

**The Role of Endotoxin, the TNF Family
of Cytokines
and
Intracellular pH
in
Perinatal Hypoxic-Ischemic Brain Injury**

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I, Giles Kendall, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

To explore the effect of endotoxin as a sensitising agent prior to neonatal hypoxia-ischemia differing doses of endotoxin (*E. coli* lipopolysaccharide, LPS) were given to neonatal mice prior to hypoxia-ischemia, with sensitising effects noted at dosages of 0.3mg/kg of LPS or higher. Varying the time interval between endotoxin administration and hypoxia-ischemia demonstrated that LPS given between 4 and 12 hours before hypoxia-ischemia had a sensitising effect on subsequent hypoxia ischemia. In contrast, LPS given at the time of or 24 hours before hypoxia-ischemia did not. To help understand the mechanism by which this sensitising effect occurs, a dose-response study of LPS alone was undertaken. Here, a dose-dependent activation of microglia was demonstrated throughout the brain, particularly in the thalamus and cortex, by 12 hours following endotoxin administration. There was also evidence of vascular endothelial activation (ICAM1) as early as 4 hours after endotoxin administration. To study the role of the TNF cluster of cytokines (TNF alpha, lymphotoxin alpha and lymphotoxin beta), animals with a deletion of the entire TNF cluster were examined. Deletion of the TNF cluster was shown to abolish both endotoxin-mediated sensitisation of the developing brain to subsequent hypoxia, and to prevent upregulation of macrophage and vascular endothelium by endotoxin alone.

This study also examined the effects of hypoxia-ischemia on intracellular pH. Increasing duration of hypoxia-ischemia resulted in a progressive intracellular acidosis within the brain, initially ipsilateral to the carotid ligation, but becoming bilateral with prolonged hypoxia. In the reoxygenation phase, there was a rebound intracellular alkalosis at 6 hours of reoxygenation across the whole forebrain. Previous studies have suggested that this alkalosis is mediated by a Na⁺/H⁺ exchanger. Blockade of this transporter with N-methyl isobutyl amiloride prior to hypoxia-ischemia was shown to confer neuroprotection in the developing brain.

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Chapter 1: General Introduction

Part 1: Epidemiology and Perspectives on Childhood Disability

A review of the historical literature regarding childhood disability shows that our understanding began with an endpoint, the identification of a pattern of “Deformities of the Human Frame” by an English Orthopaedic Surgeon, William Little in 1843. The pattern of disability described, which is now known as spastic diplegia, began our recognition of the disorder of movement and posture termed Cerebral Palsy. This endpoint defines the retrospective view on childhood disability, the perspective of the paediatrician, taking children diagnosed as suffering from conditions such as cerebral palsy, and attempting to identify the critical factors in their aetiology.

A second perspective is the prospective, obstetric view which builds on the risk factors identified from the retrospective studies. This view starts with the population of all pregnant women, and their unborn children, and tries to define those babies at risk of future disability. Following on from this an attempt is made to intervene in order to prevent long-term neurodisability.

Between these two perspectives there exists the view of the neonatologist, a specialty of medicine that has only existed for some 40 years. The neonatal perspective starts with two clinical entities, prematurity and neonatal encephalopathy, both of which have been clearly associated with childhood neurodisability. From this position, research has looked both retrospectively, to identify antecedent factors, and prospectively, looking for predictive markers in the long-term neurodevelopmental outcome of the neonate.

Paediatrics and Cerebral Palsy: A Retrospective Perspective

A clinical syndrome comprising motor difficulties and spasticity was first described by William Little in a series of articles published in the *Lancet* between 1843 and 1844 entitled "Deformities of the Human Frame". The disorder described, spastic diplegia, is one pattern of the non-progressive disorder of movement and posture, now termed cerebral palsy. Little, in a lecture and subsequent paper published in 1862, went on to propose that the principal cause of this condition was oxygen starvation at or around the time of birth (Little WJ 1862). This attributing of childhood neurodisability to perinatal events has been the subject of extensive discussion and debate ever since.

As a general principal childhood mental and physical disability may have its origins in the antenatal, perinatal or postnatal period. Antenatal causes include genetic / chromosomal disorders, and disorders of brain development and differentiation, including neuronal migration defects. Perinatal brain injury encompasses injury sustained in labour and the immediate postnatal period. Within this group are both term infants and preterm infants who demonstrate differing patterns of injury related to a maturation-dependent susceptibility to adverse stimuli. Postnatally acquired brain injury includes brain injury sustained through trauma, infection, inborn errors of metabolism, postnatal anoxic brain injury, and possibly following prolonged seizures, (Thibeault-Eybalin et al 2009).

Cerebral palsy is defined as a permanent disorder of voluntary movement or posture caused by a non-progressive lesion within the immature brain, in general cases associated with either genetic or a fetal malformation syndrome and those who have a known post-neonatal insult are excluded from the definition (Colver et al 2000, Greenwood et al 2005). Various longitudinal studies have examined the changing prevalence / incidence and severity of cerebral palsy in the United Kingdom. Pharoah et al used cerebral palsy registers covering Scotland and 6 counties of England, and birth / death registers between 1984 and 1989. Over this 5 year period the neonatal

mortality rate was 4.6 per 1000 live births. Overall the prevalence of cerebral palsy was 2.1 per 1000 neonatal survivors. When the data was stratified according to birth weight (<1000g, 1000-1499g, 1500g-2499g, ≥2500g) there was a dramatic effect of birth weight on prevalence of cerebral palsy ranging from 1.1 per 1000 neonatal survivors ≥2500g to 78.1 per 1000 neonatal survivors ≤1000g. Interestingly over the 5 year period studied there was no significant change in the incidence of cerebral palsy at any birth weight, whilst neonatal mortality fell in all birth weight groups. Colver et al examined epidemiological trends in cerebral palsy over a 30 year period 1964 to 1993 looking at annual rates of cerebral palsy stratified by both weight and severity (Colver et al 2000). Similar to the findings of Pharoah there was a fall in perinatal and neonatal mortality rates over the period studied. In those babies born ≥2500g there was no significant increase in either overall rates of cerebral palsy, or in the most severe forms. However in babies born <2500g there was an increase in overall cerebral palsy, and it was the more severe forms of cerebral palsy that increased the most.

Following on from Little's assertion, epidemiological studies have attempted to assess the proportion of cerebral palsy attributable to perinatal hypoxia. These studies suggest intrapartum hypoxia-ischemia is the predominant cause in only 3-28% of cases of cerebral palsy (Nelson & Ellenberg 1986, Blair et al 1988, Gaffney et al 1994 Hagberg et al 2001). Retrospective epidemiological studies have therefore been used in an attempt to identify other potential aetiological factors in the development of cerebral palsy. Multivariate analysis of prenatal and perinatal factors predicting cerebral palsy demonstrated that maternal mental retardation, birth weight below 2001 g, and fetal malformation were the leading predictors of subsequent cerebral palsy (Nelson and Ellenberg 1986). In this study antenatal factors were much more predictive of the development of cerebral palsy than perinatal factors. The inclusion of information about the events of birth and the neonatal period (such as asphyxia) accounted for a proportion of cerebral palsy only slightly higher (37%) than that accounted for when consideration was limited to characteristics identified before the onset of labour (34%).

In addition to low birth weight, premature birth is also significantly associated with increasing rates of cerebral palsy. In a population based study of two health districts in England during 1984-1993, 235 cases of cerebral palsy were identified. One third of the children later diagnosed with cerebral palsy were born at 32 weeks gestation or less, 15% of cases were born between 33 and 36 weeks, 54% of cases were born at 37 weeks gestation or above. When combined with population data, the prevalence of cerebral palsy was calculated at 42 per 1000 live births at 32 weeks gestation or less, 6 per 1000 live births 33 – 36 weeks gestation, and 1 per 1000 live births at 37 weeks gestation or above (Greenwood et al 2005). In this study, markers of intrapartum hypoxia and infection were associated with an increased risk of cerebral palsy in term and preterm infants: Hypoxia OR 12.2, 95%CI 1.2-119 at ≤ 32 weeks and OR 146, 95% CI 7.4-3651 at ≥ 37 weeks; Neonatal sepsis OR 3.1, 95%CI 1.8-5.4) at ≤ 32 weeks and OR 10.6, 95%CI 2.1-51.9 at ≥ 37 weeks. Interestingly, in the same study, pre-eclampsia only increased the risk of cerebral palsy in term infants.

A further factor complicating the association between cerebral palsy and perinatal events is that certain subtypes of cerebral palsy are thought to be associated with specific perinatal insults. Cerebral palsy is subdivided according to the pattern of motor involvement: Quadriplegia describes a symmetrical involvement of all 4 limbs; it is this form along with dyskinetic forms of cerebral palsy that are thought to relate to perinatal hypoxic-ischemic injury. Hemiplegia where one side of the body is affected (often the arm more than leg), is seen predominantly in term infants and thought to represent a stroke-like vascular accident. In spastic diplegia both sides of the body are affected but the arms more than legs; this form is predominantly seen in preterm or growth restricted infants.

Gaffney et al undertook a case control study of children with cerebral palsy or death occurring during labour or after birth among singletons born ≥ 37 weeks of gestation

without evidence of congenital anomaly or severe infection in a single Health Authority during 1984-1987 (Gaffney et al 1994). Antenatal risk factors more common in cases of cerebral palsy than controls include previous poor obstetric outcome, existing maternal disease, induced conception, raised α -fetoprotein, intrauterine growth retardation, pre-eclampsia, bleeding after 20 weeks, oligohydramnios, polyhydramnios, prolonged rupture of membranes and postmaturity. Indeed 48% of children with cerebral palsy had at least 1 of these antenatal characteristics. The authors described that, although many cases of cerebral palsy were managed optimally in perinatal period (75%), there was both an increased incidence of failure to respond to clinical signs of fetal distress (25%) compared with controls (7%) (OR 4.5, 95%CI 2.8-8.4). However only 7% of children diagnosed with cerebral palsy had a suboptimal response to fetal distress followed by evidence of neonatal encephalopathy. This then suggests that improving obstetric interventions in cases of fetal distress, when taken in context of the prevalence of cerebral palsy (1 in 1000 live births at ≥ 37 weeks Greenwood et al 2005), could at very best reduce the prevalence of cerebral palsy by 1 in 14000 live births.

Other than perinatal hypoxia-ischemia, epidemiological studies have demonstrated that the other major identifiable perinatal risk factor for the subsequent development of cerebral palsy is fetomaternal inflammatory response. Epidemiological studies suggest maternal / fetal infection, inflammation and possibly endotoxin-mediated cytokine release as antecedent factors in the development of white matter injury in preterm neonates (Gilles et al 1977, Leviton 1993, Nelson and Ellenberg 1985, Grether and Nelson 1997, Duncan et al 2002). A Meta-analysis of clinical chorioamnionitis in preterm infants using a random effects model also demonstrated a significant association with cerebral palsy (RR 1.9, 95%CI 1.4-2.5) and cystic periventricular leukomalacia (RR 3.0, 95%CI 2.2-4.0) When histological chorioamnionitis was studied in preterm infants the association with cerebral palsy was non-significant (RR 1.9, 95%CI 0.9-2.7) but was significantly associated with cystic periventricular leukomalacia (RR 2.1, 95%CI 1.5-2.9) (Wu and Colford 2000). In the term neonate meta-analysis of

clinical chorioamnionitis and cerebral palsy gave a summary relative risk of 4.7 (95%CI 1.3-16.2) (Wu and Colford 2000) based on two studies (Nelson and Ellenberg 1985, Grether and Nelson 1997). A study of histological chorioamnionitis in neonates with neurological impairment showed a significant independent association (OR 13.2, 95%CI 1.2-144) (Redline and O'Riordan 2000). This study, which assessed placental lesions associated with cerebral palsy and neurological impairments, also showed an independent association of other lesions generally believed to have their onset in the antenatal period: extensive avascular villi (OR 9.0, 95%CI 1.6-51) and diffuse chorioamnionic hemosiderosis (OR 74.8, 95%CI 6.3-894). The risk of neurological impairment was noted to correlate with the number of lesions present within the placenta, particularly if antenatal lesions and perinatal lesions were present in the same placenta (OR 94.2, 95%CI 11.9-747). These findings lend weight to the hypothesis of interactions of multiple antenatal and perinatal aetiological factors in cerebral palsy (Blair and Stanley 1993, Grether and Nelson 1997, Badawi et al 1998, Nelson and Grether 1998).

Animal studies have shown that either whole bacteria or endotoxin, a bacterial product, can result in neonatal brain injury when administered at critical periods of neurodevelopment (Hagberg et al 2002). There is also the potential for a transplacental effect as demonstrated by administration of a bolus dose of endotoxin to pregnant rats, which results in failure of cerebral myelination in their offspring (Cai et al 2000). The developing brain appears to be particularly sensitive to infection when it is at a premyelination stage of maturation. Whilst it is possible to cause white matter injury, typically observed in premature neonates, using endotoxin in immature animals, similar studies showing endotoxin related grey matter damage have not been reported. The relation between infection and term brain injury may therefore involve other mechanisms

The possibility that infection may play a role sensitising the fetus to the effects of hypoxia-ischemia has been suggested previously (Peebles and Wyatt 2002). This concept is explored in Figure 1.1 where the curved plot represents a hypothetical threshold which when crossed from left to right, is associated with brain injury; factors that might do this include hypoxia (indicated by fetus A) and prenatal exposure to materno-fetal infection (fetus B). In fetuses exposed to inflammatory mediators prior to the onset of labour, a relatively minor degree of intrapartum hypoxia-ischemia may have disastrous consequences (fetus C).

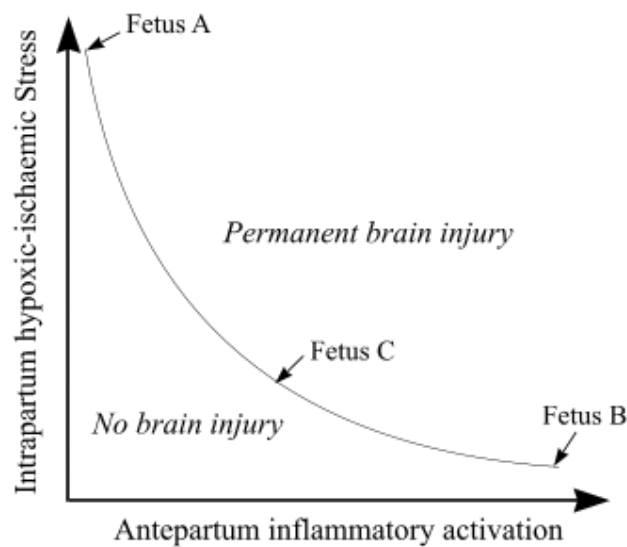


Figure 1.1 Putative relationship between brain injury, antepartum inflammatory activation and intrapartum hypoxic-ischemic stress. Fetus A can withstand substantial intrapartum stress before permanent cellular injury is initiated. Fetus B will develop permanent injury as a result of antepartum infection despite minimal intrapartum stress. Fetus C is vulnerable to intrapartum stress and will escape permanent injury provided that the ischemic threshold is not reached. From Peebles and Wyatt 2002, reproduced with permission

Evidence to support this hypothesis comes from both epidemiological and experimental studies. Epidemiological evidence suggests that exposure to combined infection and hypoxic-ischemic insults dramatically increases the risk of developing cerebral palsy compared with either insult alone (OR 78, 95%CI 4.8-406) (Nelson and Grether 1998).

Animal models have also demonstrated the synergistic effects of inflammatory responses and hypoxia-ischemia. Endotoxin (lipopolysaccharide) has been shown to sensitise and dramatically increase the lesion size in the immature rat brain following hypoxic-ischemic injury. Similar effects are seen in the neonatal mouse when exposed to E.Coli endotoxin before hypoxia ischemia. This effect is seen when endotoxin is administered systemically (Eklind et al 2001, Yang et al 2004) or intracisternally (Coumans et al 2003). Paradoxically pre-treatment with endotoxin has also been shown to reduce the volume of infarct in adult rats following regional cerebral ischemia (Ahmed et al 2000) and under certain circumstances in the neonatal rat (Eklind et al 2005). It is unclear whether this apparently conflicting neuroprotective effect of endotoxin is due to the exact time relationship of the insults, and whether there are differing effects on immature and adult brains. It is plausible that the inflammatory status of the brain, particularly in terms of the pro-/anti- inflammatory cytokine response may be quite different at different time-points following endotoxin administration. It therefore appears that a combination of antenatal and perinatal factors may act as antecedents of cerebral palsy, and it is entirely possible that there may be an interaction between antenatal / perinatal factors, and additionally specific genetic susceptibilities leading to a theory of multifactorial aetiology in the development of cerebral palsy.

Imaging of children born at term with cerebral palsy has provided little help in establishing either the aetiology or timing of the antecedents to their disability, as lesions are variable and dependent on the pattern of cerebral palsy and gestation at birth. In a systematic review of magnetic resonance imaging in children born at term diagnosed with bilateral spastic cerebral palsy 25% had normal MRI scans, 24% had grey matter lesions, 20% had periventricular white matter lesions and 18% showed patterns of brain maldevelopment. This was in contrast to preterm babies and babies with spastic hemiplegia (see Table 1.1; Krägeloh-Mann and Horber 2007). It is possible, however, that in time, with serial scans from birth through to childhood, that

the nature of some of the observed lesions can be better characterised and further information about the aetiology deduced.

	Bilateral Spastic Cerebral Palsy		Spastic Hemiplegia	
	Term	Preterm	Term	Preterm
Normal MRI	25% (33)	4% (8)	14% (6)	0% (0)
GM Lesion	24% (32)	4% (7)	41% (18)	0% (0)
PWM Lesion	20% (26)	90% (167)	20% (9)	86% (12)
Maldevelopment	18% (24)	2% (3)	16% (7)	14% (2)
Miscellaneous	5% (7)	1% (1)	9% (4)	0% (0)

Table 1.1: Distribution of MRI lesions in bilateral and unilateral spastic cerebral palsy in term and preterm infants. GM-grey matter, PWM-periventricular white matter lesion (from Krägeloh-Mann and Horber 2007)

More than 150 years after Little's original publications, it now appears that cerebral palsy is the clinical manifestation of multiple, and interacting, aetiological and pathophysiological pathways which encompass antenatal, perinatal and postnatal factors. This complex multifactorial background to cerebral palsy may explain some of the apparent conflicts in the medical literature, especially surrounding the timing of insults, and the apparent failure to impact on the incidence of childhood neurodisability.

Obstetrics and Birth Asphyxia: A Prospective Perspective

The observation that an inadequate supply of “oxygen and materials for nutrition,” or insufficient removal of “carbon and other residues” may lead to brain injury in the neonate was first made by William Little in a lecture and paper later referred to as a “learned bombshell” (Jensen et al 1991). The exact relationship between hypoxia-ischemia and brain injury has remained a topic of extensive debate ever since.

One of the significant difficulties in establishing the role and impact of hypoxia-ischemia on neuro-developmental outcome relates to the definition and use of terms in the medical literature. Hypoxia is defined as a reduction in oxygen tension which may be further defined as to where it occurs (hypoxaemia, tissue hypoxia). Ischemia is the reduction in blood delivery to the tissues which in turn leads to a reduction in substrate (glucose) delivery. The definition of birth asphyxia is very variable in the medical literature and in clinical medicine. A task force for the World Federation of Neurology Group defined asphyxia as “a condition of impaired blood gas exchange leading, if it persists, to progressive hypoxemia and hypercapnia” (Bax and Nelson 1993). Defining and quantifying these characteristics is essential to aid in the management of women in labour. Whilst epidemiological studies suggest that perinatal hypoxia is responsible for only a small proportion of childhood neurodisability, it remains the single most important cause of perinatal brain injury (du Plessis and Volpe 2002). Indeed perinatal animal models have consistently demonstrated that, in previously healthy animals, a brief period of severe and acute hypoxia-ischemia can initiate the causal sequences leading to neuronal death (Gunn et al 1992).

Even an apparently “normal” labour involves a degree of hypoxic stress on the fetus. However, the incidence of moderate or severe encephalopathy after birth is low at less than 3.8 per 1000 live births (Badawi et al 1988). The normal term fetus is able to mount a complex range of haemodynamic and metabolic compensatory mechanisms to protect the brain from hypoxia (Peebles et al 2002). Hypoxia and hypercapnia in the

fetus initially causes a redistribution of cardiac output to the brain, heart and adrenal glands mediated by the sympathetic-adrenergic nervous system. These physiological compensation mechanisms have been demonstrated in animal models. In the fetal sheep isocapnic hypoxemia for 1 hour (reduction of the maternal FiO_2 to 9 to 10%) results initially in a fall in the fetal heart rate (FHR) which then gradually returns to normal. At the same time there is a rise in mean arterial pressure (MAP) and in carotid blood flow (CaBF) with a corresponding fall in carotid vascular resistance (CaVR; see Figure 1.2). Only when these mechanisms are overwhelmed by very severe or prolonged hypoxia is there cerebral hypoperfusion and a fall in high-energy phosphate metabolism initiating a cellular and molecular cascade of events leading to neural cell death (Nijjima et al 1988, Vannucci RC 1990, Jensen and Berger 1991, Gunn et al 1992).

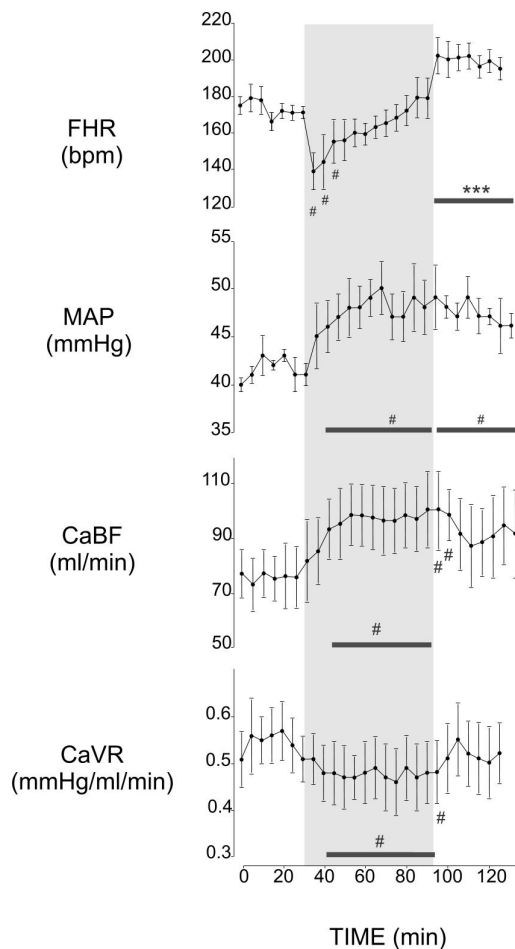


Figure 1.2 Effect of 1 hour of isocapnic hypoxia on fetal heart rate (FHR), mean arterial pressure (MAP), carotid blood flow (CaBF), and carotid vascular resistance (CaVR). The shaded region denotes the period of hypoxia. Values are means \pm SEM, # $p < 0.05$; ** $p < 0.005$, for individual time-points or intervals of data (black bars). There is a significant rise in CaBF during hypoxia, which is associated with a fall in CaVR and a rise in MAP (from Bennet et al 1998 reproduced with permission)

In contrast the responses to pure ischemia tend towards a reduction of cerebral blood flow which may exacerbate brain injury (see Figure 1.3).

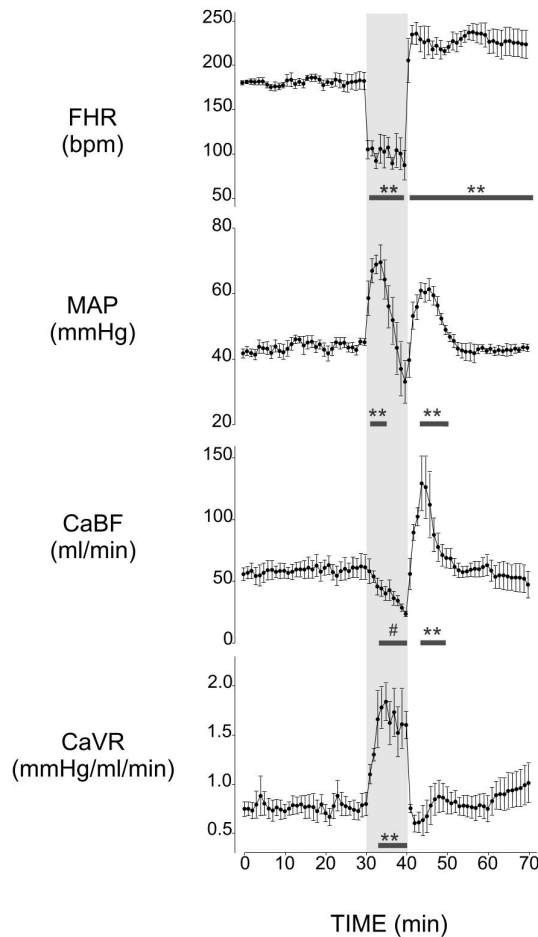


Figure 1.3 Effect of 10 min of severe asphyxia induced by umbilical cord occlusion on fetal heart rate (FHR), mean arterial pressure (MAP), carotid blood flow (CaBF), and carotid vascular resistance (CaVR). The shaded region denotes the period of asphyxia. Values are means \pm SEM (black bars, # $p < 0.05$; $p < 0.01$). Note the progressive fall in CaBF and the associated rise in CaVR at a time when MAP is significantly elevated (from Bennet et al 1998 reproduced with permission)

Whilst intrapartum events likely to cause a complete disruption of maternal-fetal gas exchange, such as cord prolapse and placental abruption do occur, and can lead to neonatal encephalopathy and brain injury, (Nelson & Ellenberg 1986) (Blair et al 1988) in clinical practice this sequence of events is rare. Hypoxia severe enough to result in brain injury is usually accompanied by evidence of other end organ damage, such as of the heart, liver and kidneys. Indeed the effects of hypoxia on cardiac contractility and hence cardiac output may be critical in disrupting the normal compensatory mechanisms to hypoxia. In animal models disruption of these compensatory mechanisms results in death or worsened brain injury. For example, impairing normal vasoconstrictor mechanisms by sympathectomy or α -adrenergic blockade results in

impaired fetal survival during acute hypoxia, and blocking A1 adenosine receptors prior to hypoxia worsens the extent of brain injury (Hunter et al 2003).

One of the key problems for obstetric practice is how to assess for the presence of asphyxia i.e. “impaired blood gas exchange leading to progressive hypoxemia and hypercapnia” severe enough to lead to damage to the fetus, allowing timely intervention. Generally in the United Kingdom assessment of the fetus in the perinatal period is by fetal heart rate monitoring often as part of a continuous cardiotocogram (CTG). In selected cases, more invasive testing in the form of fetal blood sampling for direct assessment of fetal acid base balance is used. The findings from these forms of monitoring are then compared to immediate outcome measurements in the neonate, such as condition at birth (assessed for example by the APGAR score), intermediate neonatal outcome measures such as the presence and severity of neonatal encephalopathy, and finally with long-term neurodevelopmental outcomes, such as the presence of cerebral palsy.

Fetal heart rate monitoring originated from research papers published in the 1960s describing various patterns thought to be associated with the early stages of fetal asphyxia. A number of attempts were made over the subsequent decades to formalise definitions and nomenclature for the various patterns of fetal heart rate such that appropriate interventions could be put in place. In 1997 the National Institute of Child Health and Human Development (NICHD) Research Planning Workshop published a paper attempting to describe “standardized and unambiguous definitions for fetal heart rate tracings” They described that the interpretation of the fetal heart rate tracing involves several characteristics, baseline fetal heart rate (FHR), baseline FHR variability, presence of accelerations, periodic or episodic decelerations and changes or trends of FHR patterns over time (NICHD Research Planning Workshop 1997). From a clinical perspective they described that a normal FHR tracing would demonstrate a normal baseline rate, normal [moderate] FHR variability, presence of

accelerations, and absence of decelerations. Tracings thought to be predictive of severe asphyxia, placing the fetus at risk of neurological damage or death, include recurrent late or variable decelerations or substantial bradycardia, with absent FHR variability. In a retrospective case control study, the features of a FHR tracing associated with increased risk of the subsequent development of cerebral palsy, were multiple late decelerations in the heart rate, (slowing of the heart rate well after the onset of a uterine contraction) (OR 3.9, 95%CI 1.7-9.3), and decreased FHR variability (OR 2.7, 95%CI 1.1-5.8). These features were present in 27% of children who subsequently developed cerebral palsy, but were also present in 9.3% of those who did not. However, when this is extrapolated to a population level, with an incidence (in this study) of cerebral palsy at 2 per 1000 live births (>2500g), then FHR findings of multiple late decelerations and / or decreased FHR variability would have a 99.8% false positive rate (Nelson et al 1996). Thus, the use of fetal heart rate monitoring has the potential to dramatically increase obstetric intervention rates with minimal and conflicting evidence for impact on rates of neonatal encephalopathy and long-term neurodisability.

Interestingly when pregnancies with evidence of intrauterine bacterial infection (clinical chorioamnionitis plus either positive blood cultures or signs of infection in the neonate) were studied, the cerebral palsy rate was much higher than normal at 11%. In this group of 139 pregnancies “non-reassuring” FHR patterns including recurrent late deceleration, severe variable deceleration, and prolonged deceleration occurred in 24%, but the incidence of cerebral palsy was not different according to the FHR deceleration patterns or umbilical pH values, thus suggesting that, in the presence of infection, an abnormal FHR trace may have even less predictive value for subsequent neurodisability.

Potentially the most direct assessment of fetal condition comes from direct assessment of fetal blood pH, carbon dioxide and base deficit / excess. This assessment is commonly performed in the immediate postnatal period from the umbilical cord vessels, and less commonly in the latter stages of delivery from a fetal scalp blood sample. Whilst correlation of pH to long and short term neurodevelopmental outcome is, at best, weak, the metabolic component of acidosis (using base deficit) demonstrates the strongest association (ACOG Committee Opinion 2006). Low et al studied children with base deficits of 4-8 mmol/L, 8-12 mmol/L, and 12-16 mmol/L and obtained evidence of neonatal central nervous, respiratory, cardiovascular, and renal complications which were each scored for severity. They demonstrated that an umbilical arterial base deficit of 12 mmol/L represented a threshold when moderate or severe newborn complications may occur and further worsening of metabolic acidosis was associated with increasing severity of newborn complications (Low et al 1997).

In an attempt to define an acute intrapartum hypoxic event as sufficient to cause cerebral palsy, the American Academy of Pediatrics and American College of Obstetricians and Gynecologists have defined 4 criteria all of which must be fulfilled (modified from MacLennan 1999):

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

Thus, to define childhood neurodisability as having resulted from perinatal hypoxia-ischemia, evidence is required from birth, the neonatal period and long-term follow-up.

In addition to the difficulty in identification and quantification of fetal hypoxia-ischemia, the diagnosis and interpretation of chorioamnionitis also remains a challenge. In practice chorioamnionitis may be differentiated into microbiological chorioamnionitis (culture positive), clinical chorioamnionitis, or histological chorioamnionitis. In the clinical setting, timely diagnosis may have to be made prior to the culture result being available, and, at the present time, histological chorioamnionitis is a retrospective diagnosis. The diagnosis of clinical chorioamnionitis generally includes criteria such as maternal fever $\geq 38^{\circ}\text{C}$, raised C-reactive protein, leukocytosis, uterine tenderness, foul smelling amniotic fluid, and fetal or maternal tachycardia. Whilst easy to diagnose if multiple features are present, in practice, clinical chorioamnionitis often presents a diagnostic challenge, and the decision to intervene and facilitate delivery, especially in the preterm period, remains a matter of clinical judgement.

In addition to chorioamnionitis, intrapartum fever itself has been shown to be independently associated with an increased risk of development of unexplained neonatal seizures (a strong predictor of neurological impairment in term infants) (OR 3.4 95%CI 1.03-10.9) (Lieberman et al 2000) and neonatal encephalopathy (OR 4.72, 95% CI 1.28-17.4) (Impey et al 2001). It is not clear whether these effects are just because pyrexia is serving as a marker for infection / inflammation or whether non-infectious pyrexia, related to epidural usage, may also be damaging, particularly if the fetus is hypoxic.

The challenge from the obstetric perspective remains immense. Starting with the large population of all pregnant women, most of which will have a normal outcome, the obstetrician needs to not only identify the very small number of babies with hypoxia-ischemia sufficient to risk neurological damage, but also to intervene to prevent this outcome without exposing women to unnecessary interventions, which themselves have the potential for harm.

Neonatology and Neonatal Encephalopathy: A Bidirectional Perspective

William Little described in 1861 that, in addition to prematurity and difficulties in parturition, a delay in establishing “pulmonary respiration” in the immediate postnatal period may lead to brain damage. The neonatal perspective on neurodisability is related to two main clinical entities, prematurity and neonatal encephalopathy.

Assessment of the newborn begins with an immediate assessment of condition at birth. Measurements such as Apgar scores at 1, 5 and 10 minutes, time to onset of spontaneous respirations, or need for resuscitation have been used in attempt to provide a quantifiable measure of the condition of the neonate. The Apgar score was first proposed more than 50 years ago and has become a standard method of assessing the condition of a neonate at birth (Apgar 1953). This assessment entails the scoring from 0-2 of 5 physiological characteristics: pulse, respiration, colour, muscle tone and response to stimulation. Retrospective analysis of Apgar scoring in children with cerebral palsy does demonstrate that a low score is a statistically significant risk factor for developing cerebral palsy (see Table 1.2) (Nelson and Ellenberg 1981).

	Apgar Score		
	0-3	4-6	7-10
Controls	1%	3%	96%
Cerebral Palsy	15%	12%	73%

Table 1.2: Distribution of 5-minute APGAR scores in children later diagnosed with cerebral palsy compared to controls (from Nelson and Ellenberg 1981)

Whilst it is clear that children who are later diagnosed with cerebral palsy have a greater chance of having a low Apgar score at 5 minutes, the predictive value of the score for cerebral palsy is very low. 73% of children later diagnosed with cerebral palsy had an Apgar score 7-10 at 5 minutes and, even with an Apgar score as low as 0-3 at 10 minutes, only 16.7% of children went on to develop cerebral palsy, i.e. 83.3% of

children with an Apgar score of 0-3 at 10 minutes were free of major disability at early school age (Nelson and Ellenberg 1981). The Apgar score has also been shown to correlate with neonatal mortality (death within the first 28 days of life) in both term and preterm infants (see Table 1.3). In this study the relative risk of neonatal death using the five minute Apgar score of 0-3 was 1460, 95% CI 835-2555, this was eight times the relative risk with an umbilical-artery blood pH \leq 7.0 (RR 180; 95% CI 97 to 334).

	Apgar Score		
	0-3	4-6	7-10
Preterm (26-36wk)	315	72	5
Term (\geq 37wk)	244	9	0.2

Table 1.3: Neonatal (within 28 days of birth) mortality rate per 1000 live births for term and preterm infants according to Apgar score at 5 minutes (Casey et al 2001)

In an attempt to establish the proportion of cases of low Apgar scores that can be attributed to asphyxia, Hogan et al used a case control and cohort study to look for evidence of hypoxia (Hogan et al 2007). The authors used abnormalities in CTG, interventions for fetal distress, cord pH and evidence of HIE or death (see Table 1.4).

	5 minute Apgar Score		
	<4 (n=30)	4-6 (n=143)	Controls (n=182)
Abnormal admission CTG	38%	8%	0.6%
Abnormal CTG before birth	28%	6%	0.6%
Interventions for fetal distress	83%	48%	9%
Cord artery pH below 7.15	69%	54%	7%
HIE or hypoxic death	70%	14%	0%

Table 1.4: Evidence of intrapartum hypoxia at different Apgar Scores (from Hogan et al 2007)

However, as with many studies into this area, there are some difficulties with drawing definite conclusions from these data. As discussed earlier, changes in FHR tracings

may arise from conditions other than hypoxia. Whilst cord artery acid base status is important, as described previously, use of the metabolic component (base deficit) is generally considered to be a more accurate reflection of intrapartum hypoxia-ischemia with a threshold at base deficit 12mmol/L or above (Low et al 1997) as this is the threshold above which there is significant risk of neonatal complications. In the group of neonates with an Apgar score <4, 19% had mild neonatal encephalopathy (Sarnet grade I) and 41% had moderate - severe neonatal encephalopathy (Sarnet II-III) contrasting with the group of neonates with Apgar 4-6 who had 9% with mild neonatal encephalopathy and 3% with severe neonatal encephalopathy (Hogan et al 2007).

Assessment of the newborn within minutes of delivery whilst a useful measure to alert the clinician to potential evidence of an intrapartum event severe enough to cause neonatal and long-term complication, is not able to either give prognostic information or establish the cause of any depression of consciousness noted at birth.

Neonatal Encephalopathy and Term Perinatal Brain Injury

The clinical picture of neonatal encephalopathy is well described and graded according to Sarnet and Sarnet 1976 (see Table 1.5). The relationship with hypoxia-ischemia is somewhat more complicated.

		Mild (I)	Moderate (II)	Severe (III)
Level of Consciousness		Hyperalert	Lethargic	Stuporose
Neuromuscular Control				
	Muscle tone	Normal	Mild Hypotonic	Flaccid
	Posture	Normal	Flexion	Decerebrate
	Tendon reflex	Hyper-reflexia	Hyper-reflexia	Absent
	Clonus	Present	Present	Absent
Pupils		Dilated	Constricted	Poorly reactive
Seizures		None	Common	Frequent/ intractable
Moro Reflex		Strong	Weak/ incomplete	Absent
Suck Reflex		Normal/weak	Weak/ absent	Absent

Table 1.5: Grading of neonatal encephalopathy by clinical evaluation (modified from Sarnet and Sarnet 1976)

Combining the available data from the medical literature regarding long-term neurodevelopmental outcome related to severity of neonatal encephalopathy, it is clear that there is “dose-response” effect of worsening encephalopathy with increasing long-term major disability and death (for review see Collins and Paneth 2002). Overall the risk of death or major disability in neonates diagnosed with mild encephalopathy was 2%, moderate encephalopathy 19% and severe encephalopathy was 63%. In a population based study in Sweden, moderate neonatal encephalopathy had a prevalence of 0.06%. Of the surviving individuals, 30% had cerebral palsy (similar to that reported in other studies); interestingly, however, of the 70% who were not diagnosed with cerebral palsy, 71% had definite cognitive difficulties, and 18% hearing impairment. Indeed only 8% of those individuals who had been diagnosed with grade II neonatal encephalopathy who did not have cerebral palsy were free of impairment when tested at 15-19 years (Lindström et al 2006). It is clear, therefore, that significant intrapartum hypoxia-ischemia results in neonatal encephalopathy and may, in turn, lead to long-term neurodisability.

Following on from clinical evaluation of neonatal encephalopathy, additional prognostic information has, more recently, been described from electroencephalograms (EEG) / amplitude integrated encephalograms (aEEG) and from magnetic resonance imaging and spectroscopy. Amplitude-integrated electroencephalogram (aEEG) monitoring with the cerebral function monitor (CFM) was first described almost 40 years ago (Maynard et al 1969). The aEEG allows continuous long-term monitoring of background activity, which compares well with the background on simultaneous conventional EEG. aEEG has gained widespread use in the care of neonates with encephalopathy as it has allowed early prediction of neurodevelopmental outcome, especially when used in conjunction with clinical neurological evaluation (al Naqeeb et al 1999) with a combined specificity (94%) and positive predictive value (85%) for persisting moderate to severe encephalopathy at 5 days (Shalak et al 2003). Correlation of aEEG voltage and pattern, to long-term neurodevelopmental outcome has also been demonstrated in

children with hypoxic-ischemic encephalopathy. aEEG recordings made at 3 and 6 hours after birth were compared with neurodevelopmental outcome at 30 months or older. The sensitivity of a low baseline voltage to poor outcome was 33% at 3 hours, and 42% at 6 hours, but the association was only significant at 6 hours. In contrast, sensitivity of burst suppression pattern to poor outcome was 83% at 3 hours and 75% at 6 hours (Shanay et al 2006).

The aEEG also has the potential to identify those infants who would benefit from therapeutic interventions such as therapeutic cooling (Gluckman et al 2005, Shankaran et al 2005) and may allow selection of specific neonates for different interventions. The 2-channel aEEG quantified into median values of minimum, mean, and maximum amplitude has been shown to have a negative relationship with MRI abnormalities, i.e. the lower the amplitude the worse the MRI findings (Shah et al 2006). The authors suggest that a minimum amplitude of 4 μ V gives a specificity of 75%, which may allow its use for early prognosis (in conjunction with clinical examination and neuroimaging), whereas a minimum amplitude of 6 μ V could be used for diagnostic purposes or for consideration for neuroprotective interventions. The other main use of the aEEG has been in identifying seizure activity in babies even after administration of neuromuscular blockade. Most seizure activity can be detected by the aEEG, especially with modern monitors that allow review of raw EEG data either continuously or on review of possible seizure activity. However some short or localised seizures may be missed (Hellström-Westas and Rosén 2002).

Magnetic resonance imaging is rapidly becoming a standard of care in the management of both preterm infants, and term infants with neonatal encephalopathy. Scanning of infants following hypoxic-ischemic insults can give important prognostic information and guide ongoing clinical care (for review see Rutherford 2002). MRI studies have identified basic patterns of injury following hypoxia-ischemia. Acute

severe insults result in cortico-subcortico involvement in the basal ganglia, thalami, brain stem, hippocampus and the corticospinal tracts around the central fissure.

Chronic or repetitive insults result in cortical and white matter damage in a parasagittal distribution, which represents the watershed region between major arterial supplies (Rutherford 2002). Early findings within the first week include:

- Signs of brain swelling (loss of extracerebral space, loss of sulcal markings, closure of the Sylvian fissures, narrow interhemispheric fissure, slit like anterior horns of the lateral ventricles)
- loss of normal signal in the posterior limb of the internal capsule (PLIC)
- abnormal signal intensities in the basal ganglia and thalami
- Loss of grey / white differentiation
- Cortical highlighting on T1 weighted sequences

From a prognostic perspective the presence of brain swelling alone does not necessarily represent permanent injury. If, once the swelling resolves, the underlying brain is normal, there is no association with a poor neurodevelopmental outcome. However, a loss or change in the signal intensity from myelin in the posterior limb of the internal capsule has been shown to be very sensitive (0.91) and specific (1.0) for an abnormal outcome. Furthermore these changes, when present more than 48 hours after insult, can correctly predict motor outcome in 93% of infants, born ≥ 38 weeks gestation, with grade II hypoxic-ischemic encephalopathy (Rutherford et al 1998). The severity of lesions within the basal ganglia and thalami are related to neurodevelopmental outcome. Most authors suggest that interpretation of basal ganglia and thalamic lesions should be undertaken in association with PLIC signal anomalies. Mild focal basal ganglia lesions are generally associated with normal signal intensity within the PLIC; these findings correlate with mild motor difficulties. Moderate focal basal ganglia lesions are generally associated with equivocal or abnormal signal intensity within the PLIC; these findings are associated with the development of

athetoid quadriplegia; however intellect is normally preserved. Severe diffuse lesions are always associated with abnormal signal intensity within the PLIC, these correlate with spastic quadriplegia, severe intellectual delay, secondary microcephaly and persistent refractory convulsions (Rutherford 2002, Rutherford et al 2006, Jyoti and O'Neil 2006). The other early MR findings of cortical highlighting and loss of grey / white differentiation occur in conjunction with the other findings and, as such, their extent represents part of the overall spectrum of injury detectable on MRI.

Advanced MR techniques have been used in hypoxic ischemic encephalopathy including diffusion weighted imaging, diffusion tensor imaging and magnetic resonance spectroscopy.

Diffusion weighted imaging (DWI) provides a visual image of the random motion of water within a tissue. This motion or diffusivity can be quantified as an apparent diffusion coefficient (ADC). Visual abnormalities on DWI are most obvious 1 to 4 days after hypoxic ischemic insult, when conventional imaging may appear normal. However when there has been widespread injury, DWI images may be apparently normal, possibly because there is no normal tissue for comparison. In this context, measurement of ADC values is essential to detect the presence of ischemic tissue (Rutherford et al 2005). Studies using DWI in infants following hypoxia ischemia have produced conflicting results (Forbes et al 2000, Rutherford et al 2005, Boichot et al 2006, Dağ et al 2006). ADC values are typically reduced in the first week following severe injury to white matter or basal ganglia / thalamus but normalise at the end of the first week. ADC values in the posterior limb of the internal capsule have been shown to be significantly associated with survival and to correlate with motor outcome (Hunt et al 2004). Early DWI and ADC values may, however, be normal in the presence of moderate but significant basal ganglia / thalamic lesions (Rutherford et al 2005).

Diffusion tensor imaging measures the anisotropy or directional diffusivity within a tissue. Anisotropy normally increases with age as myelination decreases radial diffusivity perpendicular to WM tracts (Rutherford et al 2005). In a series of 20 infants with hypoxic-ischemic encephalopathy Ward et al demonstrated decreased fractional anisotropy values in both severe and moderate white matter and basal ganglia thalamic injury during the first week, whereas ADC values were reduced only in severe white matter damage and some severe basal ganglia thalamic damage. In comparison to ADC values, which appear to normalise during the second week after hypoxia-ischemia, fractional anisotropy values continued to decrease (Ward et al 2006).

Magnetic resonance spectroscopy (MRS) allows quantitative analysis of chemical composition and structure using atomic nuclei such as phosphorus (^{31}P) and hydrogen-1 (proton, ^1H), the magnetic resonance signal derived can be resolved into a frequency spectrum by the mathematical Fourier Transformation. Phosphorus (^{31}P) MRS provides data on high energy phosphate molecules, including ATP; it is usually taken from the whole brain and has been used to demonstrate the biphasic nature of energy changes following hypoxic ischemic insults (see later). Additionally shift in the Pi peak (Pi relative to phosphocreatine) can derive pH_i values and has been used to demonstrate rebound alkalosis following neonatal hypoxia-ischemia (see later). Proton (^1H) MRS provides data on choline, creatine, NAA and lactate, and unlike ^{31}P MRS, ^1H MRS can give information from different brain regions. Two recent studies have looked at the prognostic value of proton (^1H) magnetic resonance spectroscopy. 11 term newborns with encephalopathy had ^1H -spectroscopy of the basal ganglia and thalami two weeks after hypoxia-ischemia. Abnormal neurodevelopmental follow-up at 9 months was detected in 45%; here there was a trend towards a low mean NAA / creatine ratio, although this was not statistically significant. Furthermore the presence of a raised lactate peak was associated with adverse neurological outcome relative risk 7.0 (95%CI 1.1-42.9) (da Silva et al 2006). A second study looked at the value of early (day 1-3) ^1H -MR spectroscopy in 17 infants with neonatal encephalopathy and 10 controls.

Neurodevelopmental outcomes were assessed at 1 year and graded as mild/ normal or severe fatal. A number of concentrations and ratios were shown to be significantly changed in the severe / fatal group compared see Table 1.6 and Figure 1.4 (Cheong et al 2006).

Quantification	Increased	Decreased
Metabolite Peak-Area Ratios	Lactate / NAA Lactate /Choline, Lactate / Creatine	NAA/ Creatine NAA/ choline
Metabolite T2 Relaxation	Lactate , NAA, Creatine	
Metabolite Absolute Concentrations	[Lac]	[Cho], [Cr], [NAA]
Metabolite Concentration Ratios	[Lac]/[NAA]	

Table 1.6: Changes in ¹H-MR spectroscopy values in neonates with neonatal encephalopathy that died or had a severe neurodevelopmental disability at 1 year compared with control (Cheong et al 2006).

Comparison of the normal/mild outcome group with controls revealed no differences in peak-area ratios, relaxation times, or concentration ratios, but decreased absolute concentrations of [NAA], [Cho], and [Cr]. Conventional and advanced magnetic techniques can act as complementary tools alongside clinical and electroencephalogram evaluation for predicting the individual outcomes of term neonates with neonatal encephalopathy.

