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The biological basis of injury and neuroprotection in the fetal and neonatal brain

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Abstract

A compromised intrauterine environment that delivers low levels of oxygen and/or nutrients, or is infected or inflammatory, can result in fetal brain injury, abnormal brain development and in cases of chronic compromise, intrauterine growth restriction. Preterm birth can also be associated with injury to the developing brain and affect the normal trajectory of brain growth. This review will focus on the effects that episodes of perinatal hypoxia (acute, chronic, associated with inflammation or as an antecedent of preterm birth) can have on the developing brain. In animal models of these conditions we have found that relatively brief (acute) periods of fetal hypoxemia can have significant effects on the fetal brain, for example death of susceptible neuronal populations (cerebellum, hippocampus, cortex) and cerebral white matter damage. Chronic placental insufficiency which includes fetal hypoxemia, nutrient restriction and altered endocrine status can result in fetal growth restriction and long-term deficits in neural connectivity in addition to altered postnatal function, for example in the auditory and visual systems. Maternal/fetal inflammation can result in fetal brain damage, particularly but not exclusively in the white matter; injury is more pronounced when associated with fetal hypoxemia. In the baboon, in which the normal trajectory of growth is affected by preterm birth, there is a direct correlation between a higher flux in oxygen saturation and a greater extent of neuropathological damage. Currently, the only established therapy for neonatal encephalopathy in full term neonates is moderate hypothermia although this only offers some protection to moderately but not severely affected brains. There is no accepted therapy for injured preterm brains. Consequently the search for more efficacious treatments continues; we discuss neuroprotective agents (erythropoietin, N-acetyl cysteine, melatonin, creatine, neurosteroids) which we have trialed in appropriate animal models. The possibility of combining hypothermia with such agents or growth factors is now being considered. A deeper understanding of causal pathways in brain injury is essential for the development of efficacious strategies for neuroprotection.

Keywords

Developmental brain injury; inflammation; intrauterine growth restriction; neuroprotective agents; prenatal hypoxia; white matter damage

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1 Introduction

During fetal life the developing brain is vulnerable to many factors which, together with genetic makeup, can contribute to a spectrum of functional and behavioural disorders that manifest throughout life. Protecting the brain from injury during the fetal, peri-partum and neonatal periods is of major importance owing to the significant number of infants who now survive very preterm birth but develop neurodevelopmental and motor disabilities. Up to 10% of infants who survive very preterm birth will develop spastic motor deficits such as cerebral palsy (Holling and Leviton, 1999; Platt et al., 2007) and a further 25-50% will suffer cognitive, behavioural, attentional and socialisation deficits (Allin et al., 2008; Larroque et al., 2008; Woodward et al., 2005). Hypoxic-ischemic encephalopathy in term neonates can also result in a range of motor and neurodevelopmental disabilities (Azzopardi et al., 2009; Gluckman et al., 2005; Shankaran et al., 2005). Currently, the only established therapy for neonatal encephalopathy in infants born at term is hypothermia and, although this has been confirmed as significant in reducing death and disability at 18 months (Edwards et al., 2010; Zhou et al., 2010), its ability to provide protection to the developing nervous system is far from complete. Further animal experimentation is required to elucidate the mechanisms underlying the etiology of perinatal brain injury to improve outcomes.

Studies of the brain of both humans and experimental animals following intrauterine insults show that the nature of the fetal neuropathology depends on the nature and severity of the insult and the gestational age of the fetus at the time of the insult. Prior to the introduction of magnetic resonance imaging (MRI) and the development of advanced histological techniques to study the injured preterm brain, the focus was on white matter damage as this was the outstanding feature in human autopsy material. However it is now recognised that cortical and subcortical grey matter can also be significantly affected. Volpe (2005; 2009) introduced the term “encephalopathy of prematurity” to encapsulate the complex constellation of events which occur in brain injury, encompassing “primary destructive events and secondary maturational and trophic disturbances”. The current view of prenatal white matter injury is that it manifests as focal and/or diffuse lesions. The focal component can consist of overt macroscopic, necrotic lesions in the periventricular white matter surrounded by astrogliosis and microgliosis and is referred to as cystic periventricular leucomalacia (PVL); this severe lesion was more common in the past but is currently seen in only about 5% of cases (Inder et al., 2003). More commonly necrosis is diffuse, microscopic and associated with activated microglia and reactive astrocytes, and is referred to as non-cystic PVL. Germinal matrix and intraventricular hemorrhages can co-exist with PVL. Vulnerable premyelinating oligodendrocytes (Pre-OLs) in the vicinity of the lesion appear to undergo cell death immediately after injury (Back et al., 2005) but the lineage compensates in the subacute and chronic stages with an increase in oligodendrocyte progenitor cells (Billiards et al., 2008). Widespread axonal injury occurs in the diffuse component of PVL, raising the question of whether white matter injury reflects a primary axonopathy (Haynes et al., 2008; Volpe, 2009), a proposition suggested previously (Dammann et al., 2001).

As indicated above, evidence is now accumulating that grey matter injury is an important component of preterm brain damage. As injury to the cerebral white matter will inevitably interrupt afferent and efferent cortical connections it is not unexpected that cortical neuronal damage will ensue, either as a direct consequence of axonal and or somal destruction or subsequent to neuronal de-afferentation. Recent pathological studies have reported neuronal loss and gliosis in at least one third of PVL cases (cystic and diffuse damage) in basal ganglia and dentate cerebellar nuclei (Pierson et al., 2007) and thalamus (Ligam et al., 2009). Furthermore, the density of pyramidal neurons in layer 5 in the sensory and associative cortices was reduced by 38% compared to controls (Andiman et al., 2010).

These neuropathological findings are supported by MRI studies demonstrating diminished volumes of the thalamus and basal ganglia (Inder et al., 2003; Inder et al., 2005) hippocampus (Isaacs et al., 2000) and cerebellum (Allin et al., 2001). In term infants, neuronal injury was thought to predominate over injury to the white matter. However, recent assessment of the brains of term infants suggests that the same range of grey and white matter injury observed in preterm brains is sustained in term infants (Iwata et al., 2010), particularly in those with congenital heart disease (Kinney et al., 2005).

This review will address how the developing brain responds to acute and chronic hypoxia, including situations when intrauterine inflammation is associated with hypoxia. We will describe studies using animal models that have been developed in an attempt to advance our understanding of the mechanisms underlying brain injury and altered development, and discuss how this knowledge is being used to develop therapies. Finally we will examine the success of these potential therapies in their translation to sick or “at risk” human infants.

2 Acute or chronic hypoxia and brain injury

During fetal life, the supply of oxygen and nutrients to the fetus can occur either acutely by umbilical cord occlusion or more chronically due to impaired placental function (chronic placental insufficiency). Placental insufficiency often results from maternal hypertension, maternal tobacco smoking, partial placental detachment, placental villus edema, or uterine artery narrowing, and can result in intrauterine growth restriction (IUGR). The systemic fetal hypoxemia that usually results from these acute and chronic intrauterine insults is likely to lead to a reduction in oxygen delivery to the brain, although the reduced delivery of other nutrients such as glucose and amino acids, with effects on immature neurons and neuroglia, cannot be ruled out. Other factors can contribute to reductions in fetal cerebral oxygenation such as a reduction in fetal cerebral perfusion due to a failure of cerebral hypoxic vasodilatation, cardiac decompensation or stroke (Lee et al., 2005).

