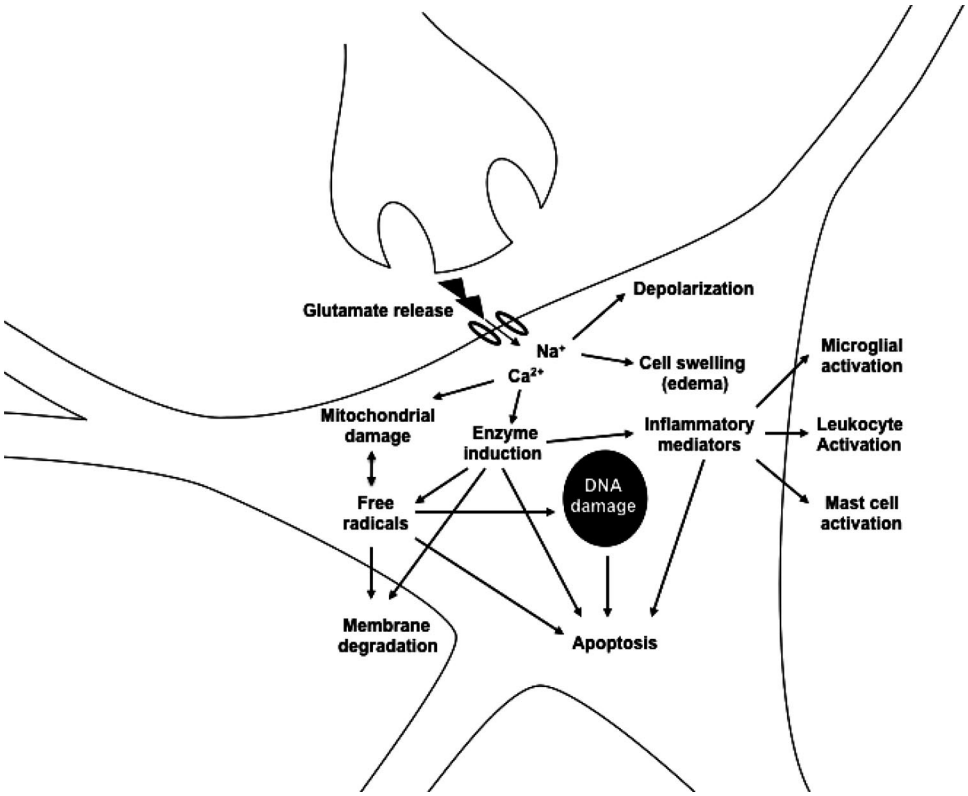

Fig. 2. Overview of the pathogenesis of hypoxic–ischemic brain injury. Excitotoxic brain injury after hypoxic–ischemia occurs because of overstimulation of neurons after excess glutamate release. Influx of sodium and calcium leads to cellular depolarization and swelling and activation of multiple injury cascades that lead to cell death. Critical mediators include the generation of free radicals and activation of enzymes that leads to membrane damage, inflammation, and apoptosis. Mitochondrial energy failure and calcium overload further contribute to the generation of free radicals and stimulation of apoptotic cascades through the release of cytochrome *C*.

themselves induce pathologic neuroapoptosis in the immature brain.[22–25] Indeed, we have recently discussed this "double-edged sword" of the use of anesthetics and antiepileptics for neuroprotection in the young.[25] However, the anesthetic (xenon) and antiepileptic (topiramate) do not induce neuroapoptotic toxicity[25] and provide synergistic neuroprotection with hypothermia in animal models of HIE and, thus, warrant further investigation as neuroprotectants for HIE.[26,27]

Role of inflammation in Hypoxic–Ischemic Encephalopathy

Maternal intrapartum fever of greater than 38°C persisting for greater than 1 h is a clinical indicator of chorioamnionitis, and although there is a surprisingly weak correlation between clinical and histologic chorioamnionitis, there is an increasing realization that perinatal infection is linked with later brain injury.[28,29] Intrapartum fever increases the risk of perinatal brain injury independent of infection,[30] and intrapartum fever was associated with a 4-fold increase in early onset neonatal seizures at term.[31] Infection exacerbates hypoxia–ischemia–induced central nervous system white matter injury, cerebral palsy, and increased blood-brain barrier permeability.[32] Inflammatory markers in amniotic fluid in women in preterm labor or in umbilical blood at birth have been associated with subsequent development of periventricular leukomalacia and cerebral palsy.[33] Animal models have shown that the administration of lipopolysaccharide (an inflammatory stimulus) before a hypoxic–ischemic insult increases subsequent neuronal injury[34] consistent with the view that the fetal inflammatory response seems to play a greater role than the maternal in the resultant injury.[32] Inflammation may contribute to increased levels of oxidative stress and apoptosis in the neonatal brain (fig. 2). However, interleukin-6 has also been reported as neuroprotective,[35] and thus, the consequences of the inflammatory milieu are complex. Further study of how infection and the inflammatory cascade impact on subsequent hypoxic–ischemic injury in the neonate is required.

Secondary energy failure

A second phase of injury starts to occur within 6 h of hypoxia–ischemia, characterized by another wave of cerebral energy failure with a decrease in the phosphocreatine to inorganic phosphate ratio (fig. 1). Studies in the newborn piglet using phosphorus 31 magnetic resonance spectroscopy suggest that the duration of the latent phase (period when the cerebral energetics appear normal) shortens with increasingly severe hypoxia–ischemia.[36] In children, the degree of the secondary

energy failure correlates with adverse neurologic outcome assessed at 1 and 4 yr,[37] intracellular brain alkalosis, increased lactate to creatine ratio, and more severe neurologic outcome.[38] Indeed, although during birth, fetal blood samples indicating acidosis are an indicator of impaired perfusion, postnatally intraneuronal alkalosis seems a particular problem. Intracellular alkalosis may exacerbate excitotoxic injury, mitochondrial permeability, protease activation, and apoptosis potentiating the ongoing pathology. As reperfusion proceeds, further inflammation and oxidative stress occur, potentiating the ongoing injury. The use of preconditioning strategies may allow the initial injury phase (primary energy failure) to be targeted. Difficulty in preempting the first phase of injury has led to strategies to combat this second phase of injury.