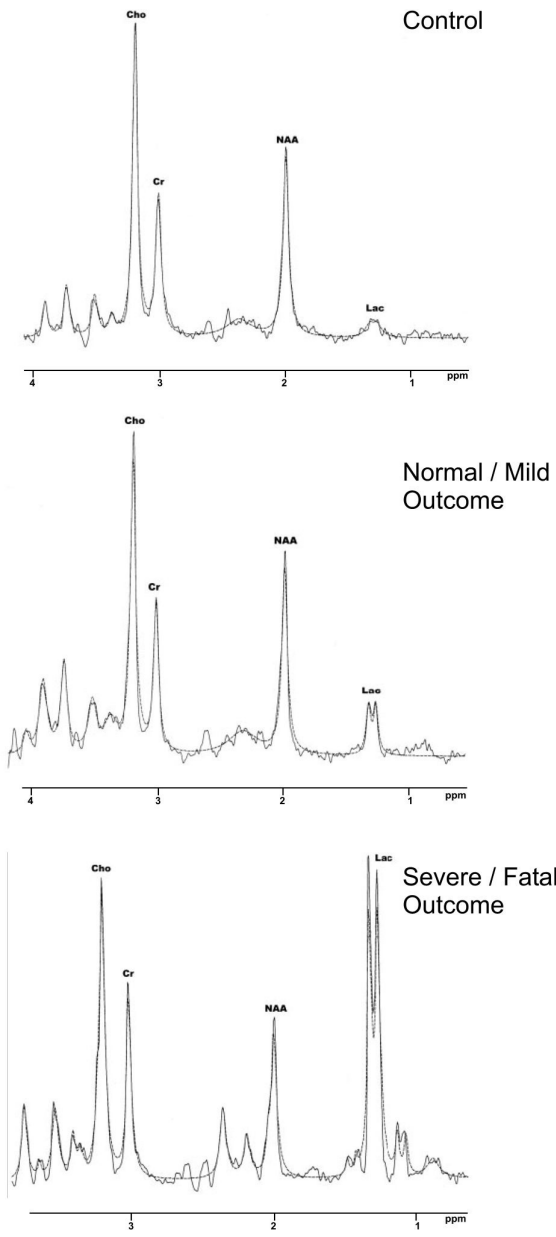


Figure 1.4: Representative brain spectra from a control infant, and 2 neonates with neonatal encephalopathy acquired from an 8-mL voxel centered on the thalami. The dashed lines are the spectrum analysis Lorentzian profiles fitted to the peaks (from Cheong et al 2006 reproduced with permission)

The aetiology and timing of the brain injury resulting in neonatal encephalopathy has been subject to more debate. In 1998 Badawi et al published two papers in the British Medical Journal describing antepartum and intrapartum risk factors for neonatal encephalopathy in Western Australia (Badawi et al 1998). 164 cases of moderate or severe encephalopathy were identified over a 2 year period and compared to 400 randomly selected unmatched controls. In this study the birth prevalence for neonatal encephalopathy was 3.8 per 1000 term live births, and there was a fatality rate of 9.1%. In the preconception and antenatal periods are shown in Table 1.7

		Adjusted OR	95% CI
Maternal Characteristics	Unemployed	3.6	1.1 - 11.8
	Unskilled manual worker	3.8	1.4 - 10.3
	Housewife	2.5	1.1 - 5.4
Family History	Seizures	2.6	1.3 - 4.9
	Neurological disorders	2.7	1.2 - 6.4
Pregnancy Features	Infertility treatment	4.4	1.1 - 17.6
	Maternal thyroid disorder	9.7	2.0 - 47.9
	Severe pre-eclampsia	6.3	2.3 - 17.6
	Bleeding during pregnancy	3.6	1.3 - 9.9
	Clinical diagnosis viral illness	3.0	1.5 - 5.8
	Not drinking alcohol	2.9	1.7 - 5.0
	Abnormal placenta	2.1	1.2 - 3.7
Birth weight	Between 3 rd and 9 th centile	4.4	1.4 - 13.4
	Below 3 rd centile	38.2	9.4 - 154.8

Table 1.7: Antenatal Risk Factors for Newborn Encephalopathy: the Western Australian Case-Control Study (Badawi et al 1998)

In the accompanying paper Badawi et al also looked at intrapartum risk factors, after adjustments for all the antepartum risk factors previously identified. In this paper they identified maternal pyrexia, persistent occipito-posterior position and acute intrapartum events (haemorrhage, maternal convulsions, uterine rupture, snapped cord, birth before arrival to hospital) as independent risk factors for the development of neonatal encephalopathy. The authors attempted to estimate the proportion of infants exposed

to significant intrapartum hypoxia, using fetal heart rate tracings, presence of fresh meconium at delivery and Apgar scores. Cord pH was not included in their assessment as it was not routinely performed at that time. Using these features, the authors estimated that only 29% of infants with moderate – severe neonatal encephalopathy had evidence of intrapartum hypoxia. Conversely 94% of infants had at least one of the antenatal risk factors identified in the previous study (Badawi et al 1998) see Figure 1.5.

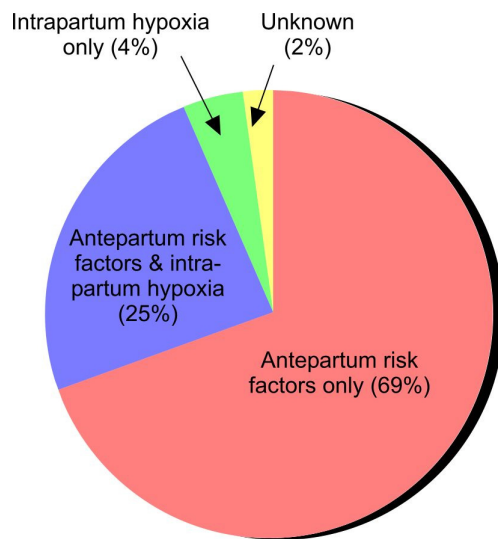


Figure 1.5: Distribution of risk factors in newborn encephalopathy. The Western Australia Case Control Study (Badawi et al 1998)

This study questioned the widespread view that most risk factors for newborn encephalopathy occurred in the intrapartum period. However, it should be noted that the definition of neonatal encephalopathy used was broad. Moderate or severe encephalopathy included all babies with either seizures alone or any two of the following lasting for longer than 24 hours: abnormal consciousness, difficulty maintaining respiration (of presumed central origin), difficulty feeding (of presumed central origin) or abnormal tone and reflexes. Furthermore, the authors included babies who developed these signs within the first week of life. This relatively broad definition may potentially underestimate the role of intra-partum hypoxia in the development of neonatal encephalopathy. More recent evidence suggests that whilst the risk factors may be predominantly antenatal, the timing of the brain injury itself remains clearly during the acute perinatal period. In 2003 Cowan et al used magnetic resonance

imaging to study neonates with neonatal encephalopathy and evidence of intrapartum hypoxia, and infants with early seizures without other features of neonatal encephalopathy. Neonatal encephalopathy was defined by abnormal tone pattern, feeding difficulties, and altered alertness. Evidence of intrapartum hypoxia with at least three of:

1. late decelerations on fetal monitoring or meconium staining
2. delayed onset of respiration
3. arterial cord blood pH less than 7.1
4. Apgar scores less than 7 at 5 min
5. multi-organ failure.

Scans were performed within 2 weeks of birth, most in the first week. In the group of neonates with encephalopathy and evidence of intrapartum hypoxia, 80% of neonates had evidence of acutely evolving lesions that were compatible with a hypoxic-ischemic insult, whereas established injury was seen in less than 1%. The lesions most commonly seen were bilateral abnormalities in basal ganglia, thalami, cortex, or white matter. In the second group of infants with seizures in the first 72 hours of life but not fulfilling the criteria for neonatal encephalopathy and preceding hypoxia-ischemia, acute ischemic or haemorrhagic lesions were seen in 69% of infants. Within this group, evidence of concomitant antenatal injury was present in only 3% of cases. In the remaining 31%, almost half had scan findings indicating a specific, non-hypoxic non-ischemic diagnosis. Of these, most had either hypoglycaemia or what was later diagnosed to be a persisting seizure disorder. Overall 90% of term infants with neonatal encephalopathy, seizures, or both, had evidence of acute perinatal brain injury and there was a very low rate of established antenatal brain injury (Cowan et al 2003).

Seemingly, neonatal encephalopathy, like cerebral palsy, may therefore represent a combination of antepartum and intrapartum risk factors, but the evidence from magnetic resonance imaging suggest that the damage itself occurs in the perinatal period. It is entirely possible that antepartum and genetic factors predispose to both a

difficult delivery, and increased susceptibility to intrapartum events that would not normally be enough to result in injury. This multifactorial / multi-hit hypothesis may go some way to explain the apparent conflicts in the medical literature with regard to the timing and aetiology of neonatal encephalopathy.

Premature Birth and Brain Injury

The epidemiological studies of Colver, Pharoah and others have demonstrated that, over the last 30 years, not only is cerebral palsy rising in incidence and severity but this rise is almost entirely within low birth weight and premature neonates (Pharoah et al 1998, Colver et al 2000). Viewed from the neonatal perspective, it is clear that there is an increase in prevalence and severity of neurodisability with increasing prematurity. The EPICure study followed the 1995 cohort of all babies born in the UK and Republic of Ireland born below 26 weeks. Their neurodevelopmental outcome has been studied and published at discharge, 30 months and 6 years (Wood et al 2000, Costeloe et al 2000, Wood et al 2005, Marlow et al 2005) see Table 1.8. Currently, the 2006 cohort of all babies born in the UK and Republic of Ireland born below 26 weeks are being studied, to see if there have been improvements in outcome over the last decade.

Gestation	≤23 weeks	24 weeks	25 weeks
No Disability	12%	14%	24%
Mild Disability (e.g. low normal IQ, wears glasses & has squint, mild hearing loss, minor neurological abnormalities)	25%	36%	35%
Moderate Disability (e.g. moderate learning problems, cerebral palsy but walking, hearing aids, some visual deficit)	38%	22%	22%
Severe Disability (e.g. severe learning problems, cerebral palsy and not walking, profound deafness, blindness)	25%	29%	18%

Table 1.8: Neurodevelopmental outcomes of the 1995 cohort of surviving babies born in the UK and Republic of Ireland below 26 weeks. The EPICure study group (Wood et al 2000, Costeloe et al 2000, Wood et al 2005, Marlow et al 2005)

In an attempt to identify specific risk factors for the development of cerebral palsy in the preterm population, Murphy et al used a case control analysis of all children born over a 6 year period in 2 regions of England. 59 cases of cerebral palsy were identified in singleton infants born below 32 weeks, and 235 random unmatched controls (approximately 4 controls per case) were identified born below 32 weeks without evidence of cerebral palsy. Overall the survival rates of preterm infants increased with increasing gestational age and birth weight. The rates of cerebral palsy among surviving infants decreased with increasing gestational age and birth weight. There was a complex relationship between cerebral palsy and method of delivery. Caesarean delivery without labour was associated with a decreased risk of cerebral palsy (OR 0.3, 95%CI 0.2-0.7). Conversely, caesarean delivery in labour was associated with an increased risk of cerebral palsy (OR 2.7, 95%CI 1.2-6.1). Of note there was no significant difference in the number of abnormal fetal heart rate tracings (OR 1.0, 95%CI 0.9-1.1) or umbilical artery pH ≤ 7.10 (OR 2.3, 95%CI 0.8-6.8). The other risk factors for cerebral palsy identified are shown in Table 1.9; however, it is important to note that only 3% of cases and 6% of controls received antenatal steroid therapy (Murphy et al 1995)

		OR	95% CI
Maternal Factors	Parity >5	4.3	1.2-15
Antenatal Factors	Prolonged rupture of membranes	2.3	1.2-4.2
	Chorioamnionitis	4.2	1.4-12.0
	Maternal Infection	2.3	1.2-4.5
Neonatal Factors	Apgar Score ≤ 3 at 5 Mins	5.3	1.4-21

Table 1.9: Antenatal and Intrapartum Risk Factors for Cerebral Palsy in Very Preterm Singleton Babies (Murphy et al 1995)

Whilst it is clear that increasing prematurity is associated with increasing risks of cerebral palsy and brain injury, premature neonates are a heterogeneous group of patients. Epidemiological studies have identified a number of factors associated with

the onset of preterm labour including black race, teenage or older mothers, low education and low socio-economic status, cigarette smoking, unmarried or not living with a partner, heavy and/or stressful occupation, low maternal pre-pregnancy body mass index. Medical and obstetric complications associated with preterm birth include multi-fetal pregnancy, gestational or pre-existing diabetes, pregnancy-induced or pre-existing hypertension, placenta praevia and placental abruption (Wen et al 2004). However, many cases of preterm birth related to either maternal illness or developing fetal compromise are iatrogenic. In North West Thames during 1988-2000 almost 50% of babies delivered between 28-31 weeks resulted from induced labour or elective caesarean section (Steer 2005).

The single most identifiable clinical risk factor for spontaneous preterm labour is infection, which is particularly prevalent in babies born below 30 weeks with histological involvement of the fetal membranes (see Figure 1.6; Goldberg et al 2000).

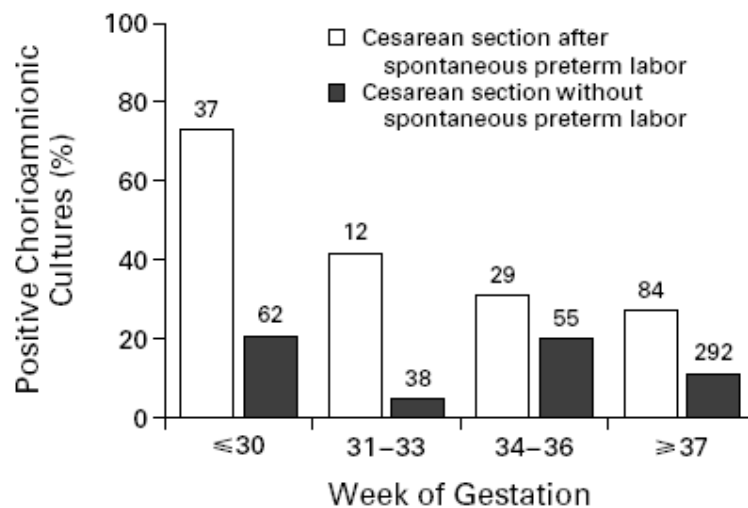


Figure 1.6: Frequency of positive chorioamnionic culture at varying gestation among women delivering by caesarean section in spontaneous labour with intact fetal membranes, compared to those delivering by caesarean section without onset of labour (Goldberg et al 2000). Copyright © [2000] Massachusetts Medical Society. All rights reserved. Reproduced with permission

Bacterial invasion of the chorio-decidual space results in the production of cytokines, including interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor (TNF- α) and granulocyte colony stimulating factor (GCSF) by the deciduas and fetal membranes. There is also synthesis of prostaglandin, which stimulates uterine contractions, and metalloprotease release which can both lead to cervical softening and rupture of the chorioamniotic membranes (see Figure 1.7; Goldenberg et al 2000).

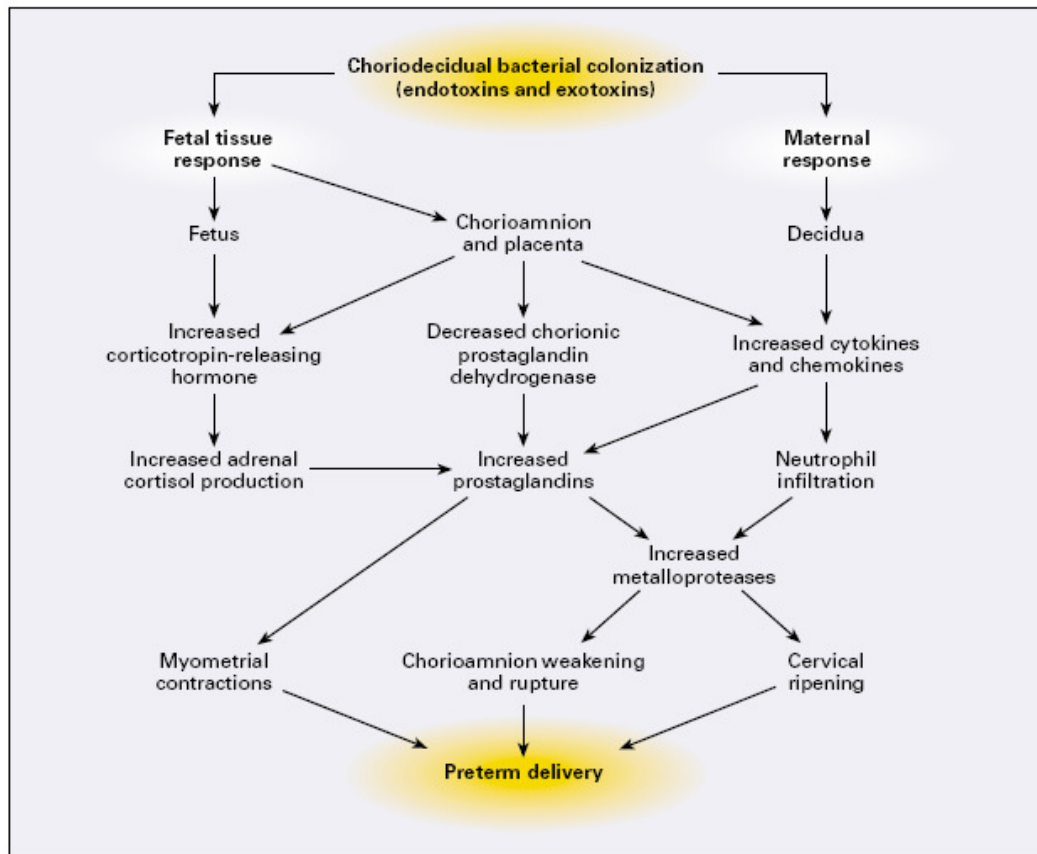


Figure 1.7: Potential Pathways from Choriodecidual Bacterial Colonization to Preterm Delivery (from Goldenberg et al 2000) Copyright © [2000] Massachusetts Medical Society. All rights reserved. Reproduced with permission

Bacterial components, such as endotoxin, are potent activators of the cytokine network and it is now accepted that pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α , of both fetal and maternal origin play a critical dual role in initiating preterm labour as well as directly contributing to perinatal brain injury (Duggan et al 2001, Yoon et al

1996, Gomez et al 1998). It has also been demonstrated that single nucleotide polymorphisms within tumour necrosis factor, interleukin-1 β , and IL-6, are associated with spontaneous preterm labour (Engel et al 2005) and preterm birth and may also make the preterm neonate susceptible to brain injury (Varner et al 2005) (see later: The role of pro- and anti-inflammatory cytokines).

Summary

The relationship between intrapartum hypoxia-ischemia, neonatal encephalopathy and cerebral palsy is more complex than that originally described by Little 150 years ago. Hypoxia-ischemia is detectable in up to 2.5% of pregnancies, 15% of which is defined as moderate-to-severe. Moderate-to-severe neonatal encephalopathy has a prevalence of 3.8 per 1000 live term births (Badawi et al 1998). Cerebral palsy has a prevalence of 1 per 1000 live term births (Greenwood et al 2005). There is considerable overlap between these conditions, with up to 30% of cerebral palsy having hypoxia-ischemia as a significant antecedent, but 7% of children with cerebral palsy and evidence of intrapartum hypoxia ischemia not having evidence of neonatal encephalopathy (Gaffney et al 1994). Neonatal encephalopathy may arise without any evidence of intrapartum hypoxia-ischemia (Badawi et al 1998). An attempt to summarise the overlap between intrapartum hypoxia-ischemia, neonatal encephalopathy and cerebral palsy is shown in figure 1.8. The estimate of the overlap between neonatal encephalopathy and intrapartum hypoxia-ischemia, is taken from the data from Cowan's MRI imaging (Cowan et al 2003), rather than the data from the Western Australia Case Control Study (Badawi et al 1998). It is important to recognise that, whilst this study was not population based and relied on timing of injury from MR imaging, a more rigorous definition was used for neonatal encephalopathy.

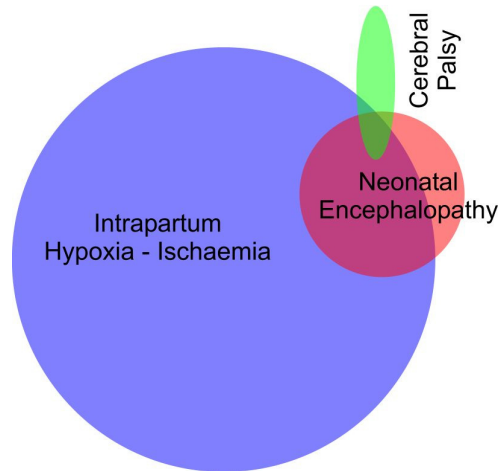


Figure 1.8: Overlap of intrapartum hypoxia-ischemia, neonatal encephalopathy and cerebral palsy.

Perinatal brain injury in term infants is consistently associated with the presence of hypoxia-ischemia and fetomaternal inflammation. Prematurity is commonly associated with the presence of bacteria in the uterus, the consequence of which may not only lead directly to preterm birth but also to neuronal damage and perinatal brain injury.

Part 2: Patterns of Perinatal Brain Injury

A number of patterns of injury have been described in the neonatal brain. Different patterns of injury are thought to arise due to a combination of differing insults, and a maturation-dependent selective vulnerability of differing cell populations.

White matter injury in neonates was first described by Virchow in 1867 from post-mortem specimens with macroscopically pale, softened areas of degeneration within the periventricular region, which he termed congenital encephalomyelitis (Virchow 1967). The term periventricular leukomalacia was first introduced in 1962 by Banker and Larroche, again from post-mortem specimens. They described microscopic lesions consisting of coagulative necrosis surrounded by areas of liquefaction and a highly vascularised periphery. Lesions occurred within the subcallosal white matter, superior fronto-occipital & superior longitudinal fasciculi; the external and internal sagittal strata of the temporal and occipital horns of the lateral ventricles; and to a lesser extent the corona radiata. Key to the understanding of the neuropathology and aetiology of periventricular leukomalacia is the recognition of a specific window of sensitivity between 23-32 weeks of gestation (Rezaie and Dean 2002). At these dates the developing brain is pre-myelination and the subcortical white matter is predominantly populated with oligodendrocyte precursors (McQuillen and Ferriero 2004), and it is these precursors that are thought to be particularly sensitive to injury (Volpe 2001). Much of the research into periventricular leukomalacia during the 4 decades since the description by Baker and Larroche has focused on the focal necrotic cystic lesions they described and specifically used cranial ultrasound scans to demonstrate their presence (Volpe 2001). More recently, however, advances using magnetic resonance techniques have demonstrated diffuse white matter injury manifest as diffuse excessive high signal intensity (DEHSI) and increased apparent diffusion coefficient (ADC) as a much more frequent finding in preterm infants at term-equivalent age (Maalouf et al 1999), (Counsell et al 2003).

In a study comparing serial cranial ultrasound scans with magnetic resonance imaging at term, in babies born below 1500g (gestational age 27.9 ± 2.4 weeks) only 4% of neonates had evidence of cystic periventricular leukomalacia, all of which was identifiable on cranial ultrasound scan prior to MRI at term; 35% of infants had evidence of non cystic white matter injury. Of note, the cranial ultrasound finding of prolonged (>7 days) echodensity in the white matter (often termed periventricular flare) had a low sensitivity (26%) and low positive predictive value (36%) for diffuse periventricular leukomalacia (Inder et al 2003). In a study of 119 infants born between 22 and 30 weeks, Dyet et al found that 80% of infants had diffuse excessive high signal intensity within the white matter on T2 weighted MRI scans at term. Developmental follow-up using the Griffiths Mental Development Scales showed a significant relationship between the presence of DEHSI and overall developmental quotient (no DEHSI: 111 ± 20 , DEHSI: 94 ± 11.6 , severe DEHSI: 92 ± 7.5 ; $P = 0.027$). When infants with both DEHSI and other abnormalities were excluded there was still a significant difference in developmental quotient between infants with and without DEHSI (normal scan: 105, DEHSI: 91, $P = 0.023$) (Dyet et al 2006).

The recognition of this distinct diffuse pattern of injury in addition to the previously described focal cystic lesions have led some authors to suggest a change in terminology from periventricular leukomalacia to “cerebral leukoencephalopathy” to more accurately encompass both patterns of injury (Volpe 2003), or more recently encephalopathy of prematurity (Volpe 2009). However, the terms cystic PVL and diffuse PVL remain in common use both in the literature and in clinical practice. From a clinical perspective the major sequelae of focal cystic periventricular leukomalacia is spastic diplegia, as a result of disruption to the corticospinal tracts within the posterior limb of the internal capsule, whereas the more diffuse white matter damage is probably the cause of cognitive and behavioural abnormalities that are seen commonly after preterm birth (Volpe 2001, Marlow et al 2005).

From a neuropathological perspective, it is thought that white matter injury results from a combination of incomplete development of the vascular supply to the cerebral white matter, a maturation-dependent impairment of cerebral blood flow regulation and a gestational dependent specific vulnerability of oligodendrocyte precursors, as compared to mature myelinating oligodendrocytes, to a variety of pro-apoptotic stimuli such as free radicals, excessive glutamate and pro-inflammatory cytokines (Volpe 2001). It is likely that inflammation, ischemia and subsequent reperfusion may be key features in the underlying pathophysiology (Volpe 2001). However, recent evidence from the preterm sheep model has challenged the existence of these vascular boundary zones (McClure et al 2008). In addition to cellular damage, disruption of axons within the white matter was also described in the original classification of PVL (Banker and Larroche 1962). It is, at present, unclear whether the primary insult is to axons, oligodendrocyte precursors or both (see Figure 1.9) and it is possible that differing insults affect axons and oligodendrocyte precursors to differing degrees, but with the final common outcome being diffuse or cystic periventricular leukomalacia dependent on the type and severity of insult.

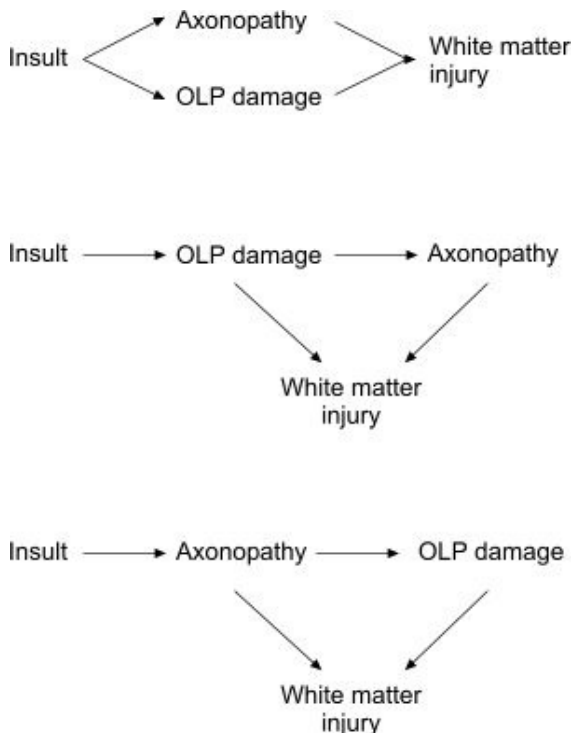


Figure 1.9: Three possible pathways by which an insult may lead to white matter injury via axonal damage and damage to oligodendrocyte precursors (OLP) (from Dammann et al 2001) reproduced with permission

Other than the gestational age, other factors have been identified in the aetiology of PVL. Many of these relate to episodes of reduced cerebral perfusion, such as hypoxic-ischemic insults, apnoea with hypoxia, and episodes of bradycardia (Rezaie and Dean 2002). Epidemiological studies have consistently identified hypocarbia as a strongly associated risk factor for the development of cystic periventricular leukomalacia (Resch et al 2004) OR 3.3; 95% CI:2.6-6.5 (Saliba and Marret 2001) and it has been postulated that this may also occur as a result of hypocarbia mediated vasoconstriction and, hence, reduction in cerebral blood flow. Other than cerebral ischemia the other factor most closely linked with white matter damage is intrauterine infection (Dammann and Leviton 1997, Yoon et al Saliba and Marrett 2001, Resch 2000, Rezaie and Dean 2002), and it is the dual role of infection as a stimulus for preterm labour and perinatal brain injury that is gaining increasing recognition.

Further potential clues to the aetiology of white matter injury can be drawn from the animal models used in the study of white matter injury (for review see Hagberg and Peebles 2002). These have used infectious, hypoxic-ischemic, excitotoxic or combination insults to generate injury resembling cystic periventricular leukomalacia. Studies of the effect of inflammation using endotoxin, a potent initiator of the inflammatory response, have demonstrated a transient hypoxemia and hypotension, with a fall in arterial pH and PaO₂, and a rise in lactate within arterial blood in the fetal sheep, at a gestation equating to 24-25 week in the human fetus. Cerebral white matter injury was observed in histological sections of all LPS treated animals, with diffuse injury seen in all animals, and lesions characteristic of focal cystic periventricular leukomalacia in 2/6 animals (Duncan et al 2002). In rat pups, chronic ischemia achieved by bilateral carotid artery occlusion on day 1 results in ventriculomegaly and rarefaction within the corpus callosum, associated with a reduction in myelin basic protein staining and microglial activation (Cai et al 2001).

Chronic hypoxia during the prenatal period achieved by exposing pregnant rats to 10% oxygen between embryonic day 5 and 20 also results in cystic white matter changes with lipid peroxidation, activation of macrophage and delayed myelination (Baud et al 2004). In the neonatal rat models of white matter injury using either hypoxia-ischemia or excitotoxins, 6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), an AMPA receptor antagonist, has been shown to be protective to oligodendrocyte precursors and reduce white matter injury, as measured by myelin basic protein immunoreactivity (Follett et al 2000). This suggests a role for glutamate receptor-mediated oligodendrocyte damage in the aetiology of preterm white matter brain injury.

Pro and anti-inflammatory cytokines have been clearly implicated in the pathology of white matter injury (Volpe 2001). IL-18 deficient mice have been demonstrated to be protected against hypoxia-ischemia mediated white matter damage with increased neurofilament and myelin basic protein immunoreactivity 3 days after injury (Hedtjärn et al 2005). In a rat model of maternal chorioamnionitis using intrauterine injection of *Escherichia coli*, subsequent administration of intravenous IL-10 prevented the symmetrical periventricular white lesions that were seen in 29% of the offspring of dams that did not receive IL-10 (Rodts-Palenik et al 2004). These studies suggest that in both infection and hypoxia–ischemia, inflammatory responses may be a final common pathway, and the modulation of this response may provide an opportunity for therapeutic neuroprotection in the neonate. This also raises the possibility that, in keeping with the triggering of preterm labour, single nucleotide polymorphisms within the inflammatory response may make a particular infant more susceptible to white matter injury (Nelson et al 2005) in a similar way as has been demonstrated in multiple sclerosis (Pleasure et al 2006).

In contrast to the white matter injury seen in the preterm neonate, hypoxic-ischemic brain injury occurring in term infants results in damage predominantly within the deep grey matter of the thalamus, basal ganglia and para-sagittal cerebral cortex (Sie et al 2000), (Johnston et al 2001). These infants are born post-myelination, and the oligodendrocytes which have matured are relatively resistant to injury compared to the oligodendrocyte precursors that predominate in the preterm infant. Severe damage results in neuronal death due to a failure of mitochondrial oxidative phosphorylation, a process that occurs more rapidly in the brain of term compared to preterm animal models of hypoxia (Berger et al 1992). Injury that is not severe enough to immediately cause neuronal death initiates a variety of events including the neurotoxic cascade, disruption of cellular energetics and changes in intracellular pH (pH_i), which may eventually result in further neuronal loss through necrosis, apoptosis or combined mechanisms.

Part 3: Mechanisms of Perinatal Brain Injury

The Neurotoxic Cascade

Most synapses within the brain use the excitatory amino acid glutamate as their neurotransmitter. Normally, glutamate is removed from the synapse following depolarisation by perisynaptic glial glutamate reuptake pumps. Raised levels of glutamate and glycine have been demonstrated following asphyxia in animal models and in the CSF of neonates following severe hypoxic-ischemic insult (Johnston et al 2002). The energy for the perisynaptic glial glutamate reuptake pumps is derived from the anaerobic metabolism of glucose (Magistretti et al 1999). Episodes of severe hypoglycaemia have been shown to result in high levels of extracellular glutamate (Ichord et al 1999). Further evidence for the role of excitatory neurotransmitters in the pathogenesis of perinatal brain injury comes from the observation that areas of the term neonatal brain susceptible to damage have the highest density of glutamate-utilising synapses. These areas include the putamen, the thalamus and the peri Rolandic cerebral cortex (Sie et al 2000), (Johnston et al 2001). Ischemia reduces the levels of glucose within the brain resulting in the failure of reuptake of excitatory neurotransmitters. This correlates in turn with the clinical findings of hyperalertness and hyperreflexivity in neonates following hypoxic-ischemic insults (Sarnat and Sarnat 1976). Acute hypoxia causes a failure of mitochondrial oxidative phosphorylation more rapidly in the brain of term compared to preterm animal models of hypoxia (Berger et al 1992). Attempts to maintain energy supply by anaerobic metabolism are insufficient to maintain ATP such that there is a failure of transmembrane ion pumping and a reduction in the membrane potential (Johnston et al 2001).

These two signals (elevated excitatory neurotransmitters and reduced membrane potential) interact at the *N*-methyl-D-aspartate (NMDA) receptor, which functions as a transmembrane calcium channel. This receptor integrates multiple signal pathways requiring the simultaneous binding of glutamate and glycine to open. The loss of the

membrane potential prevents the voltage-dependent magnesium blockade of the NMDA receptor channel and allows the excitatory amino acids to act persistently, resulting in calcium influx and subsequent neuronal damage (Novelli et al 1988). Further evidence for the role of NMDA and AMPA (non-NMDA glutamate receptors) come from studies showing receptor blockade significantly reduces brain damage following a hypoxic-ischemic insult (Hagberg et al 1994). The stimulation of NMDA receptors and voltage-dependent AMPA receptors results in a massive influx of calcium into neurones. Normally mitochondria buffer intracellular calcium. However, the calcium 'overload' that follows the locking open of the NMDA and AMPA receptors causes mitochondrial dysfunction (Kristian et al 1996, Stout et al. 1998, Johnston et al 2002). In severe cases rapid loss of mitochondrial function and cessation of ATP production, causes loss of nuclear and cytoplasmic membranes (cell necrosis). Less severe insults without loss of mitochondrial membranes may result in apoptosis through the release of cytochrome c which activates proteases and caspases causing DNA fragmentation. Blockade of caspases has been shown to afford significant neuroprotection following hypoxic ischemic insults (Cheng et al 1998).

Calcium entering the cell has a further role in the aetiology of damage via activation of neuronal nitric oxide synthase. Newly formed nitric oxide interacts with radicals released from damaged mitochondria to form peroxynitrite (Beckman 1991). Inhibition of nitric oxide synthase has been shown to reduce infarct size following middle cerebral artery occlusion in the rat pup (Ashwal et al 1995) and neonatal mice lacking neuronal nitric oxide synthase have been shown to be less vulnerable to hypoxic-ischemic damage (Ferriero et al 1996). Furthermore, the selective inhibition of neuronal and inducible nitric oxide synthase improves long-term outcome in neonatal rats following hypoxia-ischemia (van den Tweel et al 2002). The role of free radicals in the neuronal injury has been further studied in hypoxic-ischemic injury using melatonin, a neurohormone secreted from the pineal gland which has potent anti-oxidant activity. In

adult rats following middle cerebral artery occlusion melatonin significantly reduced ischemic lesion volume and reduced levels of oxidative stress markers (malondialdehyde and reduced glutathione) (Sinha et al 2001, Pei et al 2003). In this model melatonin has also been shown to reduce nitric oxide levels without affecting the integrity of the blood-brain barrier (one suggested mechanism by which free radicals have been postulated to act in cerebral ischemia) (Pei et al 2003). In fetal rats, melatonin has been shown to have a potential neuroprotective effect on ischemia / reperfusion injury achieved by bilateral utero-ovarian artery occlusion and release with reduced oxidative mitochondrial damage in the fetal rat brains (Wakatsuki et al 2001). In addition melatonin has been shown to be neuroprotective in excitotoxic brain injury in newborn mice (Husson et al 2002). However, some of the effects of melatonin neuroprotection may result from inhibition of the inflammatory response (Pei et al 2004).

In summary hypoxia-ischemia triggers a cascade of neurotoxic events that centres on the excitotoxins glutamate and glycine which, along with a reduced membrane potential, act to lock open the NMDA receptor and allow an influx of calcium into cells. This leads to further mitochondrial dysfunction and production of free radical species which combine to initiate further neuronal damage through both necrosis and apoptosis (see Figure 1.10).

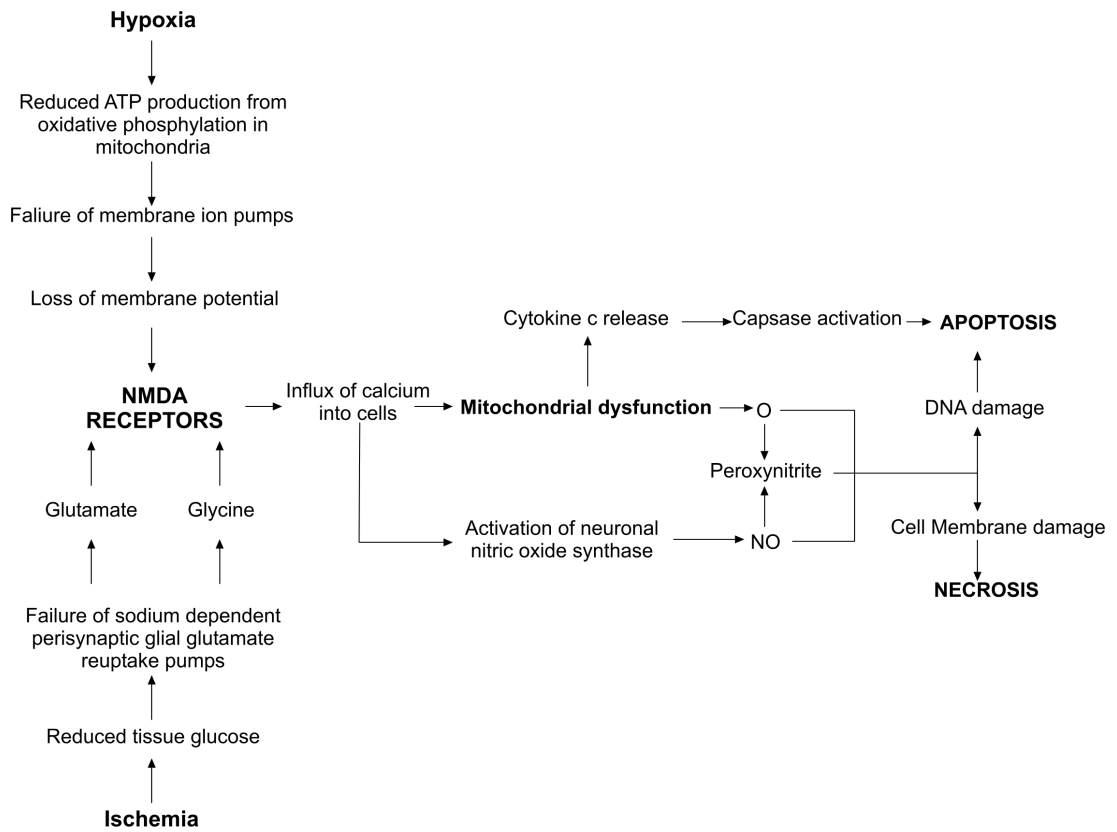


Figure 1.10: Summary of the neurotoxic cascade

Energy changes after Hypoxia-Ischemia

Over the last 20 years, studies using phosphorus (^{31}P) and (^1H) proton magnetic resonance spectroscopy (MRS) in both infants with neonatal encephalopathy and experimental models have characterised the changes in cerebral energetics that occur in the hours following hypoxia-ischemia. (Hope et al 1984, Younkin et al 1984, Laptook et al 1989, Lorek et al 1994, Penrice et al 1997). During the hypoxic-ischemic insult there is a profound failure in cerebral energetics which resolves rapidly with resuscitation and reperfusion. However, 8-24 hours later there follows a progressive decline in phosphocreatine / inorganic phosphate (PCR/Pi) and adenosine triphosphate associated with a rise in lactate. These changes were termed delayed or secondary energy failure to distinguish them from the primary energy failure that occurs during the acute hypoxic-ischemic insult. The magnitude of this secondary disruption of brain energetics has been shown to correlate with long-term neurodevelopmental outcome in neonates (Hope et al 1984, Azzopardi et al 1989, Roth et al 1992).

The identification of this second phase of energy changes demonstrated that the injury following acute hypoxia-ischemia in the neonatal brain is an ongoing process after resuscitation / reperfusion. This observation introduced the concept of a therapeutic window, during which intervention has the potential to change the neuropathological course of brain injury and, hence, the long-term neurodevelopmental outcome for the infant.

Changes in Brain Intracellular pH after Hypoxia-Ischemia

Another series of changes in cellular physiology during hypoxia-ischemia and reperfusion that may play a critical role in brain injury relate to changes in intracellular pH (pH_i) of the brain. These changes were identified alongside the changes in cerebral energetics using ^{31}P and ^1H magnetic resonance spectroscopy.

Under physiological conditions, brain pH_i is maintained at around 7.03 (LaManna 1996) some 0.3 – 0.4 pH units below that of the extracellular compartment. Crucial to the regulation of intracellular pH is the Na^+/H^+ exchanger (NHE) which tightly regulates both pH_i and cell volume by extruding protons from and taking sodium up into cells (Masreel et al 2003). Tissue hypoxia results in a switch from aerobic to anaerobic respiration and results in the production of lactic acid which in turn results in an intracellular acidosis. This tissue acidosis has been demonstrated in animal models using the creatine kinase equilibrium. Middle cerebral artery occlusion (MCAO) in adult rats results in a profound intracellular acidosis which is proportional to the accumulation of intracellular lactate (Mabe et al 1983). Whilst it has been suggested that acidosis may have a direct toxic effect on neurones through denaturation of proteins and nucleic acids, *in vitro* studies using mixed neuronal and glial cell cultures only resulted in cell death on exposure to lactic acidosis of pH 5.2 for 1 hour (Goldman et al 1989). This suggests that in the clinical setting, the direct effect of acidosis is unlikely to be involved in the mechanism of brain injury (LaManna 1996). However, in the reperfusion phase following hypoxia ischemia there is a rapid normalisation of pH_i , followed by a rise in pH_i above baseline. In the adult MCAO studies this was observed after 60 minutes and continued for up to 3 hours following reperfusion (see Figure 1.11) (Mabe et al 1983).

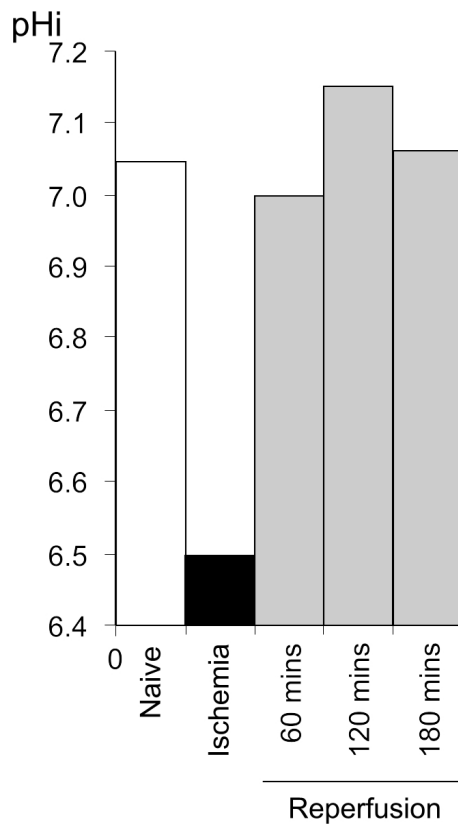


Figure 1.11: Changes in brain intracellular pH before, during and after 15 minute middle cerebral artery occlusion in the adult rat (Mabe et al 1983).

Subsequent animal studies using phosphorus (^{31}P)-MRS have shown that more severe hypoxic-ischemic insults are associated with more rapid and prolonged rebound alkalosis following tissue reperfusion (Chopp et al 1990). Adult clinical studies have demonstrated brain alkalosis in the subacute stage after traumatic brain injury (Hugg et al 1992), and in areas of chronic infarction after stroke (Levine et al 1992). These studies also demonstrated a significant correlation between the magnitude of brain alkalosis and the subsequent neurological outcome, with the worst outcome seen in patients with the greatest rebound alkalosis (Welch et al 1990).

In human neonates with neonatal encephalopathy, a similar relationship between alkalosis and the severity of brain injury has also been observed (see fig 1.12) (Robertson et al 2002). Infants with the most severe changes on MRI within the first 2 weeks after birth, and the worst neurodevelopmental outcome at one year, had the most alkaline brain pH_i . Interestingly this brain alkalosis was seen to persist for some months in those with the worst outcome (Robertson et al 1999).

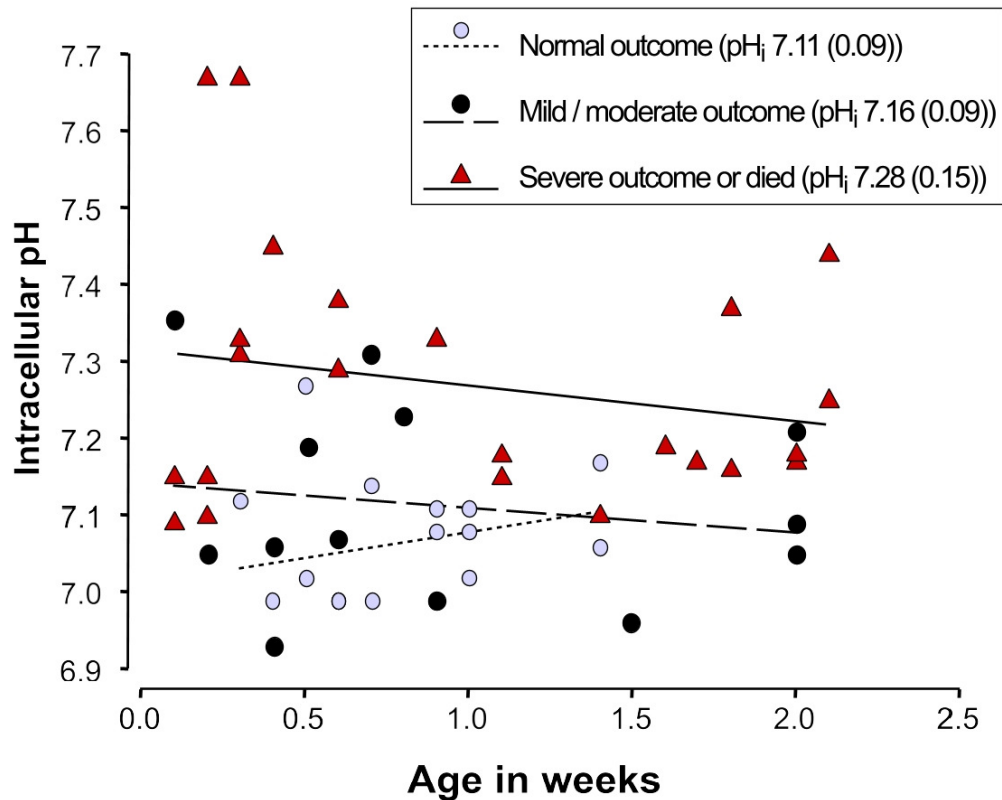


Figure 1.12: Brain pH_i in the first 2 weeks after birth according to neuro-developmental category. There was a significant difference between the normal and severe / died groups ($p < 0.05$) (from Robertson et al 2002 reproduced with permission)

It is of note that brain alkalosis occurred at a time of high lactate (normally associated with acidosis) and it has been suggested that the elevated lactate may represent ongoing mitochondrial dysfunction resulting in anaerobic respiration, and may represent continuing low-grade cellular injury months after the original hypoxic-ischemic insult (Wyatt 2002).

It is at present unclear however, whether the observed rebound alkalosis is a marker of brain injury, or is itself a contributor to the injury observed. However, if it can be demonstrated that rebound alkalosis contributes to, or exacerbates brain injury it may represent a novel mechanism for neuroprotection. There are a number of potential mechanisms by which a rise in brain intracellular pH may exacerbate injury; for example, increased N-methyl-D-aspartate (NMDA) receptor activation (Traynelis et al

1990, Giffard et al 1992) of intracellular phospholipases and proteases (Mellgren 1987), or alterations to the balance of pro- and anti-apoptotic triggers within mitochondria. The Na^+/H^+ exchanger (NHE) is also implicated in the mechanism of rebound alkalosis (LaManna et al 1987). During the lactic acidosis that accompanies hypoxia-ischemia excess intracellular protons activate the NHE, resulting in extrusion of hydrogen ions from the cell, along the sodium gradient. However it appears that this mechanism does not switch off once the pH_i of the cell has returned to normal, resulting in an alkali overshoot. The mechanism of NHE activation following hypoxia-ischemia is thought to be mediated through extracellular-signal-related-kinase (ERK1/2) and mitogen-associated protein kinase (MEK) (Luo et al 2007). MEK is known to activate the NHE through ERK (Schramek et al 1997), which phosphorylates p90 ribosomal S6 kinase, which, in turn, phosphorylates the NHE (Takahashi et al 1999).

Following the lactic acidosis that occurs during hypoxia-ischemia there is activation of NHE through the MEK-ERK pathway resulting in extrusion of hydrogen ions from the cell and a normalisation of pH_i . A failure of the NHE to switch off when the pH_i reaches normal results in an alkaline overshoot termed rebound alkalosis. The magnitude of this rebound alkalosis is associated with a worsening of neurodevelopmental outcome. This sequence of observations raises the possibility that interventions aiming to prevent or minimise rebound alkalosis, for example through blockade of the NHE following hypoxia-ischemia may confer neuroprotection following hypoxia-ischemia.

The Role of Pro- and Anti-inflammatory Cytokines

One mechanism by which both hypoxia-ischemia and infection may result in brain injury is by activation of the cytokine network. Indeed cytokines may act as a final common pathway to injury from a number of insults, such as hypoxia-ischemia and infection. This pathway may, therefore, represent one mechanism by which a number of aetiological factors may interact to result in brain injury. Studies of children with cerebral palsy have consistently shown raised levels of pro-inflammatory cytokines in the blood and CSF taken around the time of birth. Using archived blood spots from the newborn screening programme, Nelson et al compared cytokine and coagulation factors in children with spastic cerebral palsy (most born at term) with controls. Higher concentrations of interleukins 1, 6, 8, 13 and tumour necrosis factor- α , were seen in children who subsequently developed cerebral palsy than in any control child (Nelson et al 1998). This association was especially seen in children with spastic diplegia, and is consistent with previous observations relating intrauterine infection with risk of cerebral palsy. Interestingly, however, in very preterm infants, levels of inflammatory cytokines in neonatal blood have not been shown to distinguish between children who subsequently developed cerebral palsy from controls (Nelson et al 2005).

Bacterial components are known to be potent activators of the cytokine network. Pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumour necrosis factor (TNF- α) of both fetal and maternal origin are known to initiate preterm labour and to contribute to neonatal complications such as PVL (Duggan et al 2001, Yoon et al 1996, Gomez et al 1998). Pro-inflammatory cytokines are also released in response to hypoxia-ischemia, trauma and labour (Savman et al 1998, Hagberg et al 1996, Bona et al 1999, Silveira et al 2003, Shalak et al 2002). Pro-inflammatory cytokines, including TNF α , IL-1 β and interferon- γ (IFN- γ), have a variety of cerebral effects including a direct toxic effect on neurones and vulnerable oligodendrocyte precursor populations (Volpe 2001), astrogliosis with release of nitric oxide and

mitochondrial dysfunction (Bal-Price et al 2001). In addition there is microglial activation with release of nitric oxide, superoxide and a variety of other inflammation-associated molecules (Raivich et al 1999). Further evidence for the role of these cytokines comes from experimental animal models. IL-1 receptor antagonist has been shown to reduce hypoxic-ischemic brain damage. Pre-treatment reduced brain injury and post-treatment increased the proportion of animals devoid of brain injury (Hagberg et al 1996). Furthermore in animals with a deficiency in IL-1-converting enzyme, which is required for IL-1 activation, had reduced brain injury after hypoxia-ischemia compared to controls. However, this neuroprotective effect was detectable after moderate hypoxia-ischemia, but not after severe hypoxia-ischemia (Liu et al 1999). Deletion of IL-18 (a member of the IL-1 family of cytokines) has also been shown to reduce brain injury when deleted alone (Hedtjärn et al 2002) and in combination with IL-1 β (Hedtjärn et al 2005).

