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Synergistic neuroprotective therapies with hypothermia

Maria Roberta Cilio^{a,b} and Donna M. Ferriero^{a,*}

^aNewborn Brain Research Institute, University of California, San Francisco, California, USA

^bDivision of Neurology, Bambino Gesù Children's Hospital, Rome, Italy

summary

Neuroprotection is a major health care priority, given the enormous burden of human suffering and financial cost caused by perinatal brain damage. With the advent of hypothermia as therapy for term hypoxic–ischemic encephalopathy, there is hope for repair and protection of the brain after a profound neonatal insult. However, it is clear from the published clinical trials and animal studies that hypothermia alone will not provide complete protection or stimulate the repair that is necessary for normal neurodevelopmental outcome. This review critically discusses drugs used to treat seizures after hypoxia–ischemia in the neonate with attention to evidence of possible synergies for therapy. In addition, other agents such as xenon, *N*-acetylcysteine, erythropoietin, melatonin and cannabinoids are discussed as future potential therapeutic agents that might augment protection from hypothermia. Finally, compounds that might damage the developing brain or counteract the neuroprotective effects of hypothermia are discussed.

Keywords

Anticonvulsants; Hypoxia; Ischemia; Neuroprotection; Repair; Seizures

1. Introduction

Perinatal hypoxic–ischemic encephalopathy (HIE) is associated with high morbidity and mortality rates worldwide.^{1,2} Treatment and care for the sequelae of early brain hypoxic–ischemic injury imposes considerable financial and lifelong personal burdens on society and affected families. Thus, there is an urgent need to improve outcomes in these affected infants. Fortunately, promising neuroprotective strategies are emerging.

Hypothermia is rapidly becoming standard therapy for full-term neonates with moderate-to-severe HIE.^{3,4} Recent clinical trials in neonates have demonstrated that induced moderate hypothermia reduces the combined outcome of mortality and long-term neurodevelopmental disability at 12–24 months of age.^{5–7} Aside from hypothermia, no established therapies exist. Moreover, hypothermia does not completely protect an injured brain, and there is some evidence that neonates with the most severe forms of hypoxic–ischemic injury may not be able to be rescued.^{5–7} In experimental studies, clinical factors that have been shown to influence the neuroprotective efficacy of hypothermia include depth, duration, and time of onset of cooling⁸; trials suggest that birth size and degree of encephalopathy may be important.^{9–11}

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*Corresponding author. University of California San Francisco, Department of Neurology, Box 0663, 521 Parnassus Avenue C215, San Francisco, CA 94143-0663, USA. Tel.: +1 415 502 1099; fax: +1 415 502 5821. donna.ferriero@ucsf.edu (D.M. Ferriero).

Conflict of interest statement

None declared.

Drugs added during or after hypothermia that can improve neuroprotection, by extending the therapeutic window or providing long-lasting additive or synergistic protection, are needed. On the other hand, it is important to consider that drugs administered during the neonatal period may be toxic to the immature brain. Excretion of many drugs and their metabolites can be modified by hypothermia, and thus failure of liver and kidney clearance due to hypoxic–ischemic injury could exacerbate any toxicity.¹²

2. Seizures in the setting of HIE

Seizures are commonly associated with HIE. Although the majority of cases are controlled with first- or second-line therapy, many infants develop status epilepticus, requiring multiple anticonvulsants.¹³ Therefore, antiepileptic drugs (AEDs) are among the medications most commonly used in neonates with HIE. The mechanisms of action of AEDs that are critical to controlling anticonvulsant activity may also contribute to neuroprotection. That is to say, the therapeutic value of AEDs may include not only control of seizure activity, but potentially they may also independently suppress evolving injury independently of seizure control. Synaptic and cellular events initiated by acute energy deprivation caused by brain ischemia have been shown to be similar to those triggered by abnormal neuronal discharge induced by seizures.¹⁴ Thus, energy metabolism can be compromised when the hyperactive neuron is maintained in a sustained depolarized state by prolonged epileptic discharges. On the basis of the similarity of the cascade of synaptic and intracellular events exhibited by epilepsy and acute brain ischemia, AEDs have been tested as a possible neuroprotective strategy in ischemic brain injury.¹⁴