It is often during this secondary phase of energy failure that clinicians typically institute supportive therapy, including hemodynamic and ventilatory support and glycemic and temperature control. The need to avoid abnormal glycemic levels and in particular hypoglycemia has been recognized for some time[39] and is not considered further here. The avoidance of further hypoxia is clearly important; however, so is the avoidance of hyperoxia (discussed in the Postinsult Therapies, Avoiding Hyperoxia and the use of Antioxidants and Free Radical Scavengers) and hypocapnia (discussed in the Postinsult Therapies, Avoidance of Hypocapnia). All these factors can influence cerebral autoregulation and, therefore, blood supply to, and reperfusion of, ischemic areas. However, after HIE, the limited, available clinical evidence suggests that cerebral autoregulation is impaired[40,41] and that the cerebral circulation becomes dependent on arterial pressure to maintain adequate perfusion. This highlights the critical role of hemodynamic support of critically ill neonates. Nonetheless, further research is required to assess the effects of HIE on cerebral autoregulation.

Intrapartum Monitoring and the Diagnosis of Perinatal Hypoxic–Ischemic Encephalopathy

Antenatal screening identifies the risk factors that may predispose the fetus to central nervous system injury, and these include antenatally acquired infections, preeclampsia, and thyroid disease[42]; however, further research is required to define the relative risks for these conditions more precisely. Intrapartum the causal chain resulting in HIE is complex and far from completely understood. In a study of infants with HIE either referred or inborn at a tertiary referral center, 80% had morphologic injury consistent with an acute insult with no evidence of chronic injury or atrophy.[43] Certain intrapartum risk factors for HIE have been similarly identified such as maternal pyrexia and persistent occipitoposterior position.[29–30] Unfortunately, the contemporaneous detection of perinatal hypoxic–ischemic injury is hampered by the lack of monitors that can reliably provide the required specificity and sensitivity. The definition of perinatal asphyxia

Table 1. Criteria to Define an Acute Intrapartum Hypoxic Event as Sufficient to Cause Cerebral Palsy[44]

1	Evidence of metabolic acidosis in fetal umbilical cord arterial blood obtained at delivery (pH <7 and base deficit ≥ 12 mmol/l)
2	Early onset of severe or moderate neonatal encephalopathy in infants born at 34 or more weeks of gestation
3	Cerebral palsy of the spastic quadriplegic or dyskinetic type
4	Exclusion of other identifiable etiologies, such as trauma, coagulation disorders, infectious conditions, or genetic disorders

from a recent task force at the American Academy of Pediatrics and the American College of Obstetrics and Gynecology is a clinical situation of damaging acidemia, hypoxia, and metabolic acidosis with a sentinel event capable of interrupting oxygen supply to the fetus (table 1).[44] Accurate identification of this sentinel event is often problematic. The desire to identify the fetus at risk has led to increased rates of cardiotocography monitoring in Western countries; for example, 85% of live births in the United States in 2002 were monitored using cardiotocography.[45] The cardiotocography patterns of reduced fetal heart rate variability and moderate to severe variable or late decelerations have been shown to correlate with episodes of fetal acidemia.[46] However, cardiotocography suffers from large intraoperator and interoperator variability, and although it is a sensitive tool, it lacks specificity. Cardiotocography only has a positive predictive value of 0.2% for the prediction of cerebral palsy[47] and positive predictive value of approximately 2.6% for the prediction of HIE during standard practice.[48] Indeed, animal research suggests that the fetal heart rate patterns during ischemia are not predictive of neurologic outcome.[49] Meta-analysis of 13 randomized control trials by the Cochrane group concluded that continuous cardiotocography if combined with a fetal blood sampling reduced the incidence of neonatal seizures but had no effect on the incidence of cerebral palsy or perinatal death.[50] It should be noted that neonatal seizures are associated with neonatal cerebral infarction,[43] and therefore, this does represent an important advance in predicting perinatal brain injury. Another meta-analysis demonstrated that continuous use of cardiotocography (*vs.* intermittent ausculatation) was accompanied by a reduction in perinatal mortality.[51] Although current monitoring strategies have had an impact on perinatal outcome, the HIE incidence has not changed greatly in 50 yr, and further developments are required to improve the identification of a fetus developing HIE.

To reduce the high false-positive rates of cardiotocography, fetal blood sampling has been advocated with a fetal scalp pH of less than or equal to 7.21 and lactate greater than 4.2–4.8 mmol/l shown to enhance the detection of a compromised fetus.[52] Scalp lactate may be more successful than pH sampling because of the smaller volume of blood re-

quired.[53] However, these tests still suffer from high false-positive rates as a fetus may undergo transient episodes of asphyxia with no adverse consequences[54] (indeed, these episodes may prove protective as they may represent preconditioning of the fetus [explained in greater detail below in the Preconditioning section]). Recently, it has been proposed that an umbilical arterial pH less than 7.0 or a base deficit of 12 mM are appropriate levels for the risk of neonatal neurologic injury.[54] By using an umbilical arterial pH <7.0 to define intrapartum asphyxia, this condition was identified in 3.7 of 1,000 live-term births; 23% of these patients had abnormal neurology or died.