Although the insult to the fetal brain is often referred to as “hypoxia-ischemia” it is still unclear how severely underperfused the brain needs to be before irreversible brain damage occurs. Experimental interventions such as maternal hypoxemia, fetal asphyxia, or fetal exposure to endotoxin, may not lead to decreases in fetal cerebral blood flow; indeed fetal hypoxemia usually leads to an increase in cerebral blood flow due to reflex vasodilatation and to direct effects on the fetal cerebral endothelium (Pearce, 2006). Even in the immature fetus, autoregulatory mechanisms are sufficiently well developed to allow for stabilisation of cerebral blood flow under conditions where arterial pressure is reduced (Szymonowicz et al., 1990). In cases where reduction of fetal cerebral blood flow has been observed, this has occurred in conjunction with decreased cerebral metabolism (Blood et al., 2003; Jensen et al., 2006), suggesting that cerebral metabolism and perfusion remain coupled. However it is not clear when the reduction in cerebral oxygen delivery is such that cellular respiration is no longer adequate for normal tissue function: that is, when true tissue hypoxia occurs.

Severe fetal hypoxemia, whatever its cause, is widely considered to be a major factor underlying fetal brain injury (MacLennan, 1999; Volpe, 2009). However, the causes and timing of fetal hypoxemia are usually not known in individual cases; it is also unclear if it is hypoxemia alone or hypoxemia in combination with other physiological changes that render the fetal brain vulnerable to damage. As indicated above, placental insufficiency, perturbations to fetal-placental blood flow and perhaps also adverse fetal cardiac reactions to proinflammatory or cytotoxic substances in the fetal circulation could compromise cerebral perfusion and delivery of oxygen to the brain. The continuing uncertainty as to how the brain damage in the human fetus actually occurs makes it difficult to determine the most

appropriate animal models of pregnancy-induced fetal brain damage, as we will discuss below.

3 Maternal-fetal infection, fetal hypoxemia and brain injury

Maternal or intrauterine infection/inflammation is now thought to be the other major factor underlying perinatal brain damage (Dammann and Leviton, 1997; Volpe, 2009). Recent clinical and epidemiological studies have shown significant associations between maternal infection, neonatal brain damage and increased levels of proinflammatory cytokines in the amniotic fluid (Yoon et al., 1997; Yoon et al., 2000), umbilical cord plasma (Yoon et al., 1996) and fetal brain (Deguchi et al., 1996; Kadhim et al., 2001). Intrauterine infection/inflammation can manifest either as overt clinical chorioamnionitis or fetal inflammatory response syndrome (FIRS), both of which can be life-threatening to the fetus, or as histological chorioamnionitis that may be clinically silent (see review by Thomas and Speer, 2011). There is unequivocal evidence that chorioamnionitis is a major risk factor for spontaneous preterm birth (Goldenberg et al., 2000) and contributes to the high morbidity and mortality of preterm infants. The relationship between infection, white matter injury and cerebral palsy is still being debated, with two meta-analyses concluding that clinical chorioamnionitis is a risk factor for both cerebral palsy and cystic PVL (Shatrov et al., 2010; Wu, 2002); Shatrov et al (2010) also identified a significant association between histological chorioamnionitis and cerebral palsy. Whether chorioamnionitis is a determining factor for non-cystic PVL and cerebral palsy in preterm infants is not yet clear. Few studies have examined these relationships in full term infants but it appears that exposure to chorioamnionitis also carries an increased risk of cerebral palsy in these infants (Wu, 2002).

It is considered likely that most cases of intrauterine infection or FIRS arise due to invading microorganisms entering the amniotic cavity and the fetus by ascending from the vagina and cervix (Goldenberg et al., 2000; Romero et al., 2003; Romero et al., 2007). Here they induce an innate immune response and cause inflammation of the chorion and amniotic membranes and the production of proinflammatory cytokines. The cytokines and/or other inflammatory mediators then gain access to the fetus via swallowed amniotic fluid or via the fetal lungs, eyes or nasal membranes. These agents may increase the permeability of the blood-brain barrier with enhanced leukocyte infiltration of the brain mediated by brain chemokines. The observation that brain microglia and astrocytes often show an activated phenotype in the presence of chorioamnionitis has been taken as further evidence of the presence of infection-induced cytokine production. Furthermore, inflammation can interrupt hemodynamic stability in the fetus; hence it is likely that hypoxic and inflammatory pathways interact to augment brain damage (discussed below).

Non-infectious inflammation can be provoked by the release of intracellular contents from damaged and necrotic cells. For example inflammation resulting from ischemic-reperfusion injury typically occurs in the absence of any micro-organisms and is termed ’sterile ’inflammation. As with microbially-induced inflammation, sterile inflammation is marked by the recruitment of neutrophils and macrophages and the production of proinflammatory cytokines and chemokines (Chen and Nuñez, 2010).

4 Placental infection and brain injury

Studies of placental pathology following preterm birth have suggested that chronic placental infection may alter placental vascular structure, but this may not be sufficient to impair fetal oxygenation (Salafia et al., 1995). In contrast, acute intrauterine infections may adversely affect placental function leading to fetal hypoxemia and even hypoxic fetal death (Dalitz et al., 2003; Garnier et al., 2001). Indeed the placenta itself may be a key factor in the etiology of perinatal brain damage, since it has inflammatory reactions to bacterial and viral

infection. The importance of placental reaction to infection was recently shown by the fact that small doses of LPS instilled into the utero-placental circulation of pregnant sheep resulted in microglial activation, macrophage infiltration and increased numbers of acidophilic cells in the fetal brain, even though no endotoxin could be detected in the fetal circulation (Hutton et al., 2008). Thus fetal brain damage might arise not only because oxygen delivery to the brain is impaired but because of the accumulation of reactive products in the fetal circulation released from the placenta that affect fetal cerebral vascular resistance and cerebral metabolism.

5 Animal models of hypoxia, inflammation, placental insufficiency, and preterm birth

MRI has proven valuable for defining brain injury in the neonate but animal models are still required to identify the causative mechanisms and to develop neuroprotective therapies. It is now clear that for animal models to closely replicate possible scenarios of human brain injury the insult needs to be delivered prenatally (Back et al., 2006; Derrick et al., 2007; Northington, 2006). Epidemiological studies have shown that, in relation to cerebral palsy (Blair and Stanley, 1988) and neonatal encephalopathy (Badawi et al., 1998) the etiology of 70-80% of cases is likely to be due to an adverse antepartum environment, and not intra-or postpartum events. However others have argued more recently that most of the overt encephalopathy arises in the immediate perinatal period (Cowan et al., 2003; Gunn and Bennet, 2008). Perhaps antenatal factors (infection, hypoxia) initiate causal pathways or reduce the threshold at which stress or hypoxia/ischemia at parturition becomes neurotoxic (Kendall and Peebles, 2005); brain injury could then continue to evolve after birth. Recently Mann et al (2009) reported an association between maternal urinary tract infections prior to the third trimester and an increased risk of cerebral palsy in births before 37 weeks. Little is usually known about intrauterine exposure of the fetus to infection or inflammation during the course of pregnancy prior to admission for delivery.