3. Antiepileptic drugs

3.1. Topiramate

Topiramate (TPM) is an effective, clinically available, anticonvulsant that has shown some synergy with hypothermia if used immediately after hypoxia–ischemia (HI) in animal models.¹⁵ The anticonvulsant effects of TPM appear to be mediated through multiple mechanisms. TPM inhibits several carbonic anhydrase isozymes and modulates AMPA/kainate and gamma-aminobutyric acid (GABA)_A-activated ion channels as well as voltage-activated Na⁺ and Ca²⁺ channels.^{16–18} TPM may also activate K⁺ conductance and inhibit depolarizing GABA_A-mediated responses.¹⁹ TPM has demonstrated neuroprotective properties in neuronal cultures exposed to oxygen-glucose deprivation,²⁰ or excitotoxic glutamate or kainate concentrations.²¹ These effects may make it a useful potential neuroprotectant acting by reducing excitatory amino acid release and calcium overload in the ischemic cells and by increasing the seizure threshold.

Follet et al. demonstrated that TPM, when administered to neonatal (postnatal day 7) rodents immediately after HI, was protective against white matter injury and decreased subsequent neuromotor deficits.²² They reported that TPM attenuated AMPA/kainate receptor-mediated cell death and Ca²⁺ influx *in vitro*, as well as kainate-evoked currents in developing oligodendrocytes, similar to the AMPA/kainate receptor antagonist 6-nitro-7-sulfamoylbenzo-(f)quinoxaline-2,3-dione (NBQX). Notably, protective doses of NBQX did not affect normal maturation and proliferation of oligodendrocytes either *in vivo* or *in vitro*, although the duration of follow-up was comparatively short (up to 10 days).²² These data suggest that AMPA/kainate receptor blockade may have potential to prevent white matter injury and that the protective efficacy of TPM is mediated at least in part by attenuation of excitotoxic injury to premyelinating oligodendrocytes in developing white matter. However, the premyelinating oligodendrocytes that are present at this age are significantly less mature than in the full-term animal. Therefore, further studies are needed to clarify whether TPM would also be beneficial for protecting white matter in term neonates.

Liu et al. hypothesized that TPM might increase the efficacy of a suboptimal (3 h) interval of delayed post-hypoxic–ischemic hypothermia in a neonatal rat stroke model.¹⁵ They reported that neither TPM nor delayed hypothermia alone conferred protection, whereas combined treatment with TPM plus delayed hypothermia improved both functional performance and the severity of brain pathology in rats compared with delayed hypothermia alone. The limitations of this study include the impracticably early initiation of treatment, 15 min after the insult, and that the duration of cooling was much shorter than the 48–72 h that is used in clinical trials, and which appears to be required for optimal protection.⁷ However, these data support promising mechanisms of neuroprotection and provide a strong impetus for further evaluation of combined drug therapy with delayed cooling after neonatal HI.

Neuroprotective effects of TPM alone after HI have also been reported in normothermic newborn piglets.²³ Newborn piglets subjected to HI by transient occlusion of the carotid artery and hypotension received saline solution or TPM administered as a loading dose 1 h post-insult followed by maintenance doses for three days. The doses used in this experimental application were consistent with the dose range known to be effective against seizures in human infants. Two different loading and maintenance TPM doses were used, either a loading dose of 20 mg/kg and a maintenance dose of 10 mg/kg/day, or a higher dose of 50 mg/kg followed by 20 mg/kg/day. TPM significantly reduced neuronal cell loss after the severe hypoxic–ischemic insult in a dose-dependent manner. Of concern, there was evidence of increased apoptosis in the white matter after high dose TPM administration, although this was not observed in the low dose-treated animals. No significant side-effects related to neurological or feeding behavior were observed with either dose. Neurological deficits were less severe in TPM-treated animals compared to placebo-treated animals. Interestingly, TPM did not significantly affect seizure frequency, and so these data indicate that the neuroprotective potential of TPM may be independent of its anticonvulsant effects.

Before TPM or any other agent can be considered for a clinical trial with hypothermia, it is essential to understand how hypothermia affects its pharmacokinetics. In a recent study of 13 asphyxiated neonates treated with therapeutic whole body hypothermia, 24 TPM was administered orally at a dose of 5 mg/kg once daily during hypothermia, which represents the low range of doses used in children for antiepileptic treatment. In most newborns this dose schedule was associated with TPM plasma concentrations within the reference range of 5–20 mg/L, showing that oral TPM absorption was maintained during hypothermia. However, TPM concentrations and half-life were markedly higher when compared with the limited previous publications from normothermic infants,²⁵ suggesting that hypothermia reduced both absorption and elimination of TPM.²⁴ There was no apparent effect of add-on phenobarbital therapy in three infants.²⁴ Interestingly, there was very good survival in this study (92%) and magnetic resonance imaging was normal in 46% of the treated infants.