New fetal monitoring techniques may offer hope for the future, for example, fetal electrocardiogram ST segment monitoring and umbilical artery and middle cerebral artery Doppler velocity are examples of more recent developments.[50,55,56] Some support for the use of fetal ST waveform analysis as an adjunct to cardiotocography exists when a decision has been made to undertake continuous electronic fetal heart rate monitoring during labor. Fetal pulse oximetry monitoring in conjunction with cardiotocography has also been investigated but did not reduce the overall cesarean section rate, and thus, its further use has not yet been endorsed by the American College of Obstetrics and Gynecology.[45] Whether these monitors will obtain the evidence base required to change practice remains to be seen. At present, we lack a clinical tool to inform accurately when the fetus enters the decompensatory phase and needs to be delivered and/or should receive neuroprotective treatment.

Postpartum Assessment and the Diagnosis of Perinatal Hypoxic–Ischemic Encephalopathy

Rapid clinical assessment of the neonate that complements the information obtained from obstetric review is required to ensure prompt diagnosis and hence the initiation of optimal treatment. However, encephalopathy represents a syndrome with multiple possible presenting symptoms and signs,[1] and hypoxia–ischemia is not the only cause (other causes include trauma, infection, coagulopathies, and genetic disorders). The presence of acidosis (pH <7.0), Apgar 0 to 3 after 5 min, neurologic dysfunction, and multisystem dysfunction[57,58] are required for the term asphyxia. Because intrapartum hypoxia–ischemia is an evolving illness with worsening clinical signs after the first 12–24 h and a slow improvement after 4–5 days, encephalopathy scores usually peak on day 3–4. Most encephalopathy scores are based on the clinical criteria developed by Sarnat and Sarnat.[59] Recent modifications have been directed at developing quantifiable scores with good reproducibility.[1] Amplitude integrated electroencephalogram has been used as supporting evidence to aid enrollment into clinical trials for hypothermic neuroprotection because it has a positive predictive value of approximately 80% when used in infants with the clinical diagnosis.[60–62] The background activity on the amplitude integrated electroencephalogram is predictive of outcome as early as 3 and 6 h after birth in HIE.[62,63] Unfortunately, although there has been interest in the development of biomarkers of injury, in particular magnetic resonance biomarkers,[64] logistical factors currently preclude the use of this technology in the hours after birth to improve early assessment of the affected neonate.[65]

Preconditioning

There is a growing interest in harnessing endogenous neuroprotective mechanisms to optimize neuroprotection. Hypoxic preconditioning is a phenomenon in which brief nonlethal episodes of hypoxia confer protection against a subsequent sustained period of lethal hypoxia–ischemia. The ability of transient hypoxic episodes to prepare a fetus for a more severe neurologic insult in the peripartum period is of particular interest.[66] In an animal model, hypoxic preconditioning is induced by exposure to 8% oxygen for 3 h followed by a pathologic hypoxic–ischemic insult 24 h later; in this setting, long-term neuroprotection (up to 80% protection 8 weeks later), antiapoptotic effect, and improved functional recovery occur.[66] These findings have also been demonstrated with *in utero* ischemia of the fetus.[67] Hypoxia inducible factor is upregulated by this form of preconditioning leading to the downstream expression of neuroprotective factors such as erythopoeitin and vascular endothelial growth factor that combat oxidative stress, excitotoxicity, inflammation, and apoptosis and inducing increased vascular density in the brain (fig. 3).[68] Hypoxic preconditioning upregulates endogenous antioxidant and antiapoptotic defense mechanisms[69] and increases glycogen stores that aids the preservation of high energy phosphate stores during the subsequent insult.[70] As the mechanisms of hypoxic preconditioning are further unraveled (fig. 3), it is anticipated that pharmacologic agents can be developed to activate these cellular defense mechanisms to mimic hypoxic preconditioning. Indeed, possible targets include activators of adenosine or adenosine triphosphate-dependent potassium channels and alternate cell survival signaling pathways (fig. 3). An advantage of the use of preconditioning strategies to potentiate endogenous neuroprotective mechanisms before the insult is that they would not be reliant on rapid identification of those affected by HIE. Instead, provided an adequate safety profile is established, preconditioning agents could be administered to high-risk laboring women. Possible preconditioning agents that may activate similar pathways to hypoxia include desferroxiamine[71] or certain anesthetic agents.[72–74]

We tested whether the anesthetic gases, nitrous oxide, and xenon could precondition in a neonatal rat model of HIE. Both drugs block the NMDA receptor, and as NMDA antagonists can precondition *in vitro*, we sought to understand their effects *in vivo*.[72] Xenon reduced the neonatal brain injury and improved the animal neurology, whereas nitrous oxide lacked effect.[72] We have recently shown that xenon upregulates hypoxia-inducible factor activity and the pro-

