Title: A working model for hypothermic neuroprotection

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Guido Wassink is a research fellow working to understand why some fetuses survive oxygen deprivation and infection completely normal, whereas others suffer injury or die after apparently identical exposure. His studies have highlighted the factors determining the physiological resilience of the unborn child and the role of growth factors such as endogenous erythropoietin in helping to protect the brain after hypoxia-ischaemia.

Alistair Jan Gunn is a Paediatrician-scientist who has conducted groundbreaking basic research into ways of identifying compromised fetuses in labour, the mechanisms and treatment of asphyxial

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brain injury and the mechanisms of life threatening events in infancy. His research helped to establish mild cooling as the first ever technique to reduce brain injury due to low oxygen levels at birth.

ABSTRACT (250 words)

Therapeutic hypothermia significantly improves survival without disability in near-term and full-term newborns with moderate-severe hypoxic-ischaemic encephalopathy. However, hypothermic neuroprotection is incomplete. The challenge now is to find ways to further improve outcomes. One major limitation to progress is that the specific mechanisms of hypothermia are only partly understood. Evidence supports the concept that therapeutic cooling suppresses multiple extracellular death signals, including intracellular pathways of apoptotic and necrotic cell death and inappropriate microglial activation. Thus, the optimal depth of induced hypothermia is that which effectively suppresses the cell death pathways after hypoxia-ischaemia, but without inhibiting recovery of the cellular environment. Thus mild hypothermia needs to be continued until the cell environment has recovered until it can actively support cell survival. This review highlights that key survival cues likely include the inter-related restoration of neuronal activity and growth factor release. This working model suggests that interventions that target overlapping mechanisms such as anticonvulsants are unlikely to materially augment hypothermic neuroprotection. We suggest that further improvements are most likely to be achieved with late interventions that maximise restoration of normal cell environment after therapeutic hypothermia, such as recombinant human erythropoietin or stem cell therapy.

Keywords; hypoxia-ischaemia, encephalopathy, hypothermia, neuroprotection

INTRODUCTION

Therapeutic hypothermia is now standard-care for infants with moderate to severe hypoxic-ischaemic (HI) encephalopathy (HIE) (Azzopardi *et al.*, 2012), with compelling evidence from randomised controlled trials that it improves survival and neurological outcomes into middle childhood (Jacobs *et al.*, 2013; Natarajan *et al.*, 2016) and reduces brain damage on modern imaging (Shankaran *et al.*, 2015). Hypothermic neuroprotection is significant but incomplete, reducing the combined risk of death and severe disabilities at 18 months of age with ~12%, from 58 to 46% (Edwards *et al.*, 2010). Thus, many infants still die or survive with major debilitating handicaps, despite therapeutic hypothermia.

The empirical parameters for optimal neuroprotection are now well established, as previously reviewed in detail (Wassink *et al.*, 2014). Therapeutic hypothermia needs to be induced as soon as possible in the first 6 hours after HI, optimally reducing brain temperature by no more than 3 to 5 °C, and then continued for ~72 hours. Deeper cooling (by ~8.5C°), or shorter or longer periods of cooling than 72 hours reduces neuroprotection both in preclinical studies (Alonso-Alconada *et al.*, 2015; Davidson *et al.*, 2015c; Davidson *et al.*, 2017) and in a randomised clinical trial (Shankaran *et al.*, 2017). The precise factors underlying these now well-known empirical factors are still unclear. Further, given that current cooling protocols are near-optimal, future progress depends on finding interventions that can complement hypothermia. In this review, we propose a mechanistic working model to help understand these parameters for hypothermic neuroprotection, and discuss which post-insult phases and specific mechanisms should be targeted to further improve outcomes.

Hypoxic-ischaemic brain damage evolves over time

The seminal finding that underpinned the development and translation of therapeutic hypothermia is that perinatal brain damage after HI is a process that evolves over time rather than a 'static' event. Hope and colleagues first showed with magnetic resonance spectroscopy in term neonates with moderate-severe HIE, that high-energetic substrates (i.e. phosphocreatine to inorganic orthophosphate, and ATP to total phosphorus) often normalised shortly after birth but then deteriorated again (Hope *et al.*, 1984; Azzopardi *et al.*, 1989), despite sufficient cerebral oxygenation and perfusion. Studies in newborn piglets then demonstrated that cerebral energetic failure after HI corresponded with progressive neuronal death (Martin *et al.*, 2000).

As illustrated by the abstract Figure, during severe HI (the 'primary' phase), there is gradual depletion of high-energetic phosphates and anoxic depolarisation. As energy-dependent mechanisms that maintain cellular homeostasis (e.g. Na+/K+ ATP-dependent pumps) begin to fail, cytotoxic oedema (i.e. cellular swelling) and extracellular accumulation of excitatory amino acids (EAAs) occurs, with unregulated calcium influx into neurons. This energetic metabolism and cell swelling typically recovers to near-normal values within 30-60 min after reperfusion and are then

sustained during a 'latent' phase for the following ~6 hours (Hope *et al.*, 1984; Azzopardi *et al.*, 1989; Gunn *et al.*, 1997; Bennet *et al.*, 2007b).

After moderate-severe HI, the latent phase is followed by delayed deterioration after ~6-15 hours (the 'secondary phase'), with development of stereotypic seizures, accumulation of excitotoxins and oedema (Figure 1), and gradual mitochondrial failure and spreading cell death (Gunn *et al.*, 1997; Bennet *et al.*, 2007b). This triphasic pattern has been shown in multiple species, including rodents, piglets and humans as reviewed (Wassink *et al.*, 2014), and correlates with histological brain damage after HI (Williams *et al.*, 1992; Blumberg *et al.*, 1997; Vannucci *et al.*, 2004). In newborn humans, the severity of loss of oxidative cerebral metabolism after HI is highly associated with death and adverse outcomes (Azzopardi *et al.*, 1989; Roth *et al.*, 1997). Finally, there is evidence of a 'tertiary' phase after HI, where chronic inflammation and epigenetics impair neural and glial regeneration, synaptogenesis and neurite outgrowth (Fleiss & Gressens, 2012).