However, although neither TPM nor levetiracetam (LEV) alone induced cell death when administered to 8-day-old rat pups, there is evidence that both drugs exacerbated phenytoin-induced neurodegeneration.²⁶ Although the doses of all three AEDs used in this study were well above those used in children for anticonvulsant treatment,²⁶ more preclinical study is needed before combining AEDs and other potentially neuroprotective agents.

3.2. Levetiracetam

Preclinical studies have suggested that the 90 kDa binding site of LEV may represent a new target for therapeutic intervention in epilepsy.²⁷ Recently, LEV has been shown to regulate AMPA and NMDA receptor-mediated excitatory synaptic transmission in the dentate gyrus of the hippocampus by acting on the presynaptic P/Q-type voltage-dependent calcium channel, reducing glutamate release, and inhibiting the amplitude of excitatory postsynaptic current in the dentate gyrus.²⁸

Mazarati et al.²⁹ have reported that as well as high efficacy against self-sustaining status epilepticus, good solubility, low acute toxicity and a potent interaction with diazepam, LEV may also possess neuroprotective activity. Other studies conducted *in vitro* and *in vivo* in focal and global ischemia models produced controversial results. While Hanon et al.³⁰ have reported that LEV induces significant neuroprotection in focal cerebral ischemia, these results were not confirmed by others.³¹ Interestingly, in contrast to several traditional AEDs, such as phenobarbital and phenytoin, LEV does not induce cell death in the developing brain even in doses severalfold higher than therapeutic doses,³² although it can worsen phenytoin toxicity.²⁶ Based on this finding, LEV may be an especially good candidate for treatment of seizures in neonates, at least if used as monotherapy. However, the use of LEV in human neonates has not yet been formally evaluated, and experience – even for use as an anticonvulsant – is still limited.³³ A recently published controlled study on the efficacy and tolerability in infants and young children with partial seizures showed good tolerability and efficacy even in the presence of concomitant diseases or congenital abnormalities.³⁴

4. Other neuroprotective agents

4.1. Xenon

Xenon is approved for use as a general anesthetic in Europe, and has shown promise as a neuroprotective agent.³⁵ It is a potent anesthetic that, because of its low blood gas partition coefficient, crosses the blood–brain barrier easily and guarantees rapid induction and emergence from anesthesia.³⁵ Because of the high concentrations needed and the enormous costs, it is only administered via special respirators that allow scavenging of exhaled xenon. As an anesthetic, xenon has already been administered to a large number of patients, and in adult patients without brain pathologies it has been well tolerated and seems to be devoid of major side-effects.³⁵ As recently reviewed by Istaphanous and Loepke, however, animals data show that xenon can trigger neurodegeneration in the developing brain.³⁶ Thus, its safety in brain-injured newborns cannot be assumed.

Xenon is also a potent *N*-methyl-D-aspartate (NMDA) antagonist, and appears to be superior to other NMDA antagonists because it has additional mechanisms of action, such as inhibition of AMPA and kainate receptors, reduction of neurotransmitter release, or effect on other ion channels.^{37–39} Apart from NMDA antagonism, there may be additional modes of neuroprotective action, such as secondary induction of hypothermia.³⁷ David et al.⁴⁰ showed significant neuroprotective effects of xenon when administered at 50 vol (%) up to 4 h after intraatrial NMDA injection and up to at least 2 h after induction of transient brain ischemia in adult rats. These authors, by using *ex-vivo* and *in-vivo* models of excitotoxic insult and transient brain ischemia, demonstrated that sub-anesthetic doses of xenon were associated with global neuroprotection, with reduced neurotransmitter release during ischemia and a reduction of subsequent cell injury and neuronal death.