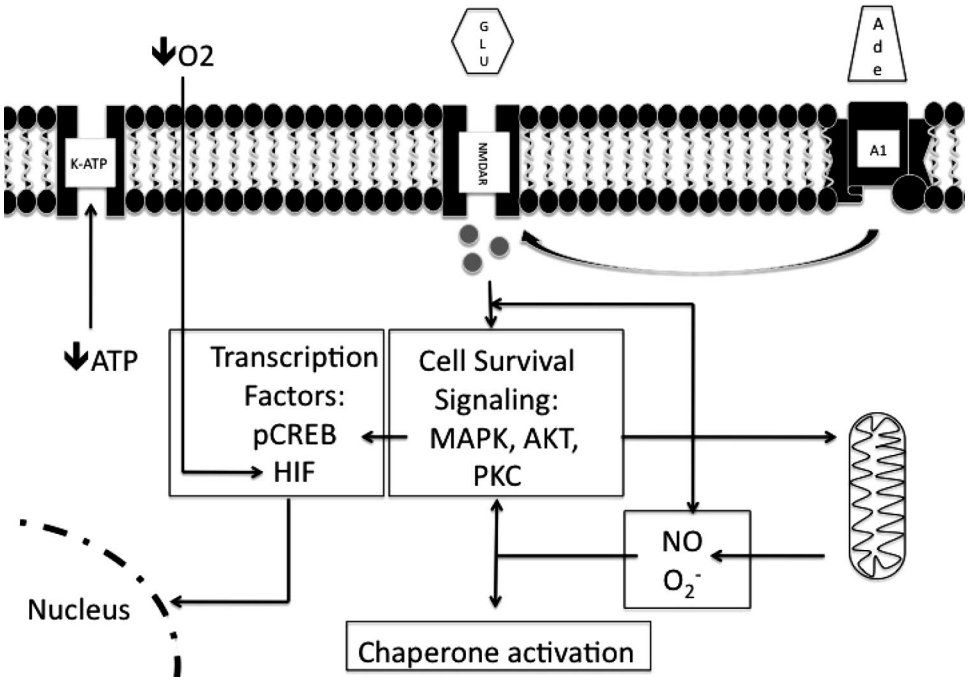

Fig. 3. Overview of the putative cellular targets of neuronal hypoxic preconditioning. Hypoxic preconditioning likely involves subinjurious stimulation occurring through pathways involved with hypoxic injury (fig. 2). The exact mechanisms of neuronal hypoxic preconditioning are slowly being unraveled; however, some of the known mediators are shown in the schematic mentioned earlier. Important receptor targets likely include ionotropic *N*-methyl-D-aspartate (NMDAR) glutamate (glu) receptors and adenosine triphosphate-dependent potassium channels (K-ATP) and G-protein-coupled adenosine (A1) receptors. Adenosine (A1) receptors (stimulated by adenosine [Ade]) likely act by reducing cellular activity during the subsequent insult. The downstream effectors distal to K-ATP signaling are unknown. Stimulation of excitatory receptors, exemplified by NMDA receptors, triggers the activation of cascades involved with cell survival and also produces reactive oxygen species (nitric oxide [NO] and superoxide [O_2^-]). The cell survival cascades include protein kinases such as mitogen-activated protein kinases (MAPK), protein kinase B (AKT), and protein kinase C (PKC; notably protein kinase Cε). The protein kinases can activate important transcription factors including phosphorylated cyclic-adenosine monophosphate response element-binding protein (pCREB). Hypoxia can also directly activate the hypoxia-inducible factor (HIF) by preventing HIF degradation allowing it to act as a transcription factor to upregulate effectors such as erythropoeitin and vascular endothelial growth factor. Finally, the activation of protein chaperones, including the heat shock proteins enhances protein stability and resists protein damage. This schematic does not portray all the targets of hypoxic preconditioning but conveys some of the important neuronal targets identified to date.

duction of erythropoietin[73] and activates adenosine triphosphate-dependent potassium channels,[75] indicating convergence on similar protective pathways whether initiated by hypoxia or xenon. Nonetheless, as NMDA antagonists can block hypoxic preconditioning in neurons[76] and have putative toxicity in the neonate,[22] clinical research is required to further understand the effects of these drugs on perinatal outcome.

Volatile anesthetics, such as isoflurane and sevoflurane, can also precondition the neonatal brain,[77,78] mimicking hypoxic preconditioning's dependence on the generation of nitric oxide (fig. 3). Because sevoflurane was proposed as an alternative to nitrous oxide for labor analgesia,[79] we have recently evaluated its preconditioning effects. Sevoflurane (1.5%) is able to precondition effectively against neonatal brain injury but had no effect at the approximate labor analgesia dose of 0.75% limiting its clinical application.[78] Interestingly, xenon (20%) and sevoflurane (0.75%) synergized in their ability to precondition in this model.[77] Xenon and sevoflurane may converge on the preconditioning pathway at different levels as xenon activates adenosine triphosphate-dependent potassium channels to induce preconditioning but sevoflurane does not.[75] This combination could not only provide analgesia for the mother but also precondition the fetus. Practically, this could provide a neuroprotective combination at a lower cost than xenon alone; however, further advances in delivery technology will be required before translation into clinical trials.

Recent data have suggested that antenatal magnesium sulfate reduces the rate of cerebral palsy in premature infants.[80,81] It is possible that antenatal magnesium administration may in part act in a preconditioning manner to decrease vulnerability of the preterm brain to subsequent injury. However, the translatability of these findings in the preterm infant to the term infant is unclear[82,83] because of the significant differences between the forms of brain injury. Indeed, there are conflicting reports from the animal literature regarding the neuroprotective efficacy of magnesium,

with lack of temperature control a frequent confound. Magnesium has recently been demonstrated to induce neurodegeneration in the neonatal rodent brain[84] similar to the observations with other NMDA antagonists.[22] Therefore, prophylactic magnesium therapy may expose many infants unnecessarily to side effects such as tocolysis with potentially prolonged labor, hypotension, and respiratory depression[1] and possible neurotoxic effects.[84] Although the neuroprotective properties of magnesium for term infants should not necessarily be dismissed, preliminary investigations for postinsult neuroprotection have not been encouraging.[2]

Postinsult Therapies

As HIE is often unpredictable, and currently preconditioning strategies are not clinically available, the primary approach has been to develop postinsult therapies to ameliorate ongoing or secondary injury. In this regard, hypothermia has proven clinically efficacious,[61,62,85,86] and subsequent strategies have focused on developing multimodal therapies that will augment hypothermic neuroprotection. We briefly review two important nonpharmacologic approaches to neuroprotection—therapeutic hypothermia and avoidance of hypocapnia—before reviewing future of pharmacologic neuroprotectants.

