J Physiol 596.23 (2018) pp 5571–5592

The Journal of Physiology

TOPICAL REVIEW

The fetus at the tipping point: modifying the outcome of fetal asphyxia

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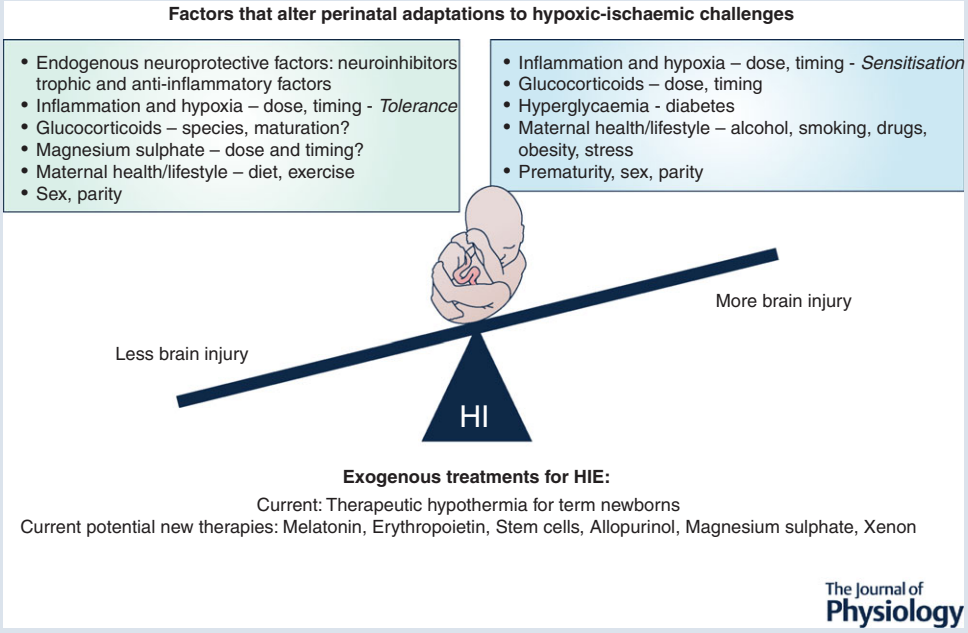
Edited by: Ole Petersen and Dino Giussani



Abstract Brain injury around birth is associated with nearly half of all cases of cerebral palsy. Although brain injury is multifactorial, particularly after preterm birth, acute hypoxia–ischaemia is a major contributor to injury. It is now well established that the severity of injury after

Simerdeep Dhillon is a PhD student working with Professor Laura Bennet to understand the role of endogenous growth factors such as erythropoietin in the brain and how best to use them to help improve neural recovery from perinatal hypoxia. **Laura Bennet** is a fetal systems physiologist whose foundation studies on preterm brain injury has led to new understanding of how the fetus adapts to key challenges such as oxygen lack, infection and common maternal therapies. She is building on this work to develop new treatments to protect and potentially rebuild the brain.

DOI: 10.1113/JP274949

hypoxia–ischaemia is determined by a dynamic balance between injurious and protective processes. In addition, mothers who are at risk of premature delivery have high rates of diabetes and antepartum infection/inflammation and are almost universally given treatments such as antenatal glucocorticoids and magnesium sulphate to reduce the risk of death and complications after preterm birth. We review evidence that these common factors affect responses to fetal asphyxia, often in unexpected ways. For example, glucocorticoid exposure dramatically increases delayed cell loss after acute hypoxia–ischaemia, largely through secondary hyperglycaemia. This critical new information is important to understand the effects of clinical treatments of women whose fetuses are at risk of perinatal asphyxia.

(Received 22 January 2018; accepted after revision 13 April 2018; first published online 8 May 2018)
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Abstract figure legend Schematic diagramme illustrating the fine balance for the fetus between surviving a period of hypoxia with little or no brain injury and experiencing brain injury that may lead to death or disability in later life. Hypoxic-ischemic brain injury occurs in a very narrow window, which is affected by many protective factors (green box, left) and harmful factors (blue box, right). Based on this knowledge, therapeutic hypothermia is now an established treatment for HI at term, and new therapies are being tested (bottom).

The global burden of hypoxic–ischaemic brain injury

Hypoxic–ischaemic (HI) events at birth and during the first 28 days of life represent the single greatest contribution to overall disability worldwide. Overall, they account for one-tenth of all disability adjusted life years (DALY) (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016), and preterm birth and neonatal encephalopathy are in the top 10 leading causes of DALY. Moreover, intrapartum-related death is the leading cause of neonatal mortality and the third leading cause of death in children under five (Liu *et al.* 2015). In practice, these statistics likely considerably underestimate the impact of HI given that many countries do not have robust maternal and perinatal mortality and morbidity databases (Blencowe *et al.* 2016).

Further, there is a need to improve precision around terms such as 'birth asphyxia', which lack specificity, to improve our understanding of the causal pathologies (Ariff *et al.* 2016). Imprecise terminology narrows our focus on *when* we consider injurious or life threatening HI may occur. The words birth and asphyxia, for example, tend to be become synonymous, leading us to focus on birth as the only time significant HI insults may happen. Yet, as we will discuss in this review, adverse events can affect the entire perinatal period, whether in isolation, acutely, chronically or in combination. For example, antenatal HI and other insults contribute to the antenatal origins of at least some cases of cerebral palsy (CP) (Tan, 2014; Shepherd *et al.* 2017) (see also Ellery *et al.* 2018, in this issue), and impaired maturation of oligodendrocytes in the preterm brain (Back, 2015). Postnatal cardiorespiratory and metabolic dysfunction (Laptook, 2013) (see also the review by Bennet *et al.* 2018c, in this issue), and intermittent or sustained systemic infection and inflammation are all significantly associated with later-life disability (Dammann & Leviton, 2014; Hagberg *et al.* 2015; Bennet *et al.* 2018*a*).

To reduce neonatal mortality and morbidity, as well as lifelong disability, we need to address the significant global challenge of ensuring timely and equitable access to obstetric and neonatal care by trained staff, particularly in developing nations, although equity remains a problem even in many resource-rich nations (Tagin *et al.* 2015; Ariff *et al.* 2016). Further, creating maternal and perinatal mortality and morbidity databases will help determine risk factors and the success of interventions (Blencowe *et al.* 2016). Importantly, however, it is the advances we must make in our scientific understanding about the adaptation of the fetus and the neonate to adverse events (both injurious and endogenously protective) that will allow us to unravel the complexity of the pathological causes underpinning perinatal mortality and morbidity. Such advances are vital if we are to truly improve our detection of the at-risk baby and for the development of treatments which will prevent death and prevent, reduce or repair injury.

The purpose of this review is to highlight the scientific challenges we face in understanding the nature and timing of adverse perinatal insults such as HI and inflammation, their interaction with each other from fetal to newborn life, and how other factors such as clinical treatments and maternal health act to modify these interactions and thus outcomes. Our primary focus for the review is on the role of HI in neonatal encephalopathy and impaired neurodevelopmental disabilities.

Hypoxic–ischaemic challenges

Hypoxia–ischaemia at birth. While the causes of brain injury and impaired brain development are complex and multifactorial (Galinsky *et al.* 2018), HI contributes to injury and impaired development in both preterm and term babies (Laptook, 2016; Gale *et al.* 2017; Huang *et al.* 2017) and represents around 50–80% of cases of neonatal encephalopathy (NE) (Ahearne *et al.* 2016). The majority of cases of NE occur in low–middle income countries (Lee *et al.* 2013; Tagin *et al.* 2015), and in developed countries the prevalence of fetal asphyxia at delivery is around 25/1000 live births (Low, 2004), of which ∼1–3/1000 live births at term will develop early onset HI encephalopathy (HIE) (Lee *et al.* 2013; Gale *et al.* 2017).