In order to model human fetal brain injury one needs a species in which the following criteria are met: a similar proportion of brain development occurs in utero; the volume of white to grey matter is similar to the human brain; an insult can be delivered in utero at an equivalent stage of development identified to be a time of vulnerability in humans; the physiological outcome of the insult can be monitored in utero, and neurobehavioural parameters can be tested after birth. Here we discuss experimental data from species which fulfill a number of these criteria (eg sheep, non-human primates, rabbits, spiny mice and guinea pigs).

Over the past two decades we, and others (e.g. Back et al., 2006; Bocking et al., 1988; Mallard et al., 2003; Penning et al., 1994; Williams et al., 1992) have used sheep which are an excellent species for studying the biological basis of injury to the developing brain. We have examined the effects of a reduction in the supply of oxygen and nutrients to the fetus, (a) for 12 hours, near mid-gestation (Rees et al., 1999; Rees et al., 1997) (b) brief single (Duncan et al., 2004a) or repeated umbilical cord occlusions in late gestation (Castillo-Melendez et al., 2004; Loeliger et al., 2003) (c) chronic placental insufficiency (20-30 days) in late gestation (Duncan et al., 2004b; Mallard et al., 1998; Nicholls et al., 2001) and (d) maternal caruncletomy which reduces the available sites for placentation, resulting in growth restricted fetuses that are chronically hypoxemic, hypoglycemic and have an altered endocrine status (Rees et al., 1988). We have developed a robust model of lipopolysaccharide (LPS)- induced inflammation in the catheterized immature ovine fetus in which it is possible to study physiological responses, peripheral inflammatory responses and neural injury within the same fetus (Duncan et al., 2002; Rees et al., 2010).

In a non-human primate model of preterm birth developed to study the pathogenesis of bronchopulmonary dysplasia (Coalson et al., 1999) we have been able to study the effects of different respiratory regimens on brain development and injury (Dieni et al., 2004); the neonatal setting and clinical interventions were similar to those received by the human preterm infant .

We have also used guinea pig models of chronic placental insufficiency induced by uterine artery ligation during the second half of gestation (term ~ 67 days) (Mallard et al., 1999; Nitsos and Rees, 1990; Rehn et al., 2004) or ablation of uterine artery branches supplying the placenta (Kelleher et al., 2011). Guinea pigs have the advantage of a relatively long gestation, allowing for intrauterine manipulations at specific developmental stages and they are amenable to postnatal behavioral and functional testing. The spiny mouse also has a long gestation and a model of birth asphyxia has been developed in this species. Compared to other rodents, the greater neurological maturity of the spiny mouse neonate at birth makes it possible to study the effects of birth asphyxia, and treatments designed to protect the brain, on various aspects of postnatal behavior including locomotion, balance, spontaneous activity, social vs non-social behavior, learning, and memory (Hutton et al., 2009b; Ireland et al., 2010; Ireland et al., 2008).

Rabbit models of intrauterine endotoxin exposure (Saadani-Makki et al., 2008) and severe placental insufficiency (Derrick et al., 2004) have recently been developed by others; in the latter model some intrauterine monitoring is possible. The evolving brain damage can be followed using sequential neuroimaging; however, the rabbit brain is not as mature as the human brain at birth.

Our studies have allowed us to reach the following generalisations:

5.1 Acute hypoxic insults in early-mid gestation, resulting in a rapid reduction in oxygen delivery to the brain at a time when neurogenesis and neural migration are at their peak, can result in the death of neurons including Purkinje cells in the cerebellum, pyramidal cells in the hippocampus and cortical neurons (Rees et al., 1999) and a slowing of neural migration, at least in the hippocampus (Rees et al., 1997). The growth of neural processes is significantly retarded in the long term if the neurons are particularly immature at the time of the insult (as in the cerebellum) but will recover, after an initial delay, if axonal and dendritic outgrowth is well established at the time of the insult (as in the hippocampus). Acute hypoxic insults and repeated LPS exposure (Duncan et al., 2002) can injure the white matter, resulting in diffuse axonal injury and cystic lesions in the periventricular area in some cases, neuropathologies resembling those observed in neonatal brain injury. Fetal hypotension was not a necessary accompaniment to the development of such lesions (Rees et al., 1997).

5.2 Acute fetal hypoxemia (umbilical cord occlusion) in late gestation results in neuronal death in the cerebral cortex and striatum (Loeliger et al., 2003), whereas hippocampal and cerebellar neurons do not appear to be affected at the gross level. The white matter is damaged but less extensively than when insults are delivered earlier in gestation (albeit in our experimental paradigm the earlier insult was more prolonged). Whether such damage persists or becomes progressively worse by term is unknown in most cases.

5.3 Chronic fetal hypoxemia, malnutrition and altered endocrine status are seen in fetuses exposed to chronic placental insufficiency, and typically arise as a result of a chronic impairment of placental exchange function. In general, placental insufficiency is caused by factors that affect uterine blood flow, umbilical blood flow or the placental exchange barrier. Experimentally we have induced chronic placental insufficiency, sufficient to restrict fetal growth, by restricting placental mass, uterine blood flow or umbilical blood flow. Our

studies have shown that chronic insults result in outcomes which differ from acute insults in several aspects. Sheep fetuses compromised throughout gestation (Rees et al., 1988) for 20 days in late gestation (Duncan et al., 2004b; Nicholls et al., 2001) or guinea pig fetuses compromised for the second half of gestation (Kelleher et al., 2011; Mallard et al., 1999; Nitsos and Rees, 1990), are growth restricted. The brain, although relatively spared in relation to other organs, is reduced in weight. There is no overt white matter damage, although axonal myelination is delayed in the central nervous system (CNS) during fetal life (Nitsos and Rees, 1990) but restored postnatally at least in the guinea pig model (M Tolcos, unpublished observations).

Chronic intrauterine insults compromise the growth of neural processes and synapses throughout the fetal sheep brain examined at term (Rees et al., 1988; Rees and Harding, 1988); similar findings have been made in the guinea pig (Dieni and Rees, 2005; Mallard et al., 1999). Neurons generally seem to survive chronic, mild intrauterine compromises, although some populations are affected; reduced cell numbers could relate to a direct effect of hypoxia on neurogenesis or alternatively to the death of postmitotic cells. For example, in sheep after 20 days of chronic placental insufficiency during late gestation, dopaminergic amacrine cells (interneurons) in the retina, which are involved in contrast sensitivity, are reduced in number but other classes of amacrine cells are not affected (Duncan et al., 2004b). Repeated LPS exposure during mid-gestation leads to a similar outcome (Loeliger et al., 2011). The loss of even small numbers of specific classes of cells could significantly affect particular neural functions. In studies in which we have been able to examine the persistence of changes in the structure of the brain and retina after birth (Duncan et al 2004b), we have found that some alterations persist (e.g. reduction in dopaminergic amacrine cells), some resolve (dendritic and axonal growth in the cerebellum catches up), while others develop after birth (reduction in dendritic growth of CA1 hippocampal neurons); these observations demonstrate the importance of long-term follow-up studies in developing a complete understanding of the effects of a prenatal insult.