Stimuli such as cerebral ischemia, excitotoxicity, trauma and various neurodegenerative disease (Raivich et al 1999) (Kiefer et al 1995) also result in the release of a second group of cytokines, including transforming growth factor beta1 (TGF- β_1) and interleukin-10 (IL-10), which have broadly anti-inflammatory properties. However, unlike the pro-inflammatory cytokines listed above, they are not up-regulated following exposure to the inflammatory mediators of bacterial infection such as lipopolysaccharide (Xiao et al 1996). TGF- β acts as an immunosuppressive factor that inhibits the activation of lymphocytes, macrophages and related cell types, such as brain microglia (Suzumura et al 1993), and also inhibits the microglial production of potentially neurotoxic molecules such as nitric oxide, superoxide and IL-1 (Vincent et al 1997). In vivo application of TGF- β_1 strongly reduces the infarct size in cerebral ischemia in adult (Vincent et al 1997) as well as in adolescent animals (Gross et al 1993). The anti-inflammatory cytokine IL-10 may also play an important role in regulating the inflammatory response to infectious and hypoxic insults. This cytokine

exerts its effect by reducing production of pro-inflammatory cytokines such as IL-1 and TNF- α (Terrone et al 2001). It too has a neuroprotective role, improving outcome after both traumatic and ischemic injury in the adult rat (Spera et al 1998). In newborn mice, systemic administration of IL-10 following intracerebral ibotenate significantly reduced the size of the excitotoxic brain lesion (Mesples et al 2003).

It therefore appears that the balance between these groups of cytokines may be critical, with a relative abundance of pro-inflammatory cytokines favouring neuronal cell death whilst a relative abundance of anti-inflammatory cytokines may be neuroprotective. Variations in this cytokine balance may therefore make a fetus more susceptible to development of brain injury, especially in the context of superimposed hypoxia-ischemia. Single gene polymorphisms within cytokines may alter the fetal inflammatory response and, hence, favour either a pro or anti-inflammatory profile (Amory et al 2001). It has also been suggested that changes in the fetal inflammatory response due to polymorphisms may account for the apparent genetic susceptibility to preterm labour in the context of the frequent association of preterm labour with histological evidence of infection/ inflammation (Vaner et al 2005). Changes in the balance between pro- and anti-inflammatory cytokines may also result from the type, timing and severity of insult. In the human neonate a two-phase inflammatory response has been described in with an antenatal wave of pro-inflammatory cytokines and a postnatal anti-inflammatory response (Dammann et al 2001). These data suggest that, in some neonates, levels of IL-1, IL-6, IL-8 and TNF α are high immediately following delivery and then fall to baseline levels by one week; conversely, anti-inflammatory cytokines (GM-CSF and TGF- β) increased continuously during the first week from low levels post delivery.

The Role of Microglia

The recognition of the role of the fetal inflammatory response as a key mediator in the onset of preterm labour, and as an important factor in the aetiology and pathophysiology of perinatal brain injury, has led to research into the role of the resident immune cell within the brain – the microglia. Activation of resident microglia has been demonstrated in neonatal autopsy specimens and animal models of periventricular leukomalacia, excitotoxic and hypoxic-ischemic brain injury (Ohno et al 1995, Rezaie et al 2002, Hagberg et al 2002).

Microglia are a specific subpopulation of cells related to monocytes and dendritic cells that have many roles within the central nervous system in health and disease. In their resting state microglia are highly ramified cells with a tertiary and quaternary branching structure which is integral to their role in immune surveillance (Raivich 2005). Transcranial two-photon laser-scanning microscopy has been used to demonstrate that, whilst the cell body of microglia is normally static, their elaborate ramifications are undergoing continuous extension and retraction (Nimmerjahn et al 2005, Davalos et al 2005) allowing them to sample the extracellular space in a seemingly random fashion (Raivich 2005). Tissue damage results in a rapid targeting of the injured area by the microglial branches from the surrounding microglia (Nimmerjahn et al 2005, Davalos et al 2005). Therefore, microglia are able to surround the injured area with a dense sphere of branches, effectively walling it off from the surrounding undamaged tissue (Raivich 2005). Microglia can exist morphologically in a number of states. In addition to the resting highly ramified cell, microglia can take on a deramified, amoeboid phagocytic phenotype. However, an intermediate or 'alert' state between the resting and phagocytic morphology has also been defined with increased integrin immunoreactivity, swelling of the microglial cell body, thickening of the proximal processes and loss of the distal processes (Kloss et al 2001). Activation of microglia may occur as a result of various stimuli, such as tissue disruption due to trauma, endotoxin, infection or ischemia. In addition to upregulation of cell adhesion molecules

which facilitate the migration of microglia to the site of injury, activation also results in the production of extracellular matrix degrading enzymes, major histocompatibility complex I & II, so that microglia can act as antigen presenting cells, cytokines such as IL1 α , IL1 β , IL6, IL12, TNF to recruit further immune cells, early components of the complement cascade and nitric oxide and prostaglandins to attack invading microorganisms (Chew et al 2006). Microglia, therefore, have specific roles in immune surveillance, cytokine production, antigen presentation, complement activation and phagocytosis within the developing central nervous system and their role in perinatal brain injury has yet to be fully understood. Further evidence of the role of microglia in the pathogenesis of perinatal brain injury has been shown using minocycline, a semi-synthetic, second-generation tetracycline antibiotic which has good CNS penetration and reduces microglial activation (Yrjänheikki et al 1998, Yrjänheikki et al 1999, Tikka et al 2001, Zemke and Majid 2004). Initial studies of minocycline in the rat model of neonatal hypoxic-ischemic injury demonstrated almost complete prevention of brain injury when administered prior to, or 2.5 hours after hypoxia-ischemia. Neuroprotection was associated with prevention of caspase-3, a known effector of apoptosis (Arvin et al 2002). However, in the same model using neonatal mice minocycline has been shown to worsen injury after hypoxia-ischemia (Tsuji et al 2004). Whilst the reason for this conflicting effect is unknown, the effect of minocycline on macrophages is different in rats (decreased prostaglandin E₂ and cyclo-oxygenase-2 production) and mice (increased prostaglandin E₂ and cyclo-oxygenase-2 production). Additionally, in the mouse, minocycline also reduces angiogenesis which may impair compensatory mechanisms after hypoxia-ischemia (Tsuji et al 2004, Diguët et al 2004).

The Role of Astrocytes

Astrocytes are characteristic star-shaped glial cells which provide mechanical and metabolic support for neurons. They are also responsible for the formation of the glial scar that occurs in areas of CNS tissue loss.

The prevalence of astrocytosis within the white matter at neonatal autopsy is 15-40% and generally occurs following hypoxia-ischemia (Rezaie et al 2002). However, the exact contribution of reactive astrocytes to brain pathology, such as periventricular leukomalacia, remains unclear. Although reactive astrogliosis is seen in damaged periventricular white matter, it is unclear whether this is a direct effect of astrocyte damage or if this is a response to damage to oligodendrocytes and axons (Sen et al 2006). It is also unknown whether the activated astrocytes result in the release of protecting or damaging factors. Astrocytes have the potential to produce free radicals and $TNF\alpha$ but, conversely, under certain circumstances, can produce anti-inflammatory factors such as $TGF\beta$ within focal PVL lesions (Meng et al 1999). It has been suggested that there may be a biphasic response by astrocytes, with early responses favouring the production of neuro- and oligo-toxins and a subacute response producing neuroprotective molecules and enhancing tissue regeneration. Establishing the exact timecourse of astrocyte activation within the neuropathology of white matter injury may help identify their role in the disease process (Sen et al 2006). Astrocyte activation is also seen in animal models of hypoxic-ischemic brain injury. In these models, however, this activation occurs some days after the activation of microglia, possibly as a response to microglial-derived interleukin-1 (IL-1) (Ohno et al 1995). In adult mice, astrocytes have a neuroprotective role following middle cerebral artery transection, with increased infarct size in mice deficient in glial fibrillary acidic protein and vimentin (Li et al 2008). However, an inhibitory effect on neural regeneration has also been demonstrated with the same deficiency (Kinouchi et al 2003).

Potential Mechanisms of Synergy

Whilst epidemiological, experimental and clinical data suggest that fetomaternal inflammation may have a sensitising effect on the neonatal brain to subsequent hypoxia-ischemia (see Figure 1.1), the mechanism by which this interaction occurs is largely unexplained.

Animal models have been used to demonstrate the synergistic effects of inflammatory responses and hypoxia-ischemia. Endotoxin (lipopolysaccharide) has been shown to sensitise and dramatically increase the lesion size in the immature rat brain following hypoxic-ischemic injury. This effect is seen when endotoxin is administered systemically (Eklind et al 2001, Yang et al 2004, Ikeda et al 2004) or intracisternally (Coumans et al 2003). There appears to be an effect of the time interval between endotoxin and subsequent hypoxia-ischemia as endotoxin has also been shown to reduce the volume of infarct in adult rats when given 24 hours prior to regional cerebral ischemia (Ahmed et al 2000). There also appears, under certain circumstances, to be a biphasic sensitising / neuroprotective response to endotoxin in the neonatal rat (Eklind 2005). These findings may correlate with the biphasic pro-inflammatory / anti-inflammatory cytokine response seen in the perinatal period (Dammann et al 2001) and raise the possibility that it is the inflammatory status of the brain, and specifically the balance between pro and inflammatory cytokines, that modulates the outcome of hypoxic-ischemic injury.

From the animal studies to date, it is likely that microglia have a key role in inflammatory sensitisation of the brain. These cells are activated in endotoxin models through toll-like receptors (TLR-4), and it is through this mechanism that endotoxin can have a direct neurotoxic effect (Lehnardt et al 2003). It is possible that the cytokines produced by endotoxin-activated microglia, therefore, sensitise the developing brain to subsequent hypoxia-ischemia. An alternative hypothesis to explain the interaction between endotoxin, microglia and hypoxia-ischemia has also been suggested. In cell

culture the addition of microglia to neuronal culture provides significant neuronal protection against oxygen-glucose deprivation involving microglial migration and close cell-to-cell contact with neurones. Interestingly this neuroprotective function is lost in both CD11a-deficient microglia and endotoxin pretreated microglia (Neumann et al 2006). This study suggests that, rather than endotoxin activation increasing neuronal damage in hypoxia-ischemia through increased neurotoxic activity, there may be loss of the normal neuroprotective function of microglia through LPS stimulation.

An alternative mechanism of the synergistic effect of the fetal inflammatory response on subsequent hypoxic-ischemic injury may be mediated through the effects of cytokines on the blood-brain barrier. Although the blood-brain barrier is thought to be intact from an early stage of fetal development, the integrity of this selective barrier may be altered by the presence of inflammatory mediators such as $TNF\alpha$ and $IL-1\beta$ (Rezaie et al 2002). Systemically administered endotoxin results in an influx of granulocytes into the brain of adult mice, a process which is dependent on the up-regulation of vascular endothelial ICAM-1. In this experimental model, endotoxin also opens the blood-brain barrier to large proteins such as albumin (Bohatschek et al 2001). It is possible, therefore, that the fetal inflammatory response affects the microenvironment within the developing brain through the action of inflammatory mediators on the blood-brain barrier, and hence this results in influx of leukocytes into the central nervous system, potentially along with other factors which make the brain sensitive to subsequent hypoxia-ischemia.

Another possible mechanism whereby inflammation could modulate the effects of hypoxia is through alterations in fetal temperature. Intrapartum fever has been shown to be independently associated with an increased risk of development of unexplained neonatal seizures (OR 3.4 95%CI 1.03-10.9) (Lieberman et al 2000) and neonatal encephalopathy (OR 4.72, 95% CI 1.28-17.4) (Impey et al 2001). It is not clear whether these effects are just because pyrexia is serving as a marker for infection/inflammation

or whether non-infectious pyrexia, for example in relation to epidural usage, may also be damaging, particularly if the fetus is hypoxic. Finally, it is also possible that the fetal inflammatory response and specifically pro-inflammatory cytokines may attenuate the normal compensatory responses exhibited by the fetus to a hypoxic insult.

Summary

In the hours following a hypoxic-ischemic insult in the neonate a number of cellular events are occurring which may eventually lead to neuronal survival or death. It seems likely that the exact nature of the insults, timing and state of the brain at the time of insult may be critical in defining the long-term neurodevelopmental outcome. Specifically, the inflammatory status of the central nervous system may affect the sensitivity of neurones to subsequent hypoxic-ischemic insults. The hours that follow such insults are characterised by a cascade of neurotoxic events, a wave of pro and possibly subsequent anti-inflammatory cytokines, a delayed failure of cerebral energetics and a relative alkalosis within the intracellular compartment of the brain. Clinically these events are characterised by the findings of neonatal encephalopathy, the magnitude of which corresponds to both the severity of perinatal insult and the long-term neurodevelopmental outcome. At the time of maximal secondary energy failure, the brain is at its most alkalotic, and clinically this corresponds to the onset of seizures, the presence of which raises the relative risk for development of cerebral palsy 71 times (Nelson and Ellenberg 1979).

However, the realisation that some of the neuronal injury that occurs following hypoxia-ischemia is delayed for at least a few hours after the initial insult has led to the potential therapeutic window during which intervention could ameliorate neuronal loss and improve the neurodevelopmental outcome after hypoxic-ischemic insults.

Part 4: Perinatal Brain Protection and Repair

An understanding of epidemiology, aetiology and molecular mechanisms involved in childhood neurodisability and perinatal brain injury raises the prospect of strategies aimed at perinatal brain protection and repair.

Primary Neuroprotection: Preventing of Damage

As described previously, the biggest challenge for primary neuroprotection in hypoxic-ischemic brain injury is the accurate and timely identification of the at-risk fetus. Currently, the main tool used is continuous fetal heart rate monitoring which, whilst sensitive in identifying babies who have early hypoxia-ischemia, carries a 99.8% false positive rate in predicting infants who will develop cerebral palsy. In the future, identification of additional risk factors in the woman and fetus, and hence stratification in the interpretation of fetal heart rate tracings, may allow more targeted intervention to prevent neurodisability whilst keeping overall interventions to a minimum.

If, as suggested by both epidemiological and animal models, there is a synergistic interaction between inflammation and hypoxia-ischemia in the development of neurodisability, then avoidance of intrapartum hypoxia might be neuroprotective in women “at risk”. In term deliveries, elective caesarean section has been shown to be associated with a highly significant reduction in term neonatal encephalopathy (OR 0.17, 95%CI 0.05-0.56) whereas emergency caesarean section is associated with an increased risk (Badawi et al 1998). A similar relationship between elective /emergency caesarean section and cerebral palsy has been shown in preterm infants (Murphy et al 1995). However, it is interesting that the incidence of cerebral palsy has not fallen despite substantial recent increases in the caesarean section rate over the last 20 years. Again, a more accurate identification of the “at risk” fetus should allow obstetric intervention to be targeted and inappropriate intervention reduced.

Materno/fetal infection and inflammation remains the other single most identifiable risk factor for development of brain injury in both the term and preterm neonate. It is therefore important to consider the potential to intervene antenatally to reduce the effects of a pro-inflammatory milieu and other harmful effects of infection. The effect of maternal antibiotics on infection-related preterm brain injury is not clear. One study has shown a correlation between maternal antibiotic use and white matter injury (Leviton et al 1999). However, this may be because antibiotic use acts as a marker of severe maternal infection, rather than that brain injury is a direct consequence of antibiotic use. A fetotoxic effect of maternal antibiotics has been demonstrated with the increased incidence of neonatal necrotising enterocolitis in pregnancies treated with co-amoxiclav, compared with erythromycin. This is thought to be a result of certain antibiotics causing a greater release of endotoxin by damaged bacteria than others (Kenyon et al 2001). Maternal antibiotics are given for intrapartum pyrexia for prevention of congenital infection with Group B streptococcus; however, there is no evidence that intrapartum antibiotics influence neonatal neurological outcome.

The significant role of the fetomaternal inflammatory response in both initiation of preterm labour and in perinatal brain injury also raises the possibility of intervention with agents that modify the inflammatory response. However, as most of the target molecules remain in a delicate balance with other pro / anti inflammatory mediators, and because all molecules within the inflammatory response have multiple functions, blocking of their activity may in fact be harmful. There has to date been no report of a clinical trial of inflammation modulators to reduce brain damage in the human neonate (Dammann and Leviton 2000).

The observation that maternal pyrexia is associated with neonatal encephalopathy and seizures raises the possibility that relatively simple interventions to aid temperature reduction may confer neuroprotection. There are a variety of theoretical reasons why trying to keep pyrexial mothers normothermic during labour may protect against

neonatal encephalopathy: maternal intrapartum pyrexia clearly increases the risk of abnormal neonatal neurology; animal studies suggest that hypothermia is neuroprotective and that hyperthermia exacerbates hypoxic brain injury. Of note the fetal temperature is on average 1°C higher than maternal temperature. However, a randomised controlled trial designed to show a neuroprotective effect of maternal cooling has yet to be performed.

Secondary Neuroprotection: Intervening in the Therapeutic Window

The observation of the biphasic nature of changes in cerebral energetics that follow neonatal hypoxic-ischemic insults has raised the possibility of a therapeutic window in the first few hours of neonatal life. As described above, many cellular and neurochemical processes occur in the hours that follow hypoxia-ischemia which potentially define whether individual neurones will survive or progress to cell death. It has long been the hypothesis that intervention to disrupt the sequence of events within this window would allow a greater number of neurones to survive and, hence, ameliorate the effects of hypoxia-ischemia. One of the most exciting advances in the field of perinatal brain injury since Little's original descriptions of 1840-1870 has been the recent publication of 2 therapeutic hypothermia trials both of which demonstrated the potential for therapeutic intervention in babies with neonatal encephalopathy to prevent long-term neurodisability.

Gluckman et al used selective head cooling using a water-filled cooling cap for 72 hours in neonates with encephalopathy following hypoxia-ischemia. (Gluckman et al 2005). At 18 month follow-up, the primary outcome was defined as unfavourable if an infant died or had severe disability defined as severe neuromotor disability, Bayley motor disability index <70, or bilateral cortical visual impairment. Infants were stratified according to the severity of their amplitude integrated EEG (aEEG) at time of randomisation. There was no benefit in the outcome in infants with severe aEEG

abnormalities at randomisation. However, in the infants with moderate abnormalities in the aEEG, there was an overall significant reduction in unfavourable outcome from 66% to 48%. There was also a reduction from 28% to 12% in incidence of severe neuromotor disability. In a subsequent paper, the authors evaluated the role of various factors in determining the response to head cooling treatment. Interestingly in the non-cooled babies weight was important with increased rates of unfavourable outcome with increasing weight, and, conversely, increased favourable outcome in smaller infants. However, cooling was highly significantly protective in infants >25th percentile but not in those <25th percentile. The authors suggested that these findings may be accounted for if either the smaller babies were over-cooled by the coolcap, and /or the possibility that larger babies tend to overheat if not cooled. These findings necessitate further studies of regional brain temperature following hypoxia-ischemia, and in babies undergoing therapeutic cooling, in an attempt to allow therapeutic hypothermia to fulfil its neuroprotective potential.

Total body cooling has also been shown to be neuroprotective following hypoxia-ischemia. (Shankaran et al 2005). In this large NIH funded multi-centre trial, infants were cooled to a rectal temperature of 33.5°C for 72 hours, after which they underwent a gradual re-warming process. Overall death or moderate-severe neurodisability was reduced in the cooled group from 62% to 44% at 18-22 month follow-up. Interestingly, in this study, aEEG studies were not performed. However, the on-going MRC funded total body hypothermia (ToBy) trial, which is soon to report, will allow stratification of outcome by aEEG in babies who have undergone total body cooling for hypoxic-ischemic encephalopathy.

In a comparison of the patterns of brain injury seen on MRI scanning in infants treated with selective head cooling or total body cooling, both methods resulted in reduced basal ganglia and thalamic injury. However, in the selective head cooling group there was also a decrease in severe cortical lesions (Rutherford et al 2005). Animal studies

of cooling suggest that we may not yet have achieved optimal results from the studies performed to date. The piglet model of neonatal hypoxia-ischemia has demonstrated that different areas of the brain require different temperatures for optimal neuroprotection (Iwata et al 2005). In this model, cooling of the brain to 35°C resulted in a 25% increase in viable neurones in the superficial cortical grey matter and a 39% increase in viable neurones in the deep grey matter; however, cooling to 33°C resulted in a 55% increase in neurones in the superficial cortical grey matter but no increase in viable neurones in the deep grey matter. These findings suggest that a temperature gradient across the brain with superficial structures cooler than deeper structures may provide optimal neuroprotection. Many questions remain unanswered with regard to the use of hypothermia in the treatment of neonatal encephalopathy, including the technique to achieve hypothermia, and the depth and duration of hypothermia. These questions have led a number of authors to conclude that therapeutic hypothermia should not be indiscriminately introduced in routine neonatal care before further research is completed (Wyatt et al 2005, Edwards et al 2006, Blackmon et al 2006)

Although to date no clinical trials have been performed for pharmacological interventions in neonatal hypoxic-ischemic encephalopathy, a number of potentially neuroprotective agents have been identified in animal models. Recently, the prospect that pharmacological agents may improve hypothermia has also been demonstrated. In rats, topiramate, whilst not neuroprotective as a single agent, was able to extend the therapeutic window for initiating hypothermia beyond the time it normally ceases to be effective (Liu et al 2004). Xenon and hypothermia in combination have been shown to provide synergistic neuroprotection following hypoxia-ischemia *in vitro* in cultured neurones and *in vivo* in neonatal rats (Ma et al 2005). Interestingly, the same group have demonstrated that the synergistic neuroprotection of xenon with hypothermia is maintained when the treatments are administered asynchronously (Martin et al 2007). In this study neither hypothermia at 35°C for 2 hours or inhalation of 20% xenon for 90 minutes were neuroprotective alone. However, if the xenon inhalation occurred either 1

or 5 hours after hypothermia there was a significant reduction in infarct volume. Combined treatment with hypothermia and xenon in the human neonate following hypoxia-ischemia are planned to start in the near future. As described previously, the neuroprotective effects of melatonin, a naturally occurring neurohormone secreted from the pineal gland, which has potent anti-oxidant activity, has been demonstrated in animal models. The effects of melatonin as a neuroprotective agent against white matter injury in preterm neonates are currently under evaluation.

Future Potential: Neonatal Brain Repair

The potential for the neonatal brain to exhibit cortical plasticity through compensation and reorganisation has been recognised for many years. For example, in the motor tracts, large unilateral lesions result in the preservation of ipsilateral projections from the uninjured hemisphere; however, as these normal physiological connections are lost in late gestation, infants with lesions occurring after this time will not demonstrate such connections. Lesions in the left hemisphere of the brain lead to a redistribution of language function to the uninjured right hemisphere without functional deficit. However, lesions to the optic radiations or visual cortex demonstrate limited compensatory potential (Krägeloh-Mann 2004). Further understanding of the mechanisms of cortical plasticity and neuronal regeneration may allow intervention to improve functional outcome and limit disability.

The other potential strategy for brain repair following injury involves the use of stem cells – cells with the ability both to self-renew and to give rise to cell types different from themselves. Much work is underway to unlock the potential of exogenous stem cells to repair injured brain tissue in animal models, particularly as in animal models stem cells transplanted into the neonatal brain migrate widely in comparison to transplantation into the adult brain (Olsson et al 1997). In various models, the potential for stem cells to produce functional and histological improvement in both injury and

neurodegenerative conditions has been well established and this strategy may provide a therapeutic avenue for perinatal brain injury in the future (Hodges et al 1997, Björklund and Lindvall 2000, Santner-Nanan et al 2005). An alternative approach to the use of exogenous stem cells arises from the recognition of endogenous stem cells which are present in the developing brain. Recent evidence from the rodent neonatal hypoxia-ischemia model demonstrates an expansion of endogenous neural stem / progenitor cells within the sub-ventricular zone on the injured side (Yang et al 2006). Furthermore, this expansion is associated with regeneration of neurones, with migration of stem cells from the sub-ventricular zone into the injured neocortex over a period of weeks to months following injury (Yang et al 2007). Of note in this and other studies, around 85% of the newly-formed neurones die before reaching maturity, raising the possibility that manipulation of the micro-environment and trophic support will allow a greater proportion of cells to reach maturity and potentially improve functional outcome following perinatal brain injury.

Summary

Although the fact that childhood neurodisability may arise from perinatal events has been recognised for over 150 years, it has only been in the last few years that postnatal intervention to ameliorate long-term brain injury has been possible. It is likely, however, that our current understanding of cooling strategies for hypoxic-ischemic injury represents only the first step in neuroprotection. Based on current research programmes, it is probable that, in the future, a combination of prevention, amelioration and regeneration / repair strategies will be increasingly available to reduce the incidence and severity of perinatal brain injury.

Aims

Inflammatory Sensitisation of the Neonatal Brain:

In order to further our understanding of the multifactorial nature of perinatal brain injury, further studies are required to understand the mechanism of injury and, hence, the potential for neuroprotective strategies. From an aetiological perspective, the role of inflammatory sensitisation to subsequent hypoxia-ischemia remains of particular interest. If this mechanism can be fully understood then further strategies for primary prevention may be identified and, hence, the incidence and severity of brain injury reduced. To build on previous studies, a number of hypotheses were formulated as the basis for the current study:

1. Endotoxin will act as a sensitising agent prior to hypoxia-ischemia in a murine model of neonatal hypoxic-ischemic brain injury; moreover, endotoxin pre-treatment will result in significant histological injury following levels of hypoxia-ischemia not normally associated with brain injury when occurring in isolation. The time interval between endotoxin administration and subsequent hypoxia-ischemia will be critical in determining the histological outcome.
2. Endotoxin administered alone will result in detectable changes within the developing brain which may be part of the process underlying the observed sensitisation to subsequent hypoxia-ischemia.
3. As pro-inflammatory cytokines, including the TNF family of cytokines, are produced in response to endotoxin and hypoxia-ischemia, and have a neurotoxic effect, these may be critical to inflammatory sensitisation. Hence, deletion of the TNF family of cytokines will prevent endotoxin mediated sensitisation of the developing brain to subsequent hypoxia-ischemia.

Intracellular pH in Perinatal Hypoxic Ischemic Brain Injury:

Current neonatal practice in the care of babies following hypoxic-ischemic brain injury aims to restore normal physiological parameters, including pH, in the hours following the hypoxic-ischemic insult. However, studies have demonstrated a rebound alkalosis during re-oxygenation following hypoxia-ischemia, the magnitude of which corresponds to long-term neurodevelopmental outcome. This may suggest that prevention of rebound alkalosis and, indeed, maintenance of a mild acidotic intracellular pH, will have a neuroprotective effect in comparison to current clinical practice. To further explore these effects a number of hypotheses were formulated:

1. Rebound alkalosis will be demonstrable within the brain of neonatal mice following hypoxia-ischemia.
2. Previous studies suggest that this alkalosis results from the action of the Na^+ / H^+ transporter which is located in the cell membrane and normally functions to maintain intracellular pH and cell volume. Blockade of this transporter prior to hypoxia-ischemia will result in reduced brain injury in comparison to hypoxia-ischemia alone.

Chapter 2: General Materials and Methods

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma.

Animals

C57/Bl6 females (Charles River laboratories UK) and males (Harlan UK) were mated in-house and the offspring were used for all experiments. The Home Office approved the animal experiments and care protocols. All animal procedures were performed according to the UK Animals (Scientific Procedures) Act 1986.

Surgical Procedures

Hypoxia-ischemia

Animals were anaesthetised with isoflurane (5% induction, 1.5% maintenance). The left common carotid artery was exposed via a midline neck incision using blunt dissection. Permanent occlusion was achieved using 8/0 polypropylene suture and the wound was closed using tissue glue. The surgical procedure was completed within 5 minutes and the total duration of anaesthesia was less than 10 minutes. Following surgery the mice were recovered at 36°C before being returned to the dam to nurse for 2 hours. The mice were then placed in a sealed hypoxia chamber and exposed to a constant flow of 2 L/min of 36°C, humidified 8% oxygen balanced with nitrogen. The temperature inside the chamber was monitored and maintained at 36°C. Following hypoxic exposure, pups were allowed to recover for 30 minutes at 36°C before being returned to their dams.

Tissue Preparation

At specific time-points, animals were anaesthetised by intraperitoneal injection with pentobarbital sodium. The heart was exposed and the animals perfused intracardially with 30 ml of phosphate buffered saline (PBS) at a rate of 6 ml per minute. The brains were extracted and post-fixed in 4% paraformaldehyde (PFA) on a rotator (8 rpm) for 1 hour at 4°C, followed by cryoprotection overnight by rotating immersion in 30% sucrose (Fluka) at 4°C as described before (Möller et al 1996). Brain tissue was mounted frozen on dry ice, and stored at -80°C until required. The brains were cut on a cryostat into 40 µm sequential coronal sections starting 1 mm from the frontal cortex. Each section was collected onto a warm 0.5% gelatin-coated slide, refrozen on dry ice and stored at -80°C until required.

Immunohistochemistry

All sections belonging to the same experiment were stained together at the same time to prevent differences in staining intensity as described before (Möller et al 1996). Sections were rehydrated in double distilled water (ddH₂O), spread with fine brushes under a dissecting microscope and allowed to dry at room temperature for 15 minutes. The sections were surrounded by a delineating pen and fixed in 4% formaldehyde in phosphate buffer (PB) for 5 minutes and then washed in 0.1M PB where they remained until all the slides had been fixed. Where appropriate (see Table 2.1) the tissue was defatted and permeabilised by passing the slides through cuvettes of 50% 100% and 50% acetone (2 minutes in each) and then washed twice in 0.1M PB and once in 0.1% bovine serum albumin in phosphate buffer (PB/BSA). The slides were placed in a humid chamber and the sections were blocked for 30 minutes in 5% goat serum to prevent non-specific binding of the secondary antibody. Sections were then incubated with the appropriate primary antibody at 4°C overnight (see Table 2.1).

Antigen	Acetone	Primary antibody	Dilution	Secondary antibody	Dilution
CD 11b (α M β 2 Integrin)	Yes	Rat anti-mouse (Serotec MCA711)	1:5000	Goat anti-rat (Vectorlabs)	1:100
CD 49d (α 4 Integrin)	No	Rat anti-mouse (Pharmingen 01271D)	1:1000	Goat anti-rat (Vectorlabs)	1:100
CD 49e (α 5 Integrin)	No	Rat anti-mouse (Pharmingen 01741D)	1:200	Goat anti-rat (Vectorlabs)	1:100
CD 49f (α 6 Integrin)	Yes	Rat anti-mouse (Serotec MCA699)	1:3000	Goat anti-rat (Vectorlabs)	1:100
CD 11c (α X Integrin)	Yes	Hamster anti-mouse (Endogen MA 11C5)	1:400	Goat anti-hamster (Vectorlabs)	1:100
β 2 Integrin	Yes	Rat anti-mouse (Serotec MCA1032G)	1:400	Goat anti-rat (Vectorlabs)	1:100
IBA-1	Yes	Rabbit anti-mouse (**)	1:400	Goat anti-rabbit (Vectorlabs)	1:100
Glial Fibrillary Acidic Protein (GFAP)	Yes	Rabbit anti cow (Dako Z0334)	1:6000	Goat anti-rabbit (Vectorlabs)	1:100
CD 54 (ICAM1)	Yes	Hamster anti-mouse (Pharmingen 01541D)	1:3000	Goat anti-hamster (Vectorlabs)	1:100
VCAM1	No	Rat anti-mouse (Serotec MCA1229)	1:400	Goat anti-rat (Vectorlabs)	1:100
Myelin Basic Protein (MBP)	Yes	Rat anti-cow (Chemicon MAB395)	1:200	Goat anti-rat (Vectorlabs)	1:100
Human Albumin	Yes	Rabbit anti-human (Sigma A-0433)	1:100000	Goat anti-rabbit (Vectorlabs)	1:100
Human Immunoglobulin	Yes	Rabbit anti-human (Sigma I-2136)	1:10000	Goat anti-rabbit (Vectorlabs)	1:100

Table 2.1: Antibodies for Immunohistochemistry

** Kind gift from Dr. Y Imai, National Institute of Neuroscience, Japan

After incubation, the sections were washed in PB/BSA, twice in 0.1M PB and once PB/BSA. The sections were then incubated for 1 hour with the appropriate biotinylated secondary antibody (see Table 1) which had been previously blocked with normal mouse serum for 30 minutes. Visualisation was achieved using the ABC system (Vector Lab). The avidin-biotin enzyme complex solution (ABC solution) was prepared as per the manufacturer's instructions. The tissue sections were washed twice in PB/BSA and twice in 0.1 M PB. The sections incubated with then incubated with the ABC solution for 1 hour at room temperature. The sections were washed four times in 10 mM PB and then were placed in 0.05% diaminobenzidine (DAB) in the presence of 0.01% hydrogen peroxide for 2-15 minutes depending on the colour visualised. The reaction was stopped by transferring the slides to 10 mM PB, and the sections were washed by transfer through 2 cuvettes of double distilled water. The sections were dehydrated in 70%, 90%, 95% and 100% ethanol, isopropanol, and twice in xylene 2 minutes in each before being covered using Depex (BDH).

Antibody Specificity

The specificity of the antibody raised against $\alpha M\beta 2$ integrin has been previously confirmed in our laboratory using appropriate knockout animals (Makwana et al 2009 *in press*). Homozygous alphaM null mice and littermate controls, obtained through collaboration with Dr Tanya Mayadas Norton (Boston, MA, USA), showed the disappearance of all microglial alphaM immunoreactivity, throughout the brain of the alphaM null mice. The antibody specificity for $\alpha 4$, $\alpha 5$ $\alpha 6$ αM and $\beta 2$ integrin subunit have been confirmed, in our laboratory, by Western blotting using un-fractionated tissue homogenates from trigeminal ganglia, brainstem, spleen and heart muscle (Makwana et al 2009 *in press*). In the case of rat monoclonal antibodies raised against alpha4 (CD49d), alpha 5 (CD49e), and alpha 6 (CD49f), previous studies have also detailed a massive upregulation of encoding mRNAs species following facial nerve axotomy in the mouse facial motor nucleus, corresponding with strong increase in the

appropriate immunoreactivity (Kloss et al., 1999; Werner et al., 2000). All the monoclonal antibodies used in the current study are well defined, with long established functional characterization for alpha4 (Holzmann and Weissman, 1989), alpha5 (Uhlenkott et al., 1996), alpha6 (Helmler et al., 1988) alphaX (Bulloch et al 2008) integrin subunits, as well as IBA1 (Okere and Kaba 2000), GFAP (Jakovcevski et al 2007), myelin basic protein (Tiwari-Woodruff et al 2007), ICAM1 (Martinez-Sanz et al 2008), VCAM1 (Matter et al 2006).

Terminal Transferase-Mediated d-UTP Nick End-Labeling (TUNEL)

TUNEL staining was performed as described previously (Gavrieli et al 1992). Sections were rehydrated, spread and fixed as described for immunohistochemistry. The slides were incubated in 3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase and then washed 4 times in 0.1M PB. Sections were defatted and permeabilised in 50%, 100% and 50% acetone (2 minutes in each) before being washed twice in 0.1M PB and once in PB/BSA. Slides were then placed in a humid chamber and incubated with terminal deoxytransferase (TdT) solution (see appendix) for 2 hours at 37°C. The reaction was stopped by incubating the slides for 10 minutes in 300 mM sodium chloride / 300 mM sodium citrate (TUNEL stop solution). Slides were washed 3 times in 0.1 M PB and then incubated for 1 hour in ABC solution (Vector Labs) at room temperature. The slides were washed 4 times in 10 mM PB and then incubated in DAB solution with nickel cobalt in the presence of 0.01% hydrogen peroxide (see appendix) for 150 seconds. The reaction was stopped in 10mM PB and the sections washed twice in double-distilled water. The sections were dehydrated in 70%, 90%, 95% and 100% ethanol, isopropanol, and twice in xylene 2 minutes in each before being covered using Depex.

Tyramide Staining

The cut tissue was spread, fixed, defatted and permeabilised as described for immunohistochemistry. The granulocyte myeloperoxidase enzyme was detected as follows. The slides were incubated with 100 µL 0.1% biotinylated tyramide solution in the presence of 0.001% hydrogen peroxide (see appendix) for 10 minutes at room temperature to allow covalent binding. The sections were washed 4 times in 0.1 M PB, the slides dried and the sections incubated with 100µL ABC solution for 1 hour at room temperature. Visualisation, dehydration and mounting of the sections were carried out as for immunohistochemistry (see above)

Nissl Staining

Cut sections were removed from the freezer onto dry ice and rehydrated with double distilled water. The tissue was spread with fine brushes and the slides were allowed to dry at room temperature overnight. The sections were then fixed in 4% PFA overnight and dehydrated in 70% alcohol overnight. The staining procedure was carried out by transferring the slides through cuvettes of: Nissl stain, ddH₂O, ddH₂O, 70% ethanol, 90% ethanol, 96% ethanol, 96% ethanol with 5-7 drops glacial acetic acid in a 300 ml cuvette for destaining, 100% ethanol, isopropanol, and 3 cuvettes of xylene. Except for the Nissl stain, in which the slides remained for 10 minutes, and the ethanol with acetic acid, in which they remained for 3-10 minutes according to colour, the slides spent 2 minutes in each of the other cuvettes. The tissue sections were covered with a coverslip using Depex, and the slides were allowed to dry in the fume cupboard.

Tissue Analysis

Infarct Size

15 sections cresyl stained sections from each forebrain (200 μm apart) were scanned and imported into Optimas 6.2 image analysis software. The areas of intact staining in the cortex, hippocampus, striatum and thalamus were outlined and measured bilaterally. Surviving brain tissue was calculated by converting the measured left (injured) and right (uninjured) areas into mm^2 and then converting to a volume by multiplying by 200 μm . The sum of these volumes was then used to calculate the percentage surviving brain tissue as left/right volume \times 100, or infarct size (right-left)/right \times 100.

Injury Score

Initial pilot studies showed that, in the neonatal mouse, hypoxic-ischemic insults were associated with histological injury, particularly severe in the hippocampus, but also in the cortex, striatum, and thalamus. Although severe injury resulted in a clearly definable infarct, which could be quantified by measurement, less severe injury resulted in a more diffuse pattern of neuronal loss. Here, infarct size, as described above, was not able to demonstrate the apparent injury seen on subjective assessment. In an attempt to detect this mild, diffuse pattern of injury, an injury score was devised. Microglia, the resident immune cell within the brain, are known to be activated, and to upregulate adhesion molecules in response to tissue damage (Ohno et al 1995, Kloss et al 1999, Raivich 2005). To identify this microglial activation, 5 coronal sections per brain (600 μm apart), were stained using immunohistochemistry for $\alpha\text{M}\beta 2$ integrin (Kloss et al 1999). The $\alpha\text{M}\beta 2$ immunoreactivity in areas of tissue damage was scored for each brain region (cortex, hippocampus, thalamus, striatum) by an observer blinded to the treatment of the individual animal (see Table 2.2 and Figure 2.1). The extent of the associated tissue damage was also scored from the cresyl stained sections, (see Table 2.2) and the two scores combined to give a total 0-7 point

injury score. Whilst this score was used in an attempt to define milder forms of brain injury, it should be recognised that the major component of this measurement was a subjective score from the cresyl stained sections, i.e. the amount of histological damage. The score may, therefore, not have additional value over the infarct measurement. Due to these limitations brain injury score was only used in addition to another analysis of injury, either infarct volume or TUNEL staining.

Score	5C6 (αMβ2-integrin) Immunoreactivity
0	No activation (see Fig 2.1A)
1	Focal activation (see Fig 2.1B)
2	Mild diffuse activation, occasional amoeboid macrophages (see Fig 2.1C)
3	Widespread activation, predominant amoeboid macrophages (see Fig 2.1D)

Score	Nissl (Cresyl Violet) Staining
0	No damage
1	Minimal evidence of damage without evidence of infarct
2	Small infarct, <50% of the affected region
3	Large infarct, >50% of the affected region
4	Total Neuronal Loss

Table 2.2: Brain Injury Scoring

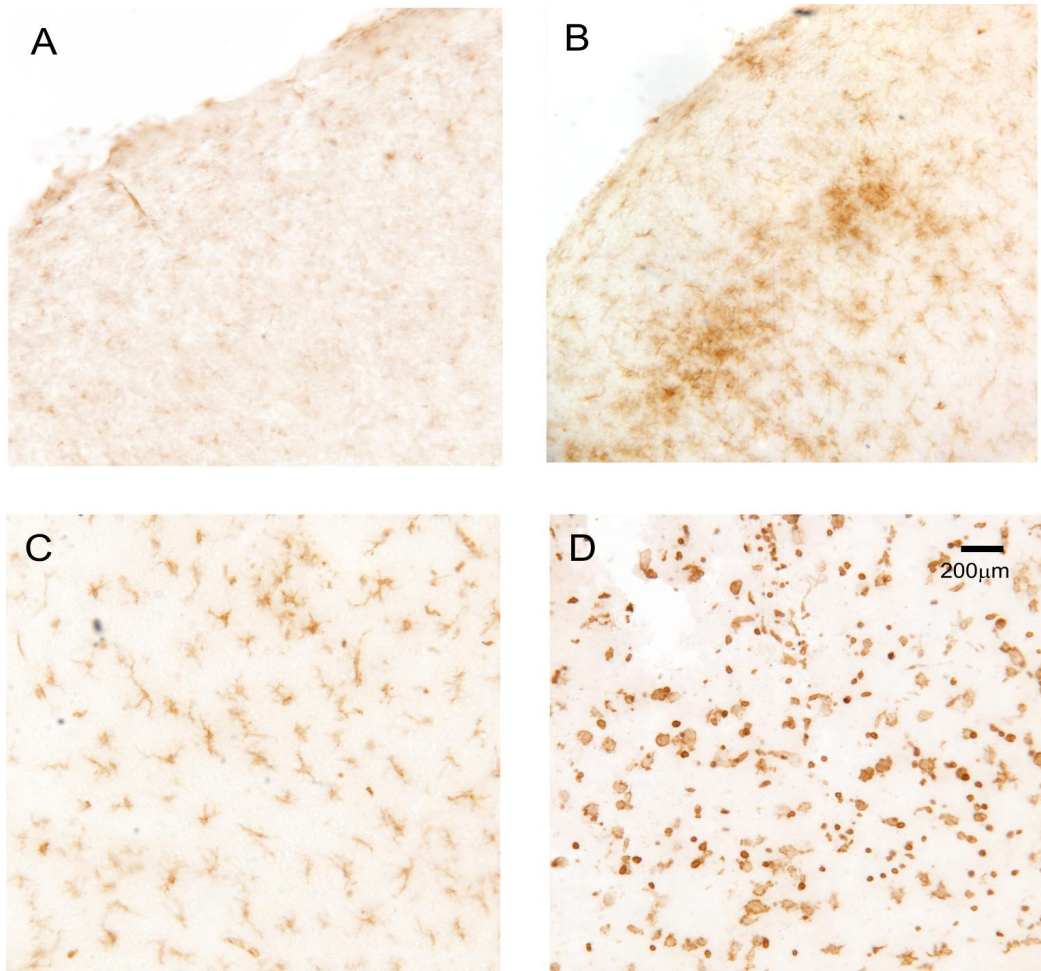


Figure 2.1: Immunohistochemistry for α M β 2-integrin expression. Brain regions were scored for microglial activation based on the different appearance as negative (A), focal activation (B), widespread activation predominant ramified morphology (C), or widespread activation predominant amoeboid morphology (D).

To quantify injury in the white matter a scoring system for microglial activation within the external capsule was devised using the 5 coronal sections per brain stained using immunohistochemistry for $\alpha\text{M}\beta$. Scores (see Table 2.3) were then allocated for left (ipsilateral) and right (contralateral) external capsule by an observer blinded to the treatment of the individual animal. The two scores were then added together to give a total 0-4 point white matter microglial activation score.

Score	5C6 ($\alpha\text{M}\beta$2-integrin) Immunoreactivity
0	No activation
1	Mild activation occasional amoeboid macrophages
2	Diffuse activation with multiple amoeboid macrophages

Table 2.3 White Matter Microglial Activation Score

Staining Intensity

For quantification of immunohistochemical staining intensity, 3 coronal sections per brain (800 μm apart) were used. From each section, images of the cortex, hippocampus, thalamus and striatum were scanned into a computer, using a Sony AVT-horn video camera. Images were then imported into Optimas 6.2 image analysis software. The region of interest was delineated using the free-hand tool, and the mean and standard deviation of the optical luminosity values were measured (see figure 2.2). The optical luminosity histogram was reviewed to ensure measured luminance values were within the measurable range (OLV; RGB 0-255).

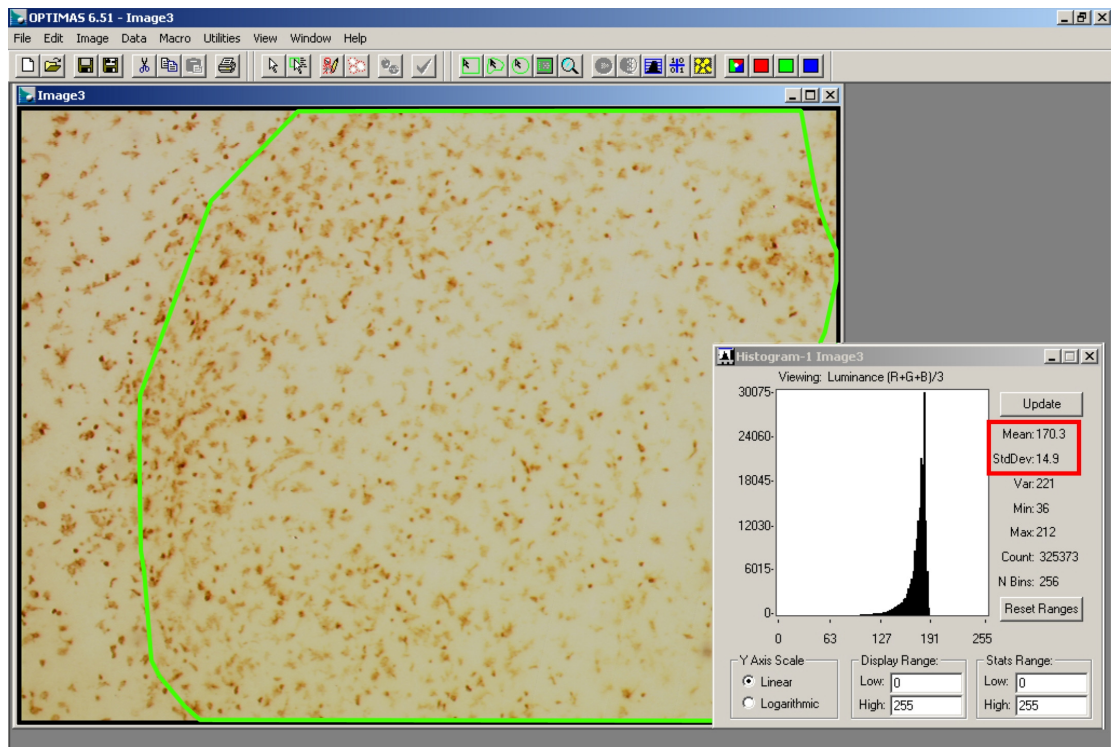


Figure 2.2: Optical luminosity measurement. The region of interest, here the striatum, was delineated using the freehand tool (shown in green). The luminance histogram was reviewed to check values were within the range 0-255. The mean and standard deviation values (shown in the red box) were transferred into an Excel spreadsheet.

The raw staining intensity was determined for each brain region, on each section, using the mean - standard deviation algorithm (Möller et al 1996, Kloss et al 2001). In addition, the background staining intensity was measured on each slide using the same algorithm. The final staining intensity was calculated by the difference between the raw staining intensity and the background staining intensity.

Cell Death

To quantify cell death involving DNA fragmentation the number of TUNEL positive nuclei was counted in each brain region. The sections were also scanned into a computer and the area in which the cells were counted was measured using Optimas 6.2 image analysis software. The results were expressed as number of TUNEL positive cells per mm².

Chapter 3: Establishing a Murine Model of Perinatal Brain Injury

Introduction

From the earliest descriptions of perinatal brain injury and the resultant neurodisability, birth asphyxia (hypoxia-ischemia) has been recognised as an important aetiological factor. However, more recently, epidemiological studies have attempted to assess the proportion of cerebral palsy attributable to pure birth asphyxia, with estimates ranging between 8% and 28% (Nelson and Ellenberg 1986, Blair and Stanley 1988, Hagberg et al 2001). There is a growing body of evidence that perinatal brain injury is of multifactorial aetiology with an interaction of various risk factors influencing eventual neurodevelopmental outcome. Other potential factors include infection / inflammation, coagulopathy and genetic causes (Nelson and Willoughby 2000).

In order to study perinatal brain injury, various animal models have been used. These have been performed in various animals and often use bacteria, endotoxin, viruses, excitotoxins or hypoxia-ischemia (for review see Hagberg et al 2002). More recently, research has begun to use combinations of such insults to better study how other factors influence the outcome following hypoxia-ischemia (Eklind et al 2001, Coumans et al 2003, Yang et al 2004) The most commonly used rodent model of perinatal hypoxia-ischemia was originally described some 25 years ago (Rice et al 1981). This model involves permanent carotid occlusion and timed exposure to hypoxia in a 7 day-old rat. Individually, carotid occlusion or hypoxia was not sufficient to cause histological damage. However, in combination, carotid occlusion followed by hypoxia led to histological evidence of brain injury in 90% of animals and an infarct in 56% (Rice et al 1981). From a neurological perspective, the seven day-old mouse or rat is considered to be a reasonable representation of the human neonate at term; specifically this relates to the process of myelination (Hagberg et al 2002). Unlike the human neonate, mice and rats are born pre-myelination, but undergo rapid myelination in the first

postnatal week. The pattern of brain injury in the 7 day-old rodent model is indeed similar to that seen in the term neonate following hypoxia-ischemia with damage to the ipsilateral cerebral cortex, thalamus, striatum, hippocampus as well as subcortical and paraventricular white matter (Rooney et al 1997)

It is, therefore, clear that in animal models and human neonates, both hypoxia-ischemia and infection / inflammation can, if severe enough, result in permanent brain injury. However, it has been suggested that much milder combinations of these insults, not sufficient alone to cause injury, may result in permanent brain injury (Peebles and Wyatt 2002). This interaction may be further complicated by the age of the animal (Ahmed et al 2000) and the exact temporal relationship of the different insults (Eklind et al 2005).