Few studies have evaluated HI events during *preterm* birth, but it has been suggested that the prevalence of asphyxia is around 73 per 1000 live births, of whom 50% are moderate or severe (Low *et al.* 2003). Some relatively small, retrospective studies, found lower rates of HIE, varying between 1.4/1000 (Chalak *et al.* 2012), 5/1000 (Schmidt & Walsh, 2010) and 9/1000 (Salhab & Perlman, 2005). However, recently, a large cohort of 115,502 deliveries in the USA between 2008 and 2011 reported that HI in preterm birth may be significantly higher with 37.3/1000 babies born before 37 weeks of gestation reported as having moderate to severe HIE (Manuck *et al.* 2016). Importantly, this study demonstrated that mortality and morbidity rates rose significantly with falling gestational age at birth, such that infants born before 28 weeks of gestation had an overall rate of HIE of 120/1000, underscoring the need to define age ranges when comparing studies.

Differences between studies may relate to the size of the cohorts studied, how HI was defined, and a lack of standardised data collection (Laptook, 2016). Further, while determining if an injurious HI event has occurred can be difficult in term births (Ahearne *et al.* 2016; Ariff *et al.* 2016; Laptook, 2016), it is much more difficult in preterm babies, particularly in infants <30 weeks of gestation (Logitharajah *et al.* 2009; Laptook, 2016). Thus, it is likely that HI at birth is underappreciated in very young preterm infants (Laptook, 2016). An example of how we may be under-reporting HIE in preterm infants is given by Logitharajah and colleagues who observed that around 30% of babies with HIE had a cord blood pH of >7.0 (Logitharajah *et al.* 2009), suggesting that studies such as that of Salhab & Perlman (2005), which included only babies with a pH of <7.0 may underestimate the number of babies affected by HI. Finally, postnatal cardio-respiratory compromise further complicates the diagnosis of HI at birth (Laptook, 2016).

Antenatal hypoxia–ischaemia. The studies discussed above have evaluated the occurrence of HI occurring around the time of birth. However, HI insults may occur both before birth (e.g. in association with intrauterine growth retardation (IUGR)/small for gestational age (SGA) or with discrete HI), as well as after birth (e.g. apnoea), particularly in preterm infants (Streimish *et al.* 2012). IUGR/SGA is defined as birth weight below the 10th percentile (Ehrenkranz, 2007) and is seen in around 3–9% of all births in high income nations, and is more than sixfold higher in low–middle income countries (Lee *et al.* 2013; Miller *et al.* 2016). While many factors contribute to IUGR/SGA, including malnutrition and fetal chromosomal abnormalities, many cases relate to placental insufficiency leading to hypoxia as well as reduced nutrition. IUGR/SGA is associated with increased risk of death and, in survivors, with impaired neurodevelopment, CP, and increased risk for cardio-metabolic diseases (Ehrenkranz, 2007; Streimish *et al.* 2012; Miller *et al.* 2016).

Acute on chronic hypoxia–ischaemia. Clinically, IUGR/SGA is associated with both chronic antenatal hypoxia, with basal hypercarbia and elevated lactate levels consistent with significant chronic placental impairment, and a higher risk of death and abnormal neurodevelopmental outcomes (Nicolaides *et al.* 1989; Arcangeli *et al.* 2012). The adverse outcomes are at least in part associated with increased vulnerability to HI at birth (Hayes *et al.* 2013). Consistent with this, in near-term fetal sheep, healthy normoxic fetuses adapt well to brief umbilical cord occlusions repeated every 5 min, a rate consistent with early labour, with minimal metabolic acidosis and stable hypertension during occlusion (Westgate *et al.* 2005). In contrast, fetuses with pre-existing but stable moderate hypoxia develop severe metabolic acidosis and hypotension (Westgate *et al.* 2005). In turn, the intermitted hypotension during occlusions was associated with greater electroencephalogram (EEG) suppression, inter-occlusion seizures and more sustained cytotoxic cerebral oedema, consistent with early onset of neural injury (Wassink *et al.* 2013). IUGR is associated with reduced stores of cardiac glycogen (Takahashi *et al.* 1995). Given that the ability of the fetus to survive prolonged asphyxia is highly associated with levels of cardiac glycogen (Shelley, 1961), it is likely that the early onset of hypotension during umbilical cord occlusion in fetuses with pre-existing hypoxia was associated with more rapid depletion of cardiac glycogen. Evolving myocardial injury may also have contributed particularly once hypotension was established during the series of occlusions (Gunn *et al.* 2000).

Tolerance to acute asphyxia falls towards term. Identifying acute HI insults *before* birth is more difficult, for obvious reasons. However, in the search for the

pathological factors which cause injury, it is important to appreciate that immature animals, both term and preterm, show high cardiac and neural tolerance to HI (for review see Bennet, 2017). An early observation of this phenomenon came from Robert Boyle and colleagues who demonstrated in 1670 that term newborn kittens could tolerate anoxia in a vacuum chamber for far longer than adult animals (Boyle, 1670). Studies in a variety of species, including humans, have since demonstrated that immature animals have greater cardiac glycogen stores that ensure the heart can continue to beat through an HI challenge (Shelley, 1961). It is notable that cardiac glycogen stores peak in fetal life around 0.5–0.6 of gestation (Shelley, 1961), suggesting the intriguing possibility that hypoxic challenges may be particularly common in preterm life. Higher glycogen stores facilitate anaerobic metabolism, and so, healthy preterm fetuses can survive much longer periods of HI induced by umbilical cord occlusion than their term counterparts (Bennet, 2017). Moreover, the preterm fetus can tolerate longer periods of hypoxia, hypoperfusion and hypotension before developing injury to the brain and other organs (Keunen *et al.* 1997; Quaedackers *et al.* 2004*a*,*b*; Wassink *et al.* 2007; Bennet, 2017).

However, even in the very preterm fetus, there comes a point when a sufficient duration of severe HI, hypoperfusion and hypotension will cause neural injury. A preterm fetus exposed to severe HI *in utero* can survive with evolving brain injury. Studies examining neural outcomes after severe HI in preterm fetal sheep have shown that by 72 h there is diffuse white matter loss, subcortical neuronal injury and no cortical neuronal loss (Bennet *et al.* 2007; Wassink *et al.* 2017). Diffuse white matter injury evolves over time involving degenerative, proliferative and arrested maturation processes. One week after prolonged cerebral ischaemia or severe HI in preterm fetal sheep, proliferation of oligodendrocyte progenitor cells restored the number of total oligodendrocytes (Riddle *et al.* 2011; Drury *et al.* 2014). However, newly formed pre-oligodendrocytes failed to differentiate into mature oligodendrocytes and there was loss of white matter volume (Riddle *et al.* 2011).

Chronic activation of microglia and astrogliosis persisted 3 weeks after severe HI in preterm fetal sheep, with reduced numbers of mature, myelin-producing oligodendrocytes, altered myelination in the subcortical white matter tracts and reduced cortical thickness (van den Heuij *et al.* 2017). Furthermore, magnetic resonance imaging (MRI) data from preterm fetal sheep, 4 weeks after prolonged cerebral ischaemia, showed altered microstructural development of grey matter with reduced dendritic arbor complexity and spine density of cortical projection neurons and medium spiny neurons of the caudate nucleus, and functional disturbances of glutamatergic signalling (Dean *et al.* 2013; McClendon *et al.* 2014). The patterns of injury seen in preterm fetal sheep after HI are highly consistent with the spectrum of injury seen in contemporary cohorts of preterm infants (Buser *et al.* 2012; Ball *et al.* 2015; Thomason *et al.* 2017).

Further, milder insults, which are less easy to diagnose, can also have long-term adverse effects on the brain. Transient moderate hypoxia in preterm fetal sheep, at 0.65–0.7 gestation (equivalent to 28–30 weeks human brain maturation), significantly impaired maturation of the fetal sub-plate neuron arborisation and activity (McClendon *et al.* 2017); the impact on maturation was related to the severity of hypoxia. Similarly, impaired brain development and delayed cerebral injury was seen after mild HI in preterm-equivalent neonatal rats at postnatal day (P) 3 (Sizonenko *et al.* 2003) and at P7 (Geddes *et al.* 2001). Thus, it is entirely feasible that HI insults can occur well before birth, which are undetected and which the fetus can survive and continue to develop until either preterm or term birth. In turn, brain injury or impairment sustained before birth may then be added to by HI events during birth. It is notable that fetal heart rate (FHR) monitoring, the gold standard for monitoring fetal well-being during labour (for review see Lear *et al.* 2016), assumes that without evidence to the contrary the fetus being monitored is neurologically intact. Research is now being undertaken to begin to determine the effect of the fetal adaptation to HI insults *in utero* on FHR parameters (see Yamaguchi *et al.* 2018 in this issue).