Hypothermic Neuroprotection

In the 1950s and 1960s, several uncontrolled case series were published in which infants not breathing spontaneously at 5 min after birth were immersed in cold water until respiration resumed and then were allowed to rewarm spontaneously.[87] In Switzerland, hypothermia was used as a standard resuscitation for newborns who showed no response to the usual method of resuscitation after 5 min.[88] However, hypothermia fell into disrepute after the recognition that even mild hypothermia was associated with increased oxygen requirements and greater mortality in premature newborns (<1,500 g)[89]; unfortunately, clinical trials of hypothermic neuroprotection were not performed at this point.

More recently, description of the biphasic pattern of energy failure observed in experimental models[9] (fig. 1) and experience in adult animal models[90] provided a basis for the realization that rescue treatment after hypoxia–ischemia might reverse or ameliorate secondary energy failure. Experimental studies using moderate hypothermia neuroprotection[91] were followed by safety studies in the newborn. Subsequent trials and meta-analyses have shown efficacy of therapeutic hypothermia in reducing death and severe disability,[61,62,85,86,92] leading to therapeutic hypothermia becoming established as a standard of care for HIE.[93]

The three largest trials had similar entry criteria, consisting of evidence of birth asphyxia and moderate or severe encephalopathy and in addition in the CoolCap and Total Body Hypothermia for Neonatal Encephalopathy (TOBY) trials abnormal amplitude integrated electroencephalogram.[61,62,86] Infants were term (at least 36 weeks gestation) and were randomized by 6 h of age. Hypothermia was maintained for 72 h, by circulating cooling fluid in a cap with a target rectal temperature of 34.5°C in the CoolCap trial, whereas in the Total Body Hypothermia for Neonatal Encephalopathy and National Institute of Child Health and Human Development Neonatal Research Network trials, whole body cooling to 33.5°C was induced by cooling blankets placed under the infants. Although each trial showed a reduction in the risk ratio for death and disability, this was statistically significant only in the National Institute of Child Health and Human Development Neonatal Research Network trial. A composite endpoint was chosen in all three trials because of concerns that cooling might increase survival with additional disability; however, this proved not to be the case. Indeed, the largest and most recent trial, the Total Body Hypothermia for Neonatal Encephalopathy trial, demonstrated a reduction in the number of children with cerebral palsy and improved more subtle cognitive and motor impairments in survivors.[62] Overall, hypothermia improves neurologic outcome in survivors without altering mortality from this devastating condition.[61,62,85,86,92] Consistent with this, neuroradiologic evidence shows that hypothermia reduces the incidence of thalamic, basal ganglia, internal capsule, and white matter lesions secondary to HIE, providing a morphologic correlate for the functional improvement.[94,95]

There are a number of possible mechanisms by which mild hypothermia may be neuroprotective after hypoxia–ischemia in the developing brain. Hypothermia reduces the metabolic rate (4–7% for a 1°C drop), decreases the release of glutamate and other excitotoxic neurotransmitters,[96] attenuates the activity of NMDA receptors,[97] reduces the production of nitric oxide and oxygen free radicals,[98] inhibits apoptosis[99] (fig. 2), and contributes to a reduction in intracranial pressure.[99] Although mild to moderate hypothermia seems to be well tolerated in experimental models and human studies,[61,62,85,86,92] there are some potentially deleterious effects that include infection, cardiac suppression, coagulopathy, arrhythmias, reduced cerebral blood flow, increased thermogenesis, and increased blood viscosity. There is likely to be a threshold temperature below which the adverse effects outweigh the beneficial effects, and further work is needed to define the optimal temperature for neuroprotection.[99]

Interestingly, adequate sedation was required to realize the benefit of hypothermic neuroprotection in one large animal model[100]; however, details about the sedative therapies used in the hypothermia trials were not reported in detail. It is likely that different sedatives will interact dissimilarly with hypothermic neuroprotection; for example, addition of methohexital to hypothermic neuroprotection showed no additional protective benefit over hypothermia alone[101] in an animal model of focal ischemia. This is in contrast to other agents such as xenon,[26] topiramate,[27] or *N*-acetylcysteine[102] that provide synergistic neuroprotection with hypothermia. Therefore, detailing which sedative agents can augment hypothermic neuroprotection has critical importance.

Avoidance of Hypocapnia

Hypocapnia has also been associated with neonatal brain damage in several observational studies,[103] although these were heterogeneous in design, the underlying conclusion remains that hypocapnia is associated with poor neurologic outcome. Hypocapnic ventilation of neonatal piglets causes perturbation of cellular energetics and apoptosis[104] that may be related to vasoconstriction and reduced tissue oxygen delivery. Hypocapnia in human infants has also been associated with a slower electroencephalogram signal and increased cerebral oxygen extraction that may reflect hypocapnic vasoconstriction.[105] During hypoxia–ischemia, hypocapnia is also associated with a detrimental effect on cerebral energetics with a reduced phosphocreatine and adenosine triphosphate relative to normocapnia or hypercapnia in the neonatal rat.[106] During reperfusion from neonatal HIE, intracellular neuronal alkalosis is associated with adverse long-term neurologic outcome,[38] and thus, hypocapnia during this phase could also further disturb local acid–base imbalance. The importance of intracellular pH during reperfusion has led to the identification of novel neuroprotective strategies such as the use of the Na^+/H^+ exchange inhibitor amiloride, to ameliorate reperfusion injury.[107] Hypocapnia should be avoided in the very young in the absence of a therapeutic indication such as raised intracranial pressure or neonatal pulmonary vascular resistance. As hypocapnia may easily occur during resuscitation (which of course may be necessary) but also during transfer from delivery suite to neonatal intensive care medical staff must be mindful to avoid overventilation of the neonate.