In order to study the effects of combinations of hypoxia-ischemia and inflammation it was first necessary to examine the effects of each insult alone, and to establish a level at which the insults produce little or no permanent brain injury. Further to these experiments, a combination of endotoxin given 4 hours prior to hypoxia-ischemia (a time interval previously shown to sensitise the developing brain to hypoxia-ischemia) (Eklind et al 2001) was used to investigate the synergy between inflammation and hypoxia-ischemia in the development of perinatal brain injury.

Materials and Methods

Lipopolysaccharide / Endotoxin (LPS) Injections

22 C57/Bl6 mouse pups at postnatal day 7 (P7) were randomly allocated to receive LPS (*Escherichia coli*, serotype 055:B5, Fluka) or vehicle (0.9% saline) intraperitoneal injections. Doses of 0.5 µg/g (n=2), 2 µg/g (n=3), 5 µg/g (n=4), 10 µg/g (n=5) and 20 µg/g (n=4) LPS were used diluted in normal saline such that all animals received a single 10 µl/g injection. The mice were then returned to their dams for 48 hours after which they were killed, perfused and brains extracted as described previously. A further 5 animals receiving normal saline, and 9 receiving 10mg/kg LPS were assessed 5 days after injection.

Hypoxia Ischemia

21 C57/Bl6 mouse pups at postnatal day 7 (P7) underwent unilateral carotid artery occlusion followed by exposure to 8% oxygen for 30 minutes (n=5), 45 minutes (n=6) or 60 minutes (n=10) as described above. At 48 hours animals were killed and their brains extracted as described previously. A further six mice that underwent unilateral carotid artery occlusion and hypoxia for 30 minutes (n=3) or 60 minutes (n=3) were examined 28 days after the insult.

Combined LPS – Hypoxia-ischemia

To explore the effect of endotoxin as a sensitising agent prior to hypoxia-ischemia, differing doses of endotoxin (LPS) were given prior to hypoxia-ischemia.

Tissue Preparation

48 hours after insult the animals were killed, their brains extracted, and amount of injury quantified as described before (see materials and methods).

Results

Endotoxin

Survival following endotoxin injection was almost 100% up to 10 $\mu\text{g/g}$ (100% 0-5 $\mu\text{g/g}$, 93% 10 $\mu\text{g/g}$) but was 0% at a dose of 20 $\mu\text{g/g}$. At 48 hours after endotoxin administration no histological damage was seen on cresyl-violet stained sections of the forebrain at any dose. TUNEL staining did not reveal any increase in TUNEL positive nuclei compared with saline treated animals. The effect of endotoxin was then assessed on microglial activation using $\alpha\text{M}\beta\text{2}$ integrin (Raivich et al 1994). Here increasing doses of endotoxin resulted in increasing $\alpha\text{M}\beta\text{2}$ integrin expression, as quantified by optical luminosity values (OLV) using a mean-S.D. algorithm as described before (Möller et al 1996) (see Figure 3.1). At the highest dose (10 $\mu\text{g/g}$) there was the focal appearance of small round non-ramified, $\alpha\text{M}\beta\text{2}$ -positive cells (arrows) in addition to the ramified microglial (hatched arrows) especially in the cerebral cortex and thalamus (see Figures 3.2).

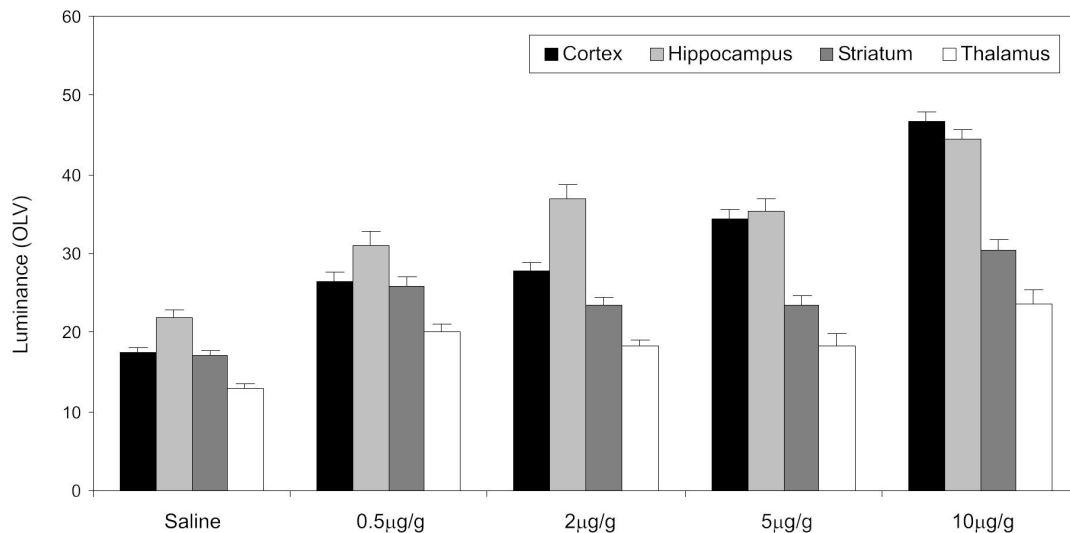


Figure 3.1: Effect of Increasing Doses of Endotoxin on $\alpha\text{M}\beta\text{2}$ Staining Intensity. Increasing doses of endotoxin resulted in increasing $\alpha\text{M}\beta\text{2}$ integrin immunoreactivity at 48 hours, most notable in the cortex and hippocampus ($p < 0.05$ ANOVA with post-hoc Tukey)

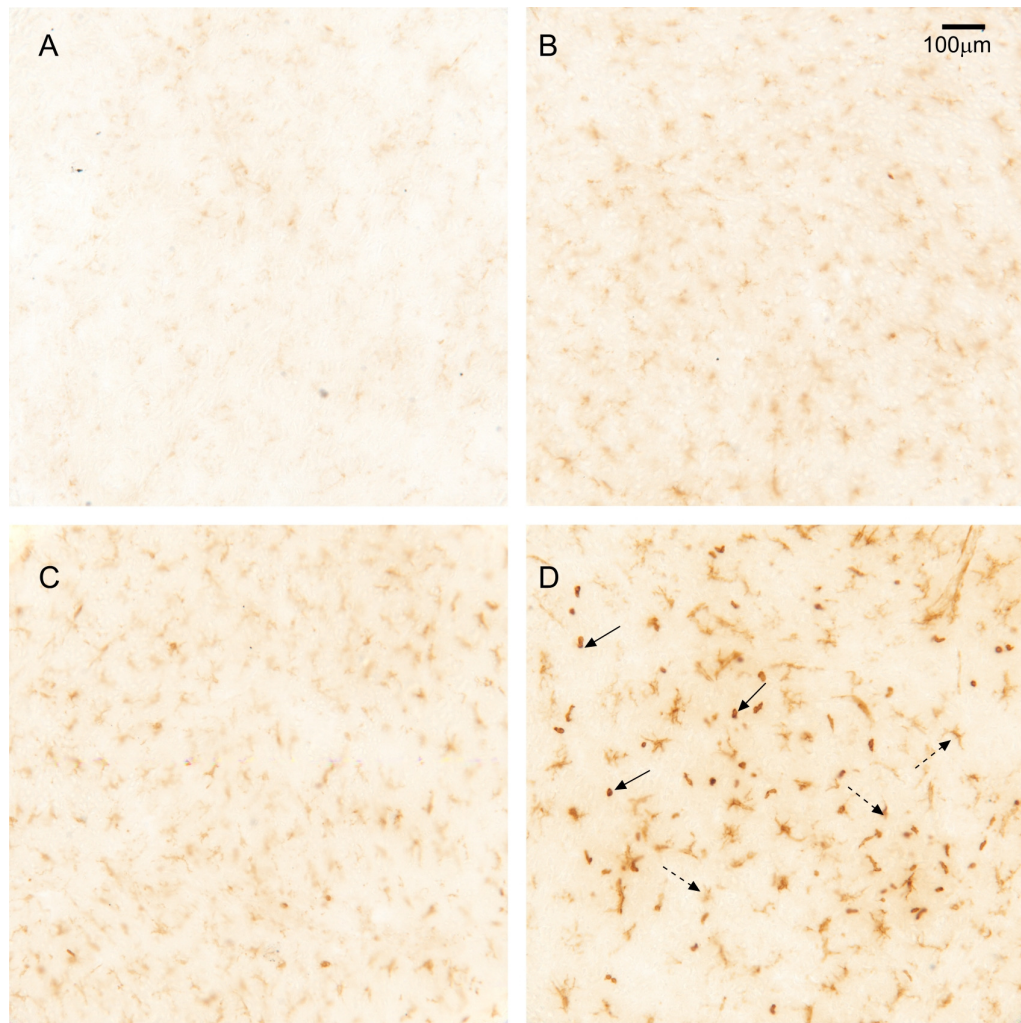


Figure 3.2: Effect of Increasing Doses of Endotoxin on $\alpha M\beta 2$ integrin Expression. A. Control, B. 2 $\mu\text{g/g}$, C. 5 $\mu\text{g/g}$, D 10 $\mu\text{g/g}$. At the highest dose (10 $\mu\text{g/g}$) there was the focal appearance of small round non-ramified, $\alpha M\beta 2$ -positive cells (arrows) in addition to the ramified microglial (hatched arrows) especially in the cerebral cortex and thalamus.

In an attempt to identify these small round non-ramified, $\alpha M\beta 2$ -positive cells, further sections were stained for the microglial / macrophage marker IBA1 and for endogenous peroxidase using tyramide, a marker of granulocytes. 48 hours after 10 $\mu\text{g/g}$ LPS there were some endogenous peroxidase-positive cells suggestive of granulocyte influx. IBA1 staining also revealed both ramified and non-ramified cells within the thalamus and cortex similar to those seen with $\alpha M\beta 2$, suggesting that some of these small round non-ramified cells were of macrophage / microglial origin (see Figure 3.3).

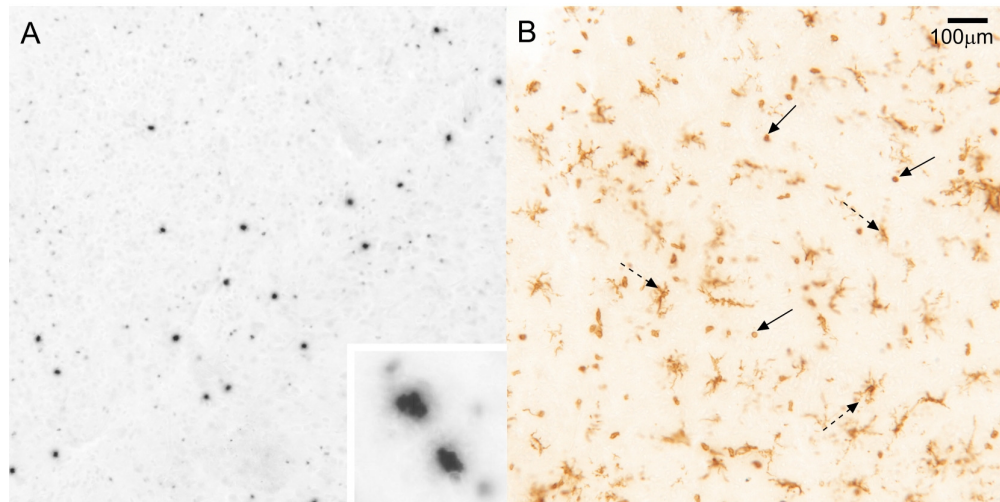


Figure 3.3. Effects of LPS 10 µg/g i.p. after 48 hours. A: Tyramide staining showed small round cells expressing endogenous peroxidase; inset high magnification view of cells with multi-lobe nucleus suggestive of granulocytes. B. IBA1 staining demonstrated both ramified (hatched arrows) and non-ramified (arrows) IBA1 positive cells in the thalamus and cortex similar to those seen with α M β 2 staining

Five days after injection with 10 µg/g LPS there was no longer any increased in α M β 2 immunoreactivity compared with saline treated animals, and the previously seen small round non-ramified α M β 2 /IBA1 expressing cells were no longer present. Sections stained with cresyl-violet showed no histological damage, and there was no increase in TUNEL positive cells compared with saline treated animals.

Hypoxia-Ischemia

Animal survival was dependent on the duration of hypoxia after carotid occlusion; 100% survival at 30 minutes, 83% at 45 minutes and 50% at 60 minutes. Infarct volume was measured 48 hours following hypoxia-ischemia in animals that had undergone carotid occlusion and hypoxia for 30 minutes (n=5), 45 minutes (n=5) and 60 minutes (n=5).

Pattern of Brain Injury following Hypoxia-Ischemia

Histological sections stained with cresyl-violet, immunohistochemistry for the microglial activation marker α M β 2 integrin, and DNA fragmentation using the TUNEL reaction were examined in animals exposed to carotid occlusion and 15 - 60 minutes hypoxia. The pattern and distribution of brain injury were noted.

Cortex

Histology (cresyl-violet) showed no evidence of cortical damage following 30 minutes hypoxia-ischemia; however, there were small focal areas of microglial activation with α M β 2 expressing ramified cells in 80% of animals. In animals exposed to 45 minutes hypoxia small foci of neuronal loss were evident, most commonly in the ventro-lateral cortex in the region supplied by the middle cerebral artery. Similarly distributed cortical foci of activated microglia were present in all 5 animals. Following 60-minute hypoxia-ischemia, all 5 animals showed a large ipsilateral cortical infarct with sparing of the parasagittal region. Throughout the cortex, there were activated macrophages of both ramified and de-ramified morphology demonstrating strong α M β 2 immunoreactivity.

Hippocampus

In animals exposed to 30 minutes hypoxia there was patchy neuronal loss in the pyramidal cell layer of the hippocampus, particularly in CA1-3. α M β 2 integrin immunoreactive and TUNEL positive cells were seen in the same area. There was consistent sparing of the dentate gyrus (see Figure 3.4).

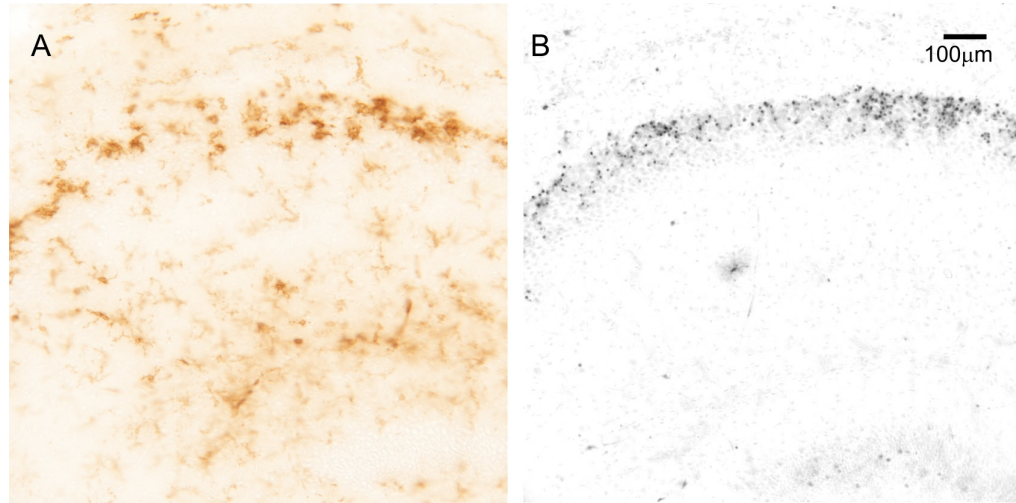


Figure 3.4: Effect of Carotid Occlusion and 30 minutes Hypoxia on the Hippocampus
A. Microglial activation (α M β 2 integrin immunoreactivity) is seen in the ipsilateral outer molecular and CA2 pyramidal cell layers. B. TUNEL staining shows DNA fragmentation in the pyramidal cell layer of the hippocampus.

Following 45-minute hypoxia, more diffuse neuronal loss was seen in CA1-3, with a small infarct and complete neuronal loss in CA1 seen in one animal. There were associated diffuse activated microglia seen in CA1-3 and the outer molecular layer in all animals. Cells were predominantly ramified, although occasional de-ramified phagocytic cells were evident. 60-minute hypoxia produced extensive hippocampal infarcts with almost total tissue loss.

Striatum

No evidence of histological damage was seen after 30 minutes of hypoxia. However, 80% of animals had diffuse activated ramified microglia evident throughout the striatum. In animals exposed to 45-minute hypoxia there was diffuse loss of neurones with a generalised decrease in neuronal density throughout the striatum. This was associated with more extensive activation of microglia, and occasional de-ramified phagocytic cells were observed. Following 60-minute hypoxia, diffuse loss of neurones progressed to the formation of a large infarct with predominantly de-ramified phagocytic microglia present.

Thalamus

No histological damage was seen in the thalamus of animals that exposed to 30 or 45 minutes hypoxia. Following 60-minute hypoxia, thalamic infarcts were seen in the superio-lateral region of all animals. These infarcts were associated with activated ramified microglia, and occasional de-ramified phagocytes.

White Matter Tracts

No histological changes were evident in the white matter tracts with any duration of hypoxia. In 60% of animals that were exposed to 30 minute hypoxia, ramified microglia were evident in the ipsilateral external capsule. TUNEL staining identified DNA fragmentation in the ipsilateral layer 7 of the cerebral cortex and caudate putamen only. Electron microscopy of the microglia within the external capsule demonstrated the presence of amorphous debris within vacuoles suggesting these cells were active phagocytes. Electron microscopy was performed by Professor Raivich (see Figure 3.5).

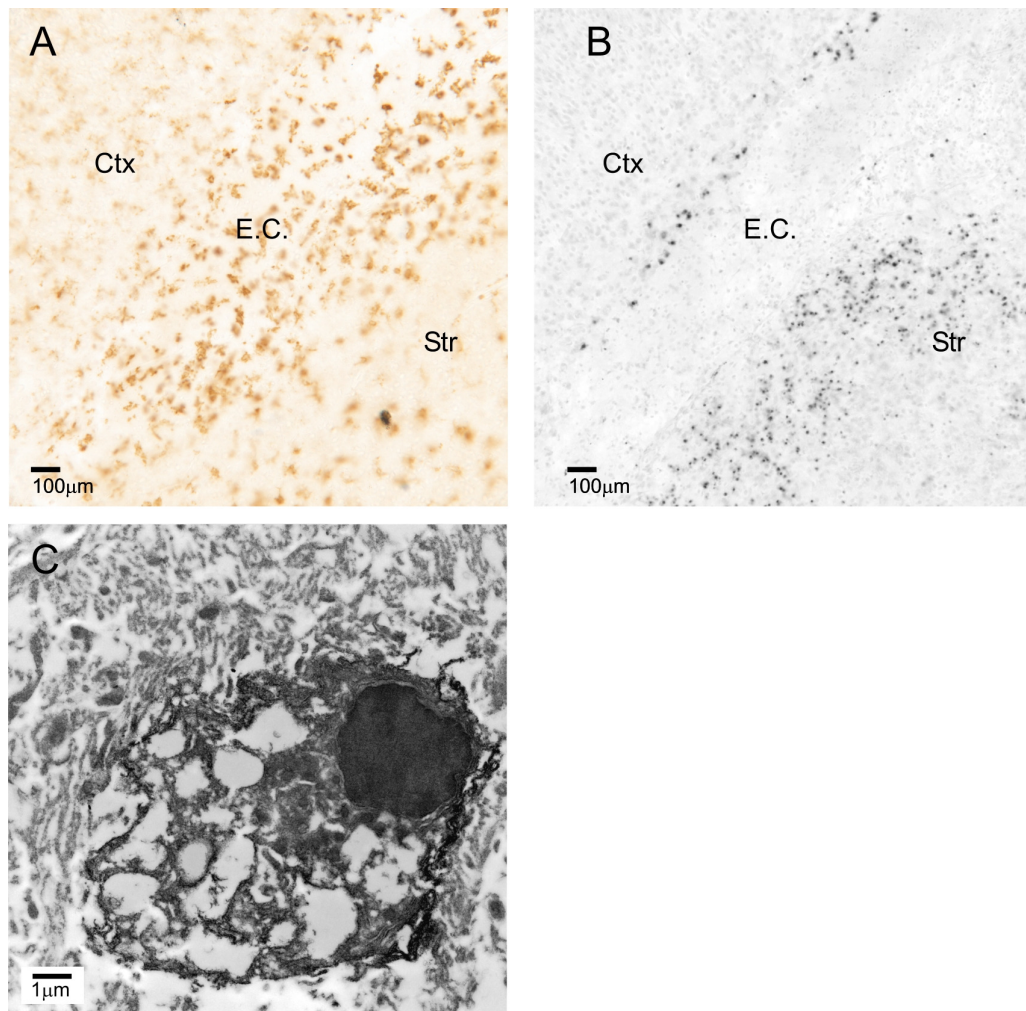


Figure 3.5: Effects of Carotid Occlusion and 30 minutes of hypoxia on Ipsilateral External Capsule. Microglial activation was noted in the external capsule (A). TUNEL staining demonstrated DNA fragmentation in the ipsilateral layer 7 of the cerebral cortex and caudate putamen only (B). Electron microscopy revealed amorphous debris within the cytoplasm of activated macrophage in the external capsule (C). (Ctx- cortex, E.C.- external capsule, Str- striatum)

More diffuse activated microglia were present in all animals following 45-minute hypoxia with involvement of the corpus callosum seen in 1 animal. Diffuse ipsilateral α M β 2 immunoreactive cells were seen in the ipsilateral external capsule extending into the contralateral tract following 60 minutes of hypoxia. This contralateral activation was most evident adjacent to the midline, becoming less evident laterally. Activated microglia were also present in the anterior commissure in two of the five animals.

Quantification of Injury following Hypoxia-ischemia

Brain injury was first quantified using infarct volume measurements (see Chapter 3: Materials and Methods). Overall, the separate regions of the forebrain (cortex, hippocampus, striatum and thalamus) showed different sensitivity in their response to the carotid occlusion and hypoxia ($F_{3,43}=14.79$ $p<0.05$) with damage particularly severe in the hippocampus compared with the cortex, striatum and thalamus. Increasing duration of hypoxia led to a significant increase in infarct volume ($F_{2,57}=101.81$, $p<0.05$ see Figure 3.6). Using the post-hoc Tukey test there was a significant difference in infarct volume between each of the three durations of hypoxia ($p<0.05$). When each forebrain region was analysed separately, a similar overall increase in infarct volume was seen: cortex $F_{2,12}=60.22$, $p<0.05$; hippocampus $F_{2,12}=20.69$, $p<0.05$; thalamus $F_{2,12}=52.32$, $p<0.05$; striatum $F_{2,12}=28.48$, $p<0.05$. Post-hoc analysis in these smaller sub-samples revealed a statistically significant difference between 60-minute hypoxia infarct volumes and 30 or 45-minute hypoxia infarct volumes. The post-hoc difference between 30 and 45-minute infarct volumes was not statistically significant.

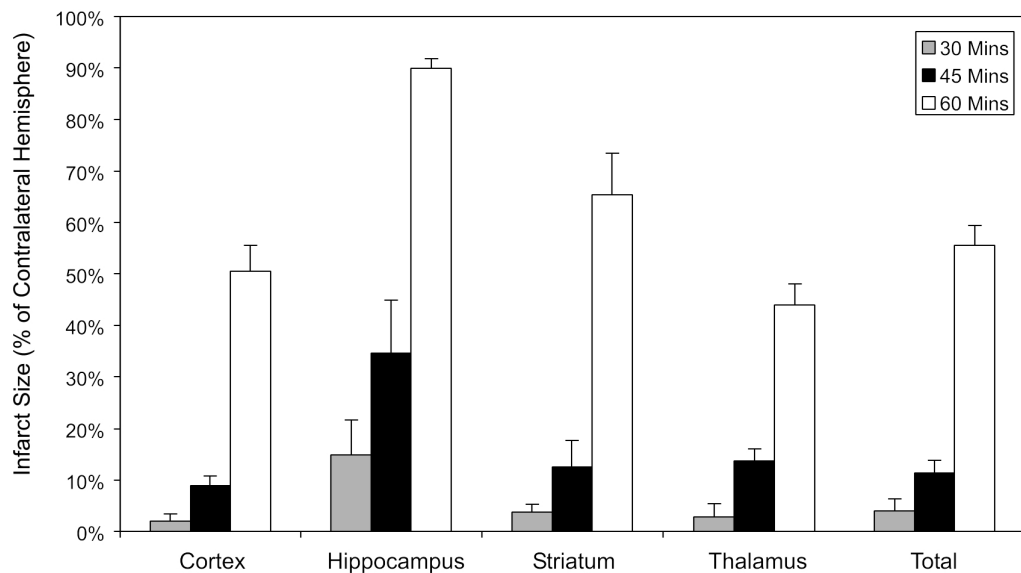


Figure 3.6: Increasing duration of hypoxia led to an overall increase in infarct volume ($p<0.05$ ANOVA with post-hoc Tukey). A similar overall increase in infarct volume was seen when each region was analysed separately <0.05 .

The animals were also analysed using the brain injury score (see Chapter 3: Materials and Methods). Here again, increasing duration of hypoxia resulted in a statistically significant worsening of the overall injury score ($F_{2,43}=216.4$, $p<0.05$ ANOVA) (see Figure 3.7), with a significant difference between the three time-points ($p<0.05$ post-hoc Tukey). When each brain region was analysed separately there was the same increase in injury score: cortex $F_{2,12}=651$, $p<0.05$, hippocampus $F_{2,12}=19.6$, $p<0.05$, thalamus $F_{2,12}=54.875$, $p<0.05$ and striatum $F_{2,12}=48.875$, $p<0.05$. As with infarct volume measurements, post-hoc analysis revealed a statistically significant difference between 60-minute hypoxia and 30 or 45 minute hypoxia injury scores. Similarly, the post-hoc difference between the 30 and 45 minute scores was not statistically significant, most likely due to the smaller difference in scores and smaller overall sample size.

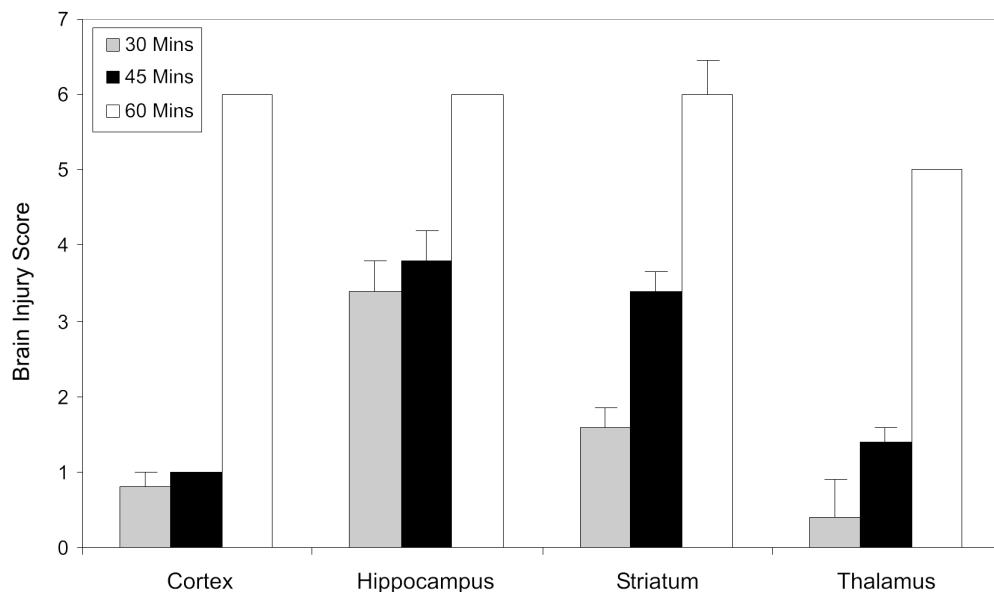


Figure 3.7: Effect of Increasing Duration of Hypoxia Following Carotid Occlusion. Increasing duration of hypoxia led to an overall increase in injury score ($p<0.05$ ANOVA with post-hoc Tukey).

To quantify the effect of hypoxia-ischemia on the white matter of the external capsule, the brain the white matter microglial score was used (see under Materials and Methods). A score was allocated to each of the 5 histological sections per animal and then an average score calculated for each animal. In keeping with the findings from the infarct volume and brain injury score, increasing duration of hypoxia following carotid occlusion resulted in increasing white matter microglial activation score (see Figure 3.8b).

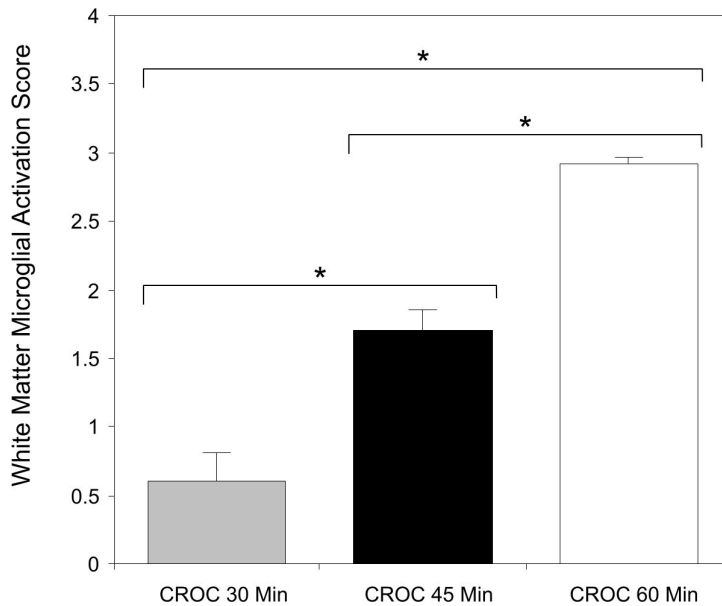


Figure 3.8b: Effect of Increasing Duration of Hypoxia on White Matter Microglial Activation Score. Increasing duration of hypoxia following carotid occlusion (CROC) resulted in a statistically significant increase in white matter microglial activation score (* $p < 0.05$ ANOVA followed by Tukey post-hoc)

Long-term effects of Carotid Occlusion and Hypoxia

To assess the long-term histological outcome following hypoxia-ischemia a further six animals that underwent unilateral carotid artery occlusion and hypoxia for 30 minutes (n=3) or 60 minutes (n=3) were examined 28 days after the initial insult.

Carotid occlusion and 30 minutes Hypoxia at 8% oxygen:

Patchy neuronal loss was seen in the pyramidal cell layer of the hippocampus especially within CA1 and 2 on cresyl violet staining. Of note there was complete preservation of the dentate gyrus in all animals. This preservation still occurred despite almost complete loss of the remaining hippocampal tissue in one animal. Staining for myelin basic protein (MBP) showed no disruption of myelination and normal architecture of the white matter tracts. No differences were noted in MBP staining between the injured and uninjured hemispheres.

Carotid occlusion and 60 minutes Hypoxia at 8% oxygen:

A large porencephalic cyst replaced the large infarcted areas seen at 48 hours after injury, when examined at 28 days after hypoxia-ischemia (see Figure 3.9). Complete absence of hippocampal tissue, except for the dentate gyrus, was seen in all animals. Extensive loss of cortical tissue was evident and the ventral lateral nuclear complex of the thalamus was absent. Staining for myelin basic protein revealed severe disruption of the external capsule white matter tract and complete loss of myelin from the inferolateral cortex (see Figure 3.9 B). Myelin was also absent from the cortex ventral to the cystic spaces. Staining with GFAP revealed extensive astrogliosis and scarring in the cortex, particularly lateral to the cystic space and lining the porencephalic cyst (see Figure 3.9 C).

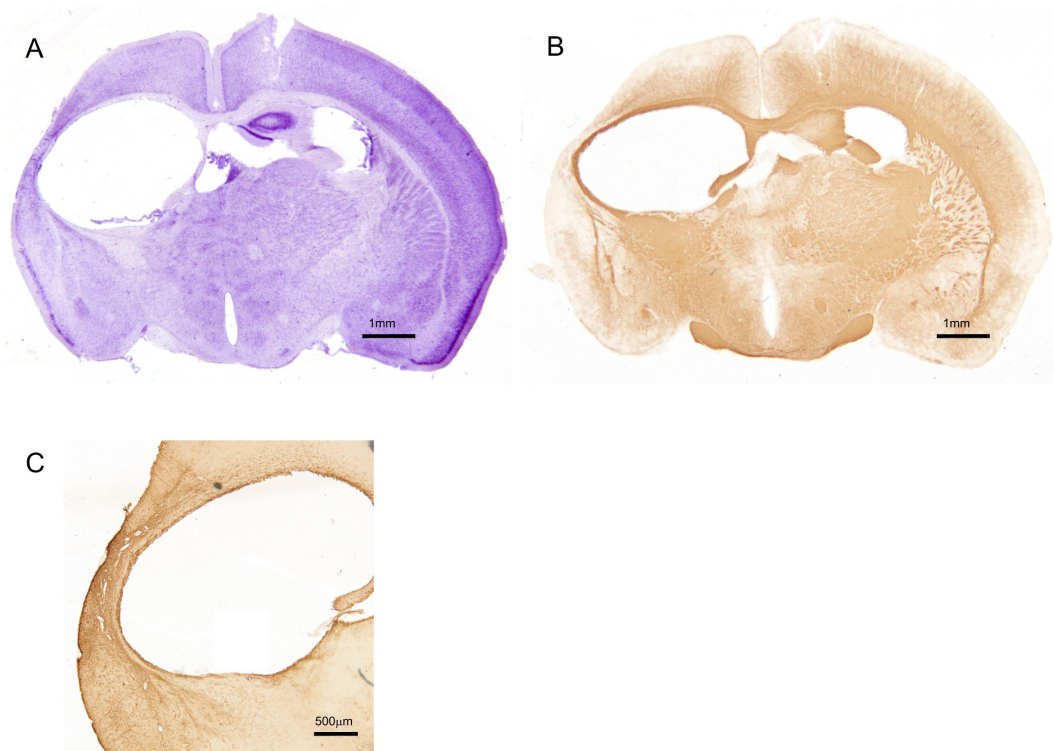


Figure 3.9: Effects of permanent left carotid occlusion and exposure to 8% oxygen for 60 minutes at 28 days.

A. Cresyl violet staining shows extensive infarction in the cortex, hippocampus thalamus and striatum previously seen at day 2 has been removed leaving a large porencephalic cyst. There is complete loss of the hippocampus and striatum, extensive loss in the cortex, and loss of ventrolateral thalamic nuclei.

B. Immunohistochemistry for myelin basic protein reveals disruption of the external capsule and complete loss of myelin from the inferolateral cortex.

C. Immunohistochemistry for glial fibrillary acidic protein demonstrates extensive astrogliosis in the cortex and lining the large porencephalic cyst.

Combined Endotoxin and Hypoxia-Ischemia

Animals were given saline (n=5) or endotoxin (0.1 µg/g n=6, 0.3 µg/g n=6, 1 µg/g n=4) 4 hours prior to carotid occlusion and hypoxia at 8% oxygen for 30 minutes. Survival was dependent on the dose of endotoxin given; 100% in saline treated animals, 83% 0.1 µg/g, 67% at 0.3 µg/g and 25% at 1 µg/g (see Figure 3.10)

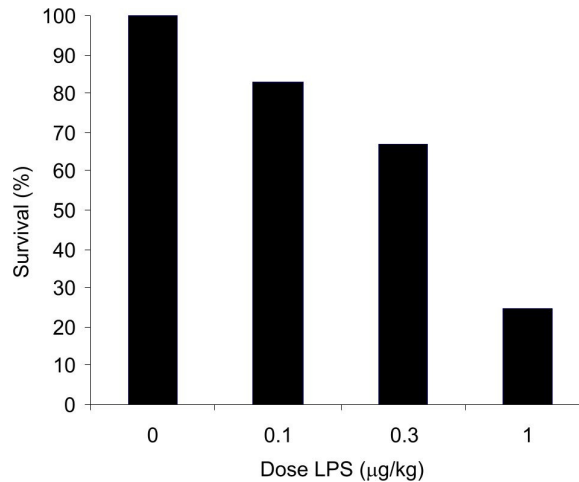


Figure 3.10: Survival in Animals Exposed to Saline or Endotoxin (0.1-1 µg/g) 4 hours prior to Carotid Occlusion and 30 minutes at 8% Oxygen.

Pretreatment with 0.3 µg/g endotoxin 4 hours prior to carotid occlusion and hypoxia resulted in increased histological damage compared to saline pre-treated animals. When damage was quantified using brain volume measurements, there was increased injury in all forebrain regions measured (cortex, hippocampus, striatum and thalamus). Overall, pretreatment with 0.3 µg/g endotoxin at 4 hours prior to hypoxia-ischemia resulted in a highly significant increase in infarct volume $F_{3,19}=6.37$ $p<0.01$ (ANOVA) see Figure 3.11

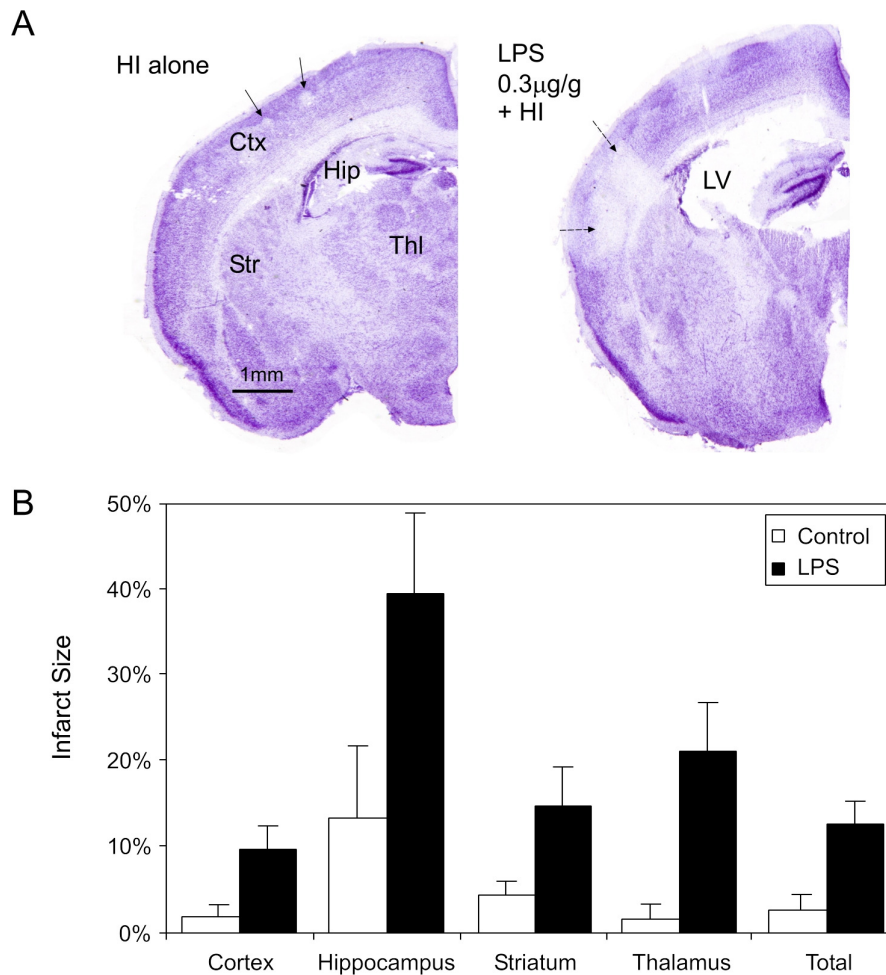


Figure 3.11: Effect of 0.3 $\mu\text{g/g}$ LPS 4 hours prior to Carotid Occlusion and 30 minutes 8% Oxygen. A. Effects of hypoxia-ischemia alone and 4 hours following LPS pre-treatment. Hypoxia-ischemia alone caused focal cortical (Ctx) damage (arrows) and loss of neurones from the pyramidal cell layer of the hippocampus (Hip). LPS pretreatment resulted in a large cortical infarct (dashed arrows) and increased damage in the hippocampus, thalamus (Thl) and striatum (Str). There was a resultant increase in size of the lateral ventricle (LV). B. LPS pretreatment resulted in an overall sensitising effect to hypoxia-ischemia with increased infarct volume expressed as percentage of contralateral hemisphere $F_{3,19}=6.37$ $p<0.01$

Discussion

The effects of systemic endotoxin (LPS) on the developing brain

Intra peritoneal injections of endotoxin (LPS) result in increased α M β 2 integrin immunoreactivity on microglia within the brain of P7 mice at 48 hours post injection. Doses of endotoxin of 10 μ g/g and below were well tolerated; however, injections of 20 μ g/g were lethal. The time-point of study (48 hours post injection) was chosen as previous studies have demonstrated that subcutaneous LPS injections in adult mice produce a dose-dependent increase of microglial α M β 2 immunoreactivity with a maximal effect at 2 days after injection (Kloss et al 2001). The current study shows a similar response in α M β 2 integrin immunoreactivity in the neonatal mouse at doses between 0.5 μ g/g and 5 μ g/g. Morphologically this staining is consistent with increased microglial α M β 2 expression. At a dose of 10 μ g/g, in addition to strongly immunoreactive ramified microglia, there were multiple small, round cells seen especially within the cortex and thalamus which also stained strongly for α M β 2 integrin. In the control animals there was a slight variability in α M β 2 integrin expression across the different forebrain regions with greatest immunoreactivity in the hippocampus and least in the thalamus. This pattern of distribution is generally conserved with increasing doses of LPS; however, at doses of 5 μ g/g and 10 μ g/g cortical staining is especially increased. Some of the observed increase in cortical staining seen at 10 μ g/g (when staining intensity exceeds that of the hippocampus) is accounted for by the presence of the small, round strongly staining cells. Previous studies have demonstrated that microglial transformation into mature phagocytes is a multi-stage process characterised by changes in morphology and cell surface markers (see Figure 3.12). This pattern of activation has been studied and characterised in mouse microglial monolayers using a co-culture on top of a confluent astrocyte monolayer (Raivich et al 1999, Kloss et al 2001).





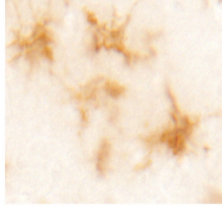
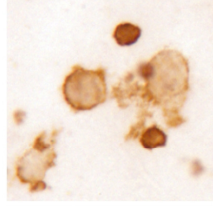
	Resting	Activated	Phagocytic
Morphology			
α M β 2 immunoreactivity			
Markers	α M β 2(w), Fc γ R(w) IgG(w), IBA1 (w)	α M β 2(s), MHC I ICAM1, IBA1 (m)	α M β 2(s), α X, B7.2 IgG, IBA1(s)

Figure 3.12: Stages of activation of microglia. In the resting microglia there is a highly branched (ramified) cell. The active or alert phenotype shows a reduction in complexity of branched structure and increase in α M β 2 immunoreactivity. Phagocytic microglia are deramified cells with an amoeboid morphology. Patterns of cell surface markers are also shown (w)-weak staining (m)- moderate staining (s)-strong staining (adapted from Raivich et al 1999)

In this *in vitro* model, addition of LPS to cultured microglia resulted in a morphological activation of the cells with an increase in the α 5, α 4, α M and β 1 immunoreactivity, and a decrease in α 6 immunoreactivity. However, even after treatment with a sub-lethal dose of LPS there was no increase in the phagocyte-associated integrin α X β 2 and there was no transformation of the ramified microglia into amoeboid phagocytes (Kloss et al 2001). In contrast, addition of brain membranes to the microglial monolayer led to rapid microglial deramification and transformation to small, rounded, macrophages with first effects at 1-3 hours, and full transformation at 24-48 hours. Removal of cell membranes led to a similarly rapid process of re-ramification. In this study, the small de-ramified microglia were demonstrated to be phagocytic, with uptake of fluorescent labelled brain membranes, whereas there was no uptake into microglia with a ramified morphology (Bohatschek et al 2001).

It must be noted, however, that α M β 2 integrin may also be expressed on monocytes and granulocytes; therefore, in the current study an alternative explanation for the presence of the small round de-ramified cells would be the recruitment of peripheral white blood cells into the brain. Granulocytes are not normally found within brain parenchyma, but their entry into the damaged central nervous system is a common feature in several brain pathologies. Granulocyte recruitment has been described in infection, stroke, and trauma. Activated granulocytes contain a large number of cytotoxic molecules and proteases, and play an important role in the phagocytic removal of pathogens and cellular debris, and in fighting infection within the central nervous system (Bohatschek et al 2001). Intraperitoneal injection of LPS in adult mice has previously been shown to result in the recruitment of granulocytes into the central nervous system with maximal effect noted between 1 and 4 days after injection. Furthermore these cells have been demonstrated to be blood-borne in origin by pre-labeling of circulating leukocytes using rhodamine B isothiocyanate (RITC) 48 h before the LPS injection. In this study, following LPS injection there was co-localisation of RITC and endogenous peroxidase, identifying these cells as granulocytes recruited into the CNS (Bohatschek et al 2001). In the current study, in order to consider the possibility of granulocytes accounting for the small round α M β 2 immunoreactive cells seen in the forebrain of the neonatal mouse 48 hours after 10 μ g/g LPS, sections were stained for endogenous peroxidase using biotinylated tyramide. Biotinylated tyramide identified some granulocyte infiltration in the cortex, but only a few granulocytes within the thalamus. Of note, at lower doses of endotoxin these cells were not seen. Therefore, whilst granulocytes are recruited into the brain of neonatal mice exposed to high-dose endotoxin the relatively small number of peroxidase-positive cells seen suggests that some of the cells may indeed be phagocytic de-ramified microglia. To confirm this hypothesis immunohistochemical staining with IBA1, a marker of macrophages, microglia and other monocyte-related cell types, was used. Here in addition to ramified microglia, multiple non-ramified IBA1-positive small round cells were seen in the cortex and thalamus in a similar pattern to that seen in the tissue

stained for α M β 2 integrin. This provides further evidence that these cells were of monocyte /microglia lineage. As discussed previously, de-ramification of resting microglia to amoeboid phagocytes requires the presence of debris such as cell membranes (Bohatschek et al 2001). However, the current study was unable to demonstrate tissue damage as defined by TUNEL-positive cells or histological injury. This raises the possibility that the observed cells are monocytes recruited from the peripheral blood, although the current study is unable to confirm this identity. 5 days after injection with 10 μ g/g LPS there was no increase in α M β 2 immunoreactivity over control, and no evidence of histological brain injury or TUNEL positive cells. There was also no persistence of the strongly activated microglia or the rounded α M β 2 / IBA1 immunoreactive cells. This suggests that there is no ongoing injury following the initial microglial changes noted at 48 hours.

These data, therefore, suggest that administration of systemic low dose LPS (0.5 μ g/g – 5 μ g/g) results in activation of microglia from a resting to alert / active phenotype without de-ramification to a phagocytic morphology, without evidence of neuronal damage and without evidence of recruitment of peripheral white blood cells into the central nervous system. Sublethal systemic LPS (10 μ g/g) additionally results in small round α M β 2 immunoreactive cells, the majority of which also stain with IBA1 suggesting microglial / monocyte lineage. In the absence of evidence of histological neuronal damage, it is possible that these represent recruitment of peripheral blood monocytes. Additionally, there is also recruitment of peripheral blood granulocytes which stain for both endogenous peroxidase and α M β 2 integrin. By 5 days after systemic LPS administration (10 μ g/g) there were no changes identifiable on histological examination (Nissl), TUNEL staining or α M β 2 immunohistochemistry.

Histological Effects of Carotid Occlusion and Timed Hypoxia

Carotid occlusion and timed hypoxia at 8% oxygen in the mouse at postnatal day 7 results in reproducible, duration-related injury in the cortex, striatum, hippocampus and thalamus. There was a highly significant increase in the forebrain infarct size and injury score with increasing duration of hypoxia. Subjectively, long-term histological outcome reflected the injury seen 48 hours post hypoxia-ischemia. These data are consistent with previous studies which show increasing infarct volume with increasing duration of hypoxia following carotid occlusion (Rice et al 1981). Previous studies have demonstrated that carotid ligation or hypoxia individually do not result in histological brain injury. However, when used in combination, brain injury occurs (Rice et al 1981). The current data also demonstrate a spectrum of injury with increasing duration of hypoxia, including damage to the cortex, hippocampus, thalamus, striatum and white matter. In the current study, there was a statistically significant difference between all three time-points when quantified using infarct volume as well as injury score, when each of the forebrain regions were considered together. When each region was analysed separately, on post-hoc analysis, there was still a statistically significant difference between 60-minute hypoxia and 30 or 45-minute time-points for infarct volume and injury scores. However, the post-hoc difference between 30 and 45-minute scores and infarct volumes was not statistically significant probably due to the small sample size.

Histological assessment of brain injury shows a close correlation between areas of neuronal loss at both 2 and 28 days and changes in microglia at 2 days, with increased α M β 2 immunoreactivity, de-ramification and transformation into phagocytes. As discussed previously, this de-ramification only occurs in the presence of cell debris and is therefore likely to be a response to cell death in the forebrain. Within the white matter tracts of the corpus callosum and external capsule there are no neuronal cell bodies. However, following hypoxia-ischemia de-ramified, α M β 2 strongly immunoreactive phagocytes were noted within these white matter tracts. Electron microscopy showed these phagocytes to contain vacuoles of amorphous debris further strengthening the suggestion that this transformation is a response to cell debris. Unsurprisingly, in the absence of neuronal cell bodies, there were no TUNEL positive nuclei noted within these white matter tracts. It can be speculated that the debris could be damaged axons, which have been subsequently phagocytosed by de-ramified microglia. This may also help to explain the presence of activated microglia extending into the contralateral tract with activation most evident adjacent to the midline and becoming less evident laterally. Alternatively, the presence of activated microglia within the white matter tracts in the absence of TUNEL-positive nuclei may result from the presence of dead or damaged cells of the oligodendrocyte lineage without DNA fragmentation. In keeping with previous studies (Liu et al, 2002), the current study suggests increasing white matter damage with increasing duration of hypoxia (as measured by white matter injury score at 48 hours and disruption in the normal pattern of myelin basic protein staining seen at 28 days).