Perinatal events are associated with approximately half of all cases of cerebral palsy (CP) (Reid *et al.* 2016). Approximately 15–20% are related to acute HIE at term (Reid *et al.* 2016), while a third of cases are related to preterm birth (Committee on Understanding Premature Birth and Assuring Healthy Outcomes, 2007). In preterm neonates, the causes of brain injury are very complex, but a recent study has demonstrated perinatal HI-related risk factors, such as acidaemia and Apgar score, are strongly associated with the development of periventricular white matter injury (Huang *et al.* 2017). Recent MRI cohort studies of children with CP show that only ∼13% have normal imaging and another 10% have malformations (Reid *et al.* 2014). Most of the remainder show overt white or grey matter injury or focal vascular insults. Thus, it is plausible that at least some children born at term with no apparent intrapartum risk factors had had undetected pre-partum HI or infection/inflammation. Further, even in infants with known acute HIE at birth, over half also had antepartum risk factors (Badawi *et al.* 1998).

In addition, there is increasing evidence that late stillbirth before the onset of labour is presumptively related to impaired placental perfusion causing fetal HI. Rates of stillbirth have been reduced by targeting fetuses who show acute reductions in fetal movements (Stacey *et al.* 2011). Moreover, the New Zealand multicentre stillbirth case-control study recently showed that when the mother went to sleep in the supine position the

risk of stillbirth was increased (adjusted odds ratio 3.7). The mechanism is likely reduced uterine perfusion. Consistent with this, in a study of healthy women in late pregnancy, the semi-recumbent and supine positions were associated with fetal sleep state switching to quiet sleep, in which fetal oxygen-consuming activity is reduced with correspondingly reduced fetal heart rate variation (Stone *et al.* 2017).

Postnatal hypoxia–ischaemia. Apnoea of prematurity and periodic breathing cause repeated, mild hypoxic insults that are associated with neurodevelopmental and motor impairments (Schmidt *et al.* 2017) (also see Bennet *et al.* 2018c, in this issue). Further, potential dysregulation of cerebral autoregulation in sick infants may lead to reduced cerebral perfusion and oxygenation (Vesoulis & Mathur, 2017). In preterm babies, perfusion and thus oxygenation may be complicated by their immature lungs and by persistent patent ductus arteriosus (Di Fiore *et al.* 2013). Periodic breathing and apnoea are more common in preterm babies, but breathing is also often irregular in term babies with HIE and may require ventilation; stable management of these infants remains a key challenge (Martinello *et al.* 2017). Cerebral desaturation during apnoeic periods may be sufficiently challenging to cerebral metabolism that there is EEG suppression (Low *et al.* 2012). The preterm brain may also be exposed to spontaneous periods of desaturation (Baerts *et al.* 2011), but correction with oxygen supplementation can lead to intermittent cerebral hyperoxia, which may itself be injurious (Baerts *et al.* 2011).

Worryingly, many preterm babies continue to have periodic breathing (Decima *et al.* 2015) and persistent apnoea (Horne *et al.* 2017) after being discharged and these events are associated with cerebral desaturation, and thus may contribute to later life neurocognitive impairment (Decima *et al.* 2015; Horne *et al.* 2017). Such events are more prevalent around 2–3 and 5–6 months of life than during the first few weeks of life (Horne *et al.* 2017). Further, individuals born preterm are 3–4 times more likely than children and adults to experience sleep disordered breathing such as snoring and obstructive sleep apnoea (Rosen *et al.* 2004). These pathological conditions can cause intermittent hypoxia and are associated with learning and behavioural difficulties (Rosen *et al.* 2004).

Preclinical data support these findings. Mild, intermittent hypoxia given to rats P2 and P12, with follow-up to P18 and P22, was associated with evident systemic and brain inflammation, impaired white matter integrity, and metabolic changes consistent with hypoxia (Darnall *et al.* 2017). Repetitive apnoea in anaesthetised newborn piglets was associated with progressive cortical deoxygenation (Schears *et al.* 2005) and evidence of cortical and subcortical injury (Mendoza-Paredes *et al.* 2008). Further, in a study where lipopolysaccharide was given to P2 rats who were then followed-up to P10, showed that inflammation may cause more episodes of periodic breathing, potentially by altering carotid body structure and function (Master *et al.* 2016).

Recovery from hypoxic–ischaemic insults

Contributing to the difficulty in determining whether an injurious insult has occurred is that injury evolves over time (Bennet *et al.* 2006; Iwata *et al.* 2008). As discussed below, external factors such as inflammation and clinical treatments can modulate how injury evolves, and affect the measurements used for diagnosis and prognosis. For example, therapeutic hypothermia for HIE significantly alters the temporal expression of seizures (Lynch *et al.* 2015; Davidson *et al.* 2015*a*). It is now well established in term infants and animals that there can be considerable cell survival after severe HI, followed by progressive evolution of bulk cell death over hours to days (Wyatt *et al.* 1989; Lorek *et al.* 1994). There are limited data on the post-HI evolution of injury in the preterm brain; however, preclinical data suggest that temporal changes in blood flow, cerebral oxygenation and seizures occur in a similar temporal pattern (Bennet *et al.* 2006, 2010, 2012*a*).

Latent phase. Following reperfusion there is recovery of depleted high energy phosphates and at least partial resolution of cellular oedema in a so-called 'latent' phase of recovery. The extent of recovery during this phase correlates with severity of injury (Iwata *et al.* 2008). The latent phase is further characterised by suppression of EEG activity, which is mediated by neuroinhibitors such as neurosteroids (Nguyen *et al.* 2004; Yawno *et al.* 2007), and upregulation of the sympathetic nervous system (Quaedackers *et al.* 2004*a*; Dean *et al.* 2006). Inhibition of these neuromodulators markedly increased cerebral injury, strongly suggesting that these endogenous responses are beneficial (Dean *et al.* 2006; Yawno *et al.* 2007). In addition to neuronal inhibition, multiple neuroendocrine responses also help protect the brain (Robertson *et al.* 2012). For example, in newborn piglets, P7 rats and fetal sheep there is release of melatonin early in the latent phase, and delayed upregulation of multiple anti-apoptotic growth factors such as erythropoietin (Epo) and insulin like growth factor 1 (IGF-1) in the secondary and tertiary phases after HI (Guan *et al.* 2003; Miller *et al.* 2005; Robertson *et al.* 2013; Ohls *et al.* 2015).

Studies of the preterm fetal brain have shown, however, that EEG suppression is not complete. Epileptiform transient activity (e.g. sharp waves) is observed throughout the latent phase, peaking around 2–3 h post-HI (Bennet *et al.* 2010). The maximum frequency of these events after HI is associated with cerebral deoxygenation and with the severity of neural injury (Bennet *et al.* 2006). These data

suggest that epileptiform transients may stress injured cells and propagate injury in a similar manner to spreading depolarisations (Hartings *et al.* 2017). Consistent with this, studies in sheep fetuses and multiple adult species (Davidson *et al.* 2012; Hartings *et al.* 2017; Kim *et al.* 2017) show that astrocytic and microglial responses contribute to spreading injury from the most severely affected regions to previously undamaged areas of the brain, in part by the opening of cell membrane channels such as connexin 43 hemichannels, leading to release of excitatory small molecules such as ATP and glutamate (Davidson *et al.* 2013; Hartings *et al.* 2017).