How does hypoxic-ischaemic brain damage spread?

One of the striking features of HI-mediated brain damage is that cell dysfunction and death spreads over time from injured regions to areas that were originally intact (Thornton *et al.*, 1998). The gap junctions that link adjacent cells to allow transport of small molecules, ions and second messengers (Davidson *et al.*, 2015a), are formed through docking of hexamer hemichannels (connexons). These hemichannels are active under physiological conditions, and signal via regulated adenosine triphosphate (ATP) release.

There is increasing evidence that severe HI triggers transient, unregulated opening of these connexon hemichannels, resulting in disrupted resting membrane potential, release of damaging ATP and glutamate levels (Ye *et al.*, 2003; Kang *et al.*, 2008), and uptake of water leading to cell swelling and rupture (Quist *et al.*, 2000; Rodriguez-Sinovas *et al.*, 2007). Supporting this concept, an intracerebroventricular infusion with a mimetic peptide that reversibly binds with the second extracellular binding loop on the connexin43 protein, at a dose that blocks hemichannels (O'Carroll *et al.*, 2008), initiated from 90 minutes until 25 hours after profound asphyxia or cerebral ischaemia in preterm and near-term fetal sheep, reduced status epilepticus, and improved EEG restoration and neural and oligodendroglial survival (Davidson *et al.*, 2012; Davidson *et al.*, 2014). These data show that connexon hemichannels have a critical role during the early latent phase in propagating damage after HI.

Mechanisms of delayed cellular death - programmed apoptosis

Multiple factors are involved in the delayed development of cell death following initial recovery of cerebral oxidative metabolism after HI. These include activation of cell death pathways, withdrawal of trophic factors and secondary inflammation. In particular, the cell death pathways are activated through unregulated influx of calcium during anoxic depolarization, exposure to reactive oxidative species during reperfusion and other factors as reviewed in detail (Thornton *et al.*, 2017).

Apoptosis can be triggered through intracellular and extracellular pathways (Figure 2), as reviewed in detail (Thornton *et al.*, 2017). The intracellular pathway involves excessive calcium influx and astrocytic growth factor withdrawal (Clawson *et al.*, 1999), leading to increase translocation and interaction of pro-apoptotic proteins at the neuronal mitochondria. These apoptotic proteins, like the Bcl_2-associated X (Bax) and truncated BH3-interacting-domain death agonist (tBid) proteins (Raemy & Martinou, 2014), produce pores in the outer mitochondrial membrane. This releases several pro-apoptogenic factors, including direct inhibitor of apoptosis-binding protein with low Pi (Diablo), second mitochondria-derived activator of caspase (Smac), apoptosis-inducing factor (AIF) and cytochrome-c from the mitochondrion (Wassink *et al.*, 2014).

Intra-mitochondrial calcium overload also facilitates cytochrome-c release through reactive oxygen species (Hagberg *et al.*, 2014), and activates brain-specific calpains that degrade intracellular structural and signalling proteins (Bevers & Neumar, 2008). In addition, hypoxia-ischaemia activates extracellular death receptors that stimulate necroptosis or caspases-8 and -3 (Giulian *et al.*, 1993). These molecular mechanisms are detailed in figure 2. In neonatal rats, caspase, Bax and cytochrome-c inhibitors all provide partial neuroprotection, supporting a pathologic role for these intracellular mechanisms (Thornton *et al.*, 2017).

Mechanisms of delayed cellular death - programmed necrosis

In the developing brain, necrosis after HI often demonstrates a variable morphology. This pattern typically involves cellular fragmentation, but there is increasing evidence that delayed necrotic cellular death is programmed (Northington *et al.*, 2007). Necroptosis, for example, is mediated via inter-connected mechanisms that involve caspase-8, receptor-interacting protein kinases (RIPK) 1 and 3 and the mixed lineage kinase domain-like pseudokinase (MLKL) (Rodriguez *et al.*, 2016). These proteins have multiple and often opposing roles that participate in both apoptosis and necrosis (Northington *et al.*, 2007). For example, RIPKs activate the inflammasome, which might underlie the robust neuro-inflammation triggered by HI (Man & Kanneganti, 2016), whereas MLKL has multiple functions, that include facilitating pore formation that cause the cell membrane to rupture (Wang *et al.*, 2014), culminating in cell death with a necrotic phenotype. Supporting these data, treatment with necrostatin-1, a non-selective necroptotic inhibitor, reduced necrotic cellular death and oxidative damage to proteins in post-HI p10 mice (Northington *et al.*, 2011a).

Summary of the mechanisms of delayed cell death

Taken together, it is clear from these findings that brain metabolism can recover to normal or near-normal levels after even severe HI, but multiple, inter-related mechanisms are triggered that ultimately lead to delayed cellular death (Thornton *et al.*, 2017).

THE MECHANISMS OF HYPOTHERMIC NEUROPROTECTION

Induced hypothermia produces a graded reduction in cerebral metabolism of approx. ~5% per °C (Laptook *et al.*, 1995). After resuscitation, tissue oxygenation and substrate delivery are restored (Gunn *et al.*, 1997), and therefore it is improbable that reduced metabolism per se would be protective. However, it is important to reflect that the neuroprotective effects of cooling *during* HI are substantially greater than would be expected from a 15 to 20% reduction in metabolism. For example, in adult rats, cooling during cerebral ischaemia was associated with a dramatic reduction in major hippocampal neuronal loss compared with normothermia (6 ± 1% vs. 90 ± 17% dead neurons), for the same duration of neural depolarisation (Bart *et al.*, 1998). This finding strongly indicates that hypothermia supports cell survival by suppressing active, intracellular cell death mechanisms rather than by reducing oxidative metabolism. There is considerable evidence that this interaction is critical for post-resuscitation neuroprotection, as discussed next.