Histological examination at 28 days after hypoxia-ischemia revealed the extent of neurological damage to be similar to that observed at 2 days after insult. There was no ongoing microglial activation at this late time-point. These data suggests that there is no progression of brain injury after 48 hours, but there is a maturation of the infarcted area with phagocytosis of dead tissue, formation of a porencephalic cyst, and an associated astrogliosis. Consistently there was also enlargement of the lateral ventricle, which may represent a compensation for loss of tissue volume in the injured hemisphere.

Combined Effects of Endotoxin and Hypoxia-Ischemia

In this pilot study, low dose of intra-peritoneal endotoxin given 4 hours prior to hypoxia-ischemia dramatically increased the lethality of the hypoxic-ischemic protocol. Survival was dependent on the dose of endotoxin, and for subsequent studies a dose of 0.3 $\mu\text{g/g}$ was used as this resulted in 67% survival. In comparison to animals pre-treated with saline, endotoxin had a highly significant sensitising effect on brain injury occurring after carotid occlusion and hypoxia at 8% oxygen for 30 minutes. This observation is in keeping with a number of previous studies (Eklind et al 2001) and was consistent across all forebrain regions assessed. This sensitisation phenomenon will be explored further in Chapters 4 and 5.

Conclusion

Carotid occlusion and timed hypoxia in the neonatal mouse at postnatal day 7 provides a reproducible model of hypoxic-ischemic brain injury. The current study suggests early histological outcome, as measured by infarct volume or brain injury score, to be representative of long-term histological outcome. Previous studies have demonstrated a correlation between long-term histological outcome and neuro-functional deficit (Ten et al 2004). This, therefore, suggests that early histological assessment may be a valuable correlate of long-term neurodevelopmental outcome, and provides an early measure appropriate for further assessment of neuro-sensitising and neuro-protective agents in the context of hypoxic-ischemic brain injury.

Chapter 4: Inflammatory Sensitisation of the Developing Brain to Hypoxia-Ischemia

Introduction

Maternal / fetal infection has been postulated as an important mediator in the development of perinatal brain injury. Epidemiological studies suggest maternal / fetal infection, inflammation and possibly endotoxin-mediated cytokine release as antecedent factors in the development of white matter injury in preterm neonates (Wu and Colford 2000, Volpe 2001, Duncan et al 2002). In the term neonate, a meta-analysis considering clinical chorioamnionitis and cerebral palsy gave a summary relative risk of 4.7 (95% CI 1.3-16.2) (Wu and Colford 2000) based on two studies (Nelson and Ellenberg 1985, Grether and Nelson 1997). A study of histological chorioamnionitis in neonates with neurological impairment showed a significant independent association (OR 13.2; 95% CI 1.2-144) (Redline and O'Riordan 2000). Additionally intrapartum fever has been shown to be independently associated with an increased risk of development of unexplained neonatal seizures (a strong predictor of neurological impairment in term infants) (OR 3.4 95%CI 1.03-10.9) (Lieberman et al 2000) and neonatal encephalopathy (OR 4.72, 95% CI 1.28-17.4) (Impey et al 2001).

Animal studies have shown that either whole bacteria or endotoxin can result in neonatal brain injury when administered at critical periods of neurodevelopment (Hagberg et al 2002). Endotoxin may be active via the transplacental route as demonstrated by the administration of a bolus dose of endotoxin to pregnant rats resulting in failure of cerebral myelination in their offspring (Cai et al 2000). The developing brain appears to be particularly sensitive to infection when it is at a premyelination stage of maturation. This maturation-dependent susceptibility is partly due the vulnerability of oligodendrocyte precursors to a variety of pro-apoptotic stimuli, including pro-inflammatory cytokines (Volpe 2001).

More recently, research has focused on the possible role of infection / inflammation in sensitising the developing brain to subsequent hypoxia-ischemia (Peebles and Wyatt 2002). Epidemiological data suggests that exposure to a combination of infection and hypoxia-ischemia dramatically increases the risk of developing cerebral palsy compared with either event alone (OR 78; 95% CI 4.8–406) (Nelson and Grether 1998). Rodent models have used endotoxin (lipopolysaccharide) as an inflammatory stimulus to sensitise the developing brain, and hence dramatically increase the lesion size following hypoxic-ischemia. This effect is seen when endotoxin is administered systemically, and or intracisternally (Eklind et al 201, Yang et al 2004, Coumans et al 2003). In these models, the time interval between the inflammatory stimulus and the hypoxic-ischemic insult has been shown to be critical in the sensitisation of the developing brain (Eklind 2005). The mechanism of the interaction between endotoxin-mediated inflammation, and hypoxia-ischemia remains unclear, although studies have highlighted the role of microglia, specifically the Toll-like receptor TLR4 (Lenhardt et al 2003). Another mechanism by which endotoxin may sensitise the brain to subsequent hypoxia-ischemia involves an opening of the blood-brain barrier possibly mediated by inflammatory cytokines. Previous studies have shown that in adult animals systemic endotoxin opens the blood-brain barrier allowing the passage of large proteins such as albumin from the circulation into the brain; endotoxin also mediates an influx of granulocytes into the brain via an ICAM1 dependent pathway (Bohatschek et al 2001).

In order to study the interaction of inflammation and hypoxia-ischemia a time-course was undertaken, the time interval between endotoxin administration and hypoxia-ischemia being varied to establish the interval that resulted in maximal histological brain injury. In an attempt to clarify the mechanism of interaction, cellular and vascular responses were examined following treatment with endotoxin alone, and the timing of the responses compared to the time-point of maximal sensitisation for subsequent hypoxia-ischemia. The effects of endotoxin on the integrity of the blood-brain barrier were also studied.

Materials and Methods

Endotoxin Hypoxia-Ischemia Time-course

56 C57/Bl6 mouse pups at postnatal day 7 (P7) underwent unilateral carotid artery occlusion followed by exposure to 8% oxygen for 30 minutes. Animals were sequentially allocated to receive LPS (*Escherichia coli*, serotype 055:B5, Fluka) 0.3 $\mu\text{g/g}$ 0 hours (n=12), 4 hours (n=12), 12 hours (n=11), or 24 hours (n=11) prior to operation, or to receive vehicle (normal saline) 4 hours prior to operation (n=10). LPS injections were diluted in normal saline such that all animals received a single 10 $\mu\text{l/g}$ injection. The mice were then returned to their dams for 48 hours after which they were killed, perfused, and their brains extracted and processed as described previously, Sections were stained using cresyl violet and immunohistochemistry for $\alpha\text{M}\beta\text{2}$ integrin for injury quantification by infarct volume and injury score as described previously (see under General Materials and Methods).

Endotoxin Time-course

The brains of 47 C57/Bl6 mice at postnatal day 7 were examined. Animals were sequentially allocated to receive LPS (*Escherichia coli*, serotype 055:B5, Fluka) 0.3 $\mu\text{g/g}$, 0.5 $\mu\text{g/g}$ or vehicle (normal saline), 4 hours (0.3 $\mu\text{g/g}$ LPS n=5, 0.5 $\mu\text{g/g}$ LPS n=5, saline n=5), 12 hours (0.3 $\mu\text{g/g}$ LPS n=5, 0.5 $\mu\text{g/g}$ LPS n=5, saline n=5), or 24 hours (0.3 $\mu\text{g/g}$ LPS n=4, 0.5 $\mu\text{g/g}$ LPS n=4, saline n=4) prior to being culled. 5 naive control animals were also studied. As before, all injections were at a volume of 10 $\mu\text{l/g}$. Following injections the mice were returned to their dams until they were killed, perfused, and their brains extracted as described previously (see under General Materials and Methods). In order to study the effects of low dose endotoxin on different cell populations, sections were stained using immunohistochemistry for microglial activation (integrins α5 , α6 , αM , αX , β2 , and the phagocytic marker IBA1) astrocyte

activation (CD44, GFAP), and vascular endothelial up-regulation (ICAM 1, VCAM 1, α 5 and α 6 integrins). Immunohistochemistry was performed as described previously (see under General Materials and Methods).

Blood-Brain Barrier Integrity

In order to study the effects of endotoxin on the blood-brain barrier, 18 C57/Bl6 mice were studied. Animals received an intra-peritoneal injection at postnatal day 6 of 50 μ L 10% human albumin (HAS) (Sigma) (n=7), 50 μ L of 10% human IgG (HIgG) (Sigma) (n=7) or 50 μ L of normal saline (n=3). 24 hours later, animals received a further injection of LPS (*Escherichia coli*, serotype 055:B5, Fluka) 0.3 μ g/g or vehicle (normal saline). The overall groups consisted of HAS – LPS (n=4), HAS – saline (n=3), saline – LPS (n=4), HIgG – LPS (n=4), HIgG – saline (n=3). 12 hours after the second injection, animals were killed, perfused, and their brains extracted as described previously. Histological sections were stained for human albumin and human immunoglobulin using immunohistochemistry (see under General Materials and Methods).

Results

Endotoxin Hypoxia- Ischemia Time-Course

The overall survival in the endotoxin hypoxia-ischemia time-course was 60% at time of perfusion (48hours). Highest lethality was seen in animals receiving endotoxin immediately prior to carotid occlusion (survival 4/12 (33%)) and there was a trend towards reduced mortality in animals with longer time intervals between endotoxin and subsequent hypoxia-ischemia (see Figure 4.1).

Infarct volume was measured as described before and expressed as percentage of the contralateral uninjured hemisphere. Overall, a sensitising effect of endotoxin on the brain to subsequent hypoxia-ischemia was seen within a time window of 4-12 hours before the onset of hypoxia, and was maximal when LPS was administered 12 hours prior to hypoxia-ischemia (see Figure 4.2).

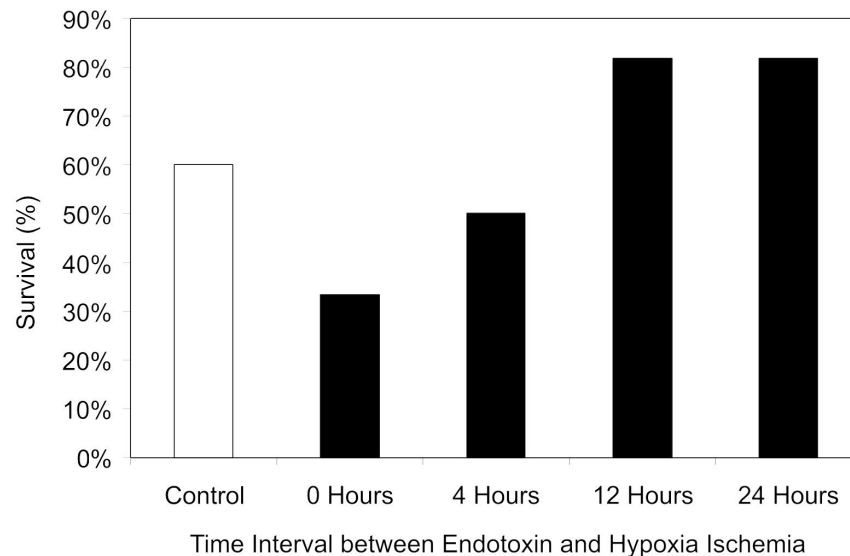


Figure 4.1: Effect of Increasing Interval between Endotoxin and Hypoxia-ischemia on Survival.

Increasing the interval between endotoxin and hypoxia-ischemia resulted in increasing survival to 48 hours. Dose intervals of 12 and 24 hours resulted in increased survival compared to animals pretreated with normal saline 4 hours prior to hypoxia-ischemia (control).

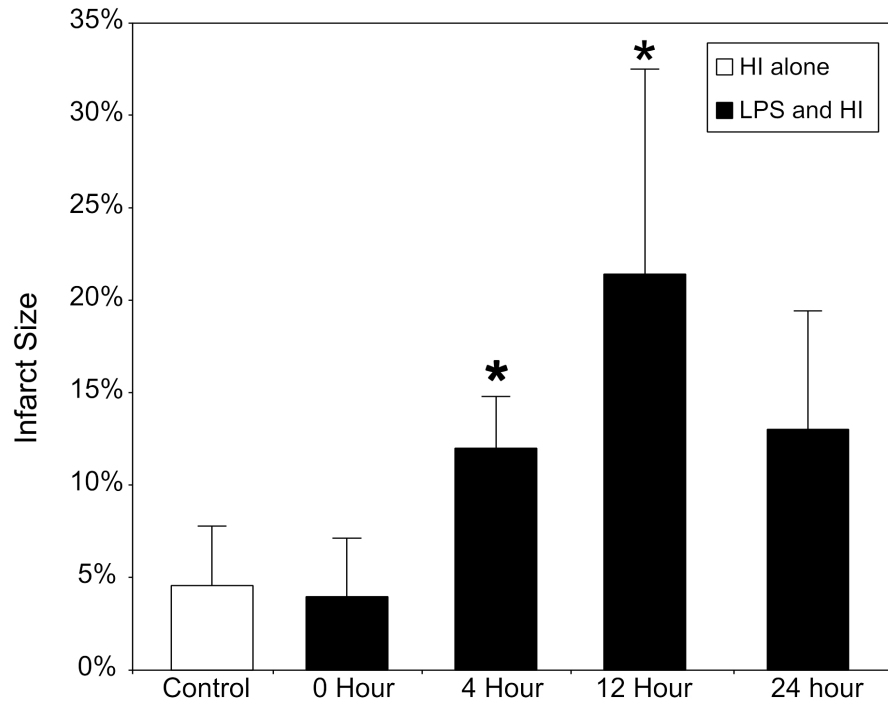


Figure 4.2: Effect of Increasing Interval between Endotoxin and Hypoxia-ischemia on Infarct Volume. Endotoxin pretreatment resulted in increased forebrain damage when administered 4 hours (n=6) and 12 hours (n=9) before hypoxia-ischemia but not at 0 hours (n=4, p=0.81) or 24 hours (n=9, p=0.16). (* p<0.05 by ANOVA with post-hoc Tukey)

When the individual forebrain regions were examined there was a trend towards sensitisation in all forebrain regions assessed (cortex, hippocampus, thalamus and striatum). The time-course of sensitisation was the same across all forebrain regions with greatest level of injury seen when endotoxin was administered 12 hours prior to hypoxia-ischemia. This analysis in the smaller sub samples did not reach statistical significance (see Figure 4.3).

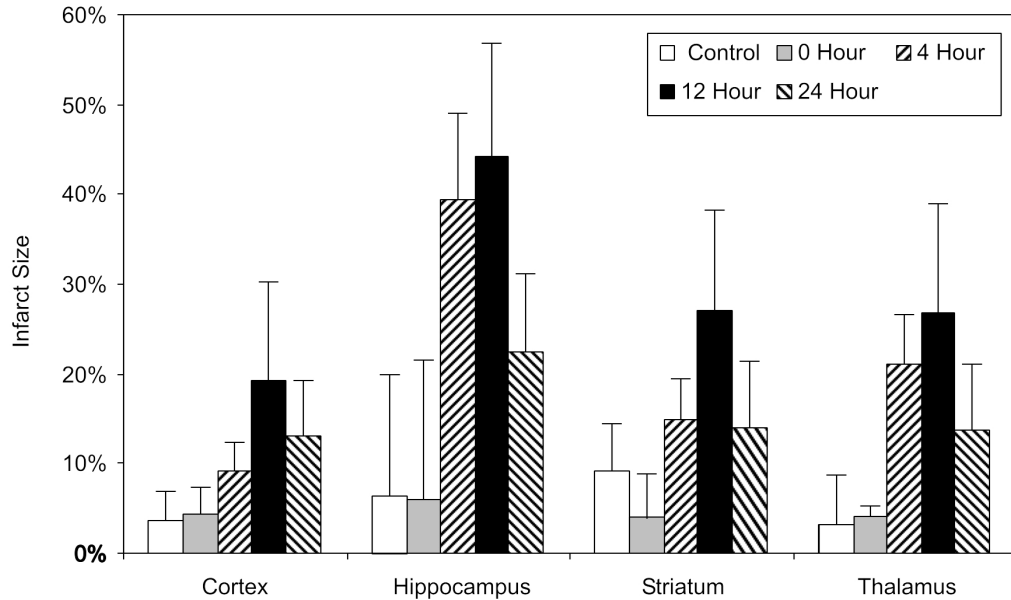


Figure 4.3: Effect of Increasing Interval between Endotoxin and Hypoxia-ischemia on Regional Infarct Volume. In all brain regions greatest brain injury was seen when LPS was administered 12 hours prior to hypoxia-ischemia

Brain injury was also quantified using injury scores. Here again the same pattern of sensitisation was seen maximally at 12 hours with similar time-course in all regions a trend towards reduced brain injury was noted when endotoxin was administered immediately before hypoxia-ischemia. In the animals treated with endotoxin 24 hours prior to hypoxia-ischemia sensitisation was no longer seen (see Figures 4.4 and 4.5).

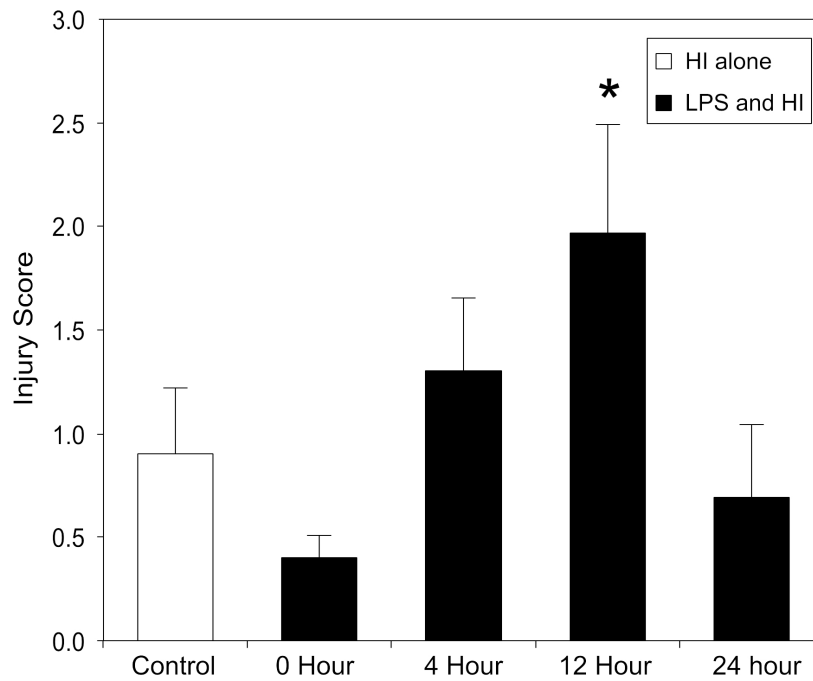


Figure 4.4: Effect of Increasing Interval between Endotoxin and Hypoxia-ischemia on Injury Score. Endotoxin pretreatment prior to hypoxia-ischemia resulted in increased forebrain injury score maximal at 12 hours prior to hypoxia-ischemia $p < 0.05$ ANOVA with post-hoc Tukey

On post-hoc Tukey analysis only the 12 hour hypoxia was significantly increased over control ($p < 0.05$). Of note, however, there was an apparent reduction in injury when endotoxin was administered immediately before hypoxia-ischemia ($P < 0.05$). When compared to the 0 hour group there was a significant increase in injury score when endotoxin was administered 4 and 12 hours before hypoxia-ischemia ($p < 0.05$) but not 24 hours prior to hypoxia-ischemia ($p = 0.40$) ANOVA with post-hoc Tukey.

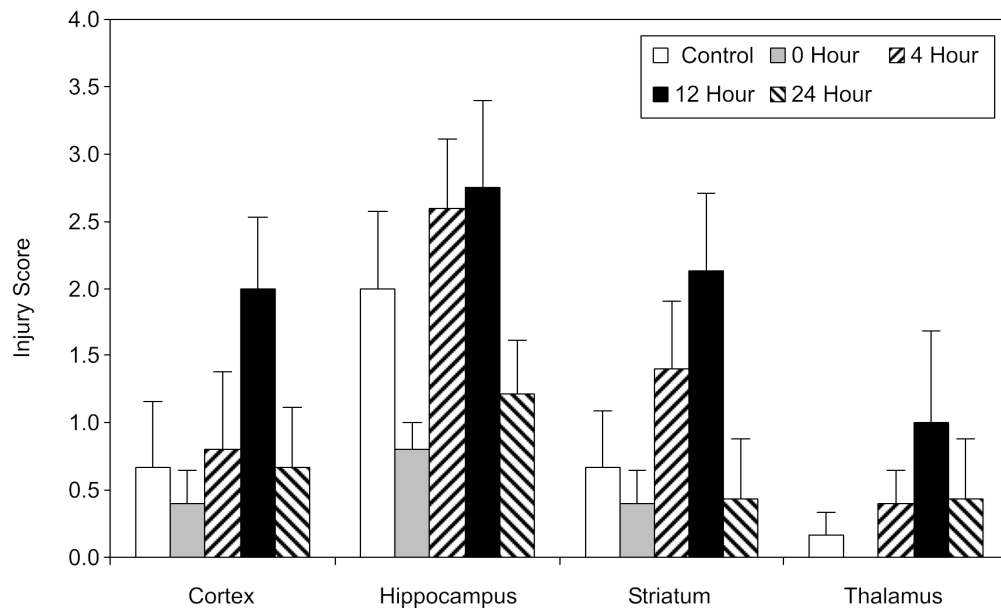


Figure 4.5: Effect of Increasing Interval between Endotoxin and Hypoxia-ischemia on Regional Injury Score. In all brain regions greatest brain injury was seen when LPS was administered 12 hours prior to hypoxia-ischemia

As noted previously, immunohistochemistry for $\alpha M\beta 2$ integrin also revealed increased microglial immunoreactivity in the white matter of the corpus callosum and internal capsule. In an attempt to quantify this effect, a second scoring system was used as previously described (see Materials and Methods). Pretreatment with endotoxin resulted in an increase in $\alpha M\beta 2$ integrin immunoreactivity in the external capsule (see Figure 4.6). The effect was seen when endotoxin pretreatment occurred 4 hours and 12 hours prior to hypoxia-ischemia.

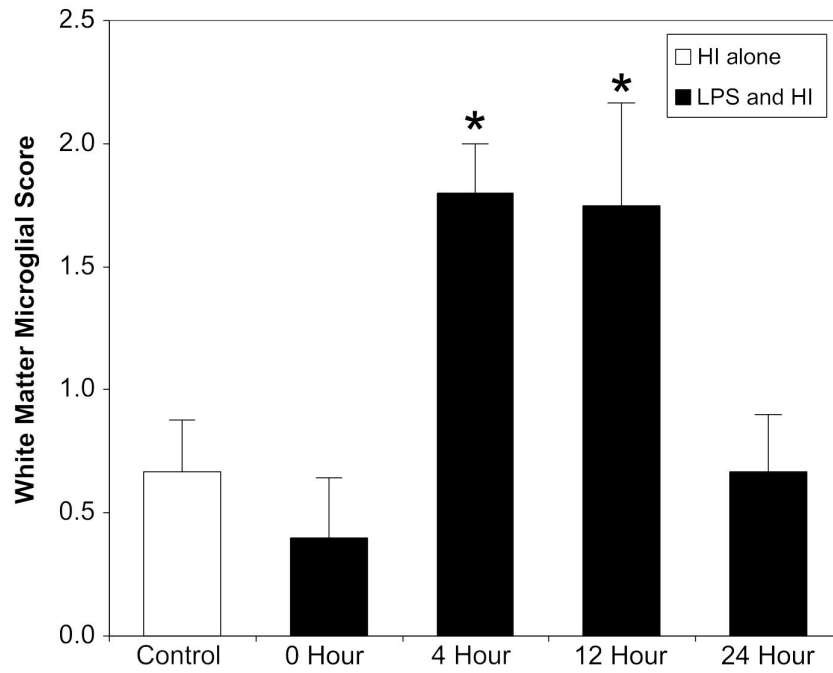


Figure 4.6: Effect of Increasing Interval between Endotoxin and Hypoxia-ischemia on White Matter Microglial Activation Score. There was a significant increase in microglial α M β 2 immunoreactivity in animals pre-treated with endotoxin 4 hours ($p < 0.05$) and 12 hours ($p < 0.05$) prior to mild hypoxia-ischemia but not when pretreatment occurred immediately or 24 hours prior to hypoxia-ischemia (one way ANOVA with post-hoc Tukey).

Endotoxin Alone Time-Course

In order to examine the cellular events that occur during the sensitisation phase following endotoxin, immunohistochemistry for $\alpha 5$, $\alpha 6$, αM , αX , and $\beta 2$ integrins (IBA1; CD44) GFAP, ICAM 1, and VCAM 1 was carried 0, 4 12 and 24 hours after administration of endotoxin alone (see Figure 4.9). All animals survived the protocol; data were analysed using ANOVA with post-hoc Tukey.

No significant difference in immunoreactivity was seen for $\alpha 5$ integrin, αX integrin or GFAP at any time-point in animals treated with 0.3 $\mu\text{g/g}$ or 0.5 $\mu\text{g/g}$ (see Figure 4.9). 4 hours after administration of endotoxin there was an increase in $\alpha 6$ immunoreactivity with 0.5 $\mu\text{g/g}$ ($p < 0.05$); although there was a trend towards increased $\alpha 6$ immunoreactivity with 0.3 $\mu\text{g/g}$ this did not reach statistical significance ($p = 0.12$). At 4 hours after administration of endotoxin there was also an increase in ICAM1 immunoreactivity in animals treated with 0.3 $\mu\text{g/g}$ and 0.5 $\mu\text{g/g}$ compared to saline treated animals. This immunoreactivity was seen in a pattern consistent with vascular endothelium (see Figure 4.7).

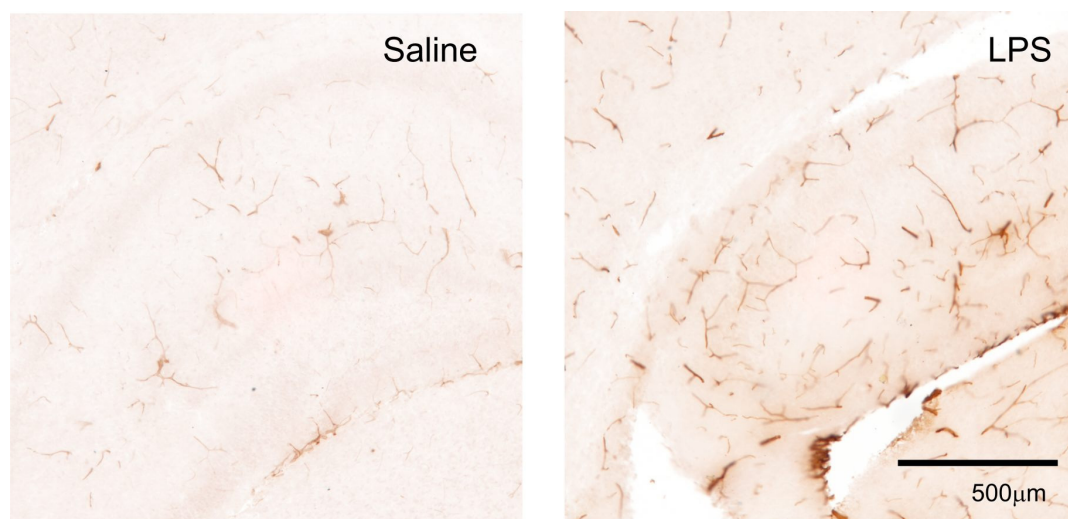


Figure 4.7: Effect of 0.5 $\mu\text{g/g}$ Endotoxin on ICAM 1 Immunoreactivity at 12 hours. Immunoreactivity was increased in a pattern consistent with vascular endothelium throughout the forebrain (here shown in the hippocampus).

12 hours after endotoxin administration there was increased immunoreactivity in α M β 2 integrin in animals treated with 0.3 μ g/g ($p < 0.05$) but not 0.5 μ g/g ($p = 0.33$). Histologically this immunoreactivity was seen in a pattern consistent with microglia (see Figure 4.8). Similarly, there was increased immunoreactivity in β 2 integrin in animals treated with 0.3 μ g/g ($p < 0.05$) but not 0.5 μ g/g ($p = 0.79$). IBA1 immunoreactivity was reduced 12 hours after endotoxin, this change was statistically significant in animals treated with 0.5 μ g/g ($p < 0.05$) but not 0.3 μ g/g ($p = 0.18$).

By 12 hours after endotoxin administration, ICAM1 immunoreactivity was increased in animals treated with 0.5 μ g/g ($p < 0.05$), a trend towards increased ICAM1 immunoreactivity was also seen at 0.3 μ g/g but this did not reach statistical significance ($p = 0.06$). Similarly VCAM1 immunoreactivity 12 hours after endotoxin administration was increased in animals treated with 0.5 μ g/g ($p < 0.05$); a trend towards increased ICAM1 immunoreactivity was also seen at 0.3 μ g/g but this did not reach statistical significance ($p = 0.06$).

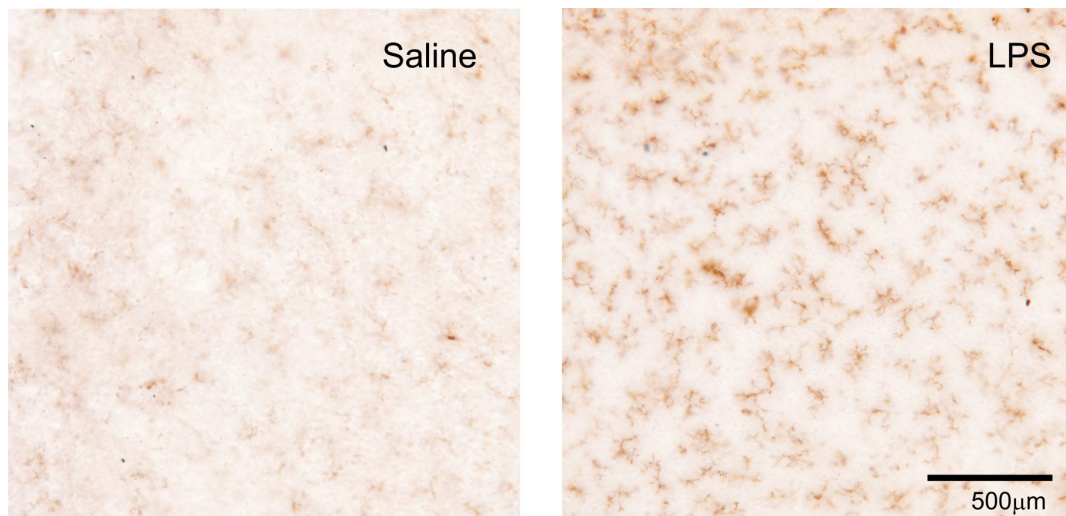


Figure 4.8: Effect of 0.3 μ g/g Endotoxin on α M β 2 Immunoreactivity at 12 hours. Immunoreactivity was seen to localise to ramified cells with the appearance of microglia.

24 hours after endotoxin administration there was only increase in $\alpha 6$ integrin immunoreactivity in animals treated with 0.3 and 0.5 $\mu\text{g/g}$ ($p < 0.05$) (see Figure 4.9).

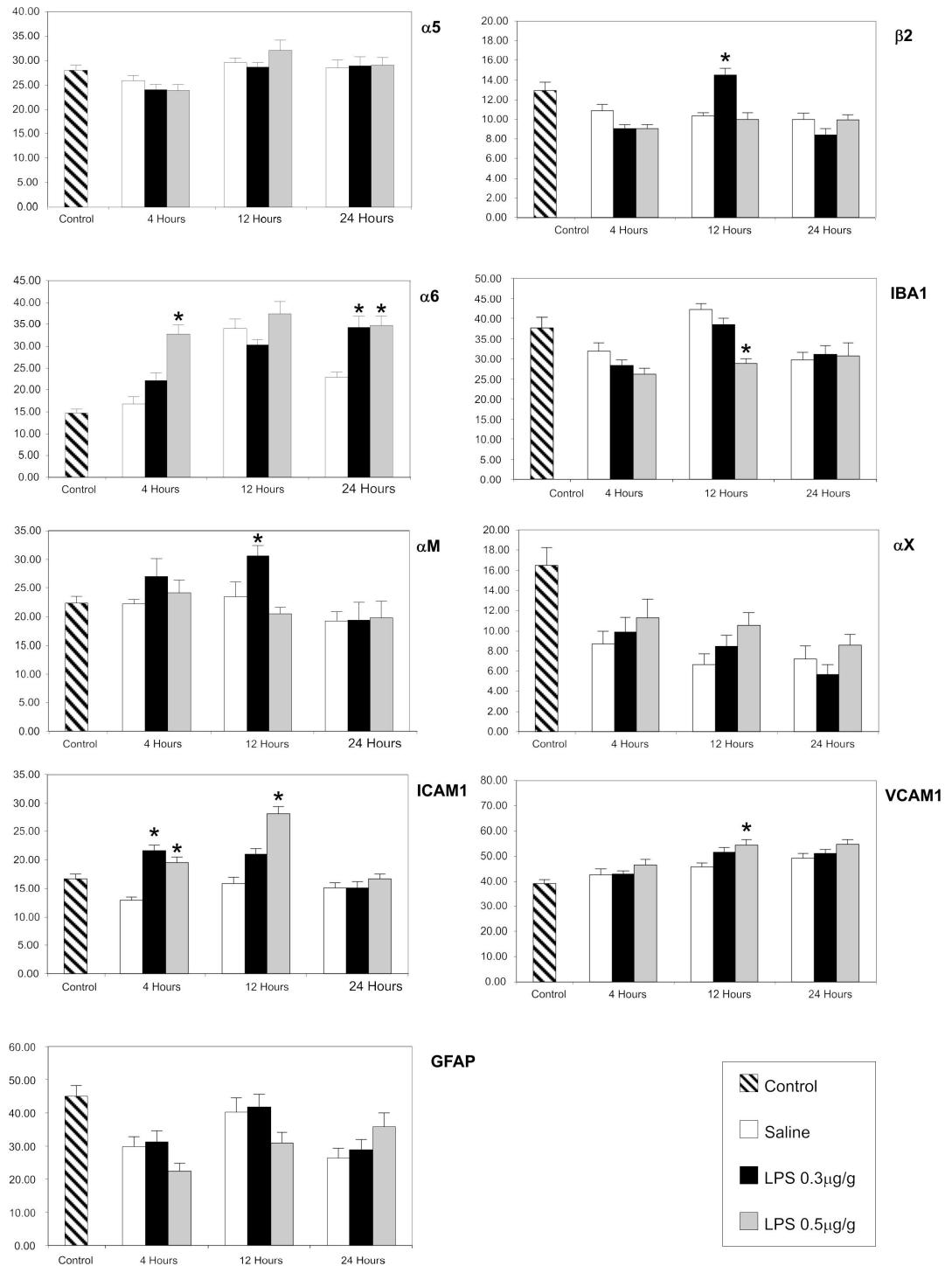


Figure 4.9: Effect of Endotoxin on Immunoreactivity at 4, 12 and 24 hours. Immunoreactivity was measured using mean – standard deviation algorithm (see Materials and Methods) * $p < 0.05$ ANOVA with post-hoc Tukey.

Blood-Brain Barrier Integrity

Pre-treatment with 50 μ L 10% human albumin (HAS) 24 hours prior to either LPS or saline resulted in a statistically significant increase in human albumin immunoreactivity throughout the forebrain both overall, and when each forebrain region was assessed individually. Post-hoc analysis (Tukey) however demonstrated that this effect was the same in animals treated subsequently with LPS or control and that there was no statistical difference if animals were treated with LPS or saline (see Figure 4.10). Similarly pre-treatment with 50 μ L 10% human IgG (HIgG) 24 hours prior to either LPS or saline resulted in a statistically significant increase in HIgG immunoreactivity throughout the forebrain both overall and when each forebrain region was assessed individually. Post-hoc analysis (Tukey) however demonstrated that this effect was the same in animals subsequently treated with LPS or control (see Figure 4.10)

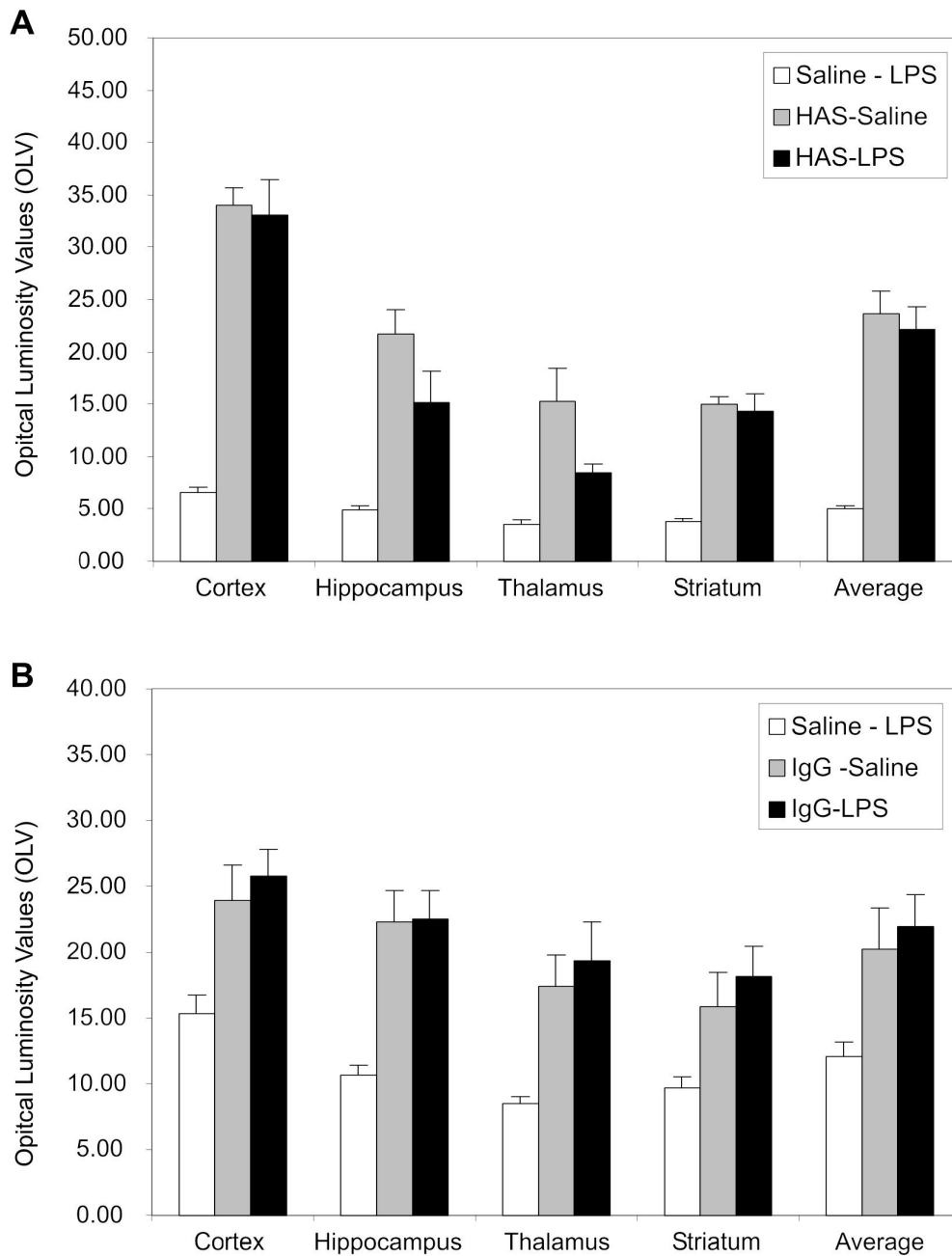


Figure 4.10: Effect of Endotoxin on the Integrity of the Blood-brain Barrier. A: animals pretreated with saline of human albumin solution (HAS) 24 hours prior to saline or LPS injections. Pretreatment with HAS led to a significant increase in HAS immunoreactivity in animals subsequently treated with saline or LPS ($p < 0.05$). There was no significant difference in the HAS immunoreactivity between animals subsequently treated with saline or LPS. B: animals pretreated with saline of human immunoglobulin (HIgG) 24 hours prior to saline or LPS injections. Pretreatment with HIgG led to a significant increase in HIgG immunoreactivity in animals subsequently treated with saline or LPS ($p < 0.05$). There was no significant difference in the HAS immunoreactivity between animals subsequently treated with saline or LPS.

Discussion

Effects of Combined Systemic Endotoxin and Hypoxia Ischemia on the Developing Brain

Pretreatment with low dose systemic endotoxin sensitises the brain to subsequent hypoxia-ischemia when administered between 4 and 12 hours before hypoxia-ischemia. Significant sensitisation was not seen when endotoxin was administered immediately, or 24 hours before hypoxia-ischemia. The overall survival in this study was 60% although this ranged in the different groups from 33% when endotoxin was administered immediately before hypoxia-ischemia to 82% when endotoxin was administered 12 or 24 hours prior to hypoxia-ischemia. This sensitisation was statistically significant when injury was quantified using infarct volume. A similar time-course of sensitisation was seen when injury was assessed using the previously described brain injury score; however, these results did not reach statistical significance using the Student t-test, possibly reflecting the relatively small sample size. It is likely that, following systemic endotoxin administration, a sequence of events occur possibly including the release of pro-inflammatory cytokines, and time is required to sensitise the brain to subsequent hypoxia-ischemia. These data suggest that the time interval between inflammatory and hypoxic-ischemic insults is critical in defining the neurological sequelae; this is in keeping with previous studies which have shown both paradoxical sensitising and neuroprotective effects of endotoxin under different circumstances. In rats at postnatal day 7, low dose endotoxin administered 4 hours prior to hypoxia ischemia has been shown to have a sensitising effect with increased brain injury following mild hypoxia ischemia (Eklind et al 2001). Conversely, in adult rats pre-treatment with endotoxin 24 hours prior to regional cerebral ischemia by middle cerebral artery occlusion has been shown to reduce the volume of infarct. It now appears that this difference in effect is, at least in part, due to the difference in time interval between the two insults. The effect of time interval between endotoxin and

hypoxia-ischemia has also been recently studied in neonatal rats (Eklind et al 2005). In this study there was difference in the effect of endotoxin pretreatment dependent on the timing of insults and the severity of hypoxia. In animals undergoing mild hypoxia ischemia (20 minutes) on postnatal day 7, endotoxin pretreatment at 6 and 72 hours prior to hypoxia-ischemia had a sensitising effect, whereas pretreatment at 2 hours or 24 hours did not. In animals undergoing a more severe hypoxic-ischemic insult (50 minutes) pretreatment at 6 hours had a sensitising effect, whereas pretreatment 2 and 72 hours did not. Intriguingly, in this severe hypoxia-ischemia group, pretreatment with endotoxin 24 hours prior to hypoxia-ischemia had a protective effect with amelioration of infarct volume. In the same study hypoxia-ischemia at day 10 in rats produced different results with no sensitisation in the mild hypoxia-ischemia group and sensitisation in the severe hypoxia-ischemia group (Eklind et al 2005). It seems therefore that the interaction of endotoxin with hypoxia-ischemia is complex in nature, with both sensitising and preconditioning effects, and possibly a biphasic interaction dependent on time interval, severity of hypoxia-ischemia and postnatal age at time of hypoxia-ischemia.

The results of the current study are, however, in keeping with those of other authors (Eklind 2001, Lenhardt 2003, Eklind 2005), with the additional finding that the peak of sensitisation occurs when endotoxin is administered 12 hours prior to hypoxia ischemia. This observation allows the further study of the cellular and humeral events occurring within the developing brain at these time-points in an attempt to understand the mechanism of sensitisation. In addition, the current study also evaluated the time-course of microglial activation within the white matter tracts of the corpus callosum and external capsule. Here there was a significant increase in microglial α M β 2 immunoreactivity in animals pre-treated with endotoxin 4 hours and 12 hours prior to mild hypoxia-ischemia but not when pretreatment occurred immediately or 24 hours prior to hypoxia-ischemia. Whilst this effect is similar to the observations in the infarct volume there was no increase in the magnitude of the effect between 4 and 12 hours.

Effects of Systemic Endotoxin on Cellular Activation within the CNS

In order to maximize the chance of detecting small changes in immunoreactivity with endotoxin alone, doses of both 0.3 $\mu\text{g/g}$ and 0.5 $\mu\text{g/g}$ were used. The effects of endotoxin on microglial activation (integrins $\alpha 5$, $\alpha 6$, αM , αX , $\beta 2$, and IBA1) astrocyte activation (CD44, GFAP), and vascular endothelial up-regulation (ICAM 1, VCAM 1, $\alpha 5$ and $\alpha 6$ integrins) were examined at the same time-points as for the sensitisation experiments. 4 hours after administration of endotoxin there was an increase in $\alpha 6$ immunoreactivity and an increase in ICAM1 immunoreactivity both in a pattern consistent with vascular endothelium. It is therefore apparent that there are detectable changes in vascular endothelium in response to endotoxin at 4 hours after systemic injection, within the same time window as sensitisation to subsequent hypoxia-ischemia was observed in the previous study. 12 hours after endotoxin administration there was increased immunoreactivity in αM and $\beta 2$ integrins in a pattern consistent with microglia. Conversely microglial IBA1 immunoreactivity was reduced 12 hours after endotoxin. Vascular ICAM1 immunoreactivity continued to be increased and there was also noted to be an increase in VCAM1. Of note in this study of the effects of endotoxin alone, the maximal increase in immunoreactivity of both vascular endothelial activation and microglial activation was seen at the 12 hour time-point, the time-point of maximal sensitisation to hypoxia-ischemia observed in the previous study. 24 hours after endotoxin administration there was only an increase in $\alpha 6$ integrin immunoreactivity. No significant difference in immunoreactivity was seen at any time-point for the astrocyte activation markers CD 44 or GFAP. Additionally there were no effects of low dose endotoxin on $\alpha 5$ integrin, αX integrin

It seems, therefore, that low dose endotoxin induces detectable changes in vascular endothelium and microglia in the developing brain in a time window consistent with sensitisation to hypoxia-ischemia.

Microglia, the resident phagocytes of the brain, have many immune functions within the central nervous system. As described previously, in their resting state microglia are highly ramified cells, which have a unique role in the CNS in immune surveillance (Raivich 2005). In response to various stimuli such as endotoxin, infection or ischemia, microglia target and surround the injured area. There is also a change in their morphology with increased integrin immunoreactivity, swelling of the microglial cell body, thickening of the proximal processes, and loss of the distal processes (Kloss et al 2001). The recognition that, in response to systemic endotoxin, microglia in the developing brain have changes in integrin immunoreactivity, which may represent up-regulation to an 'alert' phenotype, raises the possibility that these cells may be key to sensitisation of the developing brain to subsequent hypoxia-ischemia. It is at present unclear whether endotoxin travels directly to the brain and acts directly on microglia or if there is a systemic response which 'transduces' the signal to the microglia. It is plausible however, as endotoxin is a small (approximately 10KDa), highly soluble molecule, that at least some of the responses are a result of direct response to LPS within the brain. From *in vitro* studies, endotoxin is known to be able to activate microglia through the toll-like receptors (Ebert et al 2005) resulting in the production of nitric oxide and TNF α . The role of microglia and toll-like receptors has been previously studied in relation to endotoxin-mediated neuronal damage (Lehnardt et al 2003). In this study endotoxin was demonstrated *in vitro* to cause loss of axons, oligodendrocytes, and microglia when added to a mixed culture of CNS cells. Interestingly, however, endotoxin had no effect on neuronal survival when added to a pure neuronal culture. In a combined neuronal and microglial culture endotoxin resulted in almost complete loss of neurones. This suggests that the previously recognised endotoxin-mediated neuronal toxicity *in vivo* and *in vitro* may be mediated through microglia. This effect was further demonstrated using TLR4 mutant mouse with a loss of functional mutation within the C-terminal part of TLR4. This mutant is known to disrupt normal LPS-induced signaling. Mutants and wildtype animals were pretreated with endotoxin 1 hour prior to carotid occlusion and exposure to 8% oxygen for 30

minutes. In the wildtype animals there was ipsilateral axonal and neuronal loss in the external capsule and overlying cerebral cortex, whereas in the TLR4 this damage was not seen (Lehnardt et al 2003). Interestingly this *in vivo* study used a time interval between endotoxin and hypoxia-ischemia which has not been shown to sensitise the brain in other studies (Eklind et al 2005), furthermore the global TLR4 disruption does not demonstrate the cell type responsible for sensitisation. Alternative pathways for the activation of microglia within the CNS following systemic administration of endotoxin have been previously considered (Hagberg and Mallard 2005, see Figure 4.11)

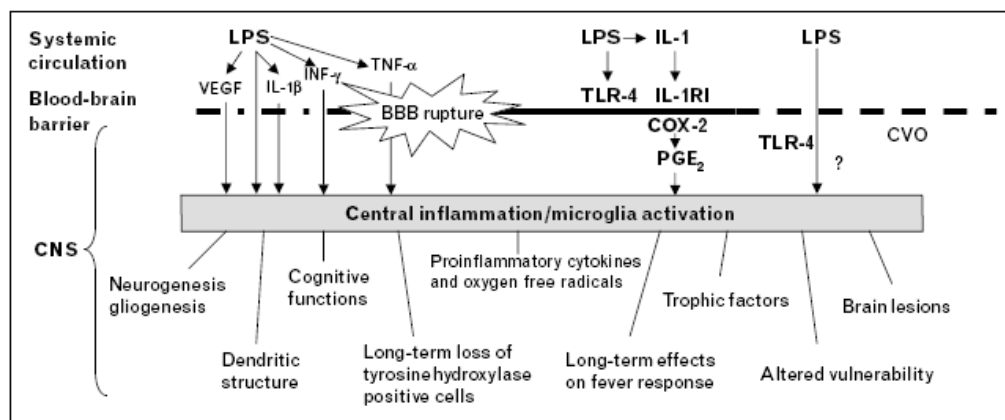


Figure 4.11: Lipopolysaccharide-induced systemic inflammation is transferred to the brain across an intact barrier via receptor-mediated prostaglandin production, areas with an incomplete barrier and through rupture of the blood–brain barrier (BBB). Central inflammation and microglia activation elicit a cytokine/ trophic factor response, affect central nervous system (CNS) functions, structure, cellular proliferation and vulnerability, and can produce brain lesions (from Hagberg and Mallard 2005 reproduced with permission). COX, cyclo-oxygenase; CVO, circumventricular organs; IL, interleukin; INF, interferon; LPS, lipopolysaccharide; PG, prostaglandin; TLR, Toll-like receptor; TNF, tumour necrosis factor.