In the guinea pig model of chronic placental insufficiency and IUGR, we have observed enlargement of the lateral ventricles, most likely resulting from reduced growth of neural processes and reduced neuronal numbers in some brain regions (Mallard et al., 1999). Ventriculomegaly is one of the most consistent findings in the brains of patients with schizophrenia (Hopkins and Lewis, 2000; Johnstone et al., 1976). Our studies have shown for the first time that such alteration can originate from an insult in utero (Mallard et al., 1999) and persist into adolescence (Rehn et al., 2004). Furthermore we have shown at adolescence, reduced brain weight, reduced basal ganglia volume, the absence of astrogliosis and sensorimotor gating deficits; these alterations parallel, to an extent, the situation in some patients with schizophrenia (Rehn et al., 2004). Sexually-dimorphic effects arising in the brain of IUGR guinea pigs, related to neurosteroid synthesis, have also been found; for example, maternal betamethasone treatment increased 5α-reductase type 1 expression in the brain of female, but not male IUGR fetuses (McKendry et al., 2010). Increased expression of neuroactive steroid enzymes may be protective in that these compounds increase cell proliferation and myelination, and failure of the male brain to increase expression of these enzymes under glucocorticoid exposure may explain some of the greater vulnerability of the male fetal brain to injury.

In the prematurely–delivered baboon, we have evaluated the effects of several respiratory regimens and procedures on brain development and injury. We have found that brain growth is delayed and subtle neuropathological alterations are evident in the brains of all animals ventilated for 2-4 weeks in neonatal intensive care compared to gestational controls (e.g. Dieni et al., 2004; Loeliger et al., 2006; Loeliger et al., 2008a). Furthermore specific regimens have differing effects on the brain. For example, conventional ventilation for 4

weeks resulted in more extensive white matter damage than did early or delayed continuous positive airway pressure (CPAP); early CPAP resulted in the best outcome (Loeliger et al., 2006). Despite attempts to maintain respiratory stability, hypoxic episodes occur in baboon newborns as they do in the human newborn. In general animals that had better ventilatory control and required less manipulation of the fraction of inspired oxygen (lower fluxes) had lower brain damage indices (Loeliger et al., 2006; Loeliger et al., 2008a).

6 Possible mechanisms underlying brain injury

The etiology of the brain damage described above is likely to be multifactorial; however as we have indicated, reduced oxygen delivery to the developing brain and its downstream effects are likely to play a major role. The principal pathways leading to cell death after cerebral hypoxia and ischemia are initiated by energy depletion followed by some or all of following events: increased neuronal release and reduced uptake of glutamate by glia; activation of glutamate receptors; accumulation of cytosolic calcium; activation of a variety of calcium-mediated deleterious events including generation of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals and nitric oxide derivatives (see Johnston et al., 2000). ROS interact with the lipid components of cellular membranes, initiating lipid peroxidation which results in the breakdown of lipid constituents into highly reactive by-products including lipid aldehydes, for example hydroxynonenal and malondialdehyde. These reactive aldehydes then bind to and modify protein creating protein adducts. Nitrosative stress results from nitric oxide (NO) released from reactive microglia reacting with superoxide anions to form peroxynitrite which targets tyrosine residues of proteins to form nitrotyrosine residues; both of these processes are highly damaging to cell membranes. These events can result in mitochondrial disruption and immediate or delayed cell death. Typically, 8-48 hours after the acute event (primary energy failure), cerebral energy is again affected (secondary energy failure) due to accumulated mitochondrial injury resulting in the release of cytotoxic enzymes and pro-apoptotic proteins from mitochondria causing delayed cell death (Iwata et al., 2008).

The late precursor cells (pre-oligodendrocytes) and immature oligodendrocytes that predominate in the developing periventricular white matter during the period of highest risk for white matter damage in humans (23-32 weeks of postconceptional age) are particularly susceptible to hypoxia-ischemia. Studies have shown that markers of oxidative and nitrosative stress can be detected in areas of injury in the periventricular white matter (Haynes et al., 2003). The sensitivity of these cells to injury is associated with a limited capacity to resist oxidative stress (Back et al., 1998) and the expression of Ca^{2+} permeable AMPA receptors on their somata (Fern and Moller, 2000) and NMDA receptors on the processes of immature oligodendrocytes (Salter and Fern, 2005). Recent studies suggest that glutamate released during hypoxia-ischemia from late precursors and immature oligodendrocytes and axons can activate glutamate receptors on adjacent oligodendrocytes leading to injury (Back et al., 2007). Riddle et al (2006) have demonstrated in immature fetal sheep that following severe cerebral hypoperfusion, white matter damage is not uniform but rather coincides with the presence of susceptible populations of oligodendrocyte progenitor cells. In regions of diffuse white matter injury caused by LPS exposure we have observed a reduction in oligodendrocyte numbers, damage and attenuation of the processes of the surviving oligodendrocytes and destruction of axons (Duncan et al., 2002; Nitsos et al., 2006).

The mechanisms underlying axonal damage are not known precisely but could involve glutamate receptor activation and a toxic influx of Ca^{2+}. In the ovine fetus it has been shown that the level of glutamate efflux in the white matter positively correlates with the degree of damage to the white matter (Loeliger et al., 2003). Axonal damage could also occur as a

result of reverse operation of ionic pumps. Energy depletion causes failure of Na^+-K^+ ATPase which allows an unopposed inward leakage of Na^+, and the ensuing membrane depolarization acts to drive the Na^+-Ca^{2+} exchanger in the Ca^{2+} import mode, leading to intracellular Ca^{2+} overload. Excess Ca^{2+} is expected to disrupt mitochondrial function, leading to axonal damage (Stys et al., 1991). If the insult is less severe, a sub-lethal influx of Ca^{2+} could significantly retard the growth of axons. Regulation of intracellular pH is also important for cellular energetics and control of cell volume. It has long been known, from activity of Na^+/H^+ in adult stroke and human neonatal encephalopathy, that there is a significant correlation between poor clinical outcome and the degree of post-ischemic cerebral alkalosis (Robertson et al., 2002; Welch et al., 1990). Intracellular pH is largely governed by plasma membrane Na^+/H^+ exchangers, but the role of these ion transporters in determining intra-cerebral pH and tissue damage in the fetal and neonatal brain is not yet known.