Pharmacologic Neuroprotection

The optimal regimen to improve neuroprotection during hypothermic therapy has not been addressed formally. The antiepileptic drug, topiramate, shows promise in this regard as it synergizes with hypothermic neuroprotection,[27] similar to xenon[26] and *N*-acetylcysteine.[102] Preclinical evidence suggests that some classes of sedative drug may be particularly effective; notable in this category are agents which antagonize the NMDA receptor or activate the α_2-adrenoceptor. NMDA receptor antagonists do, however, induce widespread apoptosis in the immature brain that may hamper their use.[22] Opioid sedation is typically used in neonatal intensive care units despite evidence that opioids have been shown to worsen hypoxic–ischemic injury in adult animal models,[108] and opioid antagonists have been investigated as neuroprotective agents.[109] Whether immature animals are similarly vulnerable to opioid-induced potentiation of hypoxic–ischemic injury warrants investigation. Supplementation of opioid sedation with benzodiazepines has also been suggested; however, midazolam administration has been associated with worse neurologic outcomes in preterm neonates.[110] Given concerns over the neurotoxicity of benzodiazepines,[24,25] investigation of whether this also pertains to term neonates should be undertaken. Alternate sedative regimens may be useful in neonates stricken by HIE. Other neuroprotective adjuncts to potentiate hypothermia may include antioxidants and antiinflammatory therapies.

Unfortunately, most neuroprotective agents tested so far have been ineffective. Mannitol therapy has not proven successful in clinical[2] or preclinical studies.[111] Calcium channel blockers are associated with decreased cerebral flow and are similarly not recommended for the treatment of perinatal HIE.[2] Dexamethasone is not recommended as it reduces cerebral perfusion pressure in line with its ability to reduce intracranial pressure.[2,112] Furthermore, the increase in intracranial pressure observed in HIE may be an epiphenomenon rather than a mechanism of injury.

Antiepileptic Therapy

Data from both animal and human studies suggest that seizures amplify neonatal hypoxic–ischemic brain damage.[113,114] In a recent study of newborns with HIE where magnetic resonance spectroscopy was used to assess tissue metabolic integrity, the severity of seizures was independently associated with brain injury.[114,115] These results provide some support for the hypothesis that effective treatment of neonatal seizures could attenuate brain injury. Barbiturates are often used in the treatment of neonatal seizures; however, it is unclear whether their antiseizure actions translate into neuroprotection. Three small clinical trials have investigated the potential role of barbiturates to ameliorate brain injury severity, but only one showed a relative risk reduction of severe developmental disability or death.[116] However, 23% patients were lost to follow-up in this trial. Two other trials[117,118] did not find thiopentone or pentobarbitone effective. Subsequent meta-analysis of the studies ($n = 77$) showed no significant effect on death or severe neurodevelopmental disability.[2] As avoidance of hypotension is desirable in the asphyxiated infant, current evidence does not support the use of prophylactic barbiturates for perinatal neuroprotection. Barbiturates still have a role in the treatment of seizures, and further study is required to investigate whether they possess neuroprotective efficacy. Similar to NMDA antagonists, there is ongoing concern that in experimental studies of rodents conventional antiepileptic drugs, including phenobarbitol, phenytoin, and diazepam, caused apoptotic neurodegeneration at plasma concentrations relevant for seizure control in human neonates.[23]

The antiepileptic, topiramate (an antagonist of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors), improves neurologic function and decreases preoligodendrocyte death, apoptosis, microglial activation, and seizure activity in animal models.[119] Unlike other antiepileptics, topiramate seems to be nontoxic.[120] Postinsult neuroprotection has also been noted after hypoxic–ischemic injury in piglets.[121] Perhaps of most significance is the discovery that topiramate potentiates hypothermic neuroprotection in rats.[27]

Recent studies have also demonstrated the neuroprotective efficacy of the NKCC1 blocker bumetanide, alone or in conjunction with phenobarbitol.[122] Further combi-

nation studies looking at the combination of prophylactic antiepileptic administration and therapeutic hypothermia are required.

α_2-Adrenoceptor Agonists

α_2-Adrenoceptor agonists, including clonidine and dexmedetomidine, have been shown to have neuroprotective potential in animal models of HIE.[123–125] Both agents reduced the size of excitotoxin-induced cortical and white matter lesions in mouse pups injected intracerebrally with ibotenate[123]; these protective effects were abolished by an α_2 antagonist confirming that these agents protect through their activity at the α_2-adrenoceptor. Clonidine has been shown to improve the outcome in preterm fetal sheep when given after the hypoxic–ischemic insult (started 15 min after a 25-min umbilical cord occlusion and continued for 4 h).[124] Interestingly, only low-dose clonidine ($10\ \mu g \cdot kg^{-1} \cdot h^{-1}$) and not the high dose ($100\ \mu g \cdot kg^{-1} \cdot h^{-1}$) was protective. This may relate to the poor α_2:α_1 selectivity ratio of clonidine resulting in loss of effect of the drug at the higher dose.