EEG suppression during the latent phase is coupled with cerebral hypoperfusion, with data suggesting that this is coupled to reduced cerebral metabolism in immature (Jensen *et al.* 2006) and adult animals (Michenfelder & Milde, 1990). Hypoperfusion is also seen in peripheral organ beds, mediated by increased vascular resistance not hypotension (for review see Bennet *et al.* 2012*a*). This is an important observation, as low blood pressure and hypoperfusion are frequently seen in preterm newborns during the first few days after birth (Dempsey, 2017), and there is debate about the contribution of this apparent 'cardiovascular instability' to evolving injury and how best to clinically manage haemodynamic changes (Dempsey, 2017). In part this relates to the variability in what is defined as normal blood pressure, but it is also clear that there is a poor relationship between blood pressure and blood flow (Dempsey, 2017), and low blood flow often does not change in response to increasing blood pressure. In some cases treating hypotension can be associated with adverse outcomes (Fanaroff *et al.* 2006; Dempsey, 2017). For some infants, low blood flow may in fact be a post-HI adaptation, as seen experimentally (Bennet *et al.* 2012*a*).

Secondary and tertiary phases. The latent phase is followed by a secondary deterioration in cerebral oxidative metabolism starting 6–15 h after birth (Azzopardi *et al.* 1989; Lorek *et al.* 1994; Gunn *et al.* 1997; Penrice *et al.* 1997), due to failure of mitochondrial function (Leaw *et al.* 2017). This phase is associated with the onset of seizures in both preterm and term fetuses, and in term fetus, greater cortical neuronal maturation, is associated with secondary cortical cytotoxic oedema (Lorek *et al.* 1994; Gunn *et al.* 1997; Penrice *et al.* 1997). The timing of energy failure after HI is tightly coupled with the appearance of histological brain damage (Blumberg *et al.* 1997; Roth *et al.* 1997; Vannucci *et al.* 2004), suggesting that it is primarily a function of evolving cell death (Fig. 1). Neuroprotection treatments such as therapeutic hypothermia, that are effective when started in the latent phase, rapidly lose effectiveness when started during the secondary phase (Gunn *et al.* 1997; Wassink *et al.* 2014). It is unclear why mitochondria become dysfunctional at a time when the supply of oxygen has normalized, but it does provide a target for treatment (Leaw *et al.* 2017).

Blood flow changes in the secondary phase can be variable. Preterm fetal sheep studies show that central and peripheral hypoperfusion may resolve or partly resolve after asphyxia (Bennet *et al.* 2012*a*). Preterm babies may be at risk of loss of cerebral autoregulation leading to impaired cerebral perfusion with low blood pressure (Vesoulis & Mathur, 2017), and blood flow can fluctuate during events such as seizures. Hypoperfusion is seen in peripheral organs like the gut during seizures in preterm fetal sheep, mediated by sympathetic activity (Bennet *et al.* 2012*a*). In younger preterm infants, increased systemic perfusion and cerebral blood flow are associated with increased risk for germinal matrix haemorrhage–intraventricular haemorrhage (GMH-IVH) (Noori *et al.* 2014). In term HIE infants and term fetal sheep, cerebral hyperaemia (increased cerebral blood flow) is observed (Meek *et al.* 1999; Greisen, 2014). Perhaps counter-intuitively, increased cerebral blood flow (CBF) correlates with adverse outcomes (Meek *et al.* 1999).

The secondary phase resolves over 3–4 days post-HI into a tertiary phase of ongoing injury, involving repair and reorganisation which may last weeks to months and even years (Hagberg *et al.* 2015; Bennet *et al.* 2018*a*), but there is also chronic inflammation and epigenetic changes lasting for weeks to months after injury that may prevent optimal neurorepair (Fleiss & Gressens, 2012; Galinsky *et al.* 2018; Bennet *et al.* 2018*a*). Key neuroprotection strategies in this phase include treating chronic inflammation and stimulation of endogenous factors which support proliferation, migration and maturation of glia and neurons (Hagberg *et al.* 2015; Bennet *et al.* 2018*a*). Treatments such as stem cell therapy have utility in this phase, given their multimodal effects in reducing inflammation and promoting release of trophic factors (Fleiss *et al.* 2014; van den Heuij *et al.* 2017; Bennet *et al.* 2018*a*). Further, there is a clear role for postnatal neurorehabilitation for optimising development of the neural network (Pitcher *et al.* 2009; Maitre, 2015) (see also Bennet *et al.* 2018c, in this issue). Importantly, as part of the challenges we face, it is clear that early and accurate diagnosis of conditions such as CP make a significant difference to providing the right neurorehabilitation treatment in a timely manner (Novak *et al.* 2017).

Modification of neural outcome by multiple insults

A recent MRI study in preterm infants demonstrated that a synergy between prenatal and postnatal insults, such as intrauterine growth restriction and prolonged mechanical ventilation had a cumulative effect on white matter injury,

as shown by lower white matter fractional anisotropy at term equivalent age, and impaired neurodevelopmental outcomes at 20 months corrected age (Barnett *et al.* 2018). In the section below we discuss how exposure to inflammation, antenatal treatments (e.g. glucocorticoids and magnesium sulphate) and maternal diabetes, obesity and other lifestyle factors may modulate fetal response to HI insults and resultant neural injury.

Inflammation. It is now recognised that fetal inflammation is associated with adverse lifelong outcomes such as impaired neurodevelopment, particularly after preterm birth (Dammann & Leviton, 2014; Back, 2015; Hagberg *et al.* 2015; Bennet *et al.* 2018*a*). Inflammation of chorionic and amniotic membranes (chorioamnionitis), for example, is reported in nearly 95% of preterm births at 21–24 weeks of gestation, and in about 10% of deliveries at 33–36 weeks (Kim *et al.* 2015). Infection of the fetus occurs in approximately 20–30% of confirmed intrauterine infections (Cordeiro *et al.* 2015). Fetal inflammation (funisitis) and early neonatal bacteraemia have been shown to be independent risk factors for encephalopathy (Tann *et al.* 2018). Late onset bacteraemia (due to factors such as long-term indwelling catheters) in preterm infants during postnatal weeks 2–4 is associated with a greater risk of neurocognitive limitations at age 10 years (Bright *et al.* 2017). Further, there is some evidence from a cohort study of 8299 women that the combination of cord blood acidosis and maternal pyrexia greatly increased the risk of neonatal encephalopathy (Impey *et al.* 2008).

Currently, the mechanisms mediating the association between infection and inflammation and neonatal encephalopathy (NE) are unclear (Hagberg *et al.* 2015). However, NE is strongly associated with elevations of pro-inflammatory mediators such as tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and IL-1β and toll-like receptors (TLRs) in plasma and in the brain (Dammann & Leviton, 2014; Hagberg *et al.* 2015; Bennet *et al.* 2018*a*). Recent data from the Extremely Low Gestational Age Newborns (ELGANs) study measured pro-inflammatory cytokine patterns in whole blood of preterm infants <28 weeks gestation in the first month of life and demonstrated that elevated systemic levels of pro-inflammatory cytokines are associated with adverse neurological outcomes up to the age of 10 years (Kuban *et al.* 2017), and that both antenatal and postnatal inflammation play a role (Yanni *et al.* 2017). Further, data suggest that in children with CP associated with white matter loss, early exposure to inflammation is associated with chronic inflammation and increased sensitivity to inflammatory mediators later in life (Lin *et al.* 2010).