The role played by hypoxia during a primarily inflammatory insult is still unclear. We know that when LPS is administered to the fetus either via a prolonged intravenous infusion (Duncan et al., 2006) or intra-amniotically (Nitsos et al., 2006), fetal hypoxemia does not ensue and the resultant brain damage is not as great as when LPS is administered acutely; we have shown that repeated bolus administration of LPS results in fetal hypoxemia, hypotension and hence reduced cerebral oxygen delivery (Duncan et al., 2002). From previous studies we know that predominantly hypoxemic insults to the fetus (without accompanying hypotension) (Rees et al., 1997) also cause white matter damage but that it is not as reproducible or generally as severe as that resulting from LPS exposure. Thus it appears that there are synergistic pathways between fetal hypoxemia and inflammation which potentiate the evolution of brain damage. In accordance with this notion, LPS has been shown to sensitize the immature rat brain to hypoxic-ischaemic injury (Eklind et al., 2001). Whether this augmentation of damage applies to human pregnancies is unclear. It has been suggested however that in the case of cerebral palsy the presence of more than one risk factor (including hypoxia and intrauterine exposure to infection) is most likely required to overwhelm defense mechanisms and cause brain injury (Nelson, 2008).

Clearly the etiology of the fetal brain damage in inflammation will involve many factors and is likely to include an increase in circulating cytokine levels. We have shown, for example, that TNF-α (Dalitz et al., 2003) and IL-6 concentrations (Duncan et al., 2002) increase within 6 hours of LPS-exposure. It has been proposed that circulating cytokines might act on cerebral endothelial cells or periventricular cells to upregulate prostaglandin synthesis, resulting in increased permeability of the blood-brain barrier (Yan et al., 2004); thus the administration of LPS to fetal sheep results in the extravasation of plasma proteins and macrophages into the brain (Yan et al., 2004). The powerful oxidative activity of macrophages, also a characteristic of the metabolism of activated microglia, not only adds to the burden of increased ROS and proinflammatory cytokines in the brain, but is likely to result in the accumulation of quinolinic acid (Heyes et al., 1996), a glutamate receptor agonist that with prolonged exposure has been shown to be neurotoxic (Stone, 1993). Quinolinic acid, a metabolite of tryptophan is known to be released from the human placenta (Manuelpillai et al., 2003) and is present in higher concentrations in cord blood of babies from pregnancies with clinical signs of infection (Manuelpillai et al., 2005). It is present in fetal sheep blood after LPS treatments (Yan et al., 2004), and is increased in blood and brain of growth restricted fetuses (Nicholls et al., 2001). The activation of microglia by low grade infection in pregnancy and release of quinolinic acid amongst other molecules may be one of the ways in which the fetal brain is made more vulnerable to the effects of hypoxia, whether this occurs subsequently in utero, during the birth process, or in the postnatal period when respiratory function may be suboptimal.

Fetal nutrient restriction and/or altered endocrine and growth factor status may also play a role in brain damage, particularly when the insult is more prolonged. For example, we have shown that the neurotrophin brain derived neurotrophic factor (BDNF) is significantly reduced in chronic placental insufficiency in the fetal hippocampus (Dieni and Rees, 2005) and retina (Loeliger et al., 2008b) thyroid and insulin-like growth factor, both hormones known to play an essential role in neuronal and neuroglial development, are also reduced in chronic placental insufficiency (see Nitsos and Rees, 1990).

7 Brain function is altered in prenatally compromised offspring

Brain injury, particularly that in the very preterm infant, occurs against a background of highly active brain development including the initiation of myelination, axonal and dendritic growth, synaptogenesis and proliferation of microglia and astrocytes. In preterm birth the neonate will be exposed to levels of light, sound, touch, olfaction, oxygen and nutrients which are different from those they would have experienced *in utero*. The developing brain is exquisitely sensitive to the appropriate levels of these factors and animal experimentation has shown that alterations in these factors can result in abnormal structural and functional development of the brain. Such alterations could account for behavioral, cognitive and functional deficits manifest postnatally in some premature infants. In our guinea pig model of chronic placental insufficiency which results in IUGR, we have been interested in testing subtle neurobehavioral and functional outcomes including those persisting in the long term. This is relevant to the observation that babies of low or very low birth weight (VLBW), many of whom are also growth restricted, are at increased risk for auditory and visual impairment including sensorineural hearing loss and deficits in visual acuity, colour vision and contrast sensitivity (Dowdeswell et al., 1995). In our model we have demonstrated long term alterations in retinal function (Bui et al., 2002), subtle deficits in neural conduction in auditory pathways (Rehn et al., 2002) and a reduction in prepulse inhibition of the startle response (Rehn et al., 2004), a behavioural paradigm that is thought to indicate the ability of the CNS to filter out extraneous stimuli. In the spiny mouse neonate following birth asphyxia we have shown deficits in learning and memory using the novel object recognition test (Hutton et al., 2009b), and deficits in formation of Long-Term Potentiation (LTP) in hippocampal pathways at postnatal day 5 (B Fliess, D Walker, H Parkington, unpublished observations). It was not the goal of all of our animal models of pre- and perinatal compromise to produce a motor phenotype equivalent to that of cerebral palsy, but this has been closely modeled in rabbit models of severe placental insufficiency (Derrick et al., 2004) and fetal endotoxin exposure (Saadani-Makki et al., 2008), where neonatal offspring exhibited abnormalities in motor control in addition to hypertonia. Taken together, these findings demonstrate that prenatal compromise can result in long term deficits in neurocognitive function and altered behaviour.

8 Potential therapeutic agents and when to administer them

In order to provide timely therapeutic intervention for the fetus or infant at risk of brain damage we need to be able to detect when an insult has occurred. As indicated above there is ongoing debate as to when this is most likely to occur. Advanced neuroimaging is now providing the opportunity to identify and then monitor the evolution of brain injury in compromised fetuses and neonates. Another approach is to identify reliable markers of brain damage in the blood of newborns (Leviton and Dammann, 2002). The presence of proteins such as S-100B (from astrocytes) or neuron-specific enolase (from neurons) is considered as evidence that these cells have been damaged and the proteins released into the blood through increased permeability of the blood-brain barrier; the search for reliable, specific markers is on-going.

Although clinical observations and animal experimentation (Iwata et al., 2008; Johnston et al., 2000) suggest that the cascade of damaging events to the developing brain can unfold over several days, extending the window of opportunity for intervention, the most successful outcome is likely to result from the earliest possible delivery of therapy. In animal models attempts have been made to inhibit the hypoxic-ischemic cascade at almost every level with, for example: NMDA receptor blockade; the use of nitric oxide synthetase inhibitors; blockade of apoptosis by inhibition of caspases; blocking the upregulation of free radical formation. Other approaches include the application of growth factors in utero (Johnston et al., 1996) and the prevention of energy depletion via mild hypothermia after birth (Williams et al., 1997). Of all these regimens, hypothermia is the only therapy that has been successfully translated from “bench to bedside” to treat human neonates with hypoxic-ischemic encephalopathy.

8.1 Hypothermia

The neuroprotective effects of moderate hypothermia for full term infants with encephalopathy has been confirmed in a recent meta-analysis of 10 randomised controlled trials, a subset of which reported neurological outcomes at 18 months (Edwards et al., 2010). Seventy two hours of hypothermia (33-35°C) induced by head cooling (Gluckman et al., 2005), or systemic cooling (Azzopardi et al., 2009; Shankaran et al., 2005) within about 6 hours of birth (based on animal experimentation (Gunn et al., 1999)), was associated with a consistent reduction in death and neurological impairment at 18 months of age. Despite this encouraging finding it is clear that hypothermia does not completely protect the injured brain; evidence suggests that it does not improve the risk of death or disability in the most severely hypoxic-ischemic neonates (Azzopardi et al., 2009; Gluckman et al., 2005; Shankaran et al., 2005).