In neonatal rodents, although delayed xenon treatment by itself was associated with relatively limited protection, the combination of xenon (20%) and hypothermia for 90min, initiated 4 h after neonatal rodent HI, provided synergistic histologic and functional protection up to 30 days after injury.⁴¹ Similarly, others have confirmed that the combination of mild hypothermia followed by delayed xenon inhalation had additional beneficial effects in rat pups that exceeded the individual benefit of either treatment alone.^{42·43}

The now well-established clinical experience with xenon, albeit not in infants with HIE, and the consistency of the apparent favorable effects of combination therapy with delayed hypothermia between groups, make this approach very attractive. However, it is important to accept that it requires special ventilators, xenon itself is expensive, none of these studies used the now ‘standard’ clinical regime of prolonged hypothermia, there are limited data on the

window of opportunity for delayed treatment, and there is as yet no evidence from a large animal model. Thus, further highly focused experimental research is essential before this attractive combination can be considered for human trial.

4.2. N-Acetylcysteine

N-Acetylcysteine (NAC) is a scavenger of oxygen radicals and restores intracellular glutathione levels, attenuating reperfusion injury and decreasing inflammation and nitric oxide (NO) production in adult models of stroke.⁴⁴ Adding NAC therapy to systemic hypothermia reduced brain volume loss after neonatal rodent HI, with increased myelin expression and improved reflexes.⁴⁵ In this study, P7 rats subjected to HI were treated with systemic hypothermia (30 °C) for 2 h and 50 mg/kg NAC once daily until killing at 2 or 4 weeks. Combined treatment preserved brain volumes at both time points and decreased the infarct area at 48 h. Further, the short-term functional outcomes of labyrinthine and cerebellar integration, assessed by cliff aversion and negative geotaxis, were also significantly improved in the treated animals.

Consistent with this, Cakir et al.⁴⁶ reported that after spinal cord ischemia, NAC and hypothermia alone were associated with limited protective effects, whereas the combination of NAC and hypothermia resulted in highly significant recovery of spinal cord function.⁴⁶

NAC may also prevent endotoxin-induced degeneration of oligodendrocyte progenitors and hypomyelination in the developing rat brain.⁴⁷ In this study, intraperitoneal pretreatment of pregnant female rats with NAC, 2 h prior administration of endotoxin lipopolysaccharide (LPS), was also associated with attenuation of the intracerebral inflammatory reaction, including reduced levels of tumor necrosis factor- α , interleukin-1 β , and inducible NO synthase. There is some preliminary evidence for clinical benefit. In a randomized clinical trial of preterm newborns, 194 infants received NAC by continuous infusion during the first 6 days after birth to prevent chronic lung disease. Although the incidence of chronic lung disease was unchanged, the authors noted a 39% decrease in periventricular leukomalacia in the NAC group.^{48,49}

4.3. Erythropoietin

Erythropoietin (Epo), the major haemopoietic growth factor, was originally identified on the basis of its role in erythropoiesis. Clinical trials demonstrated the safety and efficacy of recombinant human Epo in the prevention and treatment of anemia of prematurity.^{50,51} Numerous preliminary data suggest a neuroprotective effect on Epo in various experimental models, particularly after neuronal damage related to ischemia–reperfusion stress.⁵² In addition, systemically administered Epo is neuroprotective in neonatal brain injury models.^{53–55} Most in-vivo studies have focused on focal rather than global HI and suggest that early treatment after HI with high dose Epo (5000 U/kg) reduces gross brain injury,⁵³ reduces tissue loss preserving brain volume, and enhances neurogenesis.⁵⁴ Interestingly, this increased neurogenesis near the site of injury seems to be due to a shift from astrocytic to neuronal cell fate, rather than to newly born cells.⁵⁴

However, three months after neonatal stroke, a single dose Epo was not neuroprotective, whereas multiple doses of Epo were associated with sustained neuroprotection with reduced tissue loss.⁵⁵ Those histological findings paralleled the behavioral performance. While single-dose-treated rats did not show any improvement in spatial learning and memory performance assessed with the Morris water maze test compared with vehicle treated rats, multiple-dose-treated animals performed significantly better.⁵⁵

However, in an experimental model of acute HI in neonatal rats, lower multiple Epo doses, such as 1000 U/kg, did not result in significant neuroprotection from early neuronal damage,

even when combined with deferoxamine, an iron chelator which has been shown to decrease oxidative stress.⁵⁶ Therefore, the beneficial effects may relate to timing and dose as well as type of injury. In summary, these data suggest that Epo for neuroprotection should be used at high dose, both early and repetitively in the course of an acute brain injury. These findings must be balanced against the safety concerns that have been raised with high dose treatment in adults discussed below.