Perinatal inflammation can function as a second hit in preterm infants with SGA, acting to increase the risk of impaired neurodevelopmental outcomes (Leviton *et al.* 2013). Exposure to both sepsis and HI during the perinatal period increases the risk of cerebral palsy in very premature infants (Wang *et al.* 2014). Similarly, the combination of fetal growth restriction, denoting prenatal hypoxia and postnatal inflammation markedly increases the risk of impaired neurodevelopmental scores at 2 years of age compared to either alone (Leviton *et al.* 2013). That two injurious insults are additive is not surprising; however, considerable data suggest that inflammation can modify the responses to an HI insult in both positive (tolerance) and negative (sensitisation) ways depending on the order,

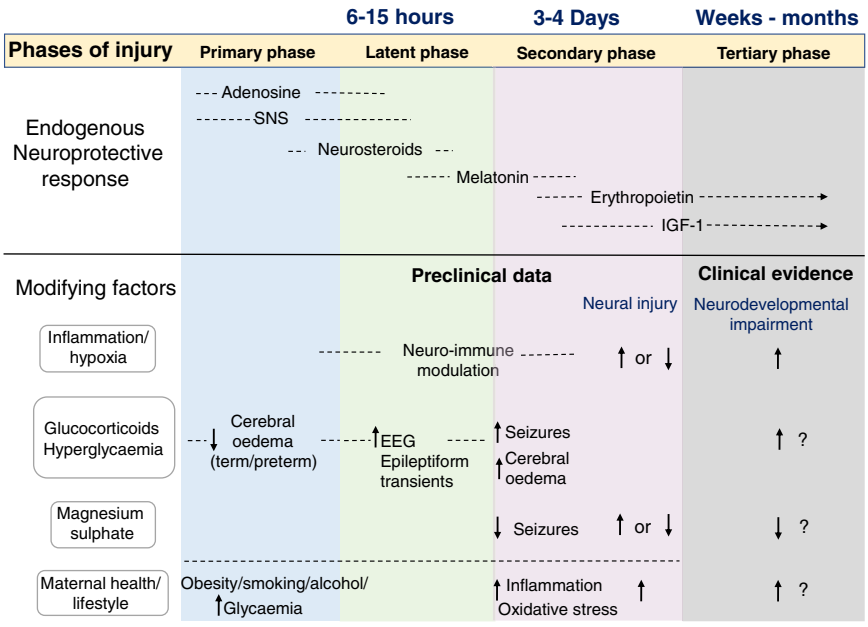

Figure 1. Schematic diagram illustrating the phases of evolving hypoxic–ischaemic (HI) brain injury
Examples of when endogenous neuroprotective factors are released are shown at the top. Examples of factors which modify the perinatal adaptation to HI are shown below. Factors that can increase neural injury or risk of neurodevelopmental impairment are denoted by up arrows, while factors associated with evidence for decreased injury and impairment are denoted by down arrows. SNS (sympathetic nervous system), insulin-like growth factor 1 (IGF-1), electroencephalographic activity (EEG).

intensity and time of the insults (Hagberg *et al.* 2015; Bennet *et al.* 2018*a*).

Sensitisation. Clinical data suggest that prior inflammatory stimulus can enhance metabolic decompensation during subsequent HI. For example, using near-infrared spectroscopy (NIRS) in preterm infants, Stark *et al.* showed that intrauterine inflammation was associated with an increase in cerebral oxygen consumption after birth (Stark *et al.* 2016). In preclinical studies, systemic inflammation induced with injection of TLR-2 agonist Pam3CSK4, given 14 h before an HI insult in P8 mice, increased loss of brain tissue and demyelination, potentially through suppression of ADP induced oxidative phosphorylation in mitochondria (Mottahedin *et al.* 2017). These data suggest that inflammation may play a role in the loss of mitochondrial function post-HI. Similarly, inflammation induced by the viral protein mimetic polyinosinic-polycytidylic acid (poly(I:C)) 14 h before HI in P8 mice also increased injury and this was associated with increased pro-inflammatory cytokines and apoptotic proteins in the brain (Stridh *et al.* 2013).

Exposure of P2 rat pups to lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, given 2 h before HI augmented microglia activation, cerebral pro-inflammatory cytokines, blood–brain barrier damage and white matter damage compared to HI alone (Wang *et al.* 2010), with similar effects observed if LPS was given at 4 h pre-HI to P7 rats (Eklind *et al.* 2001), or 2 and 72 h pre-HI (Eklind *et al.* 2005). Similar results were observed when LPS was given 14 h before HI, with evidence that injury involved TLR-4 and the recruitment of the MyD88 adaptor protein (Wang *et al.* 2009). Recently, a fetal embryonic day 18 rat model of LPS and HI exposure demonstrated that the patterns of brain injury and motor function assessed 1 month after birth were different between HI and HI + LPS, and HI with or without LPS produced patterns similar to those seen in infants with neural injury in the ELGAN study (Jantzie *et al.* 2014). All pups experienced gait abnormalities, with function worse in the HI group alone. LPS alone caused inflammation, but significantly less inflammation and injury than HI and HI + LPS, with greater acute glial activation and inflammation seen after HI + LPS. HI alone, however, was associated with greater chronic white matter and axonal injury.

Notably, in this study, loss of myelin basic protein (a marker for myelination) was observed up to P15 in both HI and HI + LPS groups, but worsened beyond this time only in the HI alone group (Jantzie *et al.* 2014). These data demonstrate the need to study the evolution of injury over time to understand the relative contributions of insults. Further, the data support the concept that HI insults alone can be associated with dysmaturation of oligodendrocytes and thus the development of subsequent myelination (Back, 2015), and may contribute to more severe or chronic white matter injury patterns. LPS appears to positively moderate the severity of HI injury, and this may occur through progressive restoration of Epo receptor and ligand expression observed in this study in the HI + LPS but not HI alone group (Jantzie *et al.* 2014).

Tolerance. In contrast to the studies demonstrating sensitisation by inflammation to greater HI mediated injury, several studies demonstrate that, depending on the insult severity, the time interval between insults, and maturational stage of the brain, the interaction between inflammation and HI insults can be protective. We have demonstrated in preterm fetal sheep that inflammation induced by chronic low-dose infusion of LPS (100–250 ng/day) for 5 days with superimposed 1 μg boluses was associated with white matter inflammation and loss of mature oligodendrocytes (Mathai *et al.* 2013; van den Heuij *et al.* 2014). However, exposure to inflammation was associated with a significant reduction in HI injury when an HI insult was given 4 h after the last bolus of LPS (van den Heuij *et al.* 2014). Upregulated plasma concentrations of the anti-inflammatory IL-10 and cortisol may have contributed to neuroprotection (van den Heuij *et al.* 2014). Further, pre-treatment of preterm fetal sheep with a bolus dose of LPS (50–100 ng/kg) led to differentially regulated TLR mRNA expression and increased protein expression of interferon-β when exposed to HI at 24 h after LPS treatment, whereas no effect was seen with a time delay of 4 h (Dhillon *et al.* 2015). In this study, LPS preconditioned fetuses had reduced loss of oligodendrocytes, with reduced microgliosis and astrogliosis, at 5 days after HI (Dhillon *et al.* 2015).

Consistent with this, in P7 rats, exposure to LPS 24 h before HI conferred protection, in contrast to increased injury seen when it was given 2, 4, 14 or 72 h before HI (Eklind *et al.* 2005). This timing likely reflects, at least in part, the time needed for upregulation of type I interferon and interferon regulatory factors (Marsh *et al.* 2009). However, the dose of the inflammatory agent and the age of exposure are also important factors. For example, tolerance to HI is observed when LPS is given at P7, P9 or P14 (Eklind *et al.* 2005; Hickey *et al.* 2011), and in response to poly(I:C) at P5 (Hickey *et al.* 2011; Shi *et al.* 2013), but not when LPS is given to P3 and P5 rat pups, or poly(I:C) given at P7 (Hickey *et al.* 2011; Shi *et al.* 2013). The differences may be due to the developmental differences in TLR induction (Shi *et al.* 2013). Consistent with this, preterm neonates (<30 weeks) have attenuated innate immune responses to TLR agonists in the first 28 days of age (Marchant *et al.* 2015). Consideration should also be given to the dose of LPS. In P7 rats, a

0.3 mg/kg bolus dose of LPS given 24 h before an HI insult increased injury, whereas a 0.05 mg/kg bolus dose reduced injury and improved neurological outcomes, and this was associated with reduced microglial activation and pro-inflammatory cytokine production (Lin *et al.* 2009).