Hypothermia suppresses programmed cell death after hypoxia-ischaemia

There is increasing evidence that induced hypothermia suppresses apoptotic and necrotic processes triggered after hypoxia-ischaemia (Wassink *et al.*, 2014). For example, in vitro, intra-hypoxic hypothermia reduced apoptotic and necrotic morphologic death in developing neurons, and hypoxia-driven protein formation (Bossenmeyer-Pourie *et al.*, 2000). Further, hypothermia also suppressed serum-deprivation and H_2O_2-induced neuronal apoptosis, with lesser caspases -3, -8 and -9 activation and cytochrome-c release, consistent with depressed intracellular and receptor-induced apoptosis (Xu *et al.*, 2002; Li *et al.*, 2012). Consistent with this, in adult rats, induced hypothermia after transient global ischaemia was associated with upregulated anti-apoptotic Bcl_2 protein, and downregulated pro-apoptotic p53 protein (Zhang *et al.*, 2010), with reduced neural necrosis and apoptosis. In adult rats with focal ischaemia, hypothermia also attenuated death receptor expression and caspase-8 activation (Liu *et al.*, 2008), supporting its interaction with extracellular apoptosis, and suppressed genes implicated in inflammation (Nagel *et al.*, 2012).

In neonatal piglets, hypothermia started after severe HI reduced apoptotic but not necrotic cell death (Edwards *et al.*, 1995), whereas hypothermic neuroprotection reduced caspase-3 and microglial activation in term-equivalent fetal sheep (Roelfsema *et al.*, 2004). In neonatal rats, acute hypothermia after HI also reduced caspase-3 and increased X-linked inhibitor of apoptosis (XIAP) in the core ischaemic lesion, but not the penumbra, whereas AIF translocation was suppressed in both regions (Askalan *et al.*, 2011), indicating that hypothermia interacts with both caspase-dependent and independent mechanisms. Finally, in neonatal rodents with HI, hypothermia attenuated macroscopic brain damage, with less necrotic and apoptotic neural death after 24 hours, and suppressed cytochrome-c release, caspase-3 and calpain activation in the cortex, hippocampus, thalamus and striatum (Ohmura *et al.*, 2005). Thus, taken together, these data suggest that hypothermic neuroprotection in the developing brain is likely achieved through both anti-apoptotic and anti-necrotic mechanisms (Northington *et al.*, 2011b).

Hypothermia suppresses inflammation after hypoxia-ischaemia

Perinatal HI triggers an inflammation-based cascade, which increases the release of cytokines and interleukins (Hagberg *et al.*, 2015). These factors potentiate developing cellular damage, either through neurotoxic-induced apoptosis or endothelial cell-propagated inflammation, with leukocytes infiltrating the post-ischaemic brain (Gunn *et al.*, 2017). In experimental paradigms, post-insult hypothermia inhibits microglial activation, chemotaxis, and interleukin and pro-inflammation cytokine release, which might provide mitochondrial protection (Wassink *et al.*, 2014). For example, cytokine-induced iNOS expression raises intracellular NO· levels, which competes with molecular oxygen for binding on cytochrome oxidase (Brown, 1997) and so depresses mitochondrial respiration. TNF-alpha and interferon-γ-mediated iNOS production also caused apoptosis and DNA damage in cultured oligodendrocytes (Druzhyna *et al.*, 2005). Critically, hypothermia has a differential effect on the glial reaction to ischaemia, demonstrating potent microglial suppression but little effect on astroglia proliferation (Si *et al.*, 1997). This suggests that hypothermic neuroprotection results, in part, from reducing 'bad' inflammation while not suppressing astroglial recovery.

Hypothermia, excitotoxins and neuronal activity

In contrast to their role during the primary and reperfusion phases, the importance of excitotoxins *after* reperfusion is questionable given that extracellular levels rapidly return to baseline values (Tan *et al.*, 1996; Thoresen *et al.*, 1997). Early studies of anti-excitotoxic agents found apparent protection but did not control for cerebral temperature (McDonald *et al.*, 1987; Hattori *et al.*, 1989). Critically, subsequent studies showed that glutamate blockade was associated with drug-induced hypothermia and controlling for temperature abolished neuroprotection (Ikonomidou *et al.*, 1989; Engidawork *et al.*, 2001). In the adult rodent, Nurse and Corbett showed that the apparent neuroprotective effect of NBQX, a glutamate antagonist administered from 1 hour after mild cerebral ischaemia, was directly associated with mild *endogenous* hypothermia for several days that developed an hour after drug administration (Nurse & Corbett, 1996), and that similar neuroprotection could be induced with application of the same hypothermia profile over 28 hours. Conversely, NBQX 'neuroprotection' was effectively abolished by maintaining normothermia. Furthermore, anti-excitotoxin therapy limited to the secondary phase did not reduce neuronal damage in the severely injured parasagittal cortex of fetal sheep, and had only limited neuroprotective effects in more mildly affected areas of the brain (Tan *et al.*, 1992; Gressens *et al.*, 2011).