It is of note that in the current study the first significant change in immunoreactivity following systemic endotoxin is the up-regulation of vascular ICAM1. This effect of endotoxin has been previously demonstrated in adult mice (Bohatschek et al 2001) and is part of a mechanism which allows granulocyte influx into the brain. Adult animal studies also indicate that the upregulation of vascular endothelium to express cell adhesion molecules and allow leukocyte recruitment in response to endotoxin is also mediated by microglia (Zhou et al 2006). In this study, intra-cerebroventricular administration of endotoxin resulted in significant rolling and adhesion of leukocytes in the brain post capillary venules. When the same study was repeated in TLR4-deficient mice this adhesion and rolling of leukocytes did not occur. Furthermore chimeric mice with TLR4 positive endothelium and microglia but TLR4 negative leukocytes had normal adhesion and rolling responses. Finally, this study showed that minocycline, a potent microglial inhibitor, was able to block adhesion and rolling responses to endotoxin *in vivo* but did not affect activation of endothelium by endotoxin *in vitro*. The authors, therefore, suggest that vascular endothelial up-regulation with increased cell adhesion molecules and hence leukocyte adhesion and rolling was a response to the activation of microglia by endotoxin and the resultant production of TNF α which then acts on the vascular endothelium (see Figure 4.12)

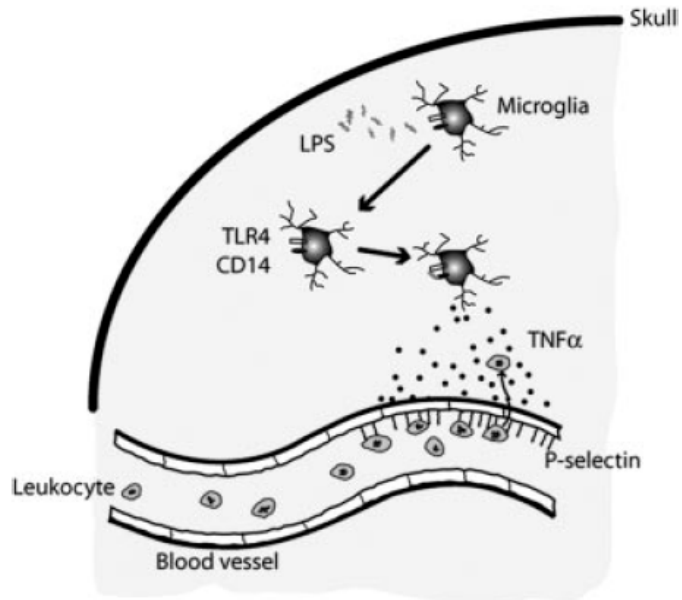


Figure 4.12: Microglia detected bacterial products and were rapidly activated and releasing $\text{TNF}\alpha$, which activates endothelium to express adhesion molecules and allows leukocytes to roll, adhere, and enter the brain parenchyma. (from Zhou et al 2006) Copyright 2006. The American Association of Immunologists, Inc. Reproduced with permission.

Once activated, microglia can produce extracellular matrix-degrading enzymes, major histocompatibility complex I & II, $\text{IL1}\alpha$, $\text{IL1}\beta$, IL6 , IL12 , TNF , early components of the complement cascade and nitric oxide and prostaglandins to attack invading microorganisms (Chew et al 2006). If there was a subsequent hypoxic-ischemic event this would then occur in a pro-inflammatory environment with the potential for the observed increase in injury resulting. This hypothesis would fit with the current and previous observations that there is a delay of at least 2 hours between administration of systemic endotoxin and the onset of sensitisation (Eklind et al 2005). Alternatively, recent *in vitro* studies have suggested that normally microglia have a neuroprotective role and activation by endotoxin results in a loss of this effect. In cell culture the addition of microglia to neuronal culture which is then exposed to oxygen-glucose deprivation results in microglial migration, close cell-to-cell contact between microglia

and neurones, and confers significant neuroprotection. Thus, it is plausible that this effect is also present *in vivo*. Microglia, in addition to immune surveillance, cytokine production and phagocytosis, may normally have a significant supporting role to damaged neurones preventing cell death and cell survival. However, when exposed to endotoxin this effect may be lost, resulting in increased brain injury.

Effects of Systemic Endotoxin on Blood-brain Barrier

The current study shows that systemically administered human albumin solution and human IgG are detectable throughout the forebrain of 7 day old mice 12 hours after injection. There was no detectable difference in immunoreactivity for human albumin or human IgG if the injection was followed by endotoxin. This study was unable to demonstrate a change in the integrity of the blood-brain barrier to the substances tested in response to endotoxin or saline. Previous studies in adult mice has shown that systemic injection of human albumin solution resulted in detectable immunoreactivity for human serum albumin restricted to brain tissue surrounding the area postrema and central canal. Animals subsequently treated with endotoxin demonstrated strong immunoreactivity for human serum albumin throughout the grey matter, and some immunoreactivity in the white matter tracts of the brain stem. These data suggest that, in adult animals, systemic endotoxin results in a disruption of the blood-brain barrier, which was accompanied by an ICAM1-mediated influx of granulocytes into the brainstem (Bohatschek et al 2001). Comparing the current study with the previous adult animal study suggests that the blood-brain barrier in the neonatal mouse is apparently more permeable to human serum albumin. The blood-brain barrier, in the form of complex tight junctions between endothelial cells, is structurally present from early embryological life. It is known that endogenous protein concentrations within the immature brain are much higher than those of adults (Habgood et al 1992). Whilst previously this was interpreted as evidence of increased blood-brain barrier permeability in the immature brain, the demonstration that, even in early development, the tight junctions form a functional barrier (Ek et al 2003) has led

to the suggestion that there may be a specific transcellular transfer process for proteins independent of that for small lipid-insoluble molecules (Johansson et al 2006). In the embryonic rat the choroid plexus tight junctions are impermeable to small molecules as early as embryological day 15, confirming a functional blood-brain barrier in early embryonic development (Johansson et al 2006). Interestingly, in this study the transfer of human serum albumin from the systemic circulation into the embryonic rat brain was 4–5 times greater than bovine serum albumin, suggesting selective blood-to-CSF transfer. The authors conclude that there are different trans-cellular mechanisms for protein and small molecule passage across the embryonic blood-brain barrier. It is, therefore, possible that the current data reflects this apparent specific transfer of human serum albumin into the brain at early stages of development rather than a reflection of the general permeability of the blood-brain barrier. The results using human IgG also demonstrates free passage into the brains of neonatal mice without subsequent addition of endotoxin. It is possible that this small molecule is able to freely pass into the CNS without the requirement of a specific transport system. The current study is unable to demonstrate any changes in blood-brain barrier integrity as a result of endotoxin; however, it also does not exclude such changes.

Conclusions

Low dose systemic endotoxin sensitises the developing brain to subsequent hypoxia-ischemia within a time window of 4-12 hours after endotoxin administration. Within this time window low dose endotoxin results in detectable up-regulation in adhesion molecules on the vascular endothelium and activation markers on microglia. Previous studies in adult animals suggest that LPS can act directly on microglia through toll-like receptors resulting in the production of cytokines, including $\text{TNF}\alpha$, which, in turn, result in the observed changes in vascular endothelium. The role of these humeral factors and the timing of their expression are crucial in understanding the mechanism of endotoxin sensitisation and will be subject to further discussion (see Chapter 5).

Chapter 5: The Role of the TNF Family of Cytokines in Inflammatory Sensitisation of the Developing Brain to Hypoxia-Ischemia

Introduction

Growing evidence from epidemiological studies and animal models suggests that there may be a synergistic relationship between infection / inflammation and hypoxia-ischemia as antecedents of perinatal brain injury and the subsequent development of cerebral palsy (Peebles and Wyatt 2002). This interaction may help explain the apparent absence of significant hypoxia-ischemia in the majority of children who develop cerebral palsy (Badawi et al 1998, Blair 1988). Mediators of bacterial infection such as the endotoxin up-regulate pro-inflammatory cytokines (Xiao et al 1996), in contrast with hypoxia-ischemia which results in the release of both pro-inflammatory and a second group of cytokines which are considered to have anti-inflammatory properties (Raivich et al 1999, Kiefer et al 1995). It is possible that the balance between these pro- and anti-inflammatory cytokines within the brain at times may be critical in defining whether an injured neuron is able to survive or is committed to cell death.

Previously, we have demonstrated the potential role of microglia and vascular endothelium in the mechanism by which endotoxin, a potent inflammatory stimulus, sensitises the developing brain to hypoxia-ischemia. *In vitro* studies also suggest that the mechanism of endotoxin-mediated neuronal toxicity is dependent on the presence of microglia through activation of Toll-like receptor 4 (TLR-4) (Lehnardt et al 2003, Zhou et al 2004). Furthermore studies have demonstrated that activation of microglia through Toll-like receptors, results in the release of both tumour necrosis factor α and nitric oxide (Ebert et al 2004) through both MyD88 -dependent and -independent pathways (Zhou et al 2003). A recent *in vitro* study demonstrated that the pathway of

endotoxin-mediated cellular toxicity is dependent on the presence or absence of different glial cells (Li et al 2008). Here, whilst microglia were essential for endotoxin mediated cellular toxicity, as shown before (Lehnardt et al 2003), the additional presence of astrocytes switched the mechanism of toxicity from one dependent on nitric oxide synthase and peroxynitrite, to one dependent on tumour necrosis factor- α (Li et al 2008). In this context, it is interesting to note that children who subsequently develop cerebral palsy have been shown to have raised levels of pro-inflammatory cytokines, including tumour necrosis factor- α , in blood samples taken in the neonatal period (Nelson et al 1998).

Tumour necrosis factor along with lymphotoxin α (LT α), and LT β form a subfamily of TNF-related ligands. These have their genes linked within a compact 2-kb cluster inside the major histocompatibility complex locus (see figure 5.1). Previous studies have demonstrated that these related cytokines have a number of distinct and overlapping functions (see figure 5.2) (Kuprash et al 2002)

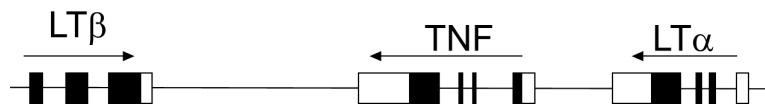


Figure 5.1 The TNF cluster which is located within the Major Histocompatibility complex

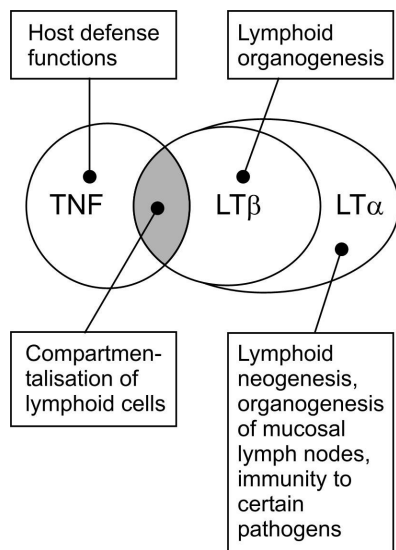


Figure 5.2 Distinct and overlapping physiological functions of the TNF / LT family. Areas on the diagram symbolise subsets of functions mediated by single molecules or by their combinations (from Kuprash et al 2002 reproduced with permission).

Single nucleotide polymorphisms in $LT\alpha$ have been demonstrated to be associated with the development of cerebral palsy (Nelson et al 2005), further implicating this family in the mechanism of perinatal brain injury.

The current study aims to explore further the role of the TNF family of cytokines ($TNF\alpha$, $LT\alpha$ and $LT\beta$) in the aetiology of perinatal brain injury. First, the expression of these cytokines was studied in neonatal mice following endotoxin alone. To study the role of the TNF family of cytokines on endotoxin-mediated microglial activation and vascular endothelial up-regulation noted previously during the window of sensitisation, animals with deletion of the entire TNF cluster were treated with endotoxin or saline and immunohistochemistry for $\alpha M\beta 2$ integrin and ICAM1 performed 12 hours after exposure. To explore further the role of the TNF family of cytokines in the mechanism of neonatal brain injury, animals with a deletion of the entire TNF cluster were exposed to endotoxin or saline 12 hours prior to carotid occlusion and hypoxia-ischemia. Finally to examine the mechanism by which the TNF family mediates endotoxin sensitisation to hypoxia-ischemia cytokine expression was compared in wildtype animals exposed to hypoxia ischemia alone, and following endotoxin pretreatment 12 hours before hypoxia-ischemia

Materials and Methods

TNF Cluster Gene Expression

To explore the role of the TNF family of cytokines in perinatal brain injury, the mRNA for TNF α , LT α and LT β was quantified using a cDNA real-time PCR 2, 4, 12 or 24 hours after endotoxin 0.3 $\mu\text{g/g}$ (n=20) and compared to naive animals (n=5). Mice were killed by decapitation, their brains quickly removed, frozen in liquid nitrogen and kept at -80°C until processed.

RNA isolation and cDNA preparation

The left (occluded) forebrain hemisphere was micro-dissected in the frozen state and total RNA isolated using the RNeasy minikit (Qiagen West Sussex, UK) according to the manufacturer's instructions. Briefly, the posterior half of the left hemisphere was suspended in RLT buffer with β -mercaptoethanol and homogenized by passing through a 23 gauge needle 10 times. 600 μl of the sample was then centrifuged and the supernatant mixed with an equal volume of 70% ethanol. The sample was then applied to an RNeasy mini column which binds the RNA. The column was washed with RWI and RPE buffers before eluting the RNA in 50 μl RNase-free water. The quality of the RNA was checked on a 2% agarose gel, and the amount of RNA quantified by measuring absorbance at 260nm (A_{260}). Concentration was calculated as $40 \times A_{260} \times$ dilution factor. The RNA was normalised to 0.12 $\mu\text{g}/\mu\text{l}$ prior to DNase treatment for 1 hour at 37°C using RQ1 RNase free DNase (Promega cat #M6101) and RNasin® (Promega cat #N2111). Samples then underwent citrate buffered phenol: chloroform extraction and RNA precipitation: Samples were mixed with an equal volume of phenol:chloroform and thoroughly vortexed prior to centrifugation. The aqueous phase was then mixed with glycogen/tRNA to act as a carrier and the RNA precipitated using 1:5 volume of 3M ammonium acetate and 2.5 volume of 100% ethanol on dry ice for 30

minutes. The RNA pellet was centrifuged, washed in 75% ethanol, dried in room air and re-suspended in 15 μ l DEPC water. The resultant RNA was reverse transcribed by heating to 75°C with random hexamers for 5 minutes, cooled on ice and then incubated at 40°C with M-MLV RT enzyme (Promega) and dNTPs. The cDNA produced by the reverse transcription reaction was checked using polymerase chain reaction (PCR) for the ubiquitous β -actin gene. The specific oligonucleotide primers (Invitrogen) used were:

Forward primer: 5'- GCTCCGGCATGTGCAA - 3'

Reverse primer: 5' – AGGATCTTCATGAGGTAGT – 3'

The annealing temperature of the primers was 60°C. The Taq polymerase used was from Applied Biosystems (5U/ μ l Cat. No. N808-0156). The resultant product molecular weight was 520 bp (See Figure 5.3).

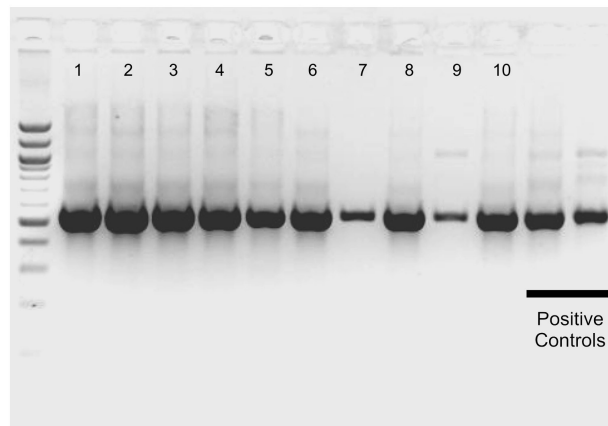


Figure 5.3: β -Actin PCR of cDNA confirmed the Presence of cDNA

The reverse transcription-PCR reaction was performed by Dr Dimitra Dafou Translational Research Laboratory, Institute for Women's Health, University College London, on an ABI 7900HT genotyper using SDS2.1 software. Samples were run in triplicate on 96 well plates with an 18S control measured in each well.

The 'relative quantification study' programme of SDS2.1 software was used for analysing data. First the cycle number at which the increase in fluorescence (and therefore cDNA) became exponential (Ct) for both the target gene and the control gene (18S) were measured and the difference in Ct between the control and target gene were calculated for each well (delta CT). The results were expressed using the comparative C_t or 2^{-[delta][delta]Ct} method, where:

$$[\text{delta}][\text{delta}]C_t = [\text{delta}]C_{t,\text{sample}} - [\text{delta}]C_{t,\text{control}} \quad \text{formula 5.1}$$

Here, $[\text{delta}]C_{T,\text{sample}}$ is the C_t value for any experimental animal normalised to the endogenous housekeeping gene and $[\text{delta}]C_{t,\text{control}}$ is the C_t value for control animals also normalised to the endogenous housekeeping gene. Expression levels of each gene were calculated in this way for each time-point as the average of the triplicates.

Effects of TNF Cluster Deletion

Animals homozygous for a deletion of the entire TNF cluster were a kind gift from K Pfeffer, Institute of Medical Microbiology, University of Dusseldorf, Germany. The generation and phenotypic analysis of these mice with combined TNF / LT α / LT β deficiency has been previously described (Kuprash et al 2002). These animals were back-crossed with C57/Bl6 (Charles River) for four generations to ensure the genetic background of the strain. The heterozygotes of F₄ were bred and their offspring were used at postnatal day 7.

To examine the effect of TNF cluster knockout on the responses to endotoxin, 63 offspring of animals heterozygous for TNF cluster deletion were studied at postnatal day 7. Animals were sequentially allocated to receive LPS (*Escherichia coli*, serotype 055:B5, Fluka) at 0.3 $\mu\text{g/g}$, or vehicle (normal saline), 12 hours prior to being culled. As before, all injections were at a volume of 10 $\mu\text{l/g}$. Following injections, the mice were returned to their dams until they were killed, perfused, and brains extracted as described previously. Following genotyping (see below) the brains of knockout and wildtype animals were cut on a cryostat and stained using immunohistochemistry for microglial activation ($\alpha\text{M}\beta\text{2}$), and vascular endothelial up-regulation (ICAM1). Immunohistochemistry and staining intensity evaluation were performed as described previously (see under General Materials and Methods).

To examine the role of the TNF cluster on endotoxin-mediated sensitisation to subsequent hypoxia-ischemia 109 animals were sequentially allocated to receive intraperitoneal endotoxin (0.3 $\mu\text{g/g}$) or vehicle (normal saline) 12 hours prior to hypoxia-ischemia as described previously. At 48 hours, the brains were perfused, extracted and post-fixed as described before. Animals were genotyped (see below) and the brains of knockout and wildtype evaluated. 15 coronal sections from each forebrain (200 μm apart) were stained with cresyl violet and the areas of intact staining in the cortex, hippocampus, striatum and thalamus were measured bilaterally. Infarct volumes were calculated and expressed as a percentage of the contralateral (uninjured) hemisphere. Statistical analysis was by ANOVA analysis of variance with post-hoc Tukey test.

Genotyping

DNA extraction from tail tips taken during perfusion-fixation was performed using the “Wizard” Genomic DNA purification system according to manufacturer’s instructions (Promega, Cat. No: A1125). Briefly, tails were suspended in chilled 0.5 M EDTA and nuclei lysis solution. Samples were incubated overnight with proteinase K (Sigma) at 55°C. 3 µl of RNase solution was added to each of the nuclear lysate samples and the samples incubated for 15-30 minutes at 37°C. 200 µl of protein precipitation solution was then added to each sample and the tubes were then thoroughly vortexed before being chilled on ice for 5 minutes. Samples were then centrifuged for 4 minutes and the DNA-containing supernatant separated off. DNA was then precipitated using isopropanol, and then washed in 70% ethanol. The DNA pellets were allowed to air dry at room temperature before being rehydrated in 100 µl of TE (Tris/EDTA) buffer. A 1:200 dilution was used for genotyping by polymerase chain reaction. For the wildtype, the specific oligonucleotide primers (Invitrogen) used were:

Forward primer: 5'- CGGGTCTCCGACCTAGAGATC - 3'

Reverse primer: 5' - CCCACGCTCGTGTACCATAAC – 3'

The annealing temperature of the primers was 56°C. The Taq polymerase used was from Applied Biosystems (5 U/µl Cat. No. N808-0156). The resultant product molecular weight was 400 bp (see Figure 5.4)

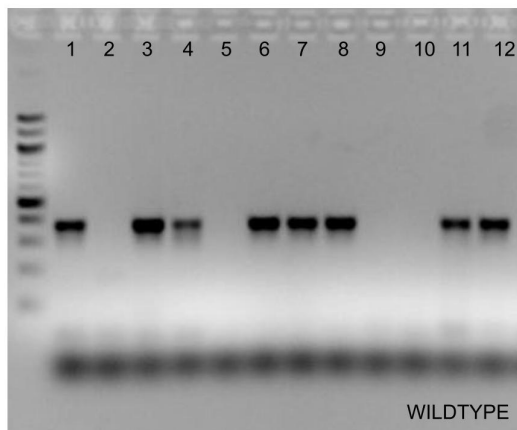


Figure 5.4: Wildtype Genotyping for Deletion of the Entire TNF Cluster of animals 1-12

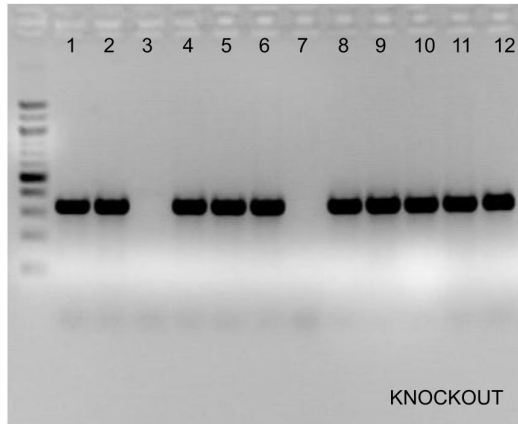


Figure 5.5: Knockout Genotyping for Deletion of the Entire TNF Cluster of animals 1-12

For the knockout the specific oligonucleotide primers (Invitrogen) used were:

Forward primer: 5'- CACCCACCCCGTTTTCTTTCTTC - 3'

Reverse primer: 5' - CCACTTGTCCAGTGCCTGCTC – 3'

The annealing temperature of the primers was 56°C. The Taq polymerase used was from Applied Biosystems (5 U/μl). The resultant product molecular weight was 320 bp (see figure 5.5).

Timing of the Effects of TNF on Endotoxin-Mediated Sensitisation

Finally, to examine the whether up-regulation of the TNF family of cytokines was critical prior to or after hypoxia-ischemia the mRNA for TNF α , LT α and LT β was quantified as before, 0-24 hours after hypoxia-ischemia alone (n=25) or following pretreatment with 0.3 μg/g endotoxin 12 hours prior to hypoxia-ischemia (n=25). Here again, levels of mRNA were compared to 5 naïve control animals.

Results

TNF family Gene Expression Following Endotoxin:

The expression of $LT\alpha$, $LT\beta$ and $TNF\alpha$ mRNA was examined 2-24 hours following intra peritoneal endotoxin administration and expressed as $2^{-[\Delta\Delta Ct]}$ (see formula 5.1). Following systemic endotoxin administration, a trend towards a rise in the expression of $TNF\alpha$ mRNA within the brain was seen 2 hours, 4 hours and 24 hours after endotoxin. In this small sample, the results did not reach statistical significance at any time-point (2 hours $p=0.10$, 4 hours $p=0.10$, 12 hours $p=0.22$). At 12 hours after endotoxin administration there was no difference in the level of TNF mRNA compared to control ($p=0.87$) by Student t-test (see Figure 5.6A). The expression of lymphotoxin α within the brain following systemic endotoxin administration appeared to be increased at 2 hours and 12 hours after endotoxin but not a 4 and 24 hours. Here again however these results in this small sample did not reach statistical significance (2 hours $p=0.21$, 4 hours $p=0.87$, 12 hours $p=0.58$, 24 hours $p=0.43$ Student t-test) (see Figure 5.6B). The expression of lymphotoxin β within the brain following systemic endotoxin administration appeared to be increased at 2 hours, 4 hours and 24 hours after endotoxin. Here again at 12 hours after endotoxin administration there was no difference in the level of lymphotoxin β mRNA compared to control. These results in this small sample did not reach statistical significance (2 hours $p=0.67$, 4 hours $p=0.37$, 12 hours $p=0.85$, 24 hours $p=0.58$ Student t-test) (see Figure 5.6C).

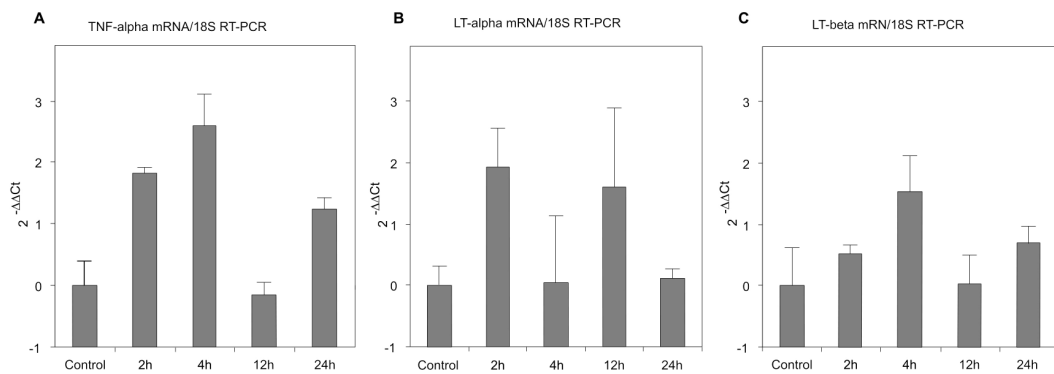


Figure 5.6: mRNA expression of TNF (A), lymphotoxin α (B) and lymphotoxin β (C) within the brain 2-24 hours after systemic endotoxin administration.

Effects of TNF Cluster Deletion on *in vivo* Endotoxin-Mediated Cellular Activation

To study the effect of TNF family of cytokines *in vivo* on microglial activation and vascular up-regulation following systemic endotoxin administration, 63 animals at postnatal day 7 were sequentially allocated to receive endotoxin or saline. All animals survived to perfusion at 12 hours and genotyping revealed 19 wildtype (30%), 16 knockout (25%) and 28 heterozygotes (44%) for the TNF cluster. As previously noted, 12 hours after endotoxin administration there was increased α M β 2 integrin immunoreactivity in the wildtype animals ($p < 0.05$). In animals with a deletion of the TNF cluster there was no increase in α M β 2 integrin following endotoxin treatment (see Figure 5.7).

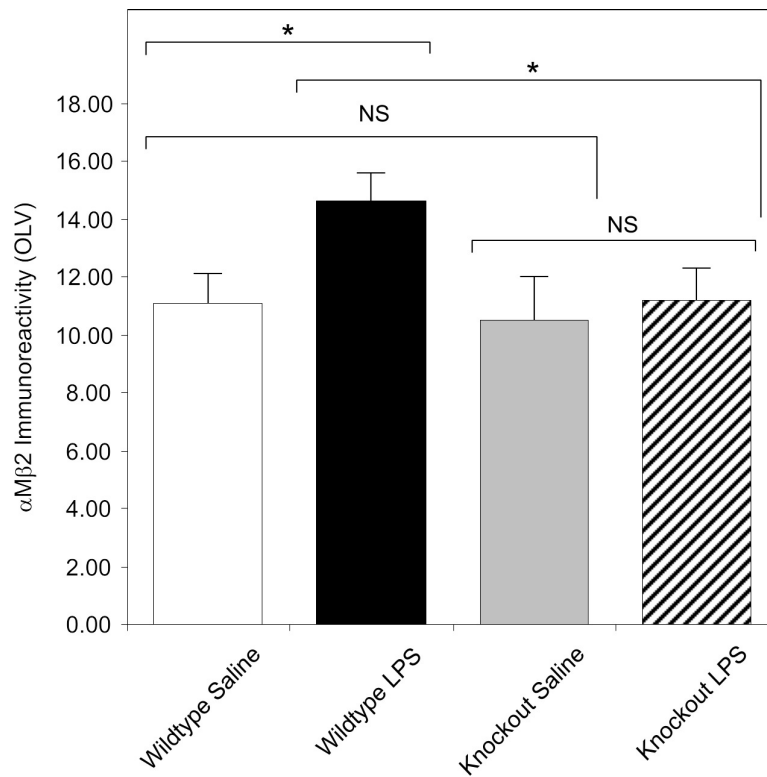


Figure 5.7: Effects of TNF cluster deletion on LPS-mediated microglial activation. 12 hours after endotoxin application there was a significant increase in α M β 2 integrin immunoreactivity in wildtype animals. In animals with a deletion of the TNF cluster there was no LPS-mediated microglial activation compared to saline-treated animals. * $p < 0.05$ (ANOVA with post-hoc Tukey); NS – Non Significant.

When the different forebrain regions were analysed independently the same trend to increased α M β 2 integrin immunoreactivity was seen in the cortex ($p = 0.11$) hippocampus ($p = 0.18$) and striatum ($p = 0.12$), as well as a significant increase in the thalamus $p < 0.05$. No LPS-mediated microglial activation seen in the animals with deletion of the TNF cluster (see Figure 5.8)

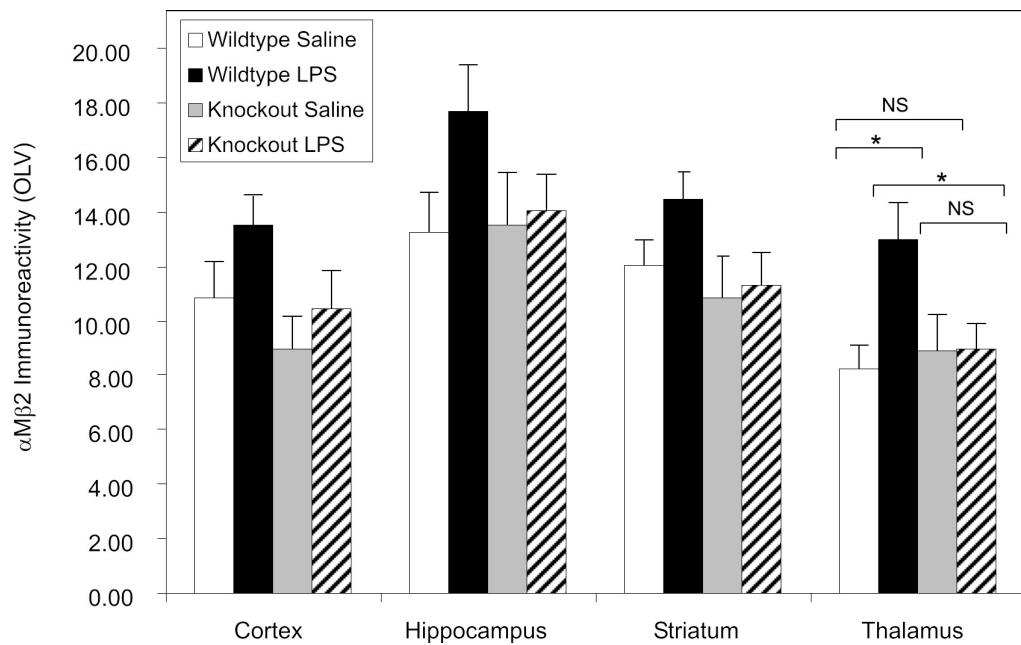


Figure 5.8: Regional Effects of TNF cluster deletion on Endotoxin mediated microglial activation. In the wildtype animals endotoxin treatment was associated with a trend towards higher $\alpha M\beta 2$ integrin immunoreactivity in all forebrain regions: cortex ($p=0.11$) hippocampus ($p=0.18$) and striatum ($p=0.12$) but this did not reach statistical significance. There, however, was a significant increase in $\alpha M\beta 2$ integrin immunoreactivity the thalamus. In animals with deletion of the TNF cluster, endotoxin treatment did not result in an increase in $\alpha M\beta 2$ integrin immunoreactivity in any region compared with saline treated controls. * $p<0.05$ (ANOVA with posthoc Tukey), NS – Non Significant

As shown in the previous study, 12 hours after endotoxin administration there was a significant increase in ICAM1 immunoreactivity in the wildtype animals ($p<0.05$). In animals with a deletion of the TNF cluster no increase in ICAM1 immunoreactivity 12 hours after endotoxin application (see Figure 5.9).

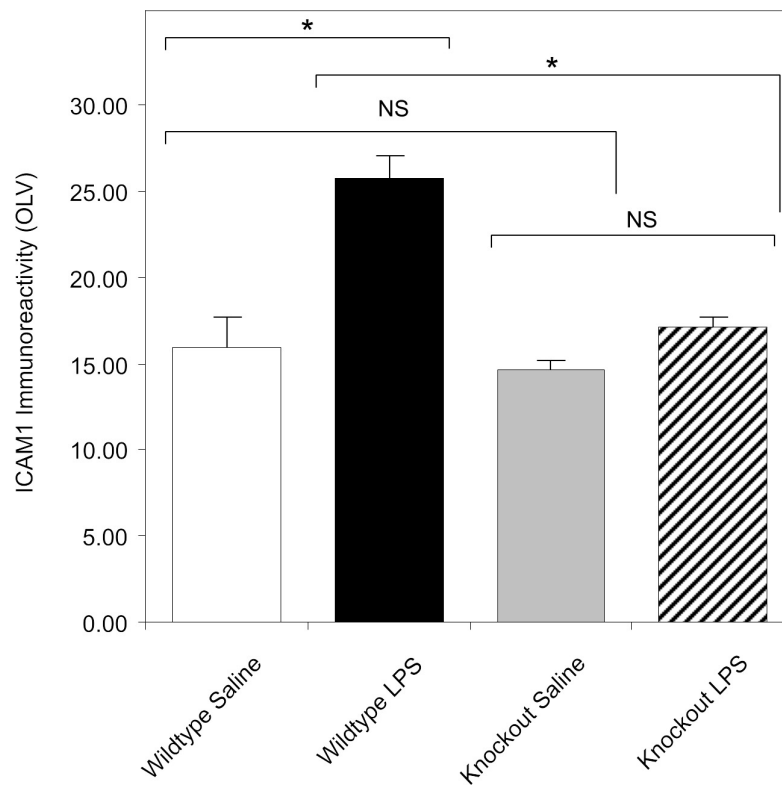


Figure 5.9: Effects of TNF cluster deletion on Endotoxin-mediated vascular endothelial ICAM1 upregulation. Endotoxin pretreatment resulted in a significant increase in ICAM1 immunoreactivity in wildtype animals. No LPS mediated ICAM1 upregulation was seen in the animals with deletion of the TNF cluster. * $p < 0.05$ (ANOVA with posthoc Tukey); NS – Non Significant.

When the different forebrain regions were analysed independently there was significant endotoxin-mediated up-regulation of ICAM1 in wildtype animals in the cortex, hippocampus, striatum and thalamus ($p < 0.05$). Here again, deletion of the TNF cluster abolished endotoxin-mediated ICAM1 up-regulation (see Figure 5.10).

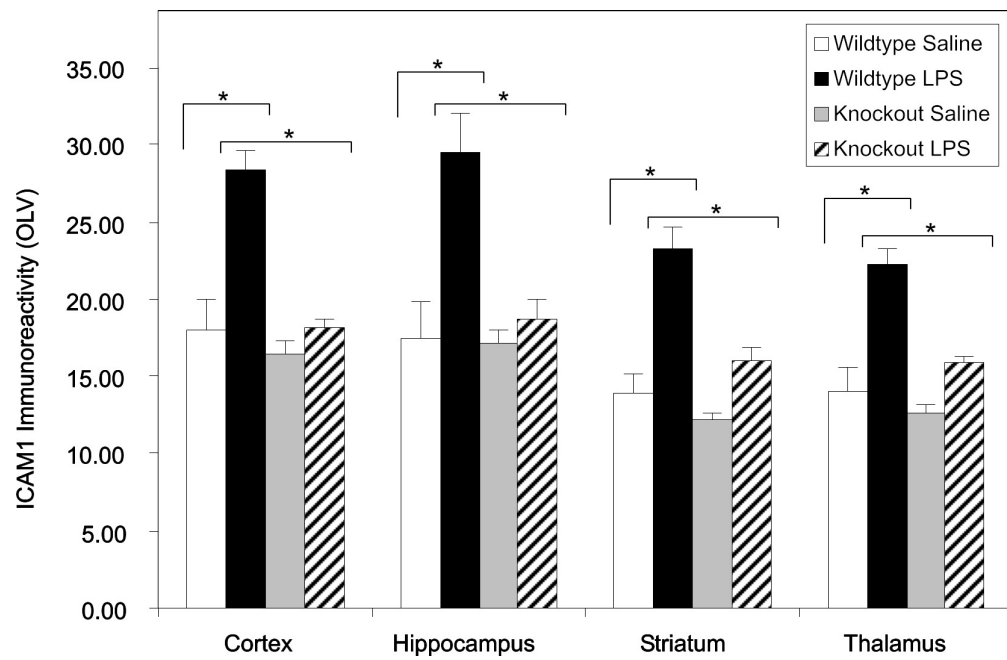


Figure 5.10: Regional Effects of TNF cluster deletion on LPS-mediated vascular Endothelial ICAM1 up-regulation. In the wildtype animals, endotoxin treatment resulted in significantly increased ICAM1 immunoreactivity in all forebrain regions analysed. In animals with deletion of the TNF cluster there was no increase in ICAM1 immunoreactivity in any of the regions compared to saline-treated control. * $p < 0.05$, all other comparisons non-significant (ANOVA with post-hoc Tukey)

To study the effect of TNF cluster deletion on endotoxin-mediated sensitisation to hypoxia-ischemia 109 animals at postnatal day 7 underwent hypoxia-ischemia 12 hours after pre-treatment with $0.3 \mu\text{g/g}$ endotoxin. There was no significant difference in weight between the animals pretreated with saline or LPS at the outset. The values were: $3.81\text{g} \pm 0.10\text{g}$ for the saline-treated group ($n=54$) versus $3.82 \pm 0.10\text{g}$ for the LPS-treated group ($n=55$) $p=0.97$ by Student t-test. Overall 35 animals died during the surgery or hypoxia (16 LPS, 19 saline). There was no significant difference in weight at time of perfusion. The values were $4.86 \pm 0.16\text{g}$ after saline treatment ($n=36$) versus $4.53 \pm 0.15\text{g}$ after LPS treatment ($n=38$) $p=0.15$ by Student t-test. Of the 74 animals surviving to perfusion genotyping revealed 14 wildtype (19%), 18 knockout (24%) and 42 heterozygotes (57%) for the TNF cluster. Once stratified for genotype, there was no

difference in the weight of animals at the time of culling in any of the 4 groups: saline wildtype, $4.75 \pm 0.55\text{g}$ (n=8); saline knockout, $4.83 \pm 0.51\text{g}$ (n=4); LPS wildtype, $4.90 \pm 0.52\text{g}$ (n=6); LPS knockout, $4.71 \pm 0.22\text{g}$ (n=14). As seen previously, pretreatment with endotoxin 12 hours prior to hypoxia-ischemia resulted in a highly significant increase in infarct volume from 3% to 22% ($p < 0.05$) in the wildtype animals. However, in the animals with a deletion of the TNF cluster, there was no overall increase in infarct volume with endotoxin pretreatment 1% saline v 5% LPS ($p = 0.58$). Overall, there was no difference in the infarct volume in the saline-treated animals between wildtype and animals with deletion of the TNF cluster 1% v 3% ($p = 0.79$) (see Figure 5.11).

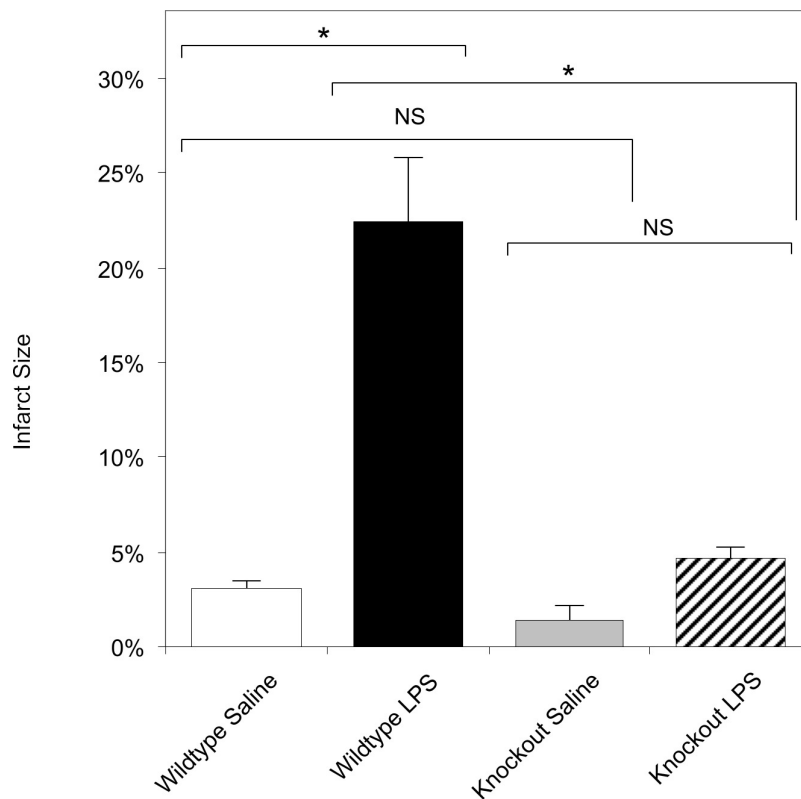


Figure 5.11: Effect of TNF cluster Deletion on Endotoxin-Mediated Sensitisation to Hypoxia-Ischemia. In the wildtype, endotoxin pretreatment resulted in a dramatic increase in brain injury following hypoxia-ischemia, as seen previously. In the animals with deletion of the TNF cluster there was no increase in injury with endotoxin pretreatment compared with hypoxia-ischemia alone. * $p < 0.05$ NS- non significant, (ANOVA with post-hoc Tukey).

When the forebrain regions were analysed separately, endotoxin pretreatment resulted in increased infarct volume in the cortex from 2% to 24% ($p < 0.05$) and striatum from 5% to 20% ($p < 0.05$) in the wildtype animals. The same trend was seen in the hippocampus 5% to 35% ($p = 0.06$) and thalamus 4% to 9% ($p = 0.56$) but these did not reach statistical significance. Here again, deletion of the TNF cluster resulted in an abolition of the sensitising effects of endotoxin to subsequent hypoxia-ischemia in all regions analysed: cortex, 0% to 4%; striatum, 4% to 6%; hippocampus, 10% to 13%; and thalamus, 5% to 4% (see Figure 5.12).

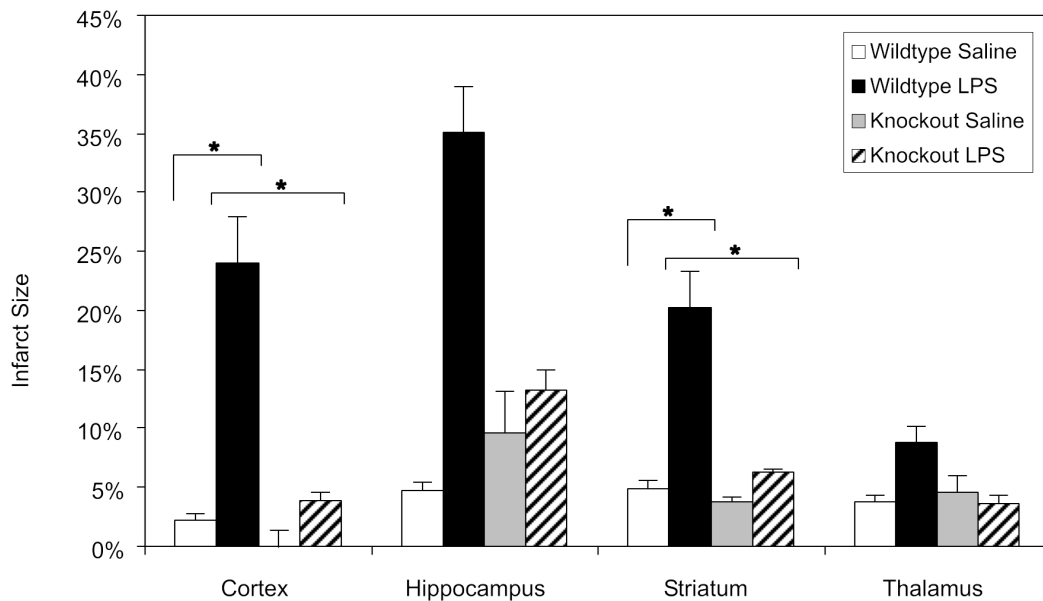


Figure 5.12: Regional Effects of Endotoxin from *E.coli* (serotype 055:B5) pretreatment prior to Hypoxia-ischemia. Endotoxin pretreatment resulted in a significant increase in infarct volume in the cortex and striatum of wildtype animals. The same trend was seen in the hippocampus ($p = 0.06$) and thalamus ($p = 0.56$). No increase in infarct volume was seen with endotoxin pretreatment in the animals with a knockout for the TNF cluster (ANOVA with posthoc Tukey)

Timing of the Effects of TNF family on Endotoxin-Mediated Sensitisation

Finally the mRNA levels of $TNF\alpha$, $LT\alpha$ and $LT\beta$ were quantified in the brains of animals 0-24 hours after pure hypoxia-ischemia or hypoxia-ischemia with pretreatment with endotoxin 12 hours prior to onset of hypoxia-ischemia. There was a significant increase in the levels of $TNF\alpha$ mRNA 2 – 24 hours after hypoxia-ischemia ($p < 0.05$) and 2-12 hours after combined endotoxin and hypoxia-ischemia ($p < 0.05$). There was a trend towards a reduction in the TNF mRNA levels 0-4 hours after hypoxia-ischemia in animals pretreated with endotoxin but this did not reach statistical significance (0 hours, $p = 0.55$; 2 hours, $p = 0.19$; 4 hours, $p = 0.33$ Student t-test) (see Figure 5.13).

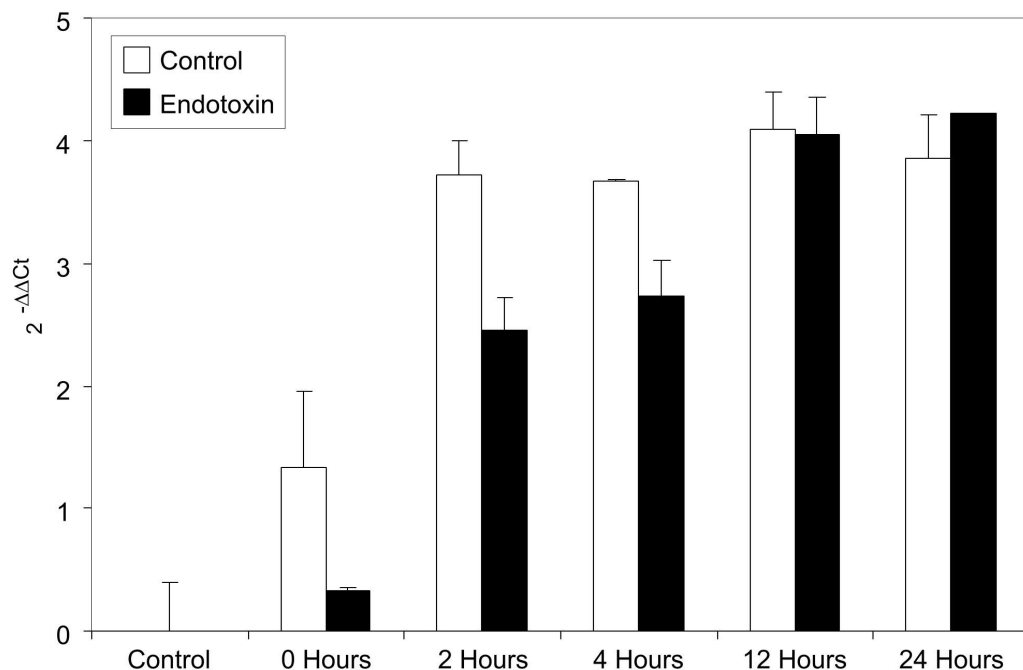


Figure 5.13: Relative quantification of $TNF\alpha$ mRNA in the hours following hypoxia-ischemia alone (control) or with endotoxin pretreatment 12 hours prior to hypoxia-ischemia (endotoxin). Note that, although levels of $TNF\alpha$ mRNA are increased in all groups following hypoxia-ischemia there is a trend towards a smaller increase in those animals pretreated with endotoxin prior to hypoxia-ischemia at 0, 2 and 4 hours.