Exposure to hypoxia can also confer tolerance to subsequent insults. The neuroinflammatory response after HI was attenuated in P7 rats preconditioned with transient asphyxia *in utero* or mild hypoxia postnatally, and the interaction between the insults was found to be neuroprotective (Park *et al.* 2011; Vlassaks *et al.* 2013). A 3 h period of 8% hypoxia alone 24 h before an HI insult in P6–7 rats suppressed glial and pro-inflammatory cytokine production (Chen *et al.* 2015; Parmar & Jones, 2015). Intermittent periods of mild hypoxia have also been shown to cause preconditioning against later injurious HI in P7 rats (Ota *et al.* 1998). However, prolonged spontaneous mild hypoxaemia for at least 5 days before carotid artery ischaemia in near-term fetal sheep did not alter brain injury (Davidson *et al.* 2015*b*). This negative finding suggests that gene induction by pre-conditioning is transient and therefore resolves during chronic hypoxia.

Modification of neural outcome by antenatal treatment

Antenatal glucocorticoids and hyperglycaemia. When considering factors which modulate the perinatal responses to HI, we should remember that the fetus and newborn often receive clinical drug treatments, ranging from routine antenatal glucocorticoids and magnesium sulphate ($MgSO_4$), to postnatal steroids, pain and anti-seizure medications, sedatives and anaesthetics, and glucose supplementation. The catch-22 is, of course, that many of the conditions being treated (e.g. seizures, pain and hypoglycaemia) themselves modulate outcomes. However, this does not mean the treatment *per se* is without effect.

Glucocorticoids are routinely given to women at risk of preterm delivery to reduce mortality and morbidity associated with complications of being born prematurely. To date, there is no clear clinical information on the interaction between antenatal glucocorticoids and HIE in preterm infants, as such cases were excluded from many randomised controlled trials (Roberts *et al.* 2017). However, it is striking how diverse data from preclinical studies are on the effect of glucocorticoids on the adaptation to HI insults. Maternal administration of dexamethasone given 48 h pre-HI had no effect on injury (Elitt *et al.* 2003). However, when given 15 min after HI in preterm fetal sheep dexamethasone increased injury (Koome *et al.* 2013), and this was associated with increased EEG activity and seizure activity, and evidence of uncoupling of CBF and cerebral metabolism as well as exacerbated hyperglycaemia (Lear *et al.* 2014). In contrast, when dexamethasone was given 4 h pre-HI, there was a significant increase in neural injury, including induction of cystic lesions, despite evidence of reduced cytotoxic oedema during HI (Lear *et al.* 2018). Interestingly, glucocorticoids given to normal healthy preterm fetal sheep, who have not had HI, also cause dysregulation of EEG activity and can induce seizures (Davidson *et al.* 2011).

Similarly, some studies in neonatal rats have also reported exacerbation of HI induced neural injury with prior dexamethasone exposure (Chang *et al.* 2013; Yeh *et al.* 2017). For example, administering a tapering course of dexamethasone 0.5, 0.3 and 0.1 mg/kg on postnatal days 1–3 in neonatal rats, and subsequently subjecting them to HI on P7, caused greater loss of oligodendrocytes, reduced myelin thickness, and worse functional outcome in the long-term as compared to animals subjected to HI alone (Yeh *et al.* 2017). A role for increased excitotoxicity is postulated as the effect of dexamethasone on HI mediated injury correlated with decreased glutamate transporter-1 (GLT-1)-mediated glutamate reuptake observed after HI (Chang *et al.* 2013).

In contrast, many studies in postnatal rats have shown neuroprotection with glucocorticoids given 4–48 h before HI (Barks *et al.* 1991; Chumas *et al.* 1993; Ekert *et al.* 1997; Dardzinski *et al.* 2000; Felszeghy *et al.* 2004; Ikeda *et al.* 2005; Feng *et al.* 2011), but no effect if given within 3 h of an HI insult. Post-treatment is associated with no effect when given immediately, 24 or 48 h post HI (Barks *et al.* 1991), or protection when given 2 h post-HI (Harding *et al.* 2016). Differences in dose, timing of glucocorticoid administration and route of administration, e.g. intracerebroventricular injection (Harding *et al.* 2016) and intraperitoneal injection (Barks *et al.* 1991) remain to be explored. It is noted, however, that clinically, antenatal steroid exposure is associated with risk of increasing adverse neurodevelopmental outcomes (Qin *et al.* 2017). Finally, Ikeda and colleagues have demonstrated in P6 rats, that the protection conferred by exposure to LPS 24 h before HI was prevented by co-administration of the glucocorticoid receptor blocker RU486 (Ikeda *et al.* 2005).

One significant effect of glucocorticoids is to increase glucose, and the differences between the perinatal rat and sheep data with regard to the effects of HI mediated injury may be explained by differences in glucose handling in the newborn period between species, which is discussed in the next section. Developmental changes in glucose handling may also explain why pre-treatment with glucocorticoids in adult rats is usually associated with increased HI mediated brain injury, as reviewed by Bennet *et al.* (2012*b*).

Glycaemia. Term infants with HIE (Nadeem *et al.* 2011) and preterm infants (McKinlay *et al.* 2017; Sharma *et al.* 2017), show highly variable blood glucose levels during the early period after birth, and a current clinical challenge is to understand what constitutes euglycaemia and how fluctuating glucose may contribute to NE and thus how best to manage changes in glucose (Ogilvy-Stuart & Beardsall, 2010). Clinical data from term infants with HIE show adverse neurodevelopmental outcomes associated with both hyperglycaemia and hypoglycaemia during the first day after birth (Chouthai *et al.* 2015; Basu *et al.* 2016). In preterm infants, hypoglycaemia is often followed by hyperglycaemia, mediated by insulin resistance and insulin deficiency (Ogilvy-Stuart & Beardsall, 2010). In preterm infants hyperglycaemia is associated with increased mortality (Alexandrou *et al.* 2010; van der Lugt *et al.* 2010), adverse neurodevelopmental outcomes (van der Lugt *et al.* 2010), and injury to white matter (Alexandrou *et al.* 2010). Currently, the role of glycaemia in modulating HI in preterm infants is not known.

As with glucocorticoids, the data on the impact of glucose on HI are variable. In preterm fetal sheep, increasing plasma glucose to similar levels to those seen after dexamethasone produced the same severe cystic injury patterns as seen with dexamethasone (Lear *et al.* 2014, 2018). Given that post-HI dexamethasone was associated with more modest injury (Koome *et al.* 2013), this suggests that increased glucose compromises cellular function during the HI insult. *In vitro* evidence supports this concept and further suggests that increased opening of connexin hemichannels may be a key factor in the detrimental effects of hyperglycaemia during HI (Orellana *et al.* 2010). Similarly, hyperglycaemia during HI exacerbates neural injury in newborn piglets (LeBlanc *et al.* 1993), term-equivalent fetal sheep (Petersson *et al.* 2004) and adult rats (Lin *et al.* 1998).

In marked contrast, hyperglycaemia is independently protective and, at least in part, mediates the protective effects of dexamethasone in P7 rats after HI (Vannucci & Mujsce, 1992; Tuor *et al.* 1997). The most likely explanation for the age-related difference in rats, and the different effects on HI compared with other species, is the much lower uptake of glucose into the neonatal rat brain (Vannucci, 1994; Vannucci *et al.* 1996). Therefore, the reassuring neuroprotective effects of dexamethasone for HI induced neural injury observed in neonatal rat studies might not translate into human infants. Consistent with this, a recent meta-analysis reported lack of evidence for antenatal glucocorticoid treatment to prevent CP (Shepherd *et al.* 2017), and that both hypoglycaemia and hyperglycaemia were associated with adverse outcomes in term infants with HIE (Basu *et al.* 2016). Intriguingly, hyperglycaemic infants with HIE birth actually showed significantly greater improvement with therapeutic hypothermia compared to normothermia (Basu *et al.* 2017). This suggests either that infants with hyperglycaemia may have been in an early, more treatable phase of injury or that the injurious effects of hyperglycaemia on the brain may be treatable. Thus, there is an urgent need to better understand the impact of glucose management in preterm and sick babies.