Nevertheless, even with normal levels of extracellular glutamate, excitotoxicity may still play an indirect injurious role. Pathological hyperexcitability of glutamate receptors has been reported in P10 rats for many hours after HI, with improved neuronal outcome after receptor blockade (Jensen *et al.*, 1998). Supporting this hypothesis, despite suppression of overall EEG activity for many hours after asphyxia, transient epileptiform activity was seen in the early recovery phase in preterm sheep fetuses that developed severe injury (George *et al.*, 2004), which was correlated with the severity of

neuronal loss in the striatum and hippocampus (Dean *et al.*, 2006b; Bennet *et al.*, 2007c). Suppression of these EEG transients with a glutamate receptor antagonist partially reduced cellular loss (Dean *et al.*, 2006a). Furthermore, neuroprotection with post-asphyxial moderate cerebral hypothermia in the preterm fetal sheep was associated with a marked reduction in the numbers of epileptiform transients in the first 6 hours after asphyxia, and reduced amplitude of delayed seizures (Bennet *et al.*, 2007a). The combination of glutamate receptor antagonist infusion and mild hypothermia after severe asphyxia in preterm fetal sheep, however, showed non-additive neuroprotection, consistent with the suggestion that cooling is partly protective by attenuating this receptor hyperactivity (George *et al.*, 2012). Further studies are needed to determine whether this is also the case after HI damage in the term-equivalent brain.

Duration of cooling and recovery of EEG activity

Recent studies in near-term fetal sheep have shown that when head cooling was started 3 hours after ischaemia, cooling until 72 hours was markedly more protective than cooling until 48 hours (Figure 3). Strikingly, rewarming at 48 hours after cerebral ischaemia was associated with marked deterioration of EEG power over the next 24 hours, and with greater numbers of microglia on histology at day 7 and substantially less improvement in overall neuronal survival compared to continued cooling until 72 hours (Davidson *et al.*, 2017). This suggests that deleterious inflammation is still continuing between 48 and 72 hours after HI, and is reactivated or exacerbated by premature rewarming. It is of particular interest that in that animal study the spectral edge frequency of the EEG was still partially suppressed at 48 hours, and did not reach control values until around 72 hours. Conversely, we have shown that extending cooling from 72 to 120 hours was not associated with any further improvement in EEG recovery, and indeed was associated with apparently impaired neuronal survival in some brain regions (Davidson *et al.*, 2015c). This suggests for the first time that normalization of EEG activity is an important biomarker for how long therapeutic hypothermia needs to be continued. Local neural interconnections, with shorter connections between neurons, lead to higher frequency activity. Thus increasing cortical EEG frequency strongly infers improved cortical function. More speculatively, it also seems to support the hypothesis that EEG activity, i.e. cross-talk between neurons, represents an important aspect of gradual normalization of the cellular environment after hypoxia-ischaemia.

Restoration of the neuronal environment: EEG activity and growth factors

The factors underlying recovery of brain activity after injury are incompletely understood. In part it is related to reversal of functional depression of injured cells, and restoration of signalling between interconnected structures (Glassman & Malamut, 1976). Neuronal activity itself is critical for cell viability, and closely interacts with trophic growth factor release.

Electrical activity is a vital part of maintaining neuronal homeostasis in target neurons (Koike *et al.*, 1989). Indeed there is some evidence that even abnormal activity can be beneficial in some settings. In rats, two electroconvulsive seizures within the first 24 hours after contusion accelerated recovery

of beam-walking, with less cerebral necrosis (Feeney *et al.*, 1987). Further, in cats, brief stimulation with d-amphetamine after bilateral frontal cortex ablation was associated with persistent improvement in beam-walking (Sutton *et al.*, 1989). Conversely, the suppression of EEG activity with gamma-aminobutyric (GABA) agonists such as diazepam and muscimol greatly impairs the recovery from cortical or striatal lesions (Schallert *et al.*, 1990), which might relate to impaired synaptogenesis. Synaptogenesis is in part dependent on brain activity (Saneyoshi *et al.*, 2008), whereas the inhibition of neuronal activity impairs synaptogenesis (van Huizen *et al.*, 1985).

Endogenous growth factors play a complementary role with neural activity in supporting neural homeostasis. As well as the direct homeostatic effects of neuronal activity (Koike *et al.*, 1989), neural stimulation also indirectly supports neuronal survival by promoting release of fibroblast growth factor (Mattson & Rychlik, 1990). Independently, during profound electrical suppression in vivo, endogenous growth factors help support neuronal survival (Anderson *et al.*, 1988). After HI brain damage in neonatal rats, neurotrophic activity is initially suppressed (Clawson *et al.*, 1999), but growth factor treatment markedly reduces post-HI brain damage in rodents and fetal sheep (Guan *et al.*, 2003). Endogenous growth factor activity increases from around 3 to 5 days, reaching maximum expression at 8 to 15 days (Nieto-Sampedro *et al.*, 1982; Guan *et al.*, 2003). This induction of growth factors might help promote stabilization of the cellular environment and long-term neurorepair.

Consistent with an important role for recovery of astrocytes in determining outcome of cerebral HI, there is some evidence in adult rodents that hypothermia after ischaemia and cardiac arrest is associated with increased expression of growth factors including glial-cell-line derived neurotrophic factor (GDNF), and brain-derived neurotropic factor (BDNF) and its tyrosine receptor kinase-B, in a temporal and regional-specific manner (Boris-Moller *et al.*, 1998; D'Cruz *et al.*, 2002; Schmidt *et al.*, 2003). Thus, at the least these data confirm that mild hypothermia does not suppress astroglial production of integral neurotrophins. Further research is essential to understand whether astroglial growth factor production is essential for long-term neurodevelopmental recovery after therapeutic hypothermia.