A recent randomized prospective study reported that repeated, low dose (300 or 500 U/kg every other day) Epo was safe and resulted in improved neurological outcome for patients with moderate HIE at 18 months of age.⁵⁷ However, the use of such expensive treatment has been questioned in a pilot study performed on adult patients with out-of-hospital cardiac arrest.⁵⁸ Patients treated with hypothermia and early high dose Epo therapy (40 000 IU) as soon as possible after resuscitation and every 12 h for the first 48 h, were compared with case-matched historical control treated with mild hypothermia only. Although a high survival rate with no or minor cerebral sequelae was observed in the Epo-treated group, adverse effects related to vascular thrombosis were detected. These safety concerns have been supported by another large multicenter phase II/III trial on 466 adult patients with acute ischemic stroke which identified the higher death rate in patients receiving Epo as compared with placebo, as well as an increased risk of serious complications: death, intracerebral hemorrhage, brain edema, and thrombotic events.⁵⁹

4.4. Melatonin

Melatonin, an endogenously produced indoleamine formed in higher amount in adults than in neonates, is a potent free radical scavenger as well as an indirect antioxidant.⁶⁰ Melatonin easily crosses the blood–brain barrier and has been shown to be safe when administered in children to improve their sleep pattern or to record sleep EEG.⁶¹ Experimental evidence regarding the neuroprotective effects of melatonin against excitotoxic and hypoxic–ischemic brain lesions in immature animals, through both short- and long-term morphologic and functional evaluations,^{62–64} provides support for the consideration of melatonin as a candidate therapy for periventricular white matter damage,⁶² as well as for the HI brain injury.⁶⁴

Although investigators have suggested several neuroprotective mechanisms for melatonin, including as an antioxidant, activation of GABAergic pathways, and antiepileptic effects, several lines of evidence suggest that the neuroprotective effects of melatonin are largely mediated by specific melatonin receptors and through adenylate cyclase inhibition.⁶²

Most of the in-vivo experimental studies demonstrating a neuroprotective effect of melatonin have used dosages between 1.25 and 20 mg/kg. In a well-defined mouse model of excitotoxic white matter cystic lesion mimicking human periventricular leukomalacia, low doses melatonin (5 mg/kg) were used in P5 mice following injection with excitotoxic ibotenate.⁶² Although melatonin did not prevent the appearance of white matter cysts, it promoted subsequent lesion repair with axonal growth and/or sprouting. Interestingly, this melatonergic effect on post-lesion plasticity has been also observed in adult animals subjected to global cerebral ischemia and melatonin prolonged high dose treatment (10 mg/kg/h for 6 h).⁶⁵ Those animals showed a peculiar rearrangement of the dendritic spines of the surviving CA1 pyramidal neurons that might explain the functional recovery of learning and memory reported in melatonin-treated animals in the immediate 6 h following brain ischemia.

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4.5. Cannabinoids

Several studies have pointed to cannabinoids as substances with a high potential as neuroprotective treatments in ischemic or traumatic brain damage. It has been suggested that

these neuroprotective effects were probably mediated both by specific mechanisms and by a cannabinoid-induced hypothermia.^{67,68} A recent study demonstrated that the non-psychoactive cannabinoid, cannabidiol (CBD), administered after HI in newborn piglets, reduced neuronal loss with no significant short-term side-effects, and therefore could be a useful neuroprotective candidate.⁶⁸ The histological improvement was associated with reduced hemodynamic impairment as measured by near-infrared spectroscopy, as well as a reduction of seizures as evaluated by amplitude-integrated electroencephalography in the first 6 h after HI.⁶⁸

However, in humans, maternal use of *Cannabis sativa* (marijuana) during pregnancy is associated with cognitive deficits in the offspring.⁶⁹ Further, the duration of follow-up after HI in the study by Alvarez et al. above was much too brief (just 6 h) to assess long-term injury or behavioral outcomes, and there are no studies on whether it can augment hypothermic neuroprotection. Therefore, it is too soon to recommend a clinical trial with CBD, and it would be important to do long-term structural and functional animal studies to determine the long-term effects of cannabinoids in the immature brain.