Magnesium sulphate. Evidence from meta-analyses and systemic reviews show that $MgSO_4$ administered to women at risk of preterm labour is associated with a small, but significant, reduction in the risk of CP at 18 months to 2 years of age (Doyle *et al.* 2009). However, the long-term follow-up studies show that $MgSO_4$ treatment is not associated with significant improvement in neurodevelopmental outcomes at school age, although these were small studies (Chollat *et al.* 2014; Doyle *et al.* 2014). Preclinical studies in term equivalent animals of effects of $MgSO_4$ for HIE have reported highly inconsistent outcomes, ranging from neuroprotection, to no effect or increased neuronal loss; it is highly likely that apparent neuroprotection was mediated by drug induced hypothermia (Galinsky *et al.* 2014).

Magnesium's primary neural effect is to inhibit glutamatergic signalling through binding its specific site on the *N*-methyl-D-aspartate receptor (Zeevalk & Nicklas, 1992). Consistent with this, reduced basal brain activity was reported in preterm infants treated with $MgSO_4$ (Stark *et al.* 2015) and in preterm fetal sheep (Galinsky *et al.* 2016). There is some evidence for anti-oxidative and anti-inflammatory effects for $MgSO_4$ (Maulik *et al.* 1999; Sugimoto *et al.* 2012). In preterm fetal sheep, $MgSO_4$ for 24 h before and after asphyxia was associated with a significant reduction in basal EEG activity and seizure burden after asphyxia (see also Bennet *et al.* 2018b, in this issue), but no effect on microglial activation, macrophage infiltration, astrogliosis or neuronal loss. Indeed, it was associated with *increased* loss of oligodendrocytes 72 h after injury (Galinsky *et al.* 2017). A recent study in P7 rats suggests that the interaction between $MgSO_4$ and HI is time dependent, with neuroprotection seen when it was administered between 6 days and 12 h before HI, but not at 3 h or 30 min before HI (Koning *et al.* 2017). This effect was likely mediated by improved mitochondrial resistance to HI. Overall, these studies suggest that the impact of $MgSO_4$ on HIE is complex and possibly time dependent. Thus, further careful investigation into the effects of $MgSO_4$ in preterm and term-equivalent translation animal models is essential before undertaking large randomised clinical trials for HIE.

Maternal health and lifestyle associated risks. A variety of maternal health and lifestyle factors can affect normal fetal development. Decades of research have confirmed the considerable potential for harm to the fetus associated with

maternal alcohol intake and smoking, including impaired fetal neurodevelopment (Polanska *et al.* 2015). Despite public health warnings, however, it remains a challenge to improve rates of cessation. It is striking, for example, how many women in both developed and developing nations binge drink before and during pregnancy (Lange *et al.* 2017). Added to these perennial health problems, maternal obesity and diabetes are increasing (Langer, 2018).

The previous section detailed experimental research which suggests that hyperglycaemia can increase the risk of perinatal brain injury after HI, suggesting that the clinical association between hyperglycaemia and adverse perinatal outcomes is at least partly causal. This is of particular concern given that there is a worldwide 'epidemic' of obesity, such that in the United States and Germany, for example, at least half of all women are overweight or obese before and during pregnancy (Dudenhausen *et al.* 2015). Obesity is associated with increased risks of miscarriage, premature birth, stillbirth and gestational diabetes (Kalliala *et al.* 2017), and both clinical and preclinical data show that maternal obesity is strongly associated with later life risk for cardiometabolic disease in offspring, highlighting the transgenerational risk of maternal obesity (Mehta *et al.* 2014; Nicholas *et al.* 2016).

Maternal obesity before and during pregnancy is associated with impaired neurodevelopmental and behavioural and psychiatric outcomes in term and preterm offspring (Mehta *et al.* 2014; Reynolds *et al.* 2014; Edlow, 2017). The greater the maternal weight, the greater risk of adverse perinatal outcomes (Smid *et al.* 2016). Maternal obesity is associated with impaired white matter development in term infants assessed 2 weeks after normal delivery (Ou *et al.* 2015), and obesity and chorioamnionitis were independently correlated with periventricular white matter injury in preterm babies (Herzog *et al.* 2015). Obesity is associated with greater complications leading to an increased risk for mortality, and adverse outcomes including seizures (Yao *et al.* 2017), this includes an increased risk of severe HI (Persson *et al.* 2014). Maternal obesity is often accompanied by an increased risk for gestational diabetes and both are also associated with poor placental perfusion and conditions such as pre-eclampsia that are associated with fetal inflammation, hypoxia and IUGR (Spradley, 2017). The factors that mediate the impact of obesity during development and which may interact with HI insults are multifactorial and include chronic neuroinflammation, oxidative stress, as well functional changes in maternal and perinatal insulin, glucose and leptin signalling (Edlow, 2017).

Collectively this overview of some of the additive physiological and clinical factors the fetus and newborn are exposed to during and after an HI insult highlights the magnitude of the challenges that we face in dissecting mechanisms of action. While the challenge is substantial, this knowledge gives us many targets to base therapeutic strategies on. Some of them are commitments to lifestyle changes such as diet and exercise, which reduce obesity and can prevent gestational diabetes leading to improved pregnancy outcomes (Brown *et al.* 2017). Others require clinical interventions. In the final section below, we address potential therapeutic targets.

Improving outcomes by augmenting endogenous protective responses

As previously discussed, HI triggers multiple endogenous protective responses. Here we review two promising examples of how we can augment these responses to protect the perinatal brain.

Melatonin. Melatonin (*N*-acetyl-5-methoxytryptamine) is released from the pineal gland and helps entrain circadian rhythms (McMillen *et al.* 1995). A role for melatonin in modulating HI injury has been demonstrated in both adult and neonatal animals through anti-oxidant, anti-inflammatory and oxygen free radical scavenging effects (Hassell *et al.* 2015). In addition, melatonin also mediates systemic effects on vascular reactivity and immune system that may provide indirect neuroprotective effects (Colella *et al.* 2016).

Fetuses receive melatonin through the placenta from the mother and therefore they have a circadian melatonin rhythm (McMillen *et al.* 1995; Seron-Ferre *et al.* 2012). This is lost at birth, and neonates have low, arrhythmic levels of melatonin in plasma, until the pineal gland begins production (Kennaway *et al.* 1992). This absence of circadian release of melatonin for the first few weeks of life may help facilitate adaptation of the newborn to the physiological demands of the postnatal environment, including the need to eat regularly both day and night (Mirmiran *et al.* 2003). Endogenous melatonin production is increased after traumatic brain injury in human adults and children (Marseglia *et al.* 2017), raising the possibility that endogenous melatonin may help protect the brain. Supporting this hypothesis, HI injury is significantly increased after pinealectomy in adult rats (Kilic *et al.* 1999), and exogenous administration of melatonin after HI reduced neural injury in a range of preclinical paradigms, as reviewed by Robertson *et al.* (2012). Similarly, an acute increase in endogenous melatonin levels was seen in newborn piglets after HI (Robertson *et al.* 2013). Interestingly, an injurious stimulus can also induce extra-pineal melatonin production in different organ systems, but it is not known if this would lead to an increase in circulating levels (Acuna-Castroviejo *et al.* 2014).

There is now consistent evidence in neonatal animals that exogenous melatonin can reduce HI brain injury

(Robertson *et al.* 2012). In preterm fetal sheep, infusion of low-dose melatonin (0.1 mg/kg bolus followed by 0.1 mg/(kg h) for 6 h) to the mother starting 15 min before severe global asphyxia induced by umbilical cord occlusion reduced microglia activation and improved survival of mature oligodendrocytes in the periventricular white matter at 7 days after asphyxia (Drury *et al.* 2014). Critically, exogenous melatonin can also be protective *after* HI. For example, high-dose melatonin (20 mg/kg) starting 10 min after asphyxia and continued for 6 h was associated with slower recovery of fetal blood pressure but reduced numbers of activated microglia and cell death (Welin *et al.* 2007). Further, melatonin given to preterm fetuses from 2 to 6 h after asphyxia reduced apoptosis, inflammation and oxidative metabolism (Yawno *et al.* 2017). Supporting this, a recent study in newborn lambs demonstrated that melatonin given either by I.V. injection or transdermal patch starting 30 min after acute asphyxia at birth reduced neuroinflammation, oxidative stress in white matter, and improved survival of mature oligodendrocytes and myelin density by 10 days after HI (Aridas *et al.* 2018). Finally, in term piglets, exogenous infusion of high-dose melatonin starting 10 min after HI significantly augmented hypothermic neuroprotection (Robertson *et al.* 2013). Brain protection was dependent on the timing and dose of intravenous melatonin in piglets, such that administration 2 h after HI was less protective than when it was given at 10 min after HI.