Thus it is evident that hypothermia alone will not provide complete protection nor potentiate the repair that is necessary for neural development to continue on a normal trajectory. This has led to the concept of synergistic neuroprotective strategies such as pharmacological agents being delivered either during or after the hypothermic treatment (Cilio and Ferriero, 2010). It will be crucial to consider drug toxicity in the injured immature brain (Cilio and Ferriero, 2010); drug excretion can be modified by hypothermia and hypoxic-ischemia can impair the ability of the liver and kidneys to clear drugs (Roka et al., 2008). It should be noted that hypothermia is contra-indicated for preterm infants, leaving this group without an established therapeutic option when hypoxia-ischemia occurs.

Here we will discuss several pharmacological agents with which our laboratories have had considerable experience; some of the agents are now in human trials either as stand-alone therapies or in combination with hypothermia. Other agents including antiepileptic drugs and xenon have been covered in recent reviews (Cilio and Ferriero, 2010; Iwata et al., 2010).

8.2 Erythropoetin

The pleiotrophic cytokine erythropoietin (EPO), which is currently used in human infants as a safe treatment for anemia of prematurity (Ohls et al., 2004), is now being studied for its neuroprotective properties. EPO has a range of actions that might underlie its putative neuroprotective functions including anti-inflammatory (Gorio et al., 2002), anti-apoptotic (Siren et al., 2001) antioxidant (Ozturk et al., 2005) and neurotrophic (Campana and Myers, 2001) effects, the reduction of glutamate (Kawakami et al., 2001) and nitric oxide toxicity (Genc et al., 2006) and enhancement of neurogenesis and angiogenesis (Wang et al., 2004).

8.2.1 Animal studies—Although the neuroprotective properties of EPO in short-gestation animal models of neonatal brain damage (Demers et al., 2005; Sola et al., 2005; Sun et al.,

2005) have been demonstrated, its therapeutic potential in a long-gestation species in which fetal brain structure and cerebrovascular function are similar to that of the very preterm infant was not known until our recent study. We have demonstrated that recombinant (rh)EPO treatment can reduce LPS-induced CNS damage in preterm fetal sheep (Rees et al., 2010). The most striking neuropathological findings were that treatment resulted in an overall reduction in axonal damage, microglial and astrocytic responses in white matter and in the expression of cortical neuronal beta-APP (a marker of neuronal stress). It also protected against a reduction in the density of oligodendrocytes (immunoreactive for myelin basic protein) in the corticospinal tract and optic nerve, thus preserving myelination. The periventricular cystic infarcts present in 12% of LPS-exposed fetuses without treatment (Duncan et al., 2002) did not occur with treatment (Rees et al., 2010). Although this was noteworthy, the reduction of diffuse white matter injury was potentially of greater significance because this is the predominant form of injury in preterm infants.

In the retina, rhEPO treatment of LPS-induced injury protected against loss of ganglion and dopaminergic amacrine cells and protected against alterations to somal size and process growth of interneurons in the inner nuclear layer (Loeliger et al., 2011). Furthermore rhEPO protected against placental and fetal liver damage in this model (Dijkstra et al., 2010), demonstrating for the first time that an agent which is protective to the brain has beneficial effects for other organs acutely exposed to an inflammatory insult. As indicated above, placental injury might contribute significantly to brain damage; therefore any agent which offers protection to this organ could be a vital aspect of prenatal treatment. The mechanisms underlying neuroprotection of the brain and retina are likely to be multifactorial and include the factors alluded to above and possibly indirect factors such as preservation of the integrity of the blood-brain-barrier (Martinez-Estrada et al., 2003).

8.2.2 Clinical trials—Recent randomized controlled studies of small groups of very preterm (Fauchere et al., 2008) and extremely low-birth weight infants (Juul et al., 2008) have shown that there were no significant adverse effects with early high-dose recombinant EPO treatment, paving the way for larger trials. The initial concern about increased risk of thrombotic events reported for adults undergoing EPO therapy for chronic kidney failure has not been borne out for the neonate (Aher and Ohlsson, 2006). The first trial of EPO in full term neonates with moderate to severe hypoxia-ischemia demonstrated that it reduced death and disability at 18 months from 44% (controls) to 25% (EPO-treated) with no adverse effects (Zhu et al., 2009). As with hypothermia EPO therapy was only effective for infants with moderate, not severe, injury. Two retrospective reports suggest that EPO treatment can improve neurodevelopmental outcome (Bierer et al., 2006; Brown et al., 2009); this is encouraging but will need to be confirmed. The combination of EPO therapy with growth factors or hypothermia warrants investigation to determine whether the level of neuroprotection can be improved (McPherson and Juul, 2010).

8.3 N- acetyl cysteine

One of the immediate consequences of severe cerebral hypoxemia and metabolic acidosis is the over-production and accumulation of oxygen free radicals, and therefore treatments based on ameliorating oxidative stress have been proposed. N-acetyl cysteine (NAC) is an antioxidant that scavenges free oxygen radicals due to its ability to interact with reactive oxygen species. It also suppresses the production of LPS-induced iNOS and pro-inflammatory cytokines (Beloosesky et al., 2006).

8.3.1 Animal studies—The efficacy of NAC as a neuroprotective agent has been examined in a number of rodent studies. For example, NAC provides neuroprotection in LPS-sensitised rat pups exposed to hypoxia-ischemia (Wang et al., 2007), and in

combination with hypothermia, reduces infarct volume after focal hypoxia-ischemic brain injury (Jatana et al., 2006). Thus although there was some evidence that NAC might be suitable as a neuroprotective agent in the developing rodent it was unknown whether NAC affected the physiological status of the fetus already exposed to an inflammatory environment; this could compromise its therapeutic potential. In experiments designed to assess this we demonstrated that NAC treatment of the LPS- exposed ovine fetus exaggerated LPS-induced fetal hypoxemia and hypotension and induced polycythemia (Probyn et al., 2010). How NAC interacts with LPS to exacerbate the deleterious physiological effects of LPS on the fetus is not clear; it is possible that it acts to delay the well known “endotoxin tolerance” (Broad et al., 2006).

8.3.2 Clinical trials—The only randomized clinical trial of preterm newborns demonstrated that continuous infusion of NAC for 6 days after birth did not improve the incidence of chronic lung disease but did appear to reduce the incidence of PVL, although this is a very preliminary study (Ahola et al., 2003). In the light of our animal studies we suggest that NAC might not be a suitable neuroprotective agent when administered antenatally in a clinical setting to a human fetus with evidence of inflammation such as chorioamnionitis.