A WORKING MODEL FOR HYPOTHERMIC NEUROPROTECTION

Taken together, these experimental studies indicate that hypothermia actively prevents delayed cell death after profound HI by suppressing apoptotic and necrotic cellular death pathways and extracellular inflammation and so stabilizing mitochondrial function. To achieve long-term neuroprotection, this hypothermia-induced suppression needs to be continued until the extracellular environment provides a sufficient level of pro-survival cues.

Key survival cues are EEG activity and growth factors. Hypothermia in part achieves this by differentially depressing microglia more than astrocytes (Si *et al.*, 1997) and so allows neurotrophin activity to recover after HI. Further, although induced hypothermia somewhat suppresses

stereographic seizures it does not significantly inhibit recovery of EEG activity (Davidson *et al.*, 2017). Critically, as discussed above, there is now compelling evidence that optimally hypothermia should be continued until high frequency EEG activity has been restored (Davidson *et al.*, 2017). It is intriguing to note that the timing of recovery of this EEG frequency to baseline values during cooling in this study at ~72 h after ischaemia, also corresponds broadly with the known time delay before endogenous growth factors begin to be induced after hypoxia-ischaemia in adult and developing rodents (Guan *et al.*, 2003).

This model is consistent with the empirical observation that optimally the brain should be cooled by 3 to 5 degrees, with loss of protection with deeper cooling (Alonso-Alconada *et al.*, 2015). This is likely, at least in part, related to the finding that mild cooling selective suppressed microglial activation, whereas deeper cooling also suppresses astrocyte function and proliferation, and so might impair endogenous restoration of growth factors (Si *et al.*, 1997). Potentially, it might also reflect greater suppression of neural function during deep hypothermia (Westover *et al.*, 2015). This need to allow recovery of the cell environment before warming is consistent with the strong observation that cooling needs to be continued until normalization of EEG frequency (Davidson *et al.*, 2015c; Davidson *et al.*, 2017).

The potential implications for combination therapies with hypothermia

This working model suggests that future combined therapies should focus on promoting cellular homeostasis after hypothermia through long-term stimulation of survival cues like neurotrophins, differential suppression / stimulation of bad / good inflammation, plus functional integration of new neurons and oligodendroglial cells (i.e. with recombinant human erythropoietin (rEpo) or stem cell therapies). First, if EEG activity is indeed critical for restoration of the normal cell environment, then high dose anticonvulsant treatment, that suppresses background activity, is both likely to overlap with the mechanisms of therapeutic hypothermia, and so not provide additional neuroprotection but also has potential to impair long-term neural recovery.

Consistent with these concerns, there is good evidence that in adult rats diazepam therapy after cerebral ischaemia does not augment hypothermic neuroprotection (Davies *et al.*, 2004), and as discussed above that prolonged suppression can impair functional recovery (Schallert *et al.*, 1990). Supporting this, the anticonvulsant topiramate (Lee *et al.*, 2000), also did not improve death or neurological disability in a small phase-II trial in hypothermia-treated neonates with HIE, compared with hypothermia-treated babies alone (Filippi *et al.*, 2018). Thus, there is an urgent need for highly targeted preclinical and clinical research that can resolve the real world impact.

Similarly, an increasing number of animal studies have shown non-additive neuroprotection during immediate co-treatment with hypothermia. For example, in fetal sheep after cerebral ischaemia, connexon blockade reduced neuronal damage and restored EEG power (Davidson *et al.*, 2012), but was non-additive to mild hypothermia (Davidson *et al.*, 2015b). Intracerebral infusion with insulin growth factor-1 (IGF-1) increased post-ischaemic astroglial and oligodendrocyte survival in near-

term fetal sheep (Guan *et al.*, 2001), but treatment with delayed IGF-1 from 4.5 hours after ischemia plus hypothermia from 5.5 to 72 hours did not provide greater protection or caspase-3 depression than cerebral cooling alone (George *et al.*, 2011). The noble gas Xenon, which has anti-apoptotic effects through the N-methyl-D-aspartate (NMDA) receptor (Zhuang *et al.*, 2012), improved hypothermic protection in neonatal piglets after HI but not in a phase-II clinical trial (Chakkarapani *et al.*, 2010; Azzopardi *et al.*, 2015). This study is not conclusive since Xenon was not started until a median of 10 hours after birth (range; 4.0-12.6). Nevertheless, these data are suggestive that non-additive neuroprotection partially resulted from overlapping mechanisms of action.

By contrast, melatonin started 15 min after HI followed by hypothermia from 2 hours improved histological outcomes and recovery of high energy phosphates on MRS compared with hypothermia alone (Robertson *et al.*, 2013). This result likely reflects melatonin's potent anti-free radical effects, which will have been maximal during reperfusion from HI (Miller *et al.*, 2005), but it is unclear whether it would have been equally effective if it had been started at the same time as hypothermia. Nevertheless, a pilot trial in human babies with HIE melatonin plus hypothermia reported that the combination was associated with improved survival at 6 months of age without neurological abnormalities compared to hypothermia alone (Aly *et al.*, 2015). These preliminary findings are encouraging but need validation in larger trials.

Neuroprotection and neurorepair - rEpo and stem cell therapies

Residual or 'persistent' inflammation has been reported during or after hypothermia (Davidson *et al.*, 2017). Thus, it is plausible that therapies with anti-inflammatory and / or pro-regenerative effects might augment hypothermic neuroprotection either during or after therapeutic hypothermia. In this respect, there is compelling preclinical evidence for benefit rEpo and stem cells (Bennet *et al.*, 2012; Juul & Pet, 2015). rEpo has anti-apoptotic, anti-oxidant, anti-excitotoxic and anti-inflammation effects in preclinical paradigms of neonatal brain damage (Rangarajan & Juul, 2014), promotes proliferation and maturation of oligodendrocytes and neurons (Sugawa *et al.*, 2002; Iwai *et al.*, 2007), and stimulates growth factors (BDNF and GDNF) and angiogenesis (Li *et al.*, 2007; Juul & Pet, 2015), which is needed for neurorepair and normal neurodevelopment.