Clinically, a small randomised control trial has assessed the feasibility of using melatonin in combination with therapeutic hypothermia after HIE at term (Aly *et al.* 2015). Melatonin was given as five daily enteral doses (10 mg/kg). The study found that melatonin during hypothermia was associated with a reduction in seizures, white matter abnormalities, and appeared to improve survival without neurological or developmental abnormalities at 6 months (Aly *et al.* 2015). These encouraging, preliminary data suggest that melatonin can improve neural outcomes, and its effects are not altered by hypothermia, which is seen with some potential treatments (Gunn & Groenendaal, 2016).

However, despite the promising neuroprotective effects of melatonin caution is needed for its use to treat neonates with HIE. First, the reader should appreciate that preclinical studies examining neuroprotection with melatonin typically dissolved melatonin in ethanol. In one study in preterm fetal sheep, even a very small amount of ethanol had regional specific effects to improve neuronal survival in the caudate nucleus, but increased neuronal loss in regions of the hippocampus (Drury *et al.* 2014). Further, in P7 rats, an alternative solvent for melatonin, dimethyl sulfoxide, also affected cerebral energy metabolism and neurotransmitter concentrations as measured by magnetic resonance spectroscopy both in sham controls and after HI (Berger *et al.* 2017). This illustrates the complexity of developing therapies for translation and highlights the urgent need for safe formulations of melatonin.

Moreover, there is limited data on the pharmacokinetics of melatonin in preterm infants, the threshold dose of melatonin and optimal route for melatonin administration required for neuroprotection in neonates (Colella *et al.* 2016). Recent studies have demonstrated a different pharmacokinetic profile of melatonin in preterm infants compared with adults and children, therefore data from adult studies should be used with caution to guide neonatal administration (Merchant *et al.* 2013). Furthermore, melatonin metabolites might have unintended side effects such as sedation (Colella *et al.* 2016). Different doses and treatment regimens of melatonin also need to be tested, to establish if substitutive or supra-physiological doses are required for an optimal neuroprotective effect. A recent study in newborn lambs showed that during the early postnatal period when endogenous melatonin levels are low, high-dose exogenous melatonin treatment interfered with the postnatal adaptation of adrenocortical function and heart development (Seron-Ferre *et al.* 2017). Melatonin injections (0.25 mg/kg) on postnatal days 1–5 altered clock-time related changes in levels of hormones and metabolic markers, affected the expression of clock genes and functional genes in adrenals and heart, and decreased heart/body weight ratio (Seron-Ferre *et al.* 2017). Although the long-term consequences of these changes are not known, these data suggest the need for more preclinical and clinical research on the systemic effects of melatonin treatment during the early postnatal period.

Erythropoietin (Epo). By contrast with the very rapid release of melatonin, the endogenous growth factor Epo shows slower upregulation that is seen mainly during the secondary and tertiary phases after HI. Epo and Epo receptor (EpoR) protein expression were increased in the injured hemisphere of P7 rats at 24 h and 1 week after HI (Sun *et al.* 2004). Similarly, Epo receptors were upregulated in the P2 rat brain after exposure to transient HI *in utero* at embryonic day 18 (Mazur *et al.* 2010). Conversely, brain-specific gene deletion (EpoR/Epo) renders neurons more susceptible to glutamate and hypoxia, and impairs cell survival after ischaemia (Chen *et al.* 2007). Moreover, there is evidence in adult mice that pre-conditioning with hypoxia before stroke was mediated by induction of endogenous Epo (Prass *et al.* 2003).

Recently, elevated serum Epo concentrations were reported in full term infants exposed to perinatal asphyxia on days 1 and 2 after birth, and were associated with severity of HIE on MRI (Sweetman *et al.* 2017). Similarly, baseline endogenous Epo levels (pre-Epo infusion and therapeutic hypothermia) in term infants with moderate

to severe injury undergoing hypothermia were positively correlated with injury in the basal ganglia and brainstem (Massaro *et al.* 2018). Endogenous upregulation of Epo after HI is delayed but prolonged, suggesting that it likely has a role both in limiting injury in the secondary phase and promoting neurorepair in the long term. However, it should be noted that milder HI brain injury in non-human primates did not stimulate Epo production, despite causing injury, and this is one rationale for exogenous treatment strategies (Traudt *et al.* 2013). Thus, exogenous Epo treatment has potential to further improve outcomes.

Studies of exogenous treatment support this hypothesis. Delayed treatment with 5000 U/kg human recombinant Epo (rEpo) at 24, 48 and 72 h after HI in P7 rats was associated with decreased neuroinflammation and improved neural outcome (Sun *et al.* 2005). Furthermore, delayed treatment with rEpo starting 48 h after HI in P7 rats did not reduce tissue volume loss, and yet increased oligodendrogenesis at 5 days after HI, with improved oligodendrocyte maturation, reduced white matter injury and increased neurogenesis at 14 days after injury (Iwai *et al.* 2010). In preterm fetal sheep, a prolonged infusion of rEpo from 30 min to 72 h after severe HI improved electrophysiological and cerebrovascular recovery in association with reduced apoptosis and inflammation, 3 days after HI (Wassink *et al.* 2017).

Clinically, postnatal treatment with Epo as monotherapy or in combination with therapeutic hypothermia improved neurodevelopmental outcomes in several trials of term neonates with hypoxic–ischaemic brain injury (Zhu *et al.* 2009; Rogers *et al.* 2014; Wu *et al.* 2016; Malla *et al.* 2017). A recent meta-analysis of 1133 very preterm infants (≤32 weeks gestation) randomised to early Epo for neuroprotection found reduced incidence of severely impaired neurodevelopmental scores at 18–24 months post-menstrual age, odds ratio 0.51 ($P < 0.005$), with a number needed to treat of 14 (Fischer *et al.* 2017).

Conclusions

Preclinical studies have provided significant evidence that interactions between multiple insults can modify neural outcomes after asphyxia and have demonstrated that time- and dose-dependent interactions can act in synergy to exacerbate or attenuate the damage induced by HI. However, there is only limited clinical data examining the effect of multiple interactions on neurodevelopmental outcome. In addition, there is a significant gap in knowledge of mechanisms underlying the interactions between various factors. There are additional factors that contribute to the modulation of HI outcomes not discussed in this review, such as the role of the peripheral immune system, fetal parity, and importantly fetal sex. Nevertheless, the evidence presented above highlights the importance of assessing the effect of multiple hits on neural outcomes in infants with HIE. Potentially, identification of high-risk groups can inform the development of future treatments. Furthermore, there is a need for more preclinical studies examining the efficacy of neuroprotective treatments for injury induced with multiple insults to examine the realistic clinical scenario. Identification of endogenous neuroprotective mechanisms has provided a rationale for exogenous treatment with these agents to further augment neuroprotective effects. It remains to be determined if multiple treatments given in a similar temporal profile to their endogenous upregulation will have an optimal neuroprotective effect.

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Additional information

Competing interests

None declared.

Author contributions

L.B., S.K.D. and A.J.G. conceptualised this topical review. C.A.L., R.G., G.W., J.O.D., S.J. and N.J.R. provided important intellectual input and preparation of figures. All authors reviewed and edited this manuscript. All authors have approved the final version as submitted to *The Journal of Physiology* and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

The author's studies discussed in this review received support from the Health Research Council of New Zealand (HRC ID nos 12/613, 14/216, 17/601) and the Neurological Foundation of New Zealand (NF1715-PG). J. O. Davidson holds a Sir Charles Hercus Fellowship from the Health Research Council of New Zealand.