8.4 Melatonin

Melatonin (5-methoxy-*N*-acetyltryptamine) is a highly effective antioxidant, both as a direct scavenger of oxygen free radicals, particularly the highly destructive hydroxyl radical, and indirectly via up-regulation of the expression of the antioxidant enzymes glutathione peroxidase, glutathione-reductase, superoxide dismutase and catalase (Reiter et al., 2000). Melatonin readily crosses both the placental and blood-brain barriers, and even at high, supra-physiological concentrations there appear to be no adverse side-effects, making it a therapy that could be administered to babies.

8.4.1 Animal studies—In fetal sheep we have shown that melatonin given to the ewe, shortly before and during a short period of severe fetal asphyxia induced by umbilical cord occlusion, substantially prevented the formation of the toxic hydroxyl radical in the fetal brain, and decreased the resultant lipid peroxidation and the extent of brain damage (Miller et al., 2005). In another study with fetal sheep, melatonin was given 10 mins after the hypoxic-acidemic episode and was found to decrease the incidence of cell death and the number of activated microglial cells present in the brain (Welin et al., 2007). These studies suggest that there is potentially a degree of latitude in the time that melatonin needs to be available, which is an advantage not shared by hypothermia as a treatment. Melatonin treatment given over an extended period in late pregnancy before experimental induction of birth asphyxia is also protective for the neonate (Hutton et al., 2009a). Subcutaneous administration of melatonin at 0.1 mg/kg/day from day 30-37 gestation in the spiny mouse (term is 39 days) resulted in significant decrease of apoptosis as measured by fractin immunocytochemistry, and indices of inflammation as measured by the number of activated microglia and macrophages in the cerebral cortex. Interestingly, this relatively long period of melatonin pre-treatment in which maternal liver concentrations were increased approximately 10-fold, had the effect of decreasing the apoptotic index in the non-asphyxiated spiny mouse fetuses at birth, and it also increased fetal body and brain weights (Hutton et al., 2009b). This is further evidence that melatonin is a physiologically important hormone during fetal development, as shown elsewhere for fetal sheep (Torres-Farfan et al., 2008).

8.4.2 Clinical studies—In a small clinical trial, melatonin has been given orally to newborn babies who had suffered birth asphyxia, and was shown to significantly reduce

plasma levels of malondialdehyde and nitrate/nitrite, two robust indicators of oxidative stress (Fulia et al., 2001). In terms of mortality, 3 of 10 asphyxiated babies died in the vehicle treated group, whereas there were no deaths in the post-asphyxia babies treated with melatonin. Importantly, this study did not report any adverse effects arising from the melatonin treatment, and clinical use of melatonin in the neonatal period has now been proposed (Gitto et al., 2009).

8.5 Neurosteroids

Progesterone and its key neuroactive metabolites, allopregnanolone and pregnanolone, have been shown to have important roles in protecting the adult brain following trauma or hypoxia-ischemia, and they are used clinically to improve outcomes in these conditions (Stein, 2008; Wright et al., 2007). These steroids exert their action by multiple pathways including modulation of $GABA_A$ and glutamate receptors as well as increased release of neurotrophins. Thus, neuroactive steroids not only influence CNS activity by their effects on GABAergic and glutamatergic synaptic activity, but also influence growth, neuronal and glial cell survival, and cell repair and regeneration after injury in the adult (Melcangi et al., 2008). These actions require the metabolism of progesterone to 5α-reduced metabolites including allopregnanolone (5α-pregnane-3α-ol-20-one) (He et al., 2004), although progesterone itself has been shown to induce synthesis and release of neurotrophins such as BNDF

8.5.1 Animal Studies—Progesterone and its neuroactive metabolites are present in remarkably high concentrations in fetal plasma and brain (Nguyen et al., 2003). All enzymes required for the synthesis of progesterone and its metabolism to allopregnanolone that are expressed in the adult brain (Melcangi et al., 2008) are also present in the fetal sheep and guinea pig brain (Kelleher et al., 2011; Nguyen et al., 2003), where 5α-reductases are the key rate-limiting enzymes for the conversion of progesterone to the GABA-A receptor competitive agonist, allopregnanolone. The type-2 5α -reductase isoform is expressed in glial cells and neurons of the fetal sheep hippocampus and cerebellum in late gestation, and in OL in the sub-cortical white matter (Petratos et al., 2000). Elsewhere, it has been proposed that allopregnanolone has a major influence on myelination in the fetal brain (Mellon, 2007). In the late gestation sheep fetus, the endogenous synthesis of neurosteroids in the brain is substantially supported by the production and release of large amounts of progesterone from the placenta. While much of this progesterone is rapidly metabolized to pregnenolone and its sulphated form on reaching the fetal circulation, the fetal brain is nevertheless exposed to considerable levels of progesterone and its metabolites throughout gestation. Not surprisingly then, in this species neurosteroids have been shown to influence fetal brain activity and behaviour. When allopregnanolone levels are suppressed increased fetal arousal is observed, but importantly for the present discussion, this procedure results in a marked potentiation of hypoxia-induced neuronal loss, an effect that is prevented by co-administration of the allopregnanolone analogue, alfaxalone (Yawno et al., 2009). These observations suggest that not only do endogenous neurosteroids regulate normal fetal brain activity, but they have an important role to play in limiting the extent of damage induced by severe hypoxia during fetal life.

8.5.2 Fetal growth restriction and neurosteriods—Abnormal fetal growth is associated with a high risk of fetal brain injury leading postnatally to motor disorders, neurodevelopmental delay and long-term cognitive impairments. As endogenous neurosteroids have been implicated in the regulation of normal brain development, it is relevant to ask if conditions that lead to IUGR also alter the synthesis of neurosteroids in the placenta and the fetal brain. Indeed, intrauterine stress and compromised placental function have been shown to influence fetal expression of the 5αR enzymes in the placenta and fetal

tissues of fetal sheep (Brewer and Wallimann, 2000) and guinea pigs (McKendry et al., 2010). In the pregnant guinea pig in which IUGR was induced from mid-pregnancy, expression of both 5α-reductase isoforms was not only *not* increased in the brain of these IUGR fetuses, but tended to be lower in male IUGR fetuses, so that the male IUGR brain had lower concentrations of allopregnanolone (McKendry et al., 2010). This sexually dimorphic effect of late gestation IUGR is interesting from the point of view that male fetuses are known to be at higher risk of adverse outcomes from gestational events such as infection, placental insufficiency, and preterm birth.

8.6 Creatine

Creatine is a metabolite synthesized from the dietary amino acids arginine, glycine and methionine. It is also acquired from a diet containing meat and dairy foods. It is required by cells for the regeneration of ATP. Supplementation of the diet with creatine has been shown to provide benefits in patients with muscular and neurological diseases, and physiological benefits in tissues with rapidly changing energy demands such as occurs with high level physical exercise, or in cases when untoward ATP depletion occurs, such as with heart attack and stroke (reviewed by Brosnan and Brosnan, 2007).

Animal models of neurodegenerative diseases, traumatic brain injury and adult stroke have shown that oral creatine supplementation has a sparing effect on neuron survival (Ferrante et al., 2000; Zhu et al., 2004). This has also been illustrated in cultured hippocampal neurons exposed to toxic levels of glutamate and beta-amyloid (Prass et al., 2006).