Multiple experimental studies have reported rEpo-mediated neuroprotection with improved long-term outcomes after HI, as reviewed (Wu & Gonzalez, 2015). For example, in preterm fetal sheep, rEpo infusion from 30 min until 72 hours after asphyxia improved neuronal and oligodendroglial loss, and electrophysiologic restoration (Wassink *et al.*, 2017). In preterm infants, a recent meta-analysis found that early, prophylactic rEpo improved neurodevelopmental outcomes at 18-24 months (Fischer *et al.*, 2017). Moreover, small randomised clinical trials in term neonates with HIE have demonstrated improved outcomes on modern imaging and neurological measures after treating with rEpo (Zhu *et al.*, 2009; Elmahdy *et al.*, 2010; Malla *et al.*, 2017). These and initial clinical phase II trials on co-treatment with hypothermia are encouraging (Wu *et al.*, 2016), but large definitive trials are awaited.

In addition, there is increasing evidence from *in vitro* and *in vivo* preclinical studies that stem / progenitor cells might have beneficial effects on outcomes after HI, as reviewed (Bennet *et al.*, 2012). For example, in newborn rabbit kits that received intrauterine ischaemia at 0.7 gestation (Drobyshevsky *et al.*, 2015), treatment with human umbilical cord blood cells at birth resulted in a dose-dependent improvement in neurobehavioural outcomes. These stem cells improved functional outcomes without significant engraftment, suggesting that their effects were mediated by trophic or immunomodulation mechanisms. Similarly, in preterm fetal sheep, intranasal infusion with human amnion epithelial cells at 1, 3 and 10 days after HI reduced neuronal and white matter loss, and suppressed gliosis and caspase-3, with improved maturation of the cortical EEG (van den Heuij *et al.*, 2017). In postnatal day 7 rats, combined administration of mesenchymal stem cells with hypothermia, from six hours after HI, was associated with greater improvement on imaging and behavioural tests than either intervention alone (Park *et al.*, 2015).

Finally, one small double-blind randomised placebo- controlled trial in 96 children with cerebral palsy reported that treatment with umbilical cord blood plus rEpo attenuated neurocognitive and motor dysfunction at 6 months more than rehabilitation with or without rEpo (Min *et al.*, 2013). Thus, stem cell therapies have potential as a treatment to improve recovery from HIE, whether in isolation or combined with hypothermia.

CONCLUSIONS AND PERSPECTIVES

The working model of the mechanisms of neuroprotection presented here suggests that immediate co-treatment of hypothermia with agents whose mechanisms overlap with those of hypothermia is unlikely to offer substantial benefit. Indeed, interventions such as high dose anticonvulsant therapy that suppress background neural activity may have potential to impair long-term neural recovery. We propose that research should focus on interventions that promote cellular homeostasis through long-term stimulation of survival cues like neurotrophins, selective suppression/stimulation of bad/good inflammation, plus integration of new functional cells. Current evidence suggests that strategies that promote these outcomes such as stem cells and erythropoietin are the most likely to further improve the outcome of therapeutic hypothermia.

AUTHOR CONTRIBUTION STATEMENT

Alistair Gunn, Guido Wassink and Joanne Davidson conceptualised this topical review. Guido Wassink undertook manuscript writing and preparation of figures. All authors reviewed and edited this manuscript, and have approved the final version as submitted to the Journal of Physiology.

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DISCLOSURE / CONFLICT OF INTEREST

The authors declare no potential conflict of interest in this article.

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FIGURE LEGEND

Abstract Figure - This graphical abstract shows the progressive phases of perinatal brain damage after severe hypoxia-ischaemia, and how interventions (i.e. hypothermia, rEpo and stem cells) interact with deleterious processes induced in these phases. Therapeutic cooling is effective at suppressing damaging mechanisms in the latent and second phases, including inflammation and trophic withdrawal, which helps stabilise neural mitochondria and thence provides neuroprotection. This hypothermia-induced suppression should be continued until cellular homeostasis and prosurvival signalling (e.g. growth factor and EEG restoration) has recovered. Future research should focus on preclinical treatments that further support these survival cues and suppress long-lasting injurious processes (i.e. persistent inflammation and epigenetic changes) in the third phase. rEpo and stem cells are promising candidates.