Similarly, there was a trend towards an increase in the levels of lymphotoxin β mRNA 0 – 12 hours after hypoxia-ischemia (0 hours, $p=0.43$; 2 hours, $p=0.1$; 4 hours, $p=0.07$; 12 hours, $p=0.06$) and a significant increase after 24 hours $p<0.05$. After combined endotoxin and hypoxia-ischemia a trend to increased levels was also seen but this did not reach statistical significance at any time-point. Here again there was a trend towards a reduction in the lymphotoxin β mRNA levels 2 - 4 hours after hypoxia-ischemia in animals pretreated with endotoxin compared to those exposed to hypoxia-ischemia alone (2 hours, $p=0.11$; 4 hours, $p=0.06$ by Student t-test), see Figure 5.14. Lymphotoxin α mRNA was not reliably detected in any animals following either hypoxia-ischemia or with endotoxin pretreatment 12 hours prior to hypoxia-ischemia

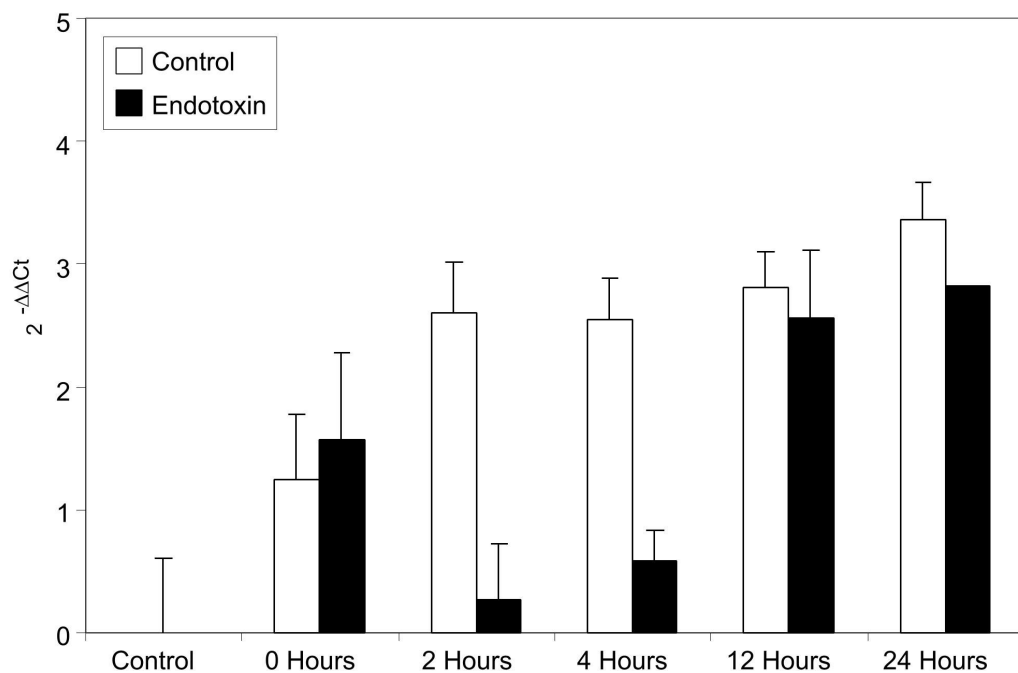


Figure 5.14: Relative quantification of Lymphotoxin β mRNA in the hours following hypoxia-ischemia alone (control) or endotoxin pretreatment 12 hours prior to hypoxia-ischemia (endotoxin). Note that although levels of lymphotoxin β mRNA are increased in all groups following hypoxia-ischemia there is a trend towards a smaller increase in those animals pretreated with endotoxin prior to hypoxia-ischemia at 2 and 4 hours.

Discussion

This study suggests a key role of the TNF family of cytokines in endotoxin-mediated sensitisation of the developing brain to subsequent hypoxia-ischemia in a mouse model of neonatal hypoxic-ischemic brain injury. In the wildtype animal, systemic administration of endotoxin resulted in the up-regulation of TNF, lymphotoxin α and lymphotoxin β mRNA in the hours following endotoxin administration. Whilst these results did not reach statistical significance this is likely to be due to the small sample sizes used in the current study. It must, however, be recognised that the presence of mRNA does not necessarily indicate the presence of protein, especially if there is disruption of protein synthesis in areas of damaged brain. However it can be speculated that the levels of these pro-inflammatory cytokines are indeed raised in the neonatal brain in the hours following endotoxin administration. Furthermore it is of particular note that there may be a biphasic response in mRNA levels following endotoxin administration and it is interesting to speculate that this may correspond to the biphasic nature of sensitisation occurring in the hours following hypoxia-ischemia demonstrated by other authors (Eklind et al 2006). Additionally, it is likely that the timing of changes in mRNA levels do not correspond exactly to the timing of changes in protein levels due to the rate of protein synthesis, and it is therefore possible that the apparent peak in mRNA levels seen at 4 hours corresponds to the peak in sensitisation shown in the current study 12 hours after endotoxin administration. Whilst the changes in mRNA levels do not reach statistical significance, deletion of the TNF cluster prevents the increase in microglial α M β 2 integrin immunoreactivity, and the increase in vascular endothelial ICAM1 immunoreactivity previously demonstrated following endotoxin administration. This suggests that these cytokines are upregulated following systemic endotoxin administration, and that these changes were mediated by a TNF family dependent pathway.

The current and previous studies have demonstrated that endotoxin has a sensitising effect on the developing brain to subsequent hypoxia-ischemia. In the previous chapter this effect was seen to be maximal when endotoxin was given 12 hours prior to hypoxia-ischemia. In the current study, the role of the TNF family of cytokines was explored at this time-point of maximal sensitisation. Here, deletion of the entire TNF family of cytokines was shown to completely abolish endotoxin mediated sensitisation of the developing brain to subsequent hypoxia-ischemia, again suggesting that this sensitisation is mediated by a TNF family-dependent pathway. This is in keeping with the findings of other authors that endotoxin mediated cellular toxicity is dependent on both microglia (Lehnardt et al 2003) and on a TNF dependent pathway (Li et al 2008). It is of note that following hypoxic-ischemic encephalopathy high levels of TNF α have previously been detected within the blood and CSF. Moreover, the presence of a high TNF α CSF / plasma ratio suggests that this cytokine may indeed be produced in the brain of term neonates with hypoxic-ischemic encephalopathy (Silveira et al 2003). Taken together, these findings provide strong evidence that endotoxin-mediated inflammatory sensitisation is a TNF family-dependent pathway. This conclusion is further supported by the clinical studies demonstrating increased incidence of spastic cerebral palsy in neonates exposed to both the pro-inflammatory environment of chorioamnionitis, and hypoxia-ischemia (Nelson et al 1998). It is of interest that no difference in infarct volume was detected in the current study between wildtype animals and those with the TNF cluster deletion when exposed to hypoxia-ischemia alone. There are at least two possible explanations for this finding. Firstly that there was a reduction in injury in the TNF cluster deletion animals, because of the small infarct size detected in the hypoxia-ischemia alone groups, the methodology was not sensitive enough to detect this difference. Alternatively, whilst it would be expected that there would be increased levels of TNF cytokines following hypoxia-ischemia in this model these cytokines may not have a direct role in neuronal injury and, hence, no difference in infarct volume would be expected between the groups.

Taken together, these data suggest a number of possible mechanisms by which endotoxin may sensitise the developing brain to subsequent hypoxia-ischemia. First endotoxin may enter the brain bind to TLR4 on microglia resulting in local production of cytokines, including the TNF family, which, in turn, further activate microglia, vascular endothelium and results in enhanced susceptibility to hypoxia-ischemia (see Figure 5.15 A). Alternatively, endotoxin may have a predominantly systemic role resulting in raised systemic levels of pro-inflammatory cytokines, including the TNF family. These mediators may then cross the blood-brain barrier and result in microglial and vascular endothelial up-regulation for a period of 4-12 hours after endotoxin administration (see Figure 5.15 B). If the brain is then exposed to hypoxia-ischemia within this critical period the up-regulated vasculature and activated microglia facilitate enhanced neuronal injury through various mechanisms. The presence of raised levels of mRNA for the TNF family of cytokines demonstrated in the current study may give further evidence for the first hypothesis. It is of particular note that, in the hours following hypoxia-ischemia, levels of mRNA for TNF and $LT\beta$ appear to be lower in animals pre-treated with endotoxin compared to those pre-treated with saline. This finding may suggest that the action of the TNF family of cytokines is predominantly in the period between endotoxin administration and hypoxia-ischemia and not in the hours following hypoxia-ischemia. This further suggests that the cellular activation previously demonstrated in the hours after endotoxin administration may be critical to the observed sensitisation phenomenon.

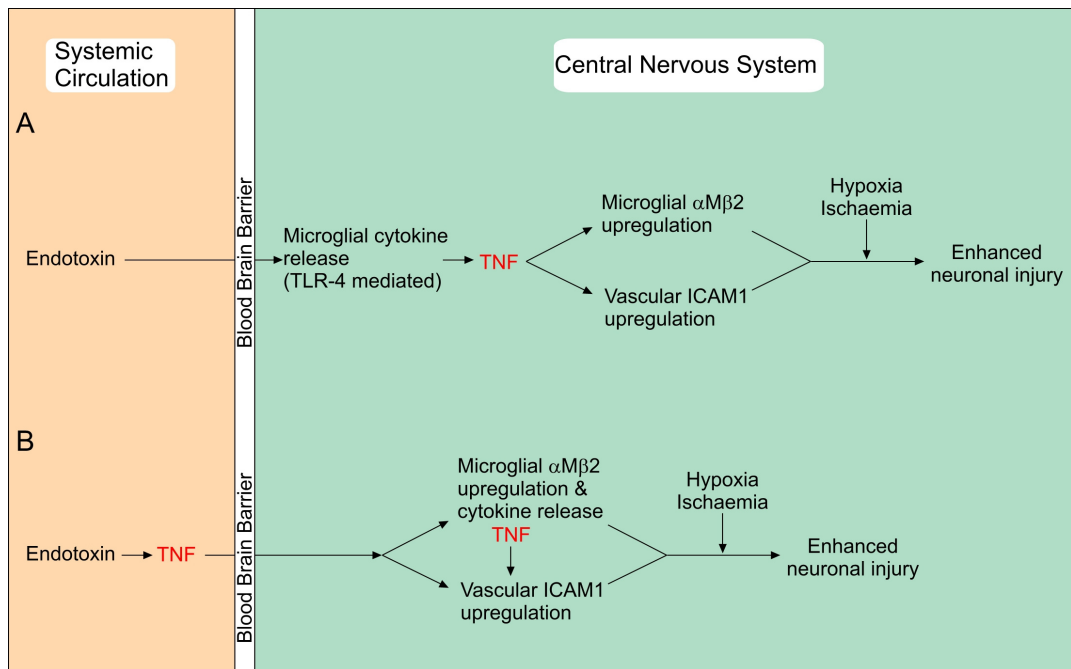


Figure 5.15: Potential mechanisms for Systemic Endotoxin-Mediated Sensitisation of the Neonatal Brain to Hypoxia-ischemia. (TNF: tumour necrosis factor, TLR-4: Toll like receptor 4)

Microglia have previously been shown to be activated by tumour necrosis factor through the TNF receptor 1, resulting in further production of TNF and other inflammatory cytokines (Kuno et al 2005). The authors suggest that this apparently autocrine activation may represent a mechanism for prolonged microglial activation following stimulation. Interestingly, TNF-mediated activation of microglia as quantified by CD11b (α M β 2 Integrin) mRNA correlates with production of mRNA for TNF p75 receptor following transient middle cerebral artery occlusion (Lambertson et al 2007). This provides further evidence that TNF, possibly from microglia, is able to further stimulate microglia with the upregulation of α M β 2 integrin. Furthermore, deficiency in TNF receptors 1 and 2 using double knockout mice has been shown to result in suppression of microglial activation following exposure to the neurotoxin MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) (Sriram et al 2006). A recent study has also demonstrated that in an *ex-vivo* model using neonatal hippocampal brain slices, deletion of TNF receptor 1 prevented endotoxin-mediated sensitisation to oxygen

glucose deprivation (Markus et al 2008). These observations provide further support for the hypotheses shown in Figure 5.15.

The identification of pro-inflammatory cytokines as critical in the synergy between infection and hypoxia-ischemia further raises the potential role of individual polymorphisms in the cytokine network. Such polymorphisms which make an individual more prone to a pro-inflammatory cytokine response may, as described earlier, be involved in both the onset of preterm labour and in sensitising the fetal brain to hypoxia-ischemia. In the context of the current study, the identification of an association between polymorphisms in the maternal lymphotoxin α gene and increased risk of the development of cerebral palsy is of particular note (Nelson et al 2006).

Conclusions

Low dose systemic endotoxin sensitises the developing brain to subsequent hypoxia-ischemia through a pathway dependent on the TNF family of cytokines. Levels of the mRNA for these cytokines are raised in the brain of neonatal mice in the hours after systemic endotoxin administration. Furthermore deletion of the genes coding for the TNF family of cytokines also prevents endotoxin-mediated up-regulation of vascular endothelium and microglia within the central nervous system.

Chapter 6: Intracellular pH in Perinatal Hypoxic-ischemic Brain Injury

Introduction

It is recognised that the most direct assessment of fetal wellbeing during labour comes from assessment of fetal blood pH, carbon dioxide and base deficit / excess. This potentially can be performed during the latter stage of labour by fetal scalp blood sampling, or in the immediate postnatal period from the umbilical cord blood. It is also recognised that the metabolic component of any acidosis (using base deficit) has the strongest association with long-term neurological outcome (ACOG Committee Opinion 2006). Clinical studies have shown that moderate or severe postnatal complications occur in infants with an umbilical arterial base deficit of more than 12mmol/L, and to be of increasing severity with increasing base deficit (Low et al 1997). Consistent with the findings of acidosis in the circulation, an intracellular brain acidosis has also been demonstrated during hypoxia-ischemia using proton magnetic resonance spectroscopy in the newborn piglet which was proportional to the rise in intra-cerebral lactate (Penrice et al 1997). This rise in lactate is a consequence of anaerobic glycolysis that occurs during periods of hypoxia-ischemia. The degree of metabolic acidosis in the blood and within the brain is related to the magnitude of the hypoxic ischemic insult, and the neurodevelopmental outcome in the neonate. Interestingly, however, acidosis *in vitro* using mixed neuronal and glial cell cultures only resulted in cell death on exposure to lactic acidosis of pH 5.2 for 1 hour (Goldman et al 1989), much more severe and prolonged than would be compatible with neonatal survival. These observations suggest that acidosis itself, whilst a marker of injury severity, may not be the direct cause of neuronal injury.

The effects of reperfusion on brain intracellular pH (pH_i) have been extensively studied using animal models of cardiac arrest and resuscitation which produces reversible total cerebral ischemia. These studies have shown that, following reperfusion, there is a rapid return of brain pH_i to normal (LaManna 1996). Following transient carotid occlusion in adult rats, there is also a rapid normalisation of brain pH_i during reperfusion, although the time required appears to relate to the magnitude of the insult (Hoffman et al 1994). In the reperfusion phase, following this normalisation of pH_i , there is a period during which the intracellular pH of the brain is relatively alkalotic compared to baseline; this is termed rebound alkalosis (Mabe et al 1983, LaManna 1996). Further studies using transient forebrain ischemia have demonstrated that the time-course of both the onset and duration of rebound alkalosis is dependent on the magnitude of the initial insult (Chopp et al 1990). This rebound alkalosis has also been demonstrated in human neonates following hypoxic-ischemic encephalopathy (Robertson et al 1999). Interestingly, the magnitude and persistence of this rebound alkalosis has been shown to correlate with the long-term neurodevelopmental outcome for the infant (Robertson et al 2002). These observations may suggest that the magnitude of acidosis predicts the magnitude and duration of alkalosis, which, in turn, is a determinant of long-term neurodevelopmental outcome. From an experimental perspective, however, the rodent models demonstrating rebound alkalosis during reperfusion after hypoxia-ischemia have all been carried out in adult animals, and the changes in pH_i in rodent models of neonatal hypoxia-ischemia have not been studied to date. The use of 3- amino-*m*-dimethylamino-2-methylphenazine hydrochloride (neutral red) (see Figure 6.1) as an intracellular indicator of pH in rodent brain was first described in 1984 (LaManna & McCracken 1984). Neutral Red is a relatively non-toxic and blood-brain barrier permeable dye which is preferentially taken up into the intracellular brain compartments after systemic (intrapertoneal) injection. It absorbs visible light in 2 different wavelength maxima depending on pH; the protonated acid form absorbs primarily at 530 - 550 nm (green), the free base form at 430 - 470 nm (blue) (see Figure 6.2).

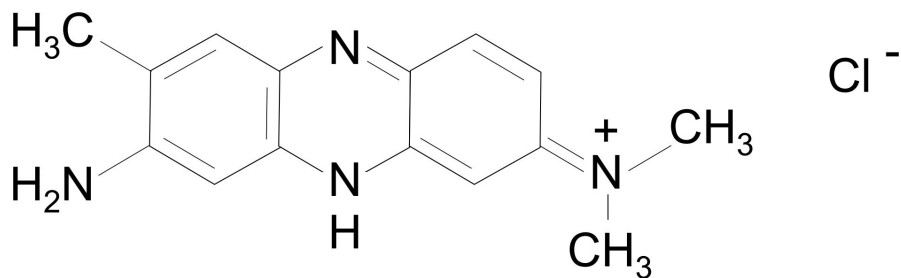


Figure 6.1: Chemical structure of 3- amino-*m*-dimethylamino-2-methylphenazine hydrochloride (neutral red)

Although first studies using neutral red were done using differential absorption of transmitted light at 550/470 on tissue slices *in vitro* (LaManna et al 1980), a similar technique based on pH-selective Neutral Red absorption was adapted to light spectra reflected from frozen tissue derived from animals injected with Neutral Red (Hoffman et al 1994). Thus, light reflected from tissue with normal or basic pH would show preferential reduction in the blue bandwidth, and light reflected from tissue with an acidotic pH would show preferential reduction in the in green bandwidth.

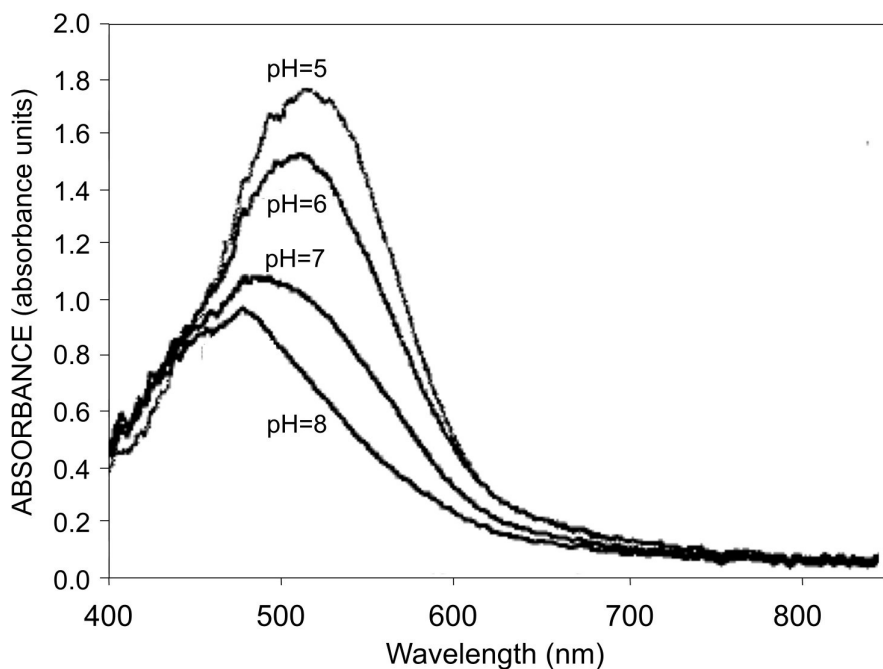


Figure 6.2: Absorbance of 3- amino-*m*-dimethylamino-2-methylphenazine hydrochloride (neutral red) at different pH (From Arregui et al 2003 © 2003 IEEE)

Mathematically, this reduction in the amount of reflected light (R) can be treated in a way similar to the more familiar, absorption-mediated decrease in transmitted light (T). Considerable help with the derivation of the following mathematical formulae was given by Professor Raivich. In a very thin slice of absorbing medium, the reduction in transmitted light – dT is proportional to light intensity, slice thickness (dx), and the concentration (c) and specific extinction coefficient (ε) of the absorbing agent, or

$$-dT = T * c * \epsilon * dx, \quad \text{or,} \quad -dT/T = c * \epsilon * dx \quad (\text{formula 6.1})$$

Integrating this over total depth (x) of absorbing medium gives rise to:

$$-\int_{T_0}^{T_x} T^{-1} dT = \int_0^x c * \epsilon * dx, \quad \text{or} \quad \ln(T_x/T_0) = -c * \epsilon * x \quad (\text{formulas 6.2, 6.3})$$

or in reverse form to:

$$T_x = T_0 * e^{-c*\epsilon*x} \quad (\text{formula 6.4})$$

In other words, specific absorption (c*ε) is proportional to the natural logarithm of transmitted (T_x) to initial light intensity (T₀). As transmitted light passes through many thin optical layers, a reverse process takes place, causing reflection of a fraction of transmitted light. Neglecting surface reflection and assuming a simple proportional factor (r_p) between reflected (R) and transmitted (T) light, light reflection becomes a function of T(x) and e^{-c*ε*x}, integrated over medium depth of x. T(x) already contains e^{-c*ε*x}, but a second term needs to be included, as some of the reflected light will become absorbed on its way back to the surface of the object:

$$R = r_p * \int_0^x T(x) * e^{-c*\epsilon*x} dx = r_p * T_0 \int_0^x e^{-2*c*\epsilon*x} dx \quad (\text{formula 6.5})$$

In the final form, this becomes:

$$R = -r_p/(c*\epsilon) * T_0 * \left| e^{-2*c*\epsilon*x} \right|_0^x = T_0 * r_p/(2*c*\epsilon) * \left| 1 - e^{-2*c*\epsilon*x} \right| \quad (\text{formula 6.6})$$

Assuming that almost all light is absorbed within the tissue and does not bounce from the back-end of the tissue back to the front (i.e. $\lim e^{-2*c*\epsilon*x} \rightarrow 0$), the formula for reflected light obtains the form of:

$$R = T_0 * r_p/(2*c*\epsilon), \text{ or simply } R = T_0 * r_p/A \quad (\text{formula 6.7})$$

with A representing the previous double absorption term $2*c*\epsilon$. In this context, light reflected within the tissue is inversely proportional to tissue absorbance (A_T). Assuming that the absorbance due to Neutral Red (A_{NR}) is simply additive, i.e. does not interfere or synergise with tissue absorbance, reflected light from tissue containing Neutral Red (R_{NR}) would be defined by the formula:

$$R_{NR} = T_0 * r_p/(A_T + A_{NR}) \quad (\text{formula 6.8})$$

and reflected light (R_T) from naïve tissue not containing Neutral Red as:

$$R_T = T_0 * r_p/A_T \quad (\text{formula 6.9})$$

Under these conditions, the ratio of reflected light from tissue not containing (R_T) to tissue containing Neutral Red (R_{NR}) will simply represent the ratio of $A_T + A_{NR}$ to A_T :

$$R_T/R_{NR} = (A_T + A_{NR})/A_T \text{ or } R_T/R_{NR} = 1 + A_{NR}/A_T \quad (\text{formulas 6.10, 6.11})$$

and subtracting 1 on both sides, this resolves into:

$$(R_T - R_{NR})/R_{NR} = A_{NR}/A_T \quad \text{(formula 6.12)}$$

In the context of current work, measuring the relative reduction in reflected light intensities at 550/470nm in tissue stained *in vivo* with neutral red, compared with unstained, “naïve” tissue, thus provides a straightforward approach to identify current tissue pH_i and/or relative changes in acid or base balance by comparing this absorbance to that in brain homogenates of known pH mixed with Neutral Red. To date this technique has been predominantly used to demonstrate changes in pH_i in the adult rat following cardiac arrest and resuscitation.

While this technique does allow regional changes in pH_i to be deduced, for example in cortex, striatum and hippocampus (Crumrine et al 1991), it has two important limitations. For reasons of consistency one needs to compare the same region treated in exactly the same way, in naïve and Neutral Red injected animals, doubling the number of animals needed. Moreover, it does not allow a high degree of structural resolution, as all calculations are performed on the basis of comparing regions in 2 different sets of brains and not on the basis of data coming from a single image.

Here, a two pronged approach was taken, studying quantitative changes in hypoxic and post-hypoxic brain using a conventional technique described above, and by developing methodology to allow generation of high resolution images on brain pH_i based on data provided by simultaneous RGB 3 channel recordings of frozen brain sections from animals injected with Neutral Red. Since Neutral Red absorbs only weakly in the part of the visible spectrum recorded by the red channel in the Sony AVT-Horn RGB video camera (hence the name, Neutral Red), a modified function of reflected red light intensity could potentially provide a useful surrogate marker for the “naïve” intensity of light illumination and tissue absorbance in green and blue channels,

in order to generate high resolution pH_i maps based on RGB channel recordings from individual brain sections. In the current study, both of these techniques were then used to provide quantitative data, and sufficient structural resolution to visualise specific local as well as global pH_i changes after hypoxia-ischemia in the P7 mouse using the Rice-Vannucci model of neonatal hypoxia-ischemia (Rice et al 1981).

Materials and Methods

Surgery

As described previously, experiments were performed at postnatal day seven (P7) on C57/Bl6 mice (see Materials and Methods). Under isoflurane anaesthesia, the left carotid artery was permanently ligated and, after two hours of recovery, the animals were exposed to 8% oxygen in 92% nitrogen for 15 to 90 minutes.

Neutral Red

100mg of 3- amino-*m*-dimethylamino-2-methylphenazine hydrochloride (neutral red) was diluted to 10ml in sterile 0.9% saline to give a 1% solution and then filtered prior to use. Animals received a 200 µl intraperitoneal injection of neutral red 2 hours prior to tissue collection. Control animals for measuring baseline light reflection without Neutral Red were injected with saline.

Tissue Preparation

pH_i measurements were made in naïve animals, immediately after carotid occlusion (without hypoxia), after 60 minutes hypoxia alone (without preceding carotid occlusion) and after combined carotid occlusion and 15-60 minutes hypoxia. Re-oxygenation was studied after carotid occlusion and 90 minutes hypoxia at 0h, 1h, 2h, 3h, 4h, 6h, 12h and 24h of re-oxygenation. Animals were killed by decapitation and their heads snap frozen in isopentane at -20°C on dry ice. Once frozen, the heads were stored at -80°C

until required. To measure pH_i , heads were mounted and cut on a cryostat at -20°C . Starting from the olfactory bulbs photographic images of the cut surface of the brain were taken at $400\ \mu\text{m}$ intervals.

Regional pH_i Quantification

A calibration curve for pH against relative green / blue absorption (A_{550}/A_{470}) was constructed using brain homogenates at known pH and a best fit algorithm defined for the range of pH 5.0 to 8.0 as described previously (LaManna & McCracken 1984). Homogenates of fresh brain tissue in 0.9% saline were prepared by passing the tissue sequentially through 20-gauge (0.9mm), 23-gauge (0.6mm) and 26-gauge (0.45mm) needles. In a microtitre plate (Nunc), $200\ \mu\text{l}$ standard brain homogenate solutions were made containing 10% brain homogenate, in 0.1M phospho-boric acid buffer corrected to known pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) with $15\ \mu\text{l}$ of 0.1% neutral red.

Images of standard brain homogenate solutions, or cut brain surface from Neutral Red or control saline injected animals were captured using a Sony AVT-Horn video camera with narrow bandwidth RGB (red 570-630nm, green 510-570nm, and blue 440-500nm) filters mounted on a Carl Zeiss dissecting microscope illuminated from above with a shadow free, ring fibre optic illuminator (Dolan – Jenner Fibre Optics). Scanned images were imported into Optimas 6.2 image analysis software, mean intensity of reflected light in the green and blue channels in anatomically defined brain regions at every $400\ \mu\text{m}$ interval were measured and averaged for each region using area-size based weighting. The relative absorbance, based on relative reduction in reflected light ($(R_T - R_{NR})/R_{NR}$, formula 6.12) in green and blue channels was determined in comparison to the same brain tissue areas from naïve animals not injected with Neutral Red. Regional tissue pH_i values were deduced based on the green to blue relative absorbance ratio using the best fit algorithm defined from the standard brain homogenates at known pH (see Figure 6.3 and Formula 6.13).

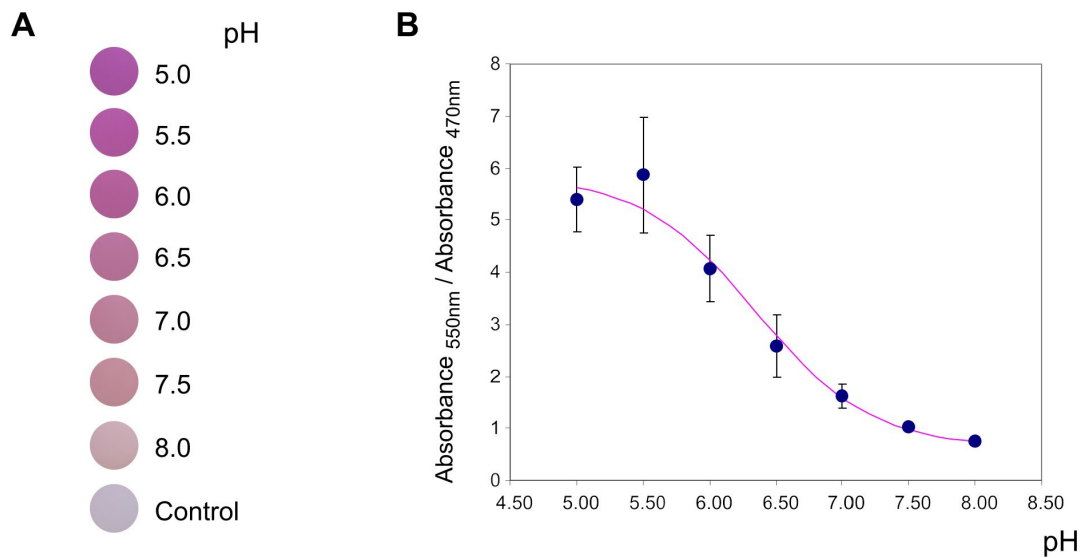


Figure 6.3: Calibration for pH measurement: A. Photographic images of 10% brain homogenate, in 0.1M phospho-boric acid buffer corrected to known pH, with 15 μ l of 0.1% neutral red. B: standard curve (black line) and best fit algorithm (red line) for absorbance at 550nm/ absorbance at 470nm against pH.

$$6.35 + \text{LOG}((5.85 - (A_{550}/A_{470})) / (-5.85 + (A_{550}/A_{470}) + 5.22)) \quad (\text{formula 6.13})$$

Intracellular pH (pH_i) was measured in all regions of the forebrain (olfactory bulbs, cortex, striatum, hippocampus and thalamus). Average measurements were taken from right and left hemispheres from 15 images. Recorded images were subsequently used to determine the colour balance (Green-Red, Blue-Red, Blue-Green) in brain section images from naïve mice without Neutral Red and to create pseudo-colour pH_i maps from mice injected with Neutral Red using Optimas 6.2 image analysis software. Blue-Red (Bb-r) balance for each individual pixel of the image (760x570pixel) was calculated by subtracting the Optical Luminosity Values (OLV, 0-255, 8bit) for Red (OLVred) from that for blue (OLVblue). Similar formulas were also used for the Green – Red and Blue – Green balance. In the case of Green -Red (Bb-r), 30 points were added, as the 8bit Optimas calculations only support operations in the 0-255 range and luminosity in green is lower than in red:

$$Bb-r = OLVblue - OLVred \quad (\text{formula 6.14})$$

$$Bb-g = OLVblue - OLVgreen \quad (\text{formula 6.15})$$

$$Bg-r = OLVgreen + 30 - OLVred \quad (\text{formula 6.16})$$

For processing RGB images from Neutral Red-injected mice for pH_i map data, red-derived Blue (rdB) value was calculated by starting with OLVred/nr and adding the Blue – Red balance, and, in case of red-derived Green (rdG), the Green – Red balance

$$rdB = OLVred/nr + Bb-r \quad (\text{formula 6.17})$$

$$rdG = OLVred/nr - 30 + Bg-r \quad (\text{formula 6.18})$$

The relative absorption in the blue bandwidth (Abl) was calculated according to formula 6.12 by subtracting OLVblue/nr from rdB and dividing it by OLVblue/nr; that for absorption in green (Agr) by subtracting and dividing it with OLVgreen/nr. Relative absorption in green to blue (Agr/bl) was obtained by dividing the two:

$$Abl = (rdB - OLVblue/nr)/OLVblue/nr \quad (\text{formula 6.19})$$

$$Agr = (rdG - OLVgreen/nr)/OLVgreen/nr \quad (\text{formula 6.20})$$

$$Agr/bl = Agr/Abl \quad (\text{formula 6.21})$$

For imaging purposes, the Agr/bl absorption ratio pixel map of the brain sections was multiplied by 32 points, the resulting map converted using a pseudo-colour filter 60 and then compared side by side with images of Neutral Red pH calibration in brain homogenates processed using the same algorithm.

Results

Timecourse of pH_i following Hypoxia-Ischemia

pH_i was measured in 40 animals in total. Measurements were made in naïve animals (n=3) after carotid occlusion alone without hypoxia (n=5), after carotid occlusion and subsequent hypoxia for 15 minutes (n=3), 30 minutes (n=3), 60 minutes (n=5) and 90 minutes (n=5). To examine the changes in pH_i during re-oxygenation, animals that had undergone carotid occlusion and subsequent hypoxia for 90 minutes were studied at 1 hour (n=3), 2 hours (n=2), 4 hours (n=2), 6 hours (n=2), 12 hours (n=2) and 24 hours (n=2) after hypoxia. An additional 3 animals were studied following 60 minutes of hypoxia alone without preceding carotid occlusion.

There was no significant difference in pH_i between naïve animals and following carotid occlusion without subsequent hypoxia in either the left (occluded) hemisphere pH_i 7.07 v 7.05 (p=0.4), or right (non occluded) hemisphere pH_i 7.07 v 7.06 (p=0.8) using the Student t-test. Additionally following carotid occlusion without subsequent hypoxia there was no significant difference in pH_i between the forebrain regions ($F_{4,32}=0.34$ p=0.88), between occluded (left) and non-occluded right hemispheres ($F_{1,32}=0.17$ p=0.71) or between individual animals ($F_{3,32}=0.40$ p=0.99) see Fig 6.4.

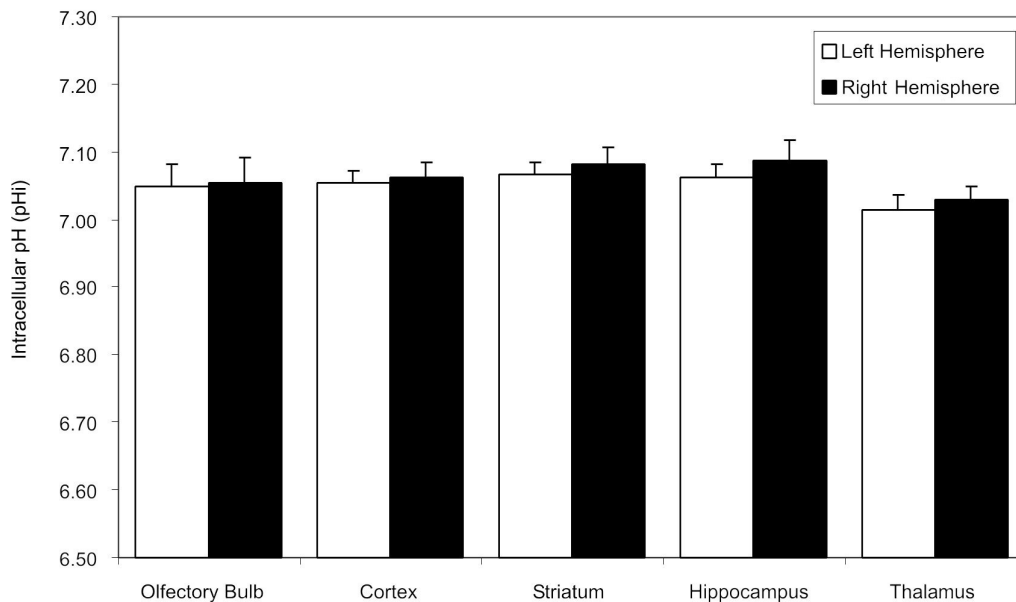


Figure 6.4: Intracellular pH (pH_i) following left sided carotid occlusion prior to hypoxia. There were no significant differences between regions, hemispheres or animals (ANOVA)

Carotid occlusion and hypoxia resulted in a significant fall in pH_i on the occluded side from 7.05 to 6.9 after 15 minutes of hypoxia, 6.9 after 30 minutes of hypoxia, 6.7 after 60 minutes of hypoxia and 6.59 after 90 minutes hypoxia. On the non-occluded side, pH_i was unchanged after 15 minutes hypoxia (7.07 to 7.05; $p=0.13$) and then fell to 7.02 after 30 minutes, 6.88 after 60 minutes and 6.67 after 90 minutes of hypoxia ($p<0.05$; Student t-test). Following re-oxygenation pH_i returned to baseline by 2 hours. By 6 hours of re-oxygenation a significant rebound alkalosis was detected on both the occluded (pH_i 7.15 $p<0.05$) and the non-occluded side (pH_i 7.16 $p<0.05$). The observed rebound alkalosis resolved by 12 hours (see Figure 6.5). Pure hypoxia for 60 minutes without preceding carotid occlusion also resulted in a significant bilateral acidosis with a pH_i of 6.96 in the left hemisphere ($p<0.05$), and pH_i of 6.97 ($p<0.05$) in the right hemisphere. Of note there was no significant difference between the two sides ($p=0.19$ see Figure 6.5).

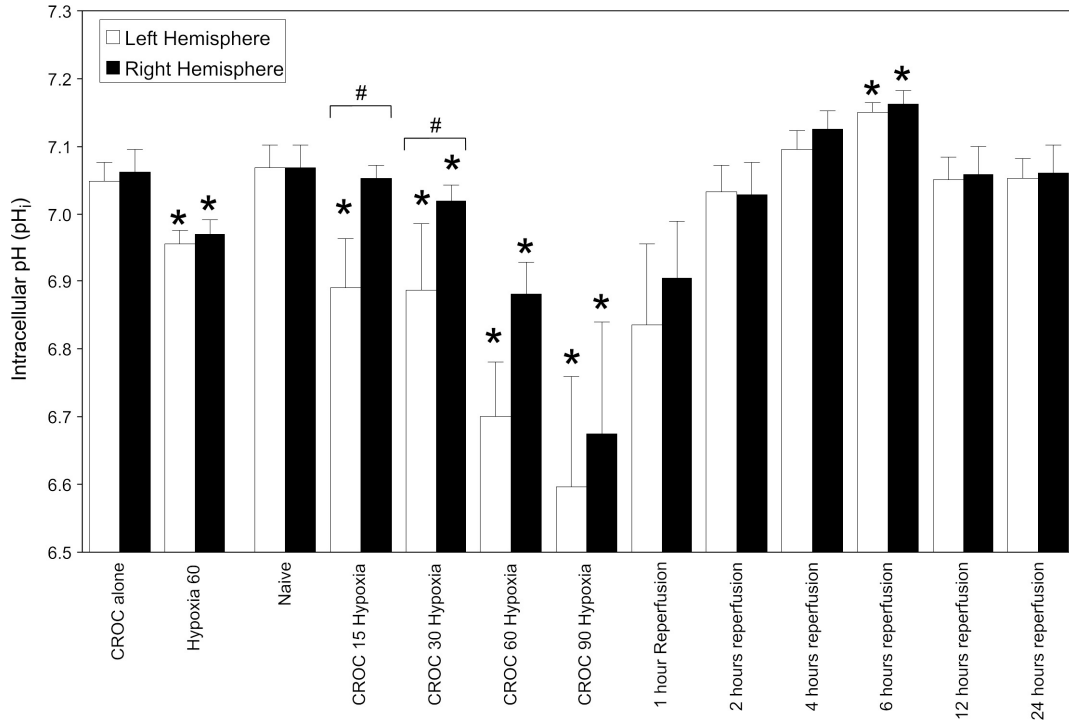


Figure 6.5 Time-course of pH_i changes following hypoxia-ischemia: Following carotid occlusion (CROC) and hypoxia there was a significant intracellular acidosis on the occluded (left) side. On the non-occluded (right side) there is relative preservation of pH_i following short duration of hypoxia (15-30 minutes). However between 60 and 90 minutes of hypoxia, significant acidosis was seen. Following re-oxygenation, pH_i rapidly recovered with a significant rebound alkalosis seen on both sides after 6 hours or re-oxygenation (* $p < 0.05$ compared with baseline, # $p < 0.05$ between occluded and non-occluded side ANOVA).

pH_i Brain Map Imaging

Single image processing technique

Since both basic and acidic forms of Neutral Red absorb only weakly in the red part of the visible spectrum compared with their maxima in blue and green, respectively, the potential of using the red channel from the same section as a surrogate marker for green and blue light reflected from tissue in the absence of Neutral Red was explored. As shown in Figure 6.6 A, colour balance analysis using brain section images from mice subjected to unilateral carotid occlusion and 90 minutes of relative hypoxia with 8% oxygen, showed very similar mean values for the blue to red balance averaged across the brain for the occluded (left) and non-occluded (right side). A similar absence of significant difference was also observed for the average green to red, and blue to green balances between the occluded and non-occluded side for the whole forebrain (see Figure 6.6 A), as well as in individual brain sections taken at different depths. Further analysis also showed very similar levels of intra-image, pixel-to-pixel standard deviations on the occluded, as well as the non-occluded side (see Figure 6.6 B). These inter-pixel standard deviations were also relatively moderate, i.e. at most 4 OLV points or just 10-20% of the absolute colour balance values.

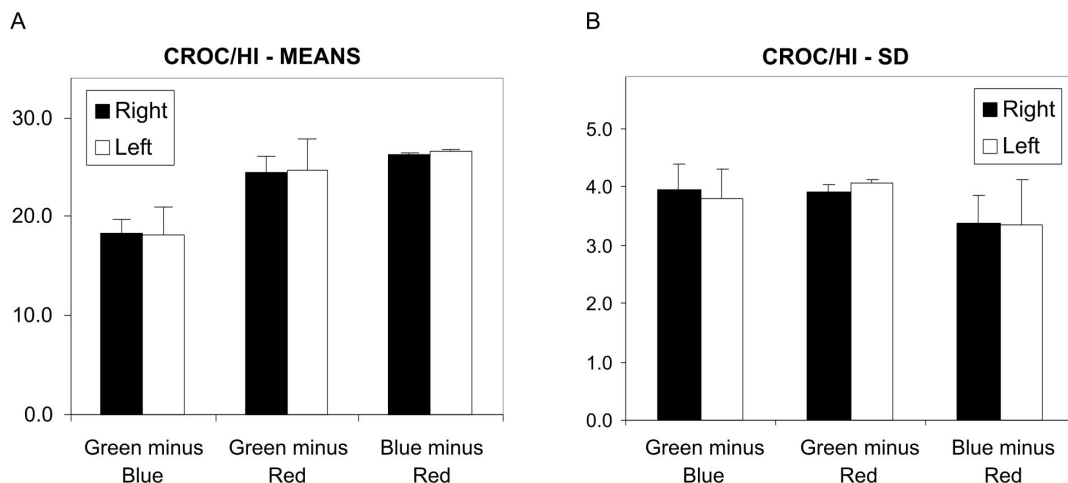


Figure 6.6: Colour Balance following Carotid Occlusion (CROC) and 90 minutes Hypoxia of the Left (occluded) and Right (non-occluded) hemispheres. There was no difference in the average green to blue, green to red, and blue to green balances between the occluded and non-occluded side for the whole forebrain A. Means, B. standard deviations (SD).

To determine whether these balances stay the same or change before, during or after a hypoxic-ischemic insult, the changes in the green-to-red, blue-to-red and green-to-blue colour balance in unoperated control animals, after carotid occlusion alone, after 90 min hypoxia and after 2, 4, 6 and 24 hours re-oxygenation were examined (see Figure 6.6A-C). Again, none of the very minor side-to-side differences were significantly different. As shown in Figure 6.7 A, there was also very little overall effect on the green-to-red balance, on the unoperated or operated side. In contrast, the blue-to-red balance increased, from approx 17.5 points for the right and left side in control animals, peaking at approx 25 points after 90min of hypoxia, decreasing to 13 points at 6h of re-oxygenation and returning to approx 23 points at 24hours (Figure 6.7 B). As expected, reverse changes were observed for the green-to-blue balance, with a peak in control animals and 6h re-oxygenation, and a trough following 90 min of hypoxia (Figure 6.7 C). To avoid systemic errors, and for reasons of consistency, all pH_i map calculations were, therefore, based on settings derived from naïve mice not injected with Neutral Red but that experienced the same hypoxic-ischemic insult and re-oxygenation conditions as the animals injected with Neutral Red.

Pseudocolour pH_i maps for animals following carotid occlusion, carotid occlusion and 15 - 90 minutes hypoxia and following 1 - 24 hours of re-oxygenation were constructed according to formulas 6.17-6.21. As shown in fig. 6.4 and 6.5, animals that underwent left sided carotid occlusion alone without subsequent hypoxia showed no change in pH_i on either the occluded or non-occluded side in the quantitative analysis. The same absence of effect was also observed with pseudocolour maps, showing a symmetrical pH_i throughout all forebrain regions with no apparent effect of the surgical ligation (fig. 6.8 A).

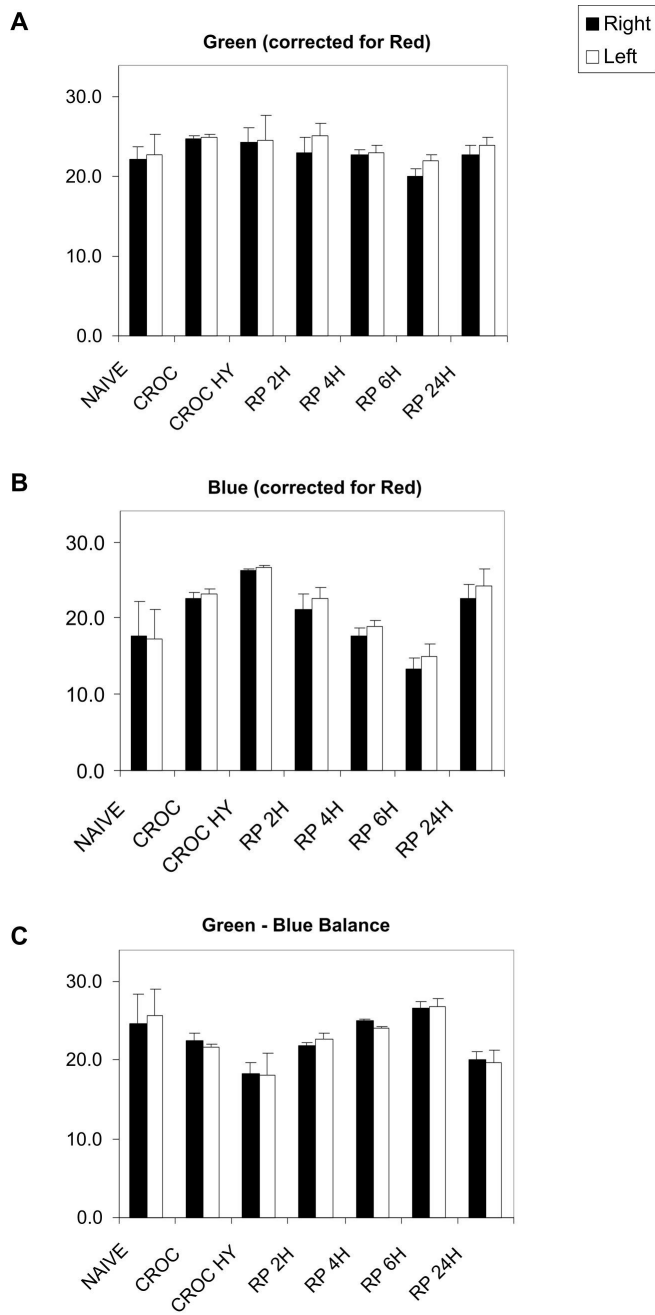


Figure 6.7: A: Green, B: Blue and C: Green-to-Blue Colour Balance in naïve animals, following carotid occlusion (CROC), carotid occlusion and 90 minutes Hypoxia (CROC HY) and following 2-24 hours re-oxygenation. No difference was seen between the Left (occluded) and Right (non-occluded) hemispheres.

The acidic shift in brain pH_i following carotid occlusion and exposure to 8% oxygen increased with time but was already observed at 15 min of hypoxia. At this early time-point, a clear acidic shift was evident in the superficial dorsal cortex on the occluded side, with milder changes in the ventral cortex, ventrolateral thalamus, and throughout the hippocampus on the occluded side. The contralateral hemisphere remained unaffected. At 30 minutes of hypoxia, the acidic shift in the ipsilateral cortex became more widespread, with clear changes in the ipsilateral hippocampus, thalamus and striatum and a start of acidosis in the contralateral superficial dorsal cortex. At 60 minutes, the ipsilateral hemisphere showed generalised acidosis, most severely in the ventral lateral cortex, hippocampus and thalamus. A generalised acidosis also extended to the contralateral hemisphere but with relative preservation of the dorsal cortex, hippocampus and thalamus. At 90 minutes, all forebrain regions showed general acidosis with no obvious difference between the hemispheres, consistent with the previous quantitative analysis (see Figure 6.7 a-e). All re-oxygenation studies were performed after carotid occlusion and 90 minutes of hypoxia at 8% oxygen. One hour of re-oxygenation resulted in a dramatic normalisation of the pH_i throughout the brain, with mild residual acidosis most notable in the ipsilateral posterior cortex hippocampus and thalamus. After 2 hours of re-oxygenation, slight residual acidosis was confined predominantly to the ipsilateral ventral cortex and hippocampus and to a lesser extent the contralateral ventral cortex and hippocampus. After 4 hours of re-oxygenation some rebound alkaline shift is seen in brain pH_i , most notable in the contralateral dorsal cortex and dorsal hippocampus. A similar, but less pronounced, effect was seen in the ipsilateral dorsal cortex and dorsal hippocampus. 6 hours of re-oxygenation was associated with generalised bilateral rebound alkalosis, most apparent in the dorsal cortex and hippocampus. After 12 hours, the general pH_i of the brain had returned to normal. However, there was an apparent relative acidic shift in the ipsilateral dorsal cortex and hippocampus in sections 8-15; the ipsilateral dorsal acidic wedge. By 24 hours, there was a generally symmetrical distribution of pH_i similar to that seen prior to hypoxia, with the reduction in the dorsal acidic wedge (see Figure 6.8 F-K).