5. Potentially harmful drugs

5.1. GABAergic drugs: phenobarbital, benzodiazepines, and MK801

Hypoxic-ischemic encephalopathy is the single most common cause of seizures in both full-term and premature infants.⁷⁰ Currently, the first-line medical treatment for neonatal seizures is composed of drugs that increase GABA subtype A (GABA_A)-receptor channel chloride currents: barbiturate and benzodiazepines. Although these drugs are effective anticonvulsant in the more mature brain, there is some evidence that the excitatory effect of GABA in immature neurons before birth renders these anticonvulsants only partially effective in controlling the electrographic seizures in the majority of neonatal patients.⁷¹ Critically, this reduced efficacy of GABA-enhancing antiepileptic drugs has been linked to neuronal chloride transport in the developing brain.⁷² This is of concern even outside the classic GABA-agonists since TPM, for example, works partly on the GABA receptor.¹⁷ Low concentration of diuretic bumetanide has been shown to alter the ion gradient that underlies the excitatory effects of GABA, and the combination of bumetanide and phenobarbital (PB) has been proven to be significantly more effective than PB alone on seizure occurrence, frequency, and duration, in an in-vitro model of neonatal seizures.⁷³

However, many compounds that suppress synaptic activity in the brain and are used as sedatives or anticonvulsants in neonatal intensive care units have been associated with widespread apoptotic neurodegeneration throughout the brain when administered to immature rodents during the period of the brain growth spurt.⁷⁴ This developmental period occurs at different times relative to birth in different species. In rats and mice, it occurs post-natally; but in humans, it extends from the sixth month of gestation to several years after birth and coincides with the developmental period of ongoing programmed cell death.⁷⁴ Compounds that may cause neuronal apoptosis in the developing brain include antagonists of NMDA receptors (ketamine), agonists of GABA_A receptors (barbiturates and benzodiazepines), and sodium-channel blockers (phenytoin, valproate).⁷⁵ Barbiturates and benzodiazepines exert their effects by acting directly at the GABA_A receptor site to allosterically influence the chloride current and augmenting GABAergic inhibition.

Investigators have found that administration of PB to rat pups results in significant decreases in brain weight and DNA, RNA, protein and cholesterol concentrations and reduced neuronal number.⁷⁴ More recently, Stefovskaja et al. administered the NMDA antagonist MK801 and the GABA_A agonists PB and diazepam to infant rats, and studied cell proliferation and neurogenesis in the brain by using 5-bromo-2'-deoxyuridine (BrdU) and doublecortin

immunochemistry and stereology.⁷⁶ Learning and memory were assessed by using water maze testing at the age of 6 months after early postnatal treatment with PB. Blockade of NMDA receptor-mediated excitation and enhancement of GABA_A receptor activation were associated with reduced cell proliferation and inhibition of neurogenesis. At the age of 6 months, PB-treated rats had fewer neurons in the dentate gyrus of the hippocampus and performed worse than saline-treated littermates in water maze learning and memory tasks.⁷⁶ These data call for caution with the use of those drugs in neonatal medicine.

6. Conclusion

Although hypothermia and single pharmacotherapies show promise, combined therapies that have been shown to exert distinct mechanisms of action may be necessary to reach different targets in the setting of an acute HI insult: prevention of acute lesions, increased therapeutic time window for protection, and enhanced repair in the long term.

This may be seen in some ways as analogous to the treatment of epilepsy, where, if monotherapy fails, combination therapy is tried in an attempt to improve efficacy. However, two important distinctions should be evident. First, in the studies described in this review, even AEDs have been given well before the onset of post-hypoxic–ischemic seizures, and thus are likely working through highly timing-dependent mechanisms of evolving injury. Second, and even more important, there is increasing preclinical evidence from immature rodents that many neuroinhibitors can impair brain development and that particular combinations of drugs that were individually harmless can exacerbate neurodegeneration in the developing brain. This sobering evidence suggests that careful preclinical and clinical testing will be essential to design strategies that would block brain damage without disrupting normal development. Finally, most recent evidence suggests that brain injury occurs over long periods of time and that neuroprotective therapies may need to be continued for some time after birth.

Practice point

- Despite the potential for treatment of seizures after HI to reduce the injury, efficacy is not yet demonstrated.

Research directions

- Potential therapies that have shown synergy in animal models may point to novel potential drug targets.
- Potential adverse effects of proposed neuroprotective treatments must be closely assessed, singly and in combination, before translation to human trials.
- Attention must be directed to those compounds that might trigger neurodegeneration in the developing brain, and thus counteract the neuroprotective effects of hypothermia.

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