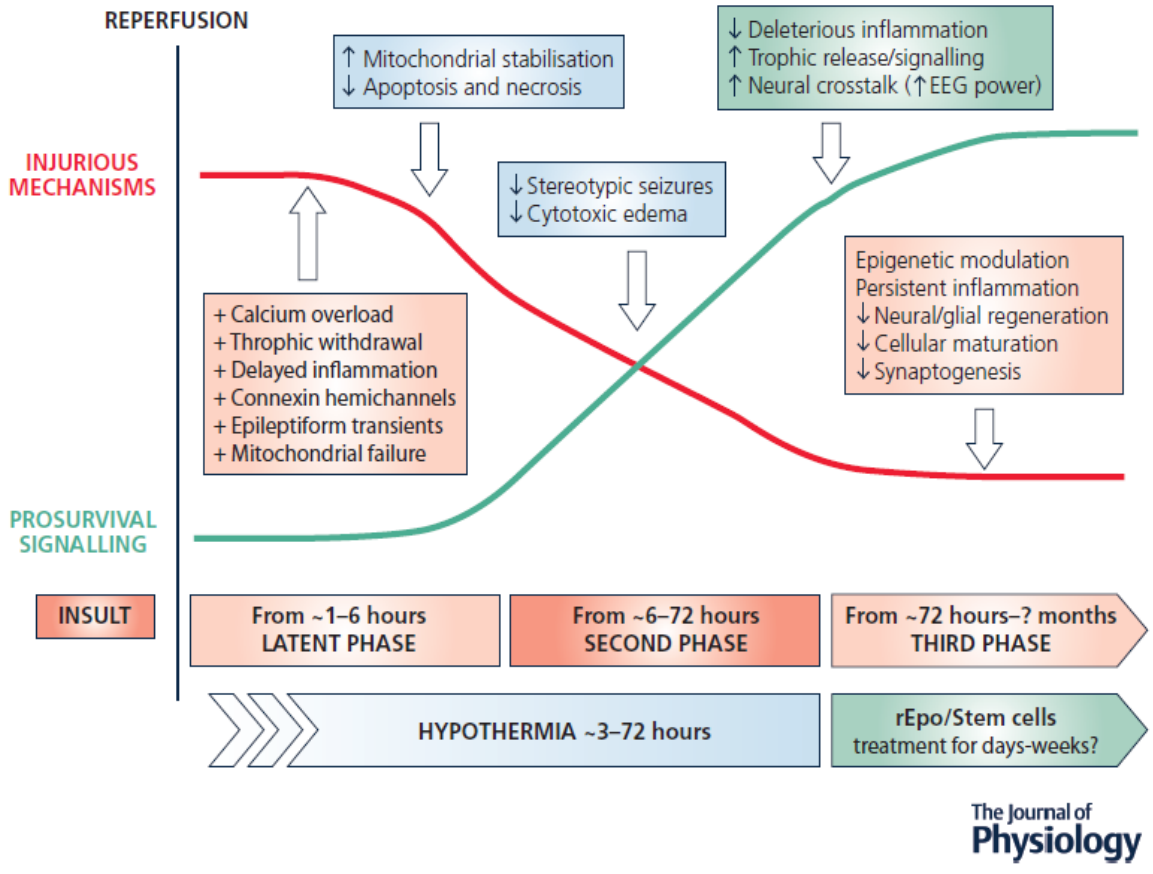

Figure 1 - The physiological effects of cerebral ischaemia for 30 min (from time zero), with or without cerebral cooling (indicated with the blue bar) induced from 3 until 72 hours after reperfusion in term-equivalent fetal sheep. The panels show, in descending order, temporal changes in extradural temperature (°C), cortical impedance (i.e. cellular swelling, as a percentage from baseline), and electroencephalographic power (EEG, decibel) in normothermia (black circles) and hypothermia groups (blue circles), compared to sham-ischaemia animals (white circles). Treatment with hypothermia suppressed the delayed rise in cytotoxic oedema (as measured with cortical impedance), and improved recovery of EEG power after resolution of the secondary seizures. Hz, hertz; dB, decibel.