The more selective α_2-adrenoceptor agonist of the two agents, dexmedetomidine, dose-dependently reduces neuronal injury *in vitro* and *in vivo* in a neonatal asphyxia rat model.[125] Dexmedetomidine, administered during the asphyxia, improved the neuromotor function when assessed 30 days later. Whether dexmedetomidine can exert neuroprotection when provided after the onset of injury is not known. However, as dexmedetomidine can target different aspects of ongoing injury, including excitotoxicity, inflammation, and apoptosis, it has the potential to be beneficial even when delivered after the initial hypoxic–ischemic insult. It should also be noted that a synergistic interaction between dexmedetomidine and the NMDA antagonist, xenon, has been noted in this model,[126] although any possible neuroprotective interaction between dexmedetomidine and hypothermia has not been investigated. However as dexmedetomidine is the more selective agent (compared with clonidine), it seems prudent to pursue this agent as the α_2-adrenoceptor agonist of choice for neuroprotection.

NMDA Receptor Antagonists

The central role of the NMDA receptor in excitotoxic injury makes it a prominent target for neuroprotective strategies (fig. 2). Indeed, the neonatal brain seems particularly vulnerable to excitotoxicity,[12] and NMDA receptor expression is upregulated after HIE[127] (in contrast to reduced γ-aminobutyric acid type A receptor expression[128]). Therefore, the application of NMDA antagonists during and after the insult seems a promising therapeutic strategy.[26,129–131] As discussed earlier (in Preconditioning), antenatal magnesium therapy has shown potential to reduce cerebral palsy when given before preterm labor.[80–81] Yet, although magnesium can act to block the NMDA receptor, there is a lack of evidence to support it as a postinsult neuroprotectant.[82,83] Other NMDA antagonists in current clinical use include the anesthetics, nitrous oxide and ketamine, that are both used in obstetric practice. Interestingly, their neuroprotective capabilities are variably reported.[132] NMDA antagonists have been associated recently with neurotoxicity (apoptotic neurodegeneration) in the very young,[22,25] and thus, despite having therapeutic properties for areas of hypoxic–ischemic injury, they may also injure the developing brain. This has led to concern both to their application for neuroprotection in the neonatal brain and their use in obstetric and pediatric anesthesia.[25]

Xenon, also an NMDA antagonist, does not produce significant apoptotic neurodegeneration in the young[133] but provides neuroprotection in several adult animal models of neuronal injury.[117] Furthermore, xenon attenuates hypoxic–ischemic neuronal damage in animal models of HIE *in vivo* and *in vitro* at concentrations of 40% and greater, and thus, xenon offers neuroprotection at subanesthetic concentrations.[26] Xenon attenuated hypoxic–ischemic damage, including apoptosis, when given up to 6 h after the injury and provided synergistic neuroprotection when given postinjury in combination with hypothermia (35°C). Remarkably, this synergistic interaction still occurs when the administration of hypothermia and xenon occurs asynchronously.[134] The neuroprotection observed correlated with improved neuromotor function at 30 days of age, indicating long-term functional protection.[26] Xenon and hypothermic therapy may converge on an antiapoptotic pathway accounting for why asynchronous application of the interventions attenuates the injury. This asynchronous administration of xenon and hypothermia could be used in a clinical context with hypothermia being instituted soon after delivery before transfer to a tertiary center for subsequent xenon therapy that requires specialized administration apparatus. Indeed, in the piglet model, therapeutic hypothermia doubles the duration of the therapeutic window for adjunctive therapies.[135] Consistent with this, we have demonstrated recently that xenon augments hypothermic neuroprotection in the piglet model improving cerebral magnetic resonance biomarkers of injury and histology.[136] This supports transition to a clinical trial (TOBYXe; NCT00934700), where the efficacy of the combination of hypothermia and xenon will be tested against cerebral magnetic resonance biomarkers and clinical outcomes.

Erythropoietin

Erythropoietin exerts trophic properties to promote neurogenesis and differentiation in the brain and induce proangiogenic effects *via* downstream effectors such as vascular endothelial growth factor. Erythropoietin also acts to inhibit oxidative stress, excitotoxicity, inflammation, and apoptosis.[137] Interestingly, erythropoietin has been reported to be upregulated in the umbilical cord blood from babies who have suffered perinatal asphyxia.[138] This may represent a defense mechanism as erythropoietin is neuroprotective when given after hypoxic–ischemic injury providing long-

term neuroprotection in preclinical models.[139,140] Research probing any interaction with hypothermic neuroprotection is required before progression to clinical trials of combined therapy. Indeed, further clinical safety data are also required as erythropoietin has poor penetration (<2%) of the blood-brain barrier, and therefore, in animal studies, large doses are required to transduce a neuroprotective effect.[141] Concerns over the use of high doses of erythropoietin include possible thrombotic and hematologic complications. Nonetheless, a randomized controlled trial has been published recently of erythropoietin therapy for HIE.[142] Despite only enrolling 167 term infants (with 9 lost to follow-up), erythropoietin treatment reduced the risk of death or disability at 18 months.[142] Two relatively low doses of erythropoietin were administered (300 or 500 UU/kg) without hematopoietic complications. Whether the higher doses can further improve the outcomes without inducing complications is unknown but is of distinct interest. Another approach is to upregulate endogenous erythropoietin; for example, xenon upregulates erythropoietin expression[73] and easily crosses the blood-brain barrier providing an alternative mechanism to harness erythropoietin protection in the central nervous system.