Little is known of the fetal and neonatal requirement for creatine, and how this changes with advancing pregnancy and in early infancy, particularly for tissues known to have a high creatine requirement in the adult such as skeletal muscle, heart and brain. In recent experiments using the precocial spiny mouse we found that the fetal synthesis of creatine was limited until late in pregnancy by low expression of hepatic enzyme g-uanidinocetate N-methyltransferase (Ireland et al., 2009), implying that the fetus relies on the maternal supply of creatine for much of gestation. If also true for the human fetus, then it is unclear how the preterm infant can meet its requirement for creatine if the major creatine-producing organs (kidney, liver) are still immature in this regard. It is unlikely that significant intestinal transfer of creatine occurs in preterm infants, particularly where necrotising enterocolitis is a risk or already developed. It is therefore possible that preterm infants are at risk of becoming creatine deficient.

Evidence for the importance of creatine in the development of the CNS comes from observations of infants born with congenital creatine deficiency syndromes (Schulze, 2003). Such conditions are usually associated with the development of neurological impairment during the first year of life. Prevention of neurological deficits, or significant neurological improvement, is achieved with early implementation of creatine supplementation, implying that creatine is essential in early infancy for the normal development of the CNS (Battini et al., 2006; Bianchi et al., 2000). There is no evidence for neurological morbidity in the fetus, or that these infants are creatine-deficient at the time of birth. These findings therefore confirm that in human pregnancy the mother sustains the fetal requirement for creatine for most or all of pregnancy, and that the congenital creatine deficiencies appearing *after* birth are the result of the inability of these infants to synthesize enough creatine to sustain normal brain function.

8.6.1 Animal studies—In the spiny mouse, supplementing the maternal diet with 5% creatine (w/w) from day 20 gestation (approx 0.5 term) has the effect of decreasing the mortality caused by experimentally-imposed asphyxia at birth, preventing the slowing of postnatal growth rate that follows this insult, and improving the behavioural deficits that

otherwise occur (Ireland et al., 2010; Ireland et al., 2008). It is by no means certain that such prolonged exposure to supplementary creatine is necessary for such benefits to occur, although experiments with adult mice have indicated that exposure for several weeks is required for the neuroprotective benefits to emerge (Zhu et al., 2004). The precise mechanism by which creatine provides neuroprotection is not well understood. Generally it is thought to be related to preserving energy levels in the brain by providing for the maintenance of mitochondrial function via phosphocreatine, although more recently it has been suggested to also improve cerebrovascular function (Prass et al., 2007).

9 Timely delivery of neuroprotective treatment

Due to the risk of adverse effects, it is unlikely that treatments would be given routinely to pregnant women without evidence that their fetus had already been compromised, or was thought to be at extreme risk of compromise. There is an understandable reluctance to treat all pregnant women prophylactically, but if the agents were endogenously occurring substances such as melatonin or creatine, with no known adverse side effects even in high doses, this might be more acceptable. Folic acid, for example, is now routinely taken during early pregnancy to ensure neural tube closure.

Many of the pharmacological treatments used in the animal studies discussed above have been delivered prenatally. Although it is conceivable that some treatments could be administered in this way to women whose fetuses are identified as being at risk of brain injury it needs to be shown that they are also efficacious when delivered to the neonate after delivery, a stage at which treatment can be more readily administered, perhaps in combination with hypothermia.

Currently there is robust debate about the efficacy of magnesium sulphate as an intrapartum treatment for fetal hypoxemic brain injury, with findings from a recent meta-analysis suggesting that it is highly successful in reducing the incidence of cerebral palsy (Doyle et al., 2009; Peebles et al., 2010). Although magnesium sulphate has been a treatment for pre-eclampsia for many years, its use in the context of fetal brain injury is still contentious.

It may be possible to improve the neuronal environment immediately after birth in neonates considered to be at risk of perinatal brain injury, to enhance the growth of axons, dendrites and synaptogenesis and myelination of axons. We know that these events can be manipulated in the period of neonatal plasticity in the months after birth. Increasing the levels of growth factors or other molecules immediately after birth, ideally by systemic injection of small molecule mimetics of neurotrophins or growth factors which can cross the blood brain barrier, might be successful in restoring appropriate brain development and function. Transplantation of neural stem cells is currently being trailed in neonatal brain injury (Sato et al., 2008); however, it is too early to know whether such therapy is likely to be successful. These manipulations could also produce aberrant or inappropriate synaptogenesis, so the outcome of such treatments, indeed of any therapeutic treatment, needs to be tested for functional improvement.

10 Concluding remarks

It is now increasingly apparent that an adverse intrauterine environment which delivers low levels of oxygen and/or nutrients to the fetus, or is infected or inflammatory, can result in fetal brain injury and abnormal brain development. Such environments likely underlie many of the neurological and behavioral deficits which manifest after birth in some infants. The timing and severity of prenatal insults are critical in determining the nature of the outcome in terms of the severity of the damage and the brain regions affected. In animal models it has been demonstrated that relatively brief periods of fetal hypoxemia can have significant

effects on the fetal brain, for example death of susceptible neuronal populations (cerebellum, hippocampus, cortex) and cerebral white matter damage. Chronic mild placental insufficiency, which includes fetal hypoxemia, nutrient restriction and altered endocrine status, can cause long-term deficits in neural connectivity as well as affecting postnatal function, for example in the auditory and visual systems. Perinatal infection with fetal cytokine exposure is now considered to be an important factor in causing brain damage; there are likely to be synergistic pathways between hypoxia and inflammation which potentiate the evolution of injury. A central factor in the many forms of fetal brain injury appears to be a severe reduction in the delivery of oxygen (and perhaps other nutrients) to the developing brain. A greater understanding of the etiology of perinatal brain damage will allow us to devise strategies to intervene and reduce the burden of perinatal brain injury which is likely to increase with the increasing survival of very preterm infants. Creating the most suitable extrauterine environment for these preterm infants will also be essential if we are to facilitate appropriate neural development postnatally. Currently the only established therapy for neonatal encephalopathy in full term neonates is moderate hypothermia although this only offers some protection to moderately but not severely affected brains. There is currently no accepted therapy for brain injury in preterm infants. Experiments are now being directed towards combined therapies of hypothermia and pharmacological agents or growth factors to improve neurological outcomes.

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Abbreviations

(BDNF)	Brain derived neurotrophic factor
(FIRS)	fetal inflammatory response syndrome
(IUGR)	intrauterine growth restriction
(IL)	interleukin
(LPS)	lipopolysaccharide
(OL)	NMDA (n- methyl -D-aspartate), oligodendrocyte
(ROS)	reactive oxygen species
(rhEPO)	recombinant erythropoietin
(TNF)	tumour necrosis factor
(VLBW)	very low birth weight

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Research (review) highlights

- Acute fetal hypoxemia can have significant effects on fetal brain development
- Chronic placental insufficiency leads to long-term neurological deficits
- Brain damage resulting from intrauterine inflammation is compounded by hypoxemia
- We review a range of agents which provide neuroprotection in animal models
- We discuss clinical trials of therapeutic approaches to neuroprotection