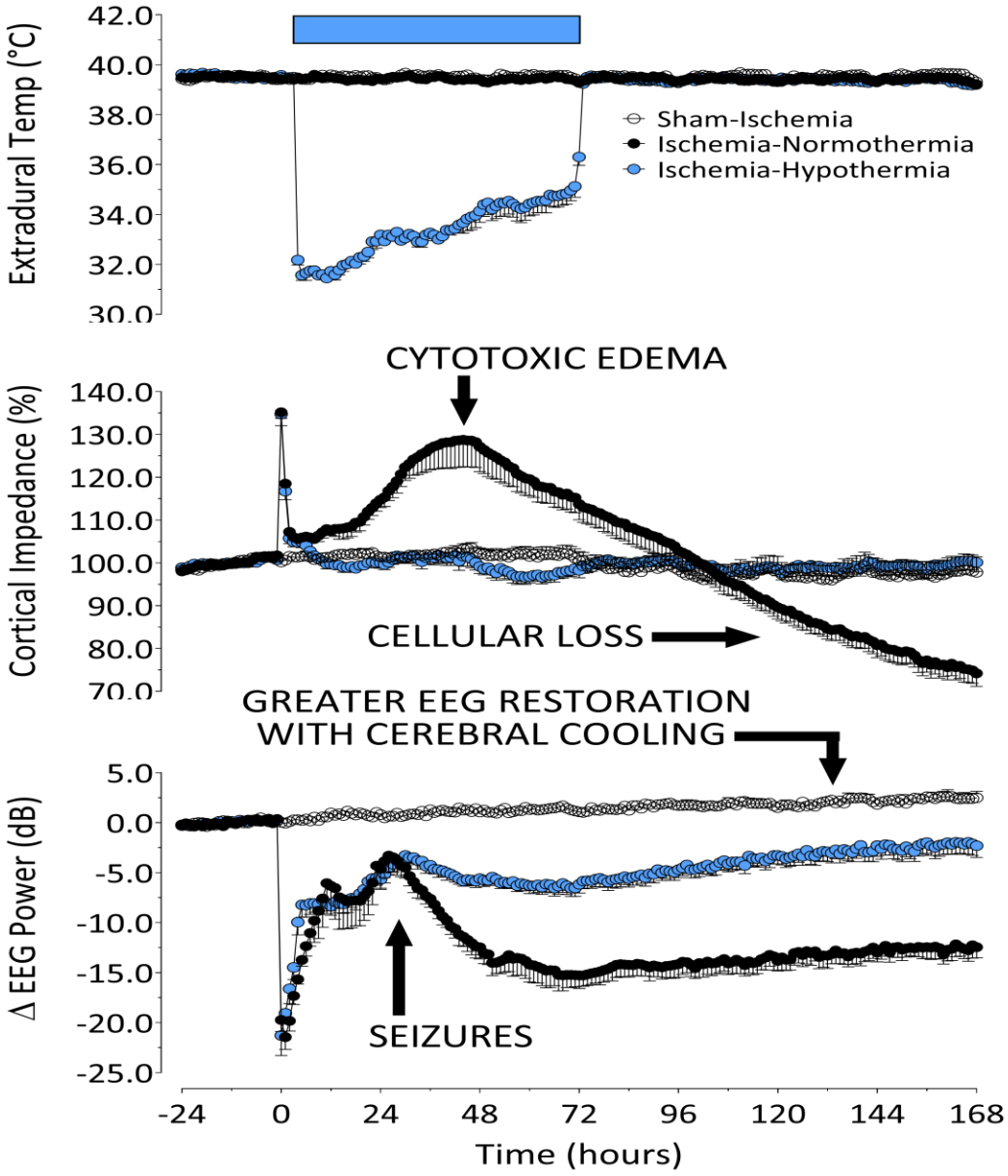

Figure 2 - Flow chart to illustrate intracellular mechanisms associated with delayed programmed cell death after HI. The snowflakes illustrate likely targets for therapeutic hypothermia. AIF (apoptosis inducing factor). APAF (apoptosis protease activating factor). BID, BH3-interacting domain death agonist. tBID, truncated BH3-interacting domain death agonist. BAX, BCL-2-associated X protein. BAK, BCL2-antagonist/killer 1. BCL2, B-Cell lymphoma 2 protein family. BCL-XL, B-cell lymphoma-extra-large. Cyto-c, cytochrome c. Diablo (direct inhibitor of apoptosis protein-binding protein). DISC, death-inducing signalling complex. Fas: first apoptosis signal receptor. MLKL, Mixed lineage kinase domain-like pseudokinase. P53, tumour protein p53. ROS, reactive oxygen species. RIPK, receptor-interacting serine/threonine-protein kinase. Smac, second mito-derived caspase activator. TNF, tumour necrosis factor receptor. TRAIL, TNF-related apoptosis-inducing ligand receptor.

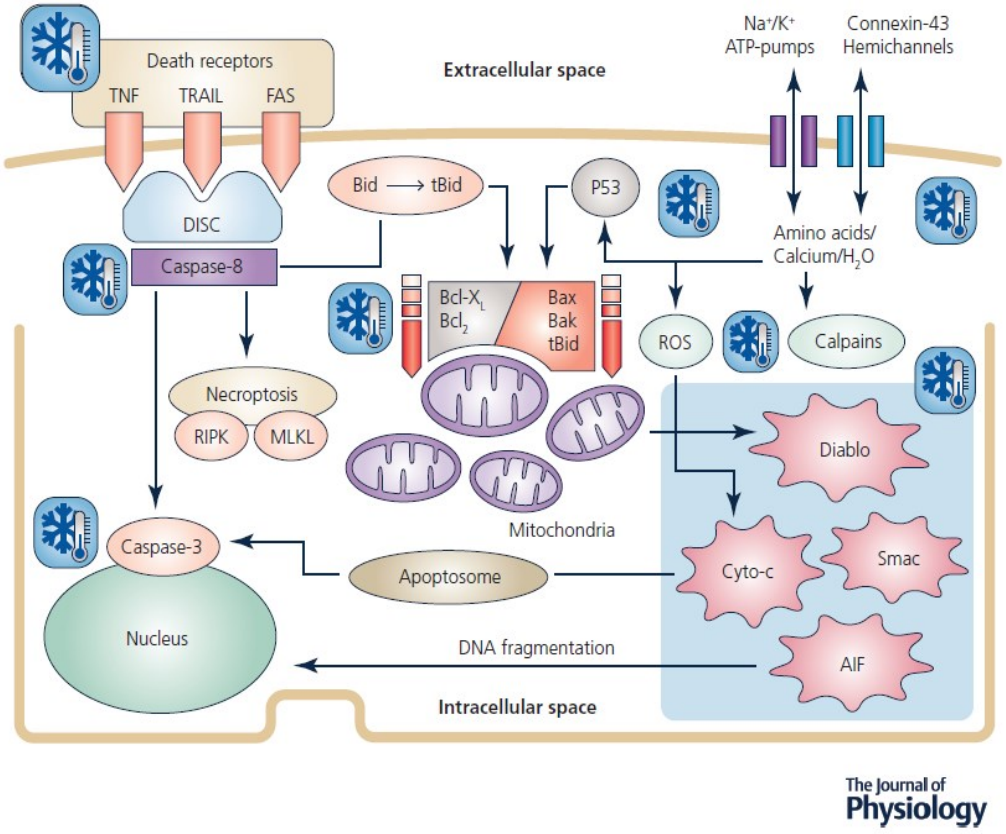

Figure 3 -The physiological effects of cerebral ischaemia for 30 min (from time zero), with or without cerebral cooling (indicated with the blue bar) induced from 3 hour until either 48 or 72 hours after reperfusion in term-equivalent fetal sheep. The panels show, in descending order, temporal changes in extradural temperature (°C), electroencephalographic power (EEG, decibel) and spectral edge frequency (Hertz) in ischaemia-normothermia (black circles), ischaemia-hypothermia 48 h (light blue

circles) and ischaemia-hypothermia 72 h groups (dark blue circles). EEG activity was suppressed in all groups during and immediately after ischaemia followed by a transient increase during seizures from 8-48 h. EEG activity in the ischaemia-normothermia group remained low for the remainder of the experiment, whereas both hypothermia groups showed a significant recovery in power and spectral frequency from 24 to 72 h ($p<0.05$). Rewarming at 48 hours was associated with loss of EEG power in the ischaemia-48 h hypothermia group, which did not occur with rewarming at 72 h ($p<0.05$). Data are mean ± SEM, Hz, hertz; dB, decibel.