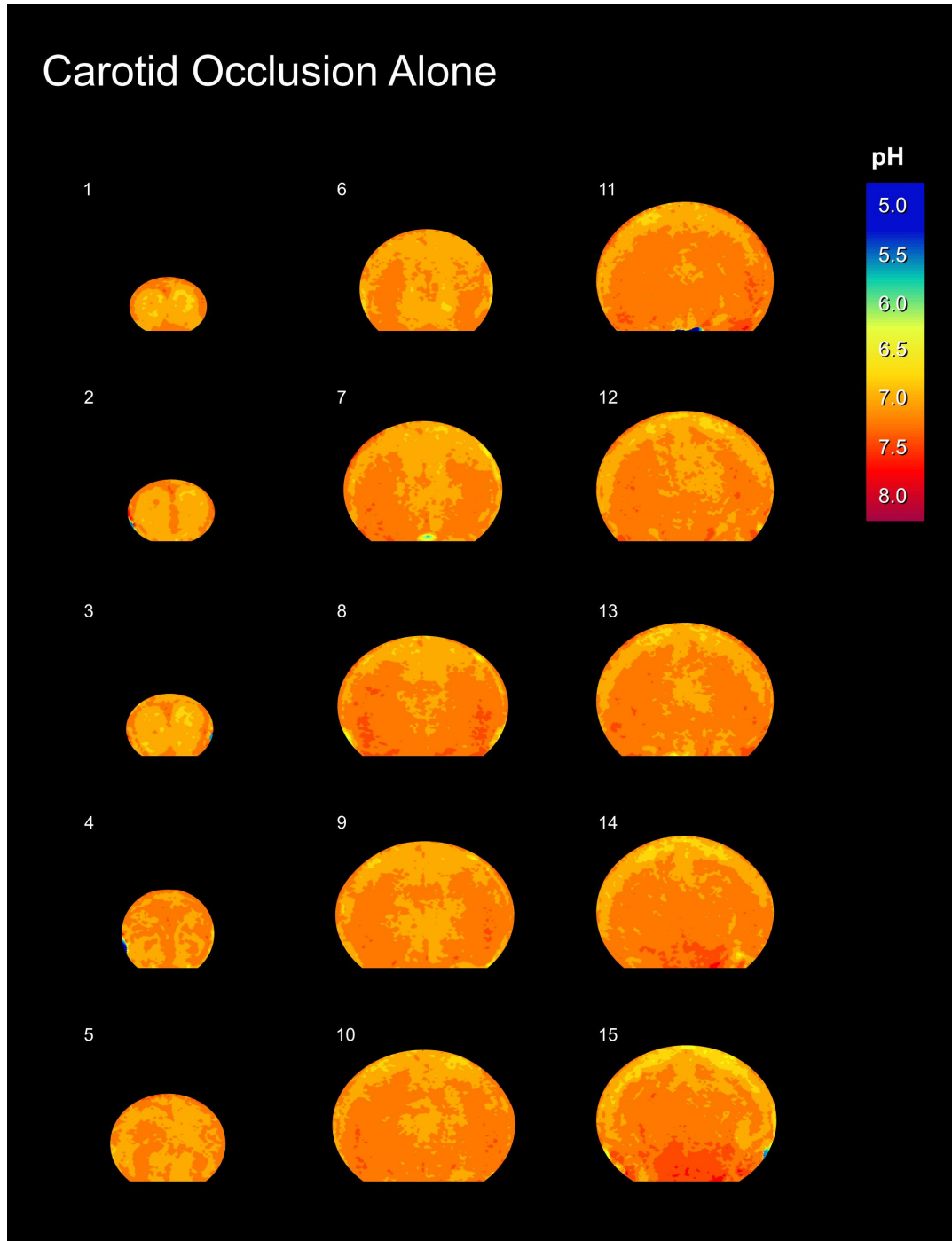


Figure 6.8A: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion alone.

Carotid Occlusion 15 Minutes 8% Oxygen

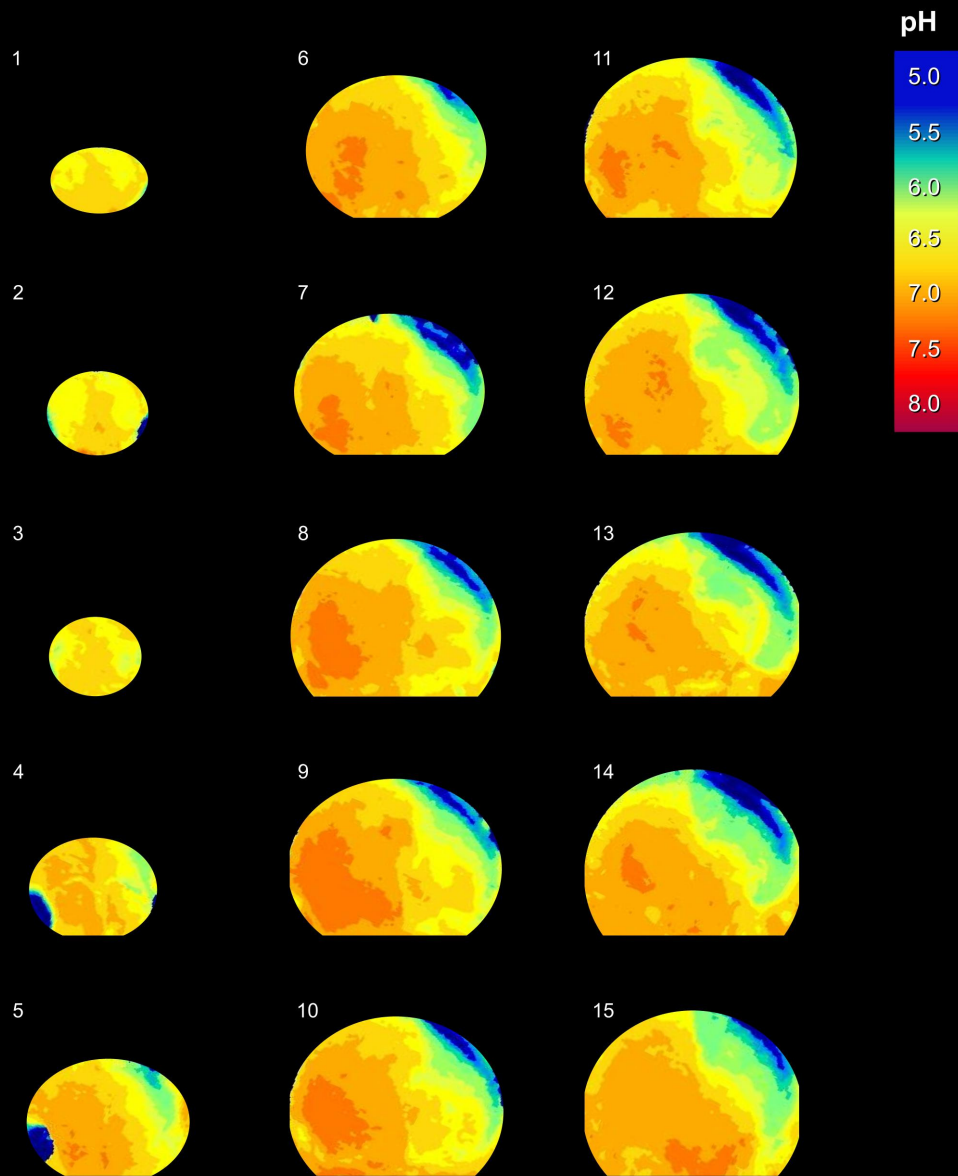


Figure 6.8 B: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion and 15 minutes of hypoxia at 8% oxygen.

Carotid Occlusion 30 Minutes 8% Oxygen

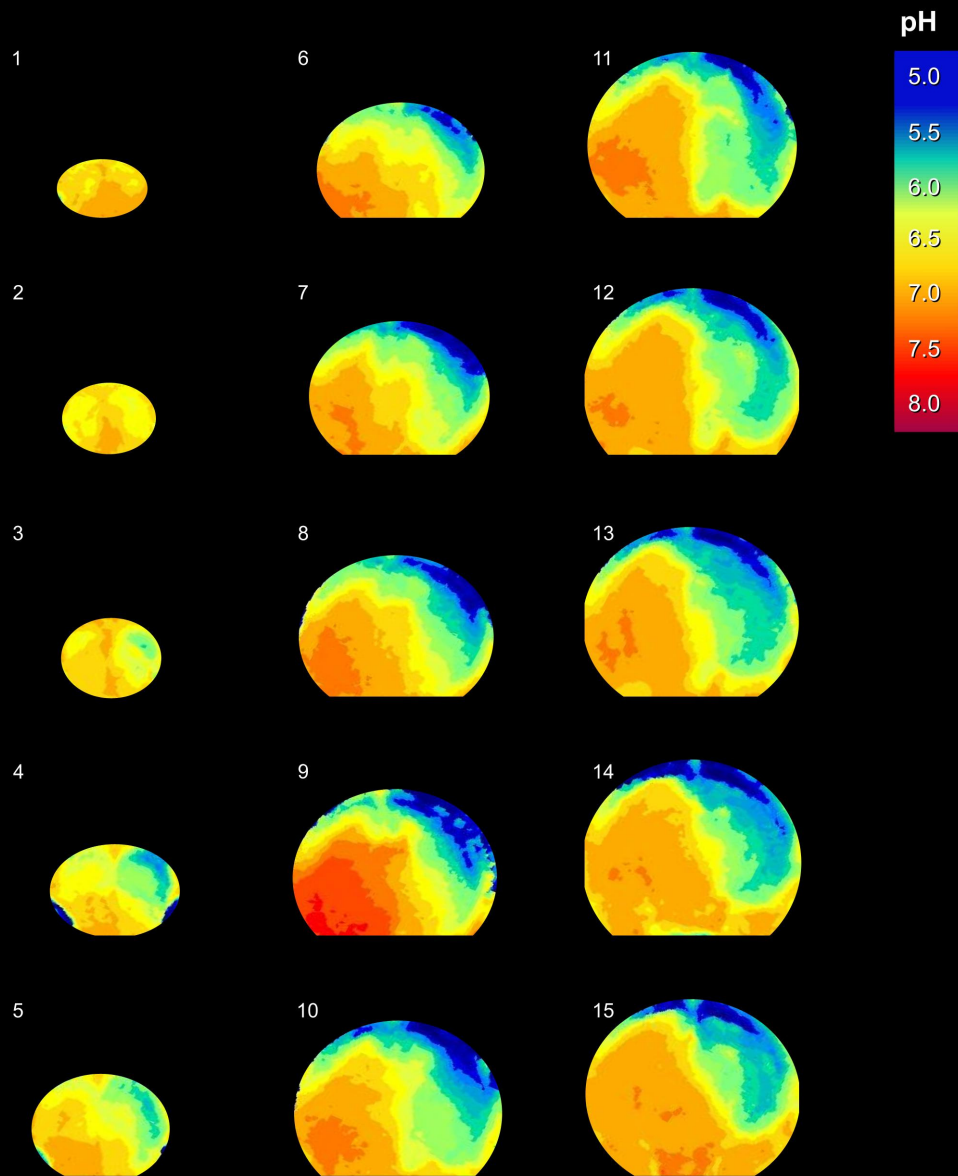


Figure 6.8 C: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion and 30 minutes of hypoxia at 8% oxygen.

Carotid Occlusion 60 Minutes 8% Oxygen

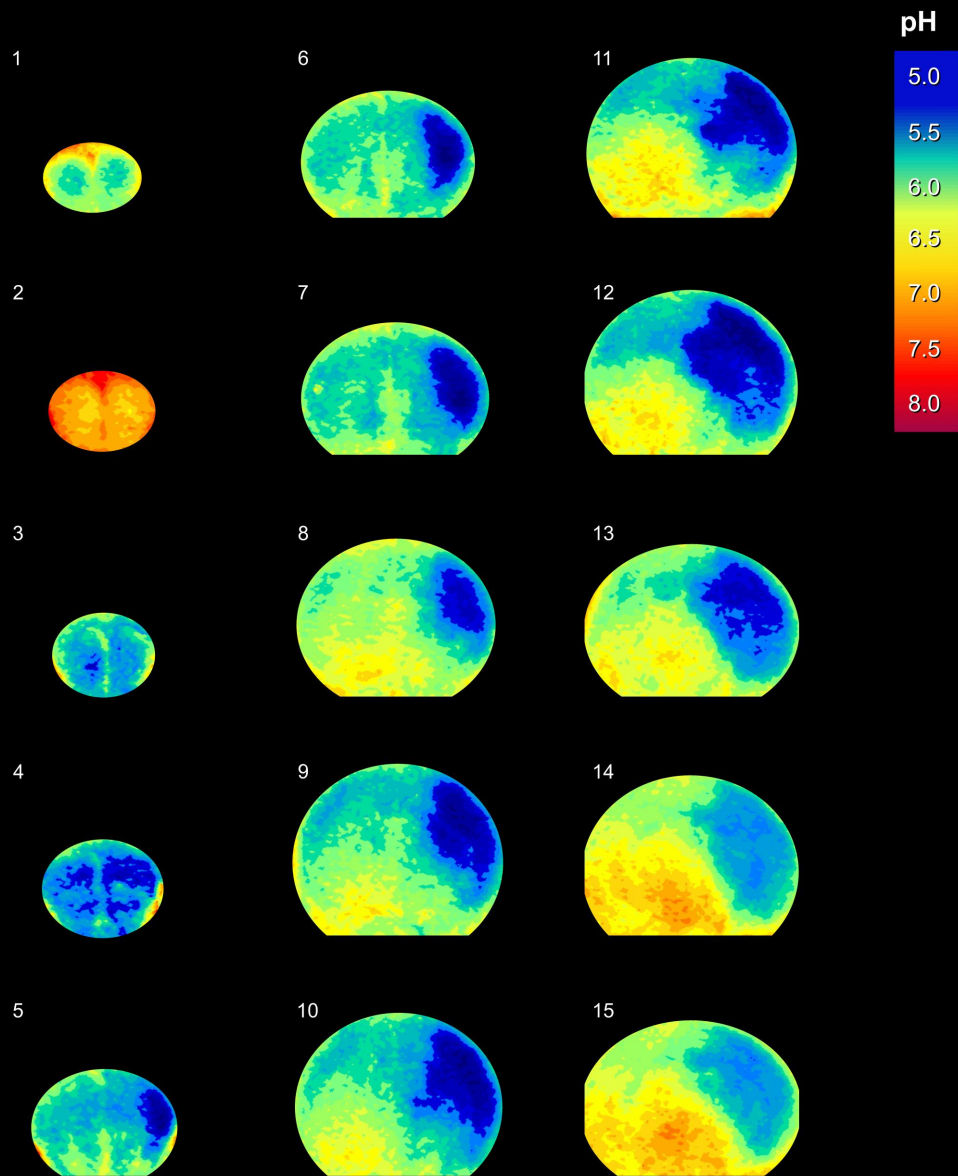


Figure 6.8 D: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion and 60 minutes of hypoxia at 8% oxygen.

Carotid Occlusion 90 Minutes 8% Oxygen

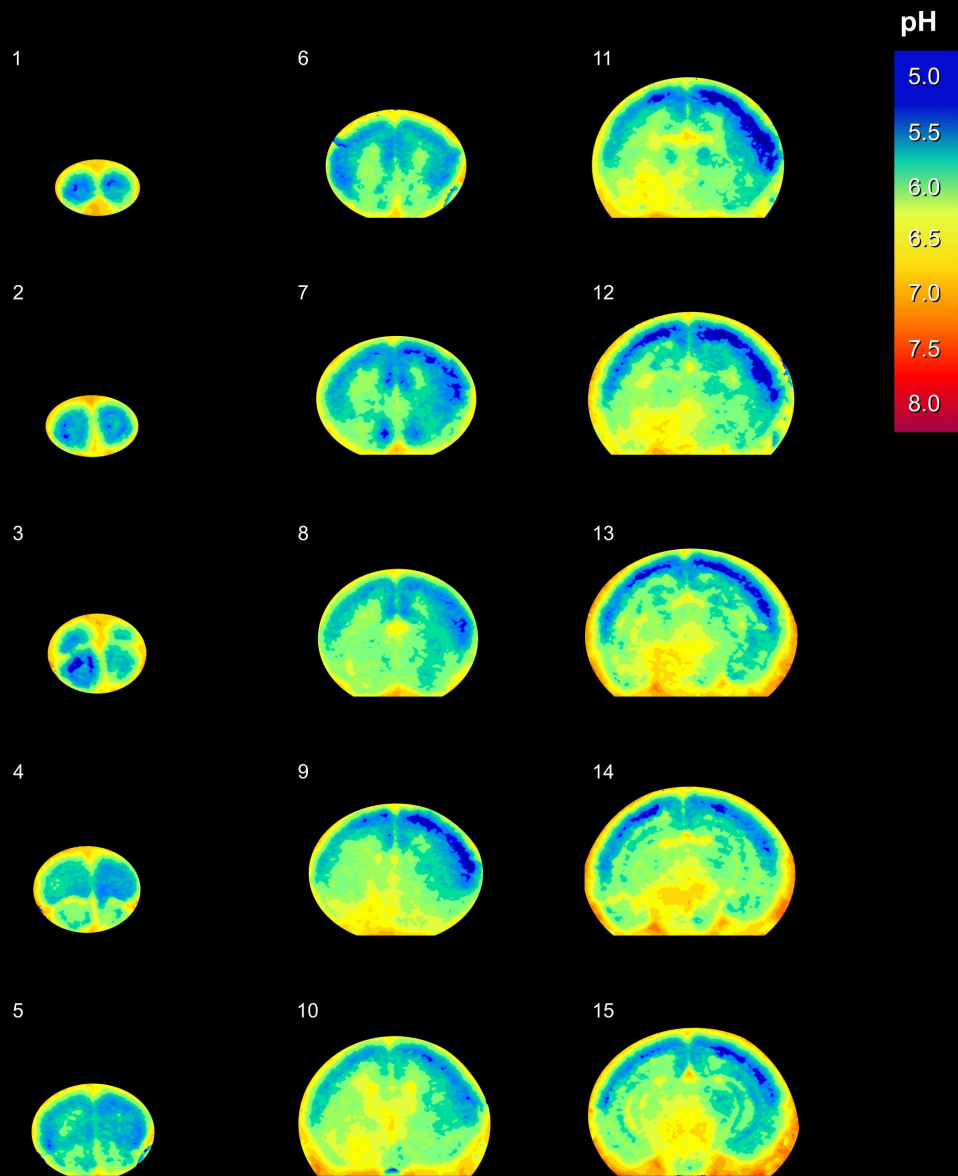


Figure 6.8 E: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion and 90 minutes of hypoxia at 8% oxygen.

Carotid Occlusion 90 minute 8% Oxygen 1 hour Re-oxygenation

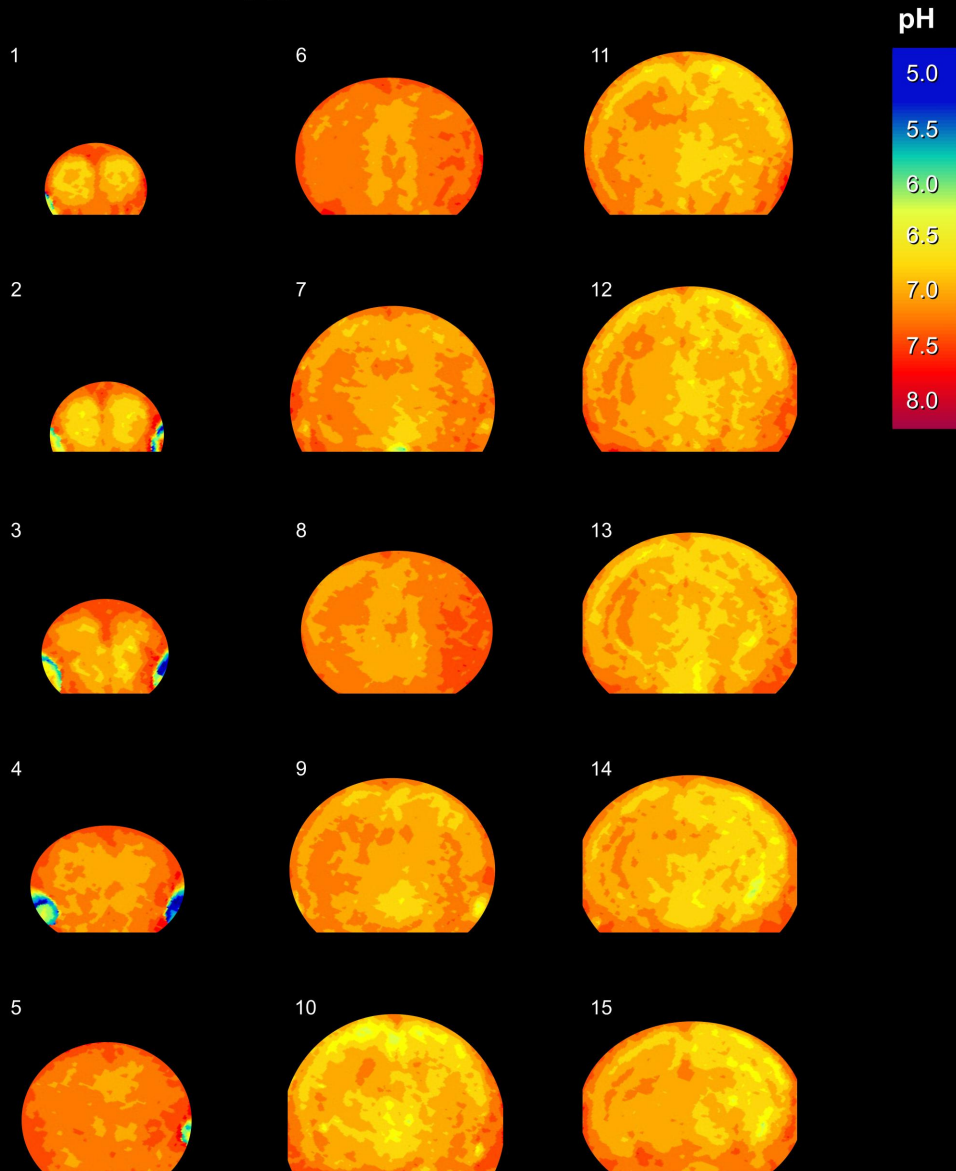


Figure 6.8 F: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion, 90 minutes of hypoxia at 8% oxygen and 1 hour of re-oxygenation.

Carotid Occlusion 90 minute 8% Oxygen 2 hours Re-oxygenation

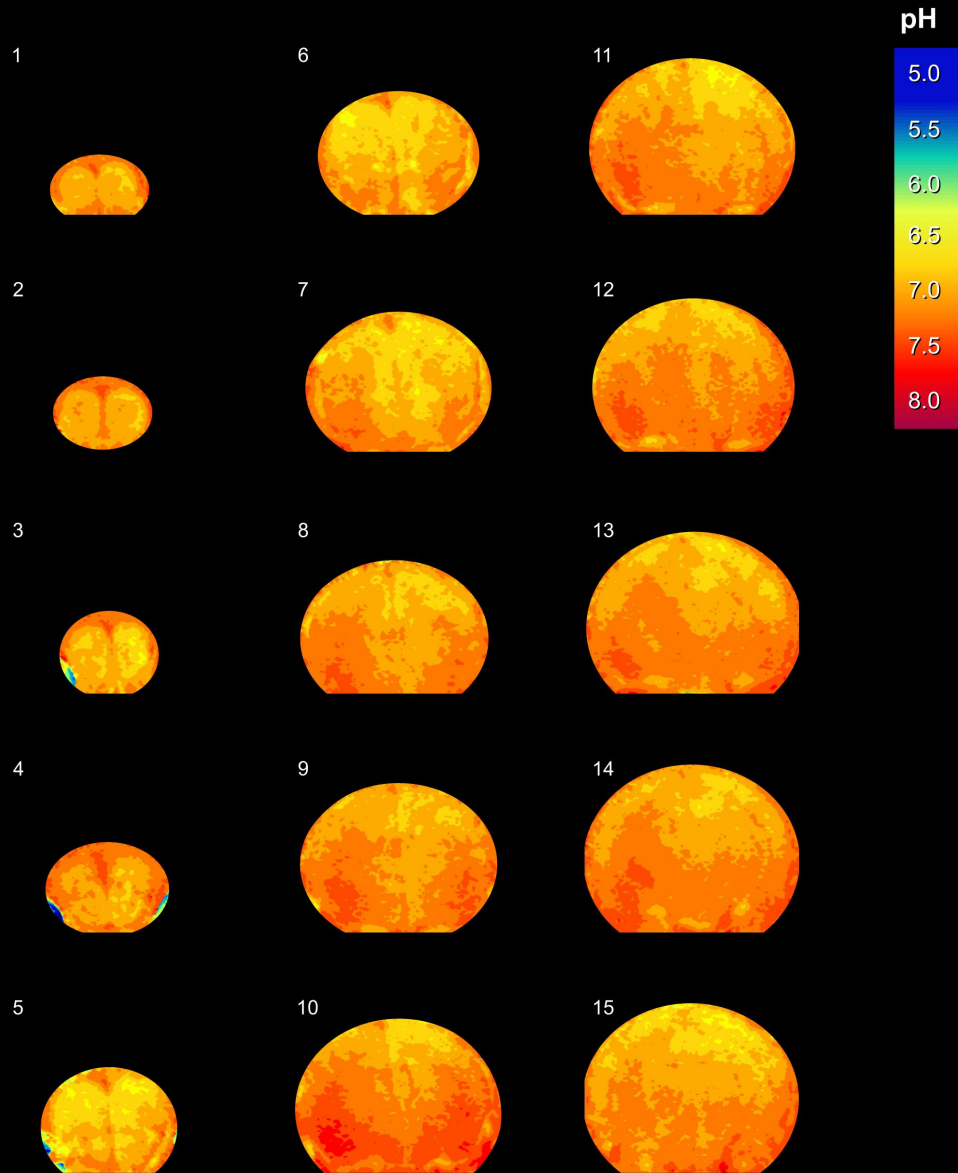


Figure 6.8 G: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion, 90 minutes of hypoxia at 8% oxygen and 2 hours of re-oxygenation.

Carotid Occlusion 90 minute 8% Oxygen 4 hours Re-oxygenation

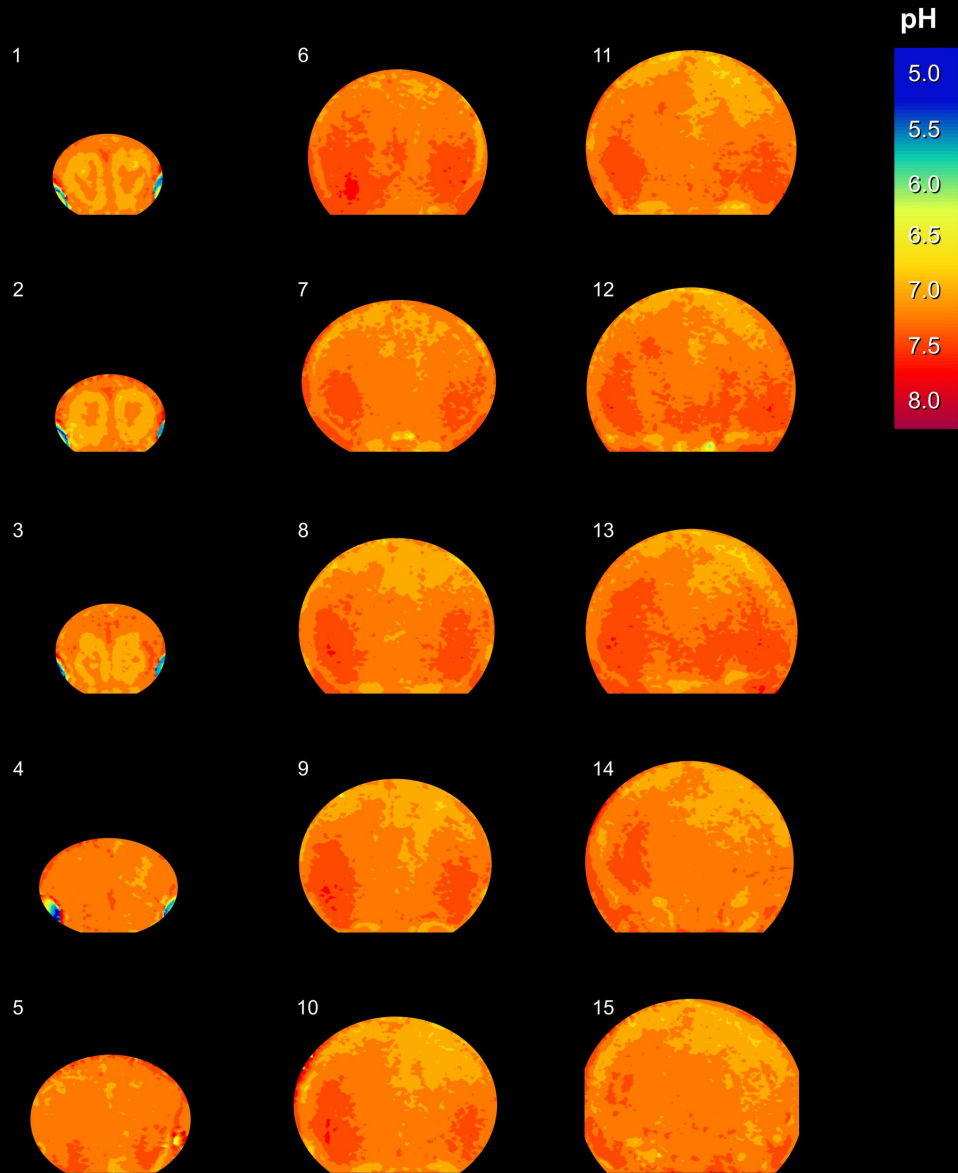


Figure 6.8 H: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion, 90 minutes of hypoxia at 8% oxygen and 4 hours of re-oxygenation.

Carotid Occlusion 90 minute 8% Oxygen 6 hours Re-oxygenation

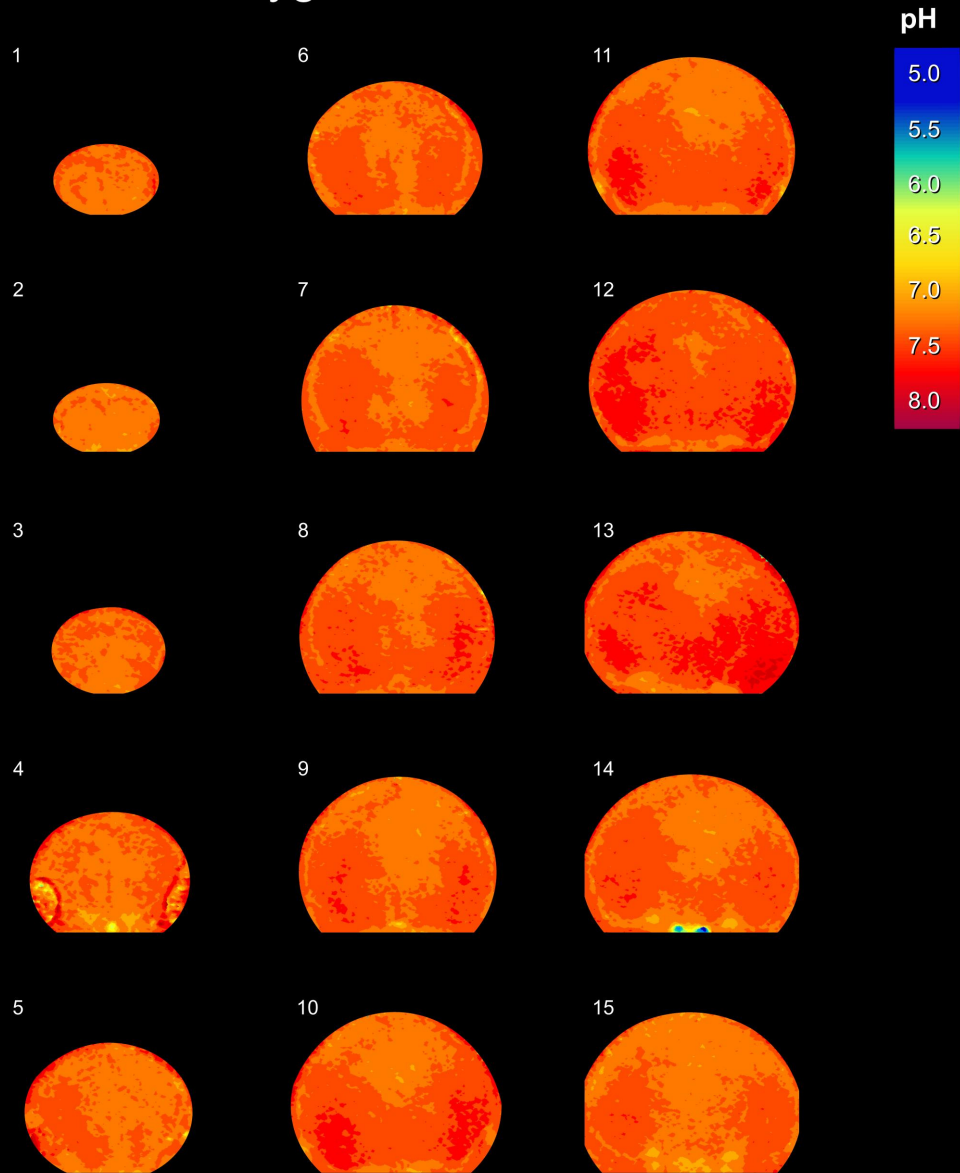


Figure 6.8 I: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion, 90 minutes of hypoxia at 8% oxygen and 6 hours of re-oxygenation.

Carotid Occlusion 90 minute 8% Oxygen 12 hours Re-oxygenation

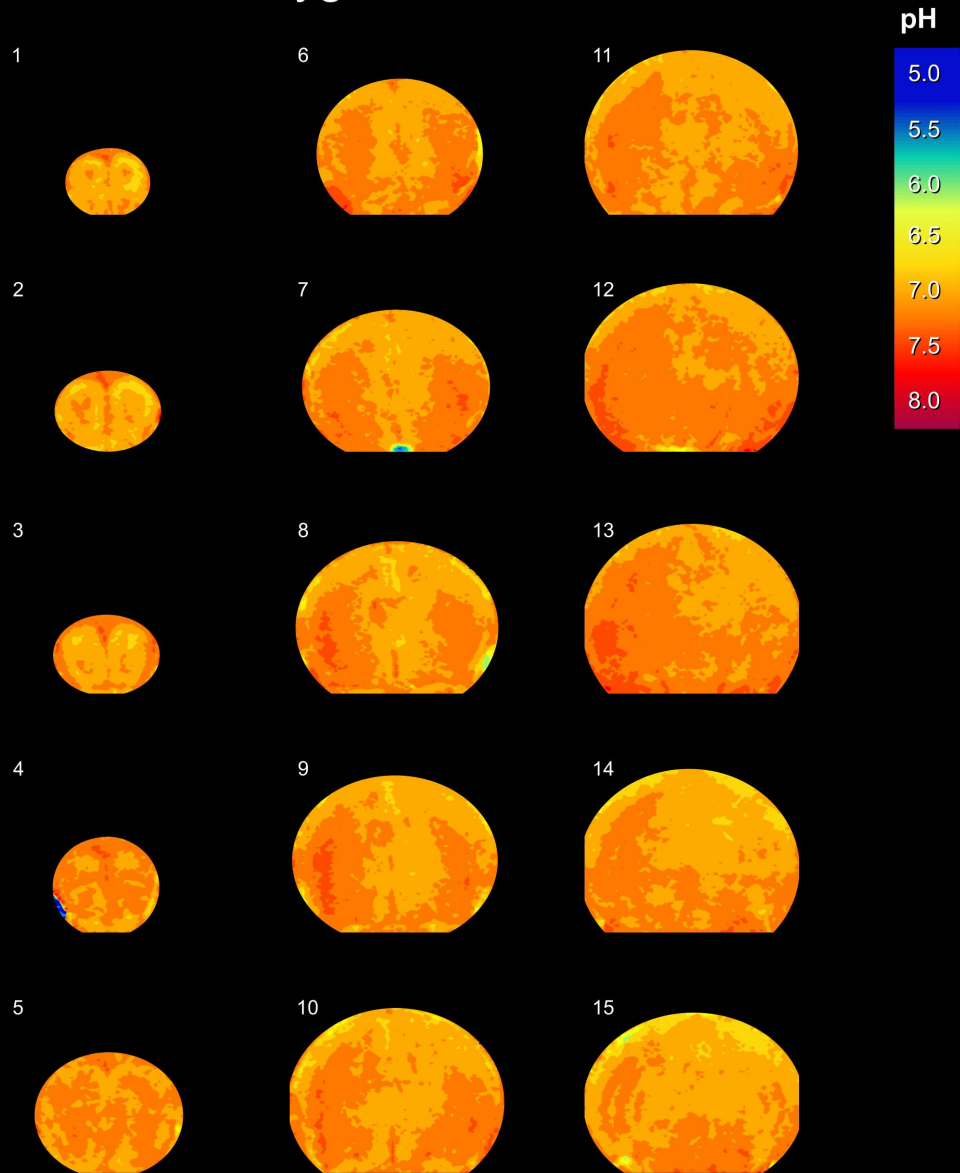


Figure 6.8 J: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion, 90 minutes of hypoxia at 8% oxygen and 12 hours of re-oxygenation.

Carotid Occlusion 90 minute 8% Oxygen 24 hours Re-oxygenation

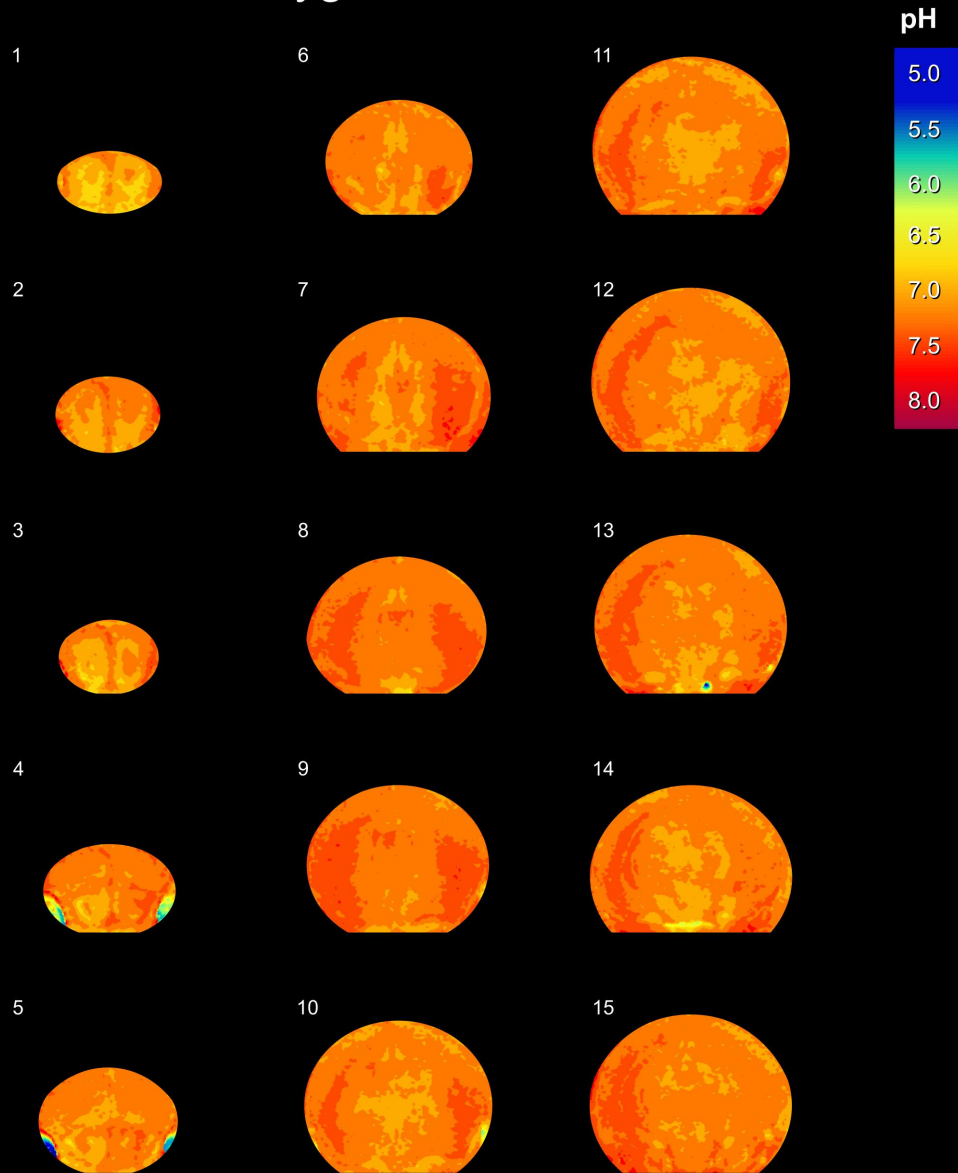


Figure 6.8 K: Pseudo-colour reconstruction of coronal brain sections demonstrating intracellular brain pH (pH_i) following carotid occlusion, 90 minutes of hypoxia at 8% oxygen and 24 hours of re-oxygenation.

Discussion

The current study used blue, green and red waveband channel light absorption by the blood-brain permeable pH-indicator Neutral Red, to assess regional brain pH_i during and after a hypoxia-ischemia. Carotid occlusion alone had no effect on pH_i whereas carotid occlusion combined with hypoxia led to a rapid, biphasic decline in ipsilateral pH_i with a first drop at 15-30, and second one at 60-90 minutes after initiation of hypoxia. The perfusion territory of the ipsilateral middle cerebral artery (Zhang et al., 1997, Maeda et al., 1998) (dorsal cortex, hippocampus, striatum and thalamus) was the most affected. The contralateral side initially showed a small decline in pH_i after 15 minutes of hypoxia, but became progressively more acidic with the increasing duration of hypoxia. Re-oxygenation after carotid occlusion combined with hypoxia resulted in a global mild alkalotic shift at 6 hours (a pH_i value 0.1-0.15 above baseline).

Intracellular Acidosis during Hypoxia-ischemia

Unsurprisingly, hypoxia-ischemia achieved by a combination of carotid occlusion and exposure to a hypoxic environment results in a progressive acidosis on the occluded side proportional to the duration of hypoxia. Previous animal studies of transient middle cerebral artery occlusion in rats (Mabe et al 1983), and transient carotid occlusion with hypoxia in piglets (Penrice et al 1997) have demonstrated a similar acidosis proportional to the accumulation in cerebral lactate. It is therefore likely that the acidosis observed in the current study also results from anaerobic glycolysis and lactate production. It is interesting to note, that carotid occlusion alone does not result in a change in pH_i , with no significant difference between occluded and non-occluded hemispheres, or between naïve and operated animals. Indeed this observation is in keeping with the current and previous studies that have not identified histological damage with carotid occlusion alone in the rat (Rice et al 1981) or mouse (Sheldon et al 1998).

Previous studies of cerebral perfusion, in immature rats, following carotid occlusion using Indian ink trapped in cerebral vessels and measurement of [^{14}C]iodoantipyrine ([^{14}C]IAP) extraction into the brain have demonstrated that permanent unilateral carotid occlusion alone reduces [^{14}C]IAP accumulation in the ipsilateral hemisphere by only 20%, whereas carotid occlusion in combination with 2 hours hypoxia at 8% reduced [^{14}C]IAP accumulation by 70% (Silverstein et al 1984). This suggests that carotid occlusion alone has only a mild impact on cerebral perfusion which is consistent with the current study showing no change in pH_i .

Mild hypoxia (15 minutes) in addition to carotid occlusion resulted in ipsilateral acidosis in the distribution of the middle cerebral artery; this may suggest that the observed preservation of anterior and posterior pH_i results from contralateral supply through the circle of Willis. More prolonged hypoxia (30 minutes) resulted in a more generalised acidosis on the ipsilateral side with a small confined area of acidosis in the contralateral dorsal cortex. It is not possible from the current study to deduce the mechanism of this contralateral acidosis. However, it may be speculated that it may result from either a vascular steal phenomenon with resultant ipsilateral perfusion failure, or from the start of a generalised cardiovascular decompensation, with a fall in systemic blood pressure and acidosis in a watershed area of the murine brain. It is clear, however, that in prolonged hypoxia (60 - 90 minutes) there is a worsening bilateral acidosis which is not significantly different between the hemispheres. The most likely explanation for this observation is cardiovascular decompensation with impairment of cardiac contractility, fall in blood pressure and reduced general cerebral perfusion. Further evidence for this mechanism can be seen following 60 minutes of pure hypoxia alone, without preceding carotid occlusion. In these animals, there is a bilateral acidosis of equal magnitude on both sides. It is interesting to correlate the pattern of changes in pH_i shown in the current study with histological injury as described earlier (see chapter 3). It is interesting to note that the areas of maximal histological injury on the ipsilateral side correspond to the areas of greatest acidosis in the current study (dorsal cortex and

dorsal hippocampus). Moreover larger cortical infarcts were only seen after 60 minutes of hypoxia-ischemia despite significant acidosis seen during shorter duration of hypoxia. Additionally, whilst contralateral acidosis is noted after a relatively short duration of hypoxia-ischemia (30 minutes) evidence of contralateral histological injury is not seen even after prolonged (60 minute) hypoxia-ischemia. Furthermore, in the human neonate, it is recognised that there is a threshold of systemic acidosis which, if exceeded results in multi-organ damage (Low et al 1997). Taken together, these observations are in keeping with the suggestion that acidosis is not the direct cause of neuronal injury and are consistent with the *in vitro* evidence that neurones are relatively resistant to lactic acidosis (Goldman et al 1989).

Previous studies have suggested that mice exposed to the same hypoxic-ischemic insult may, in the long-term, demonstrate either mild atrophy of the brain ipsilateral to the carotid occlusion with intact contralateral side, or severe cerebral atrophy with formation of a large porencephalic cyst, loss of the ipsilateral cortex, and contralateral ventriculomegaly and thinning of the cortex (Ten et al 2004). Whilst the current study was not large enough to study this phenomenon it is known that C57/Bl6 mice have differing patency of circle of Willis in C57/Bl6 (McColl et al. 2004). It can, therefore, be speculated that mice may have differing abilities to compensate for unilateral carotid occlusion by flow through the circle of Willis dependent on the arteries present (see Figure 6.8).

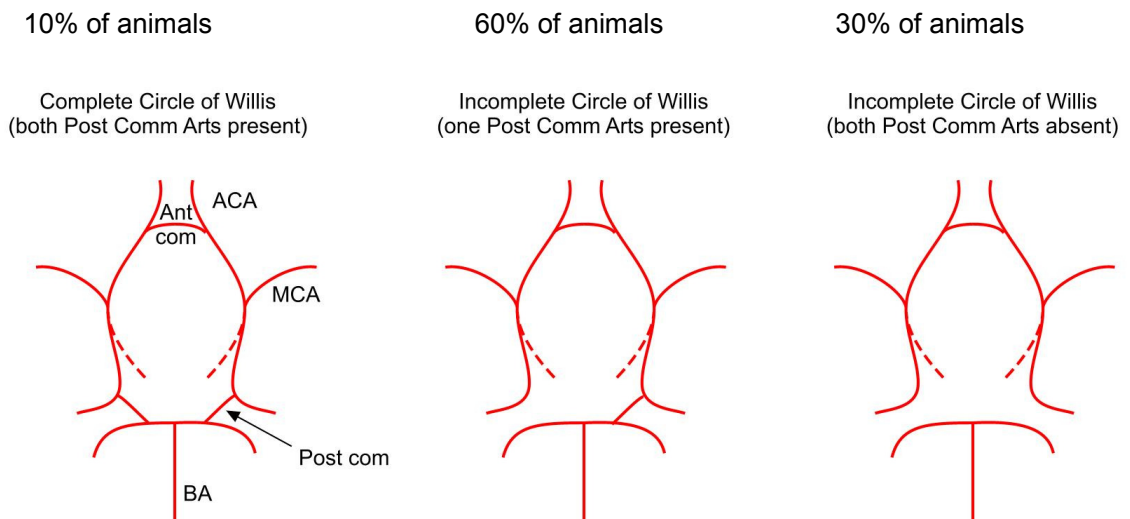


Figure 6.9 Differing patency of the Circle of Willis. Ant Com – Anterior Communicating Artery, Post Com – Posterior Communicating Artery, ACA – Anterior Cerebral Artery, MCA – Middle Cerebral Artery, BA – Basilar Artery (from McColl et al 2004, reproduced with permission).

Re-Oxygenation and Rebound Alkalosis

Consistent with clinical observations following stroke (Hugg et al 1992), neonatal encephalopathy (Robertson et al 1999, Robertson et al 2002), and adult animal models of middle cerebral artery occlusion (Mabe et al 1983, Chopp et al 1990), the current study demonstrates rebound alkalosis in neonatal mice following hypoxia-ischemia. Previous evidence suggests that the rapid normalisation of pH_i seen in animal models during reperfusion following cardiac arrest is mediated by the Na^+/H^+ exchanger, which is known to be present on the cell membranes in various isoforms (Masreel et al 2003). Furthermore, blockade of this transporter has been shown to delay normalisation of pH_i during reperfusion (Ferimer et al 1995). It has been suggested, therefore, that the rebound alkalosis following hypoxia-ischemia may arise because of a failure to switch off the Na^+/H^+ exchanger resulting in excess hydrogen ion extrusion from cells (LaManna 1996).

In the current study, a statistically significant rebound alkalosis is detected 6 hours following re-oxygenation. Previous studies in the human neonate following hypoxic-ischemic encephalopathy demonstrate that the magnitude and duration of rebound alkalosis correlates with worsening neurodevelopmental outcome (Robertson et al 2002). A number of mechanisms have been postulated by which rapid normalisation of pH_i and rebound alkalosis may be responsible for this association. Mild acidosis (both extra and intracellular) during reperfusion after transient hypoxia-ischemia has previously been shown to protect the brain (Simon et al 1993, Vannucci et al 2001). Conversely an alkaline pH_i may exacerbates excitotoxic neuronal injury due to increased *N*-methyl-D-aspartate activation (Traynelis et al 1990, Giffard et al