Avoiding Hyperoxia and the Use of Antioxidants and Free Radical Scavengers

Free radical–induced cellular damage contributes significantly to HIE, partly, because of the relative deficiency of endogenous antioxidants.[3,14] This reasoning prompted clinical trials analyzing the safety of neonatal resuscitation with air rather than oxygen. Hyperoxia induced by ventilation with 100% oxygen is associated with reduced cerebral blood flow,[143] production of free radicals such as hydrogen peroxide,[18] increased inflammation, and neuronal apoptosis[144] compounding concerns over hyperoxia-stimulated retrolental fibroplasia. Although no individual trial has shown difference in mortality with resuscitation with air rather than oxygen, in 2005, a meta-analysis found that resuscitation with air is associated with a reduction in mortality[145] (relative risk, 0.71; 95% CI, 0.54–0.94; numbers needed to treat = 20). Further meta-analysis supports this finding with a mortality benefit apparent within the first week and from 1 month onward, suggesting that it is not just short-term mortality that is affected.[146] Saugstad *et al.*[147] in their meta-analysis concluded that the relative risk was improved to a greater extent in the studies with stricter randomization protocols (relative risk, 0.32; 95% CI, 0.12–0.84). A trend toward moderate to severe HIE reduction was also seen (relative risk, 0.88; 95% CI, 0.12–0.84). At present, there are insufficient data to determine whether air resuscitation may reduce neurodevelopment delay and cerebral palsy. Although the finding of a mortality difference is remarkable, it is biologically plausible and, therefore, requires further evaluation. Further trials of other oxygen concentrations are also warranted as are trials designed at specific subgroups that may require higher oxygen concentrations such as severe asphyxia or sepsis. Furthermore, for resuscitation of babies from mothers who have recently used nitrous oxide for labor analgesia or after cesarean section conducted under general anesthesia with high concentrations of nitrous oxide it may be prudent initially to use higher concentrations of oxygen during neonatal resuscitation to avoid any nitrous oxide-induced diffusion hypoxia in the immediate postpartum period.[148]

Other strategies to reduce free radical generation include the use of xanthine oxidase inhibitors that have shown protection of cerebral energetics when administered early during the reperfusion phase but did not attenuate brain morphologic damage or markers of apoptosis.[149] However, in a further study, allopurinol did provide histologic neuroprotection.[150] Although allopurinol reduced circulating concentrations of free radicals in human neonates with HIE (using reduced malondialdehyde level as a marker of lipid peroxidation),[151] early results from one randomized controlled trial in humans were not promising[152]; however, an ongoing trial in The Netherlands, and a recent report, based on the reduction of the putative brain injury biomarker, S-100ß, have suggested more promise with maternal allopurinol therapy.[153]

Melatonin (*N*-acetyl-5-methoxytryptamine) is a natural neuroprotectant produced in the pineal gland, retina, and gastrointestinal tract; exogenously administered melatonin crosses the blood-brain barrier and acts as a potent free radical scavenger and antioxidant. In adult animal models, melatonin provides neuroprotection when administered before[154] and after hypoxia–ischemia.[155] In mice, delayed melatonin treatment reduced both gray and white matter damage and improved neurobehavioral outcome after transient focal cerebral hypoxia–ischemia[156] and prevented excitotoxic white matter lesions in newborn mice.[157] Further preclinical and clinical studies are under way to elucidate whether melatonin can play a role as a neuroprotective agent for HIE and whether it can enhance hypothermic neuroprotection.

N-Acetylcysteine is a widely used free radical scavenger and has shown utility in animal models of HIE. Notably *N*-acetylcysteine provided superior protection to melatonin with evidence for better antioxidant, antiinflammatory, and antiapoptotic effect in a rat model of lipopolysaccharide sensitized perinatal hypoxic–ischemic injury.[158] *N*-Acetylcysteine (200 mg/kg) given before and after hypoxic–ischemic injury reduced brain injury by up to 78%, whereas postinsult therapy alone reduced the injury by 41%. As both inflammation[159] and hypoxic–ischemic brain injury[160] increase blood-brain barrier permeability, it is possible that improved penetration of some neuroprotectants such as N-acetylcysteine may occur, enhancing their therapeutic potential. Nonetheless, *N*-acetylcysteine has to be administered in high doses if given systemically to overcome the limited passage across the blood-brain barrier. *N*-Acetylcysteine (50 mg/kg) has also been shown to augment hypothermic neuroprotection in one small preclinical study of HIE; however, it was inef-

fective when tested alone.[102] Further evaluation of antioxidant combination with hypothermia is required.

Antiinflammatory Agents

As described earlier, inflammation is believed to potentiate hypoxic–ischemic injury in the brain (fig. 2), explaining why maternal infection predisposes to worse outcomes from hypoxic–ischemic injury in the neonate.[30,42] This has been demonstrated in multiple animal models with the critical role of microglial activation and release of inflammatory cytokines such as tumor necrosis factor α, interleukin-1β, and interleukin-6 noticed in these settings. Caspase-1 activation of interleukin-1β and interleukin-18 (expressed in activated microglia) are important mediators of the injury as caspase-1[161] or interleukin-18[162] gene deficiency reduces injury and the interleukin-1 receptor antagonist offers protection also in the immature brain.[163] Reducing microglial activation with immune modulators, such as the tetracycline derivative, minocycline, has shown promise in multiple animal models and is under investigation as a therapeutic in adult stroke. Unfortunately, a lack of consistency in neonatal animal models has occurred with both inefficacy and increased toxicity observed.[3]

Summary

Defining the optimal strategy for perinatal neuroprotection has a potential to improve significantly the neurocognitive outcome after asphyxial injury. Further advances in the identification of the "at risk" fetus and neonate are required. In parallel, investigation of safe preconditioning strategies should continue in an attempt to improve the perinatal outcomes. Postinsult treatment should concentrate on augmenting hypothermic neuroprotection *via* the application of adjunctive agents. In this regard, combining hypothermia with xenon (that targets NMDA receptors) and topiramate (that targets α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors) may be useful. The incorporation of α_2-adrenoceptor agonists, melatonin, and *N*-acetylcysteine, which act through defined and different mechanisms, may also be of use. Significant preclinical advances in the development of neuroprotective strategies are occurring and with further studies addressing their efficacy in different animal models clinical trials could follow in the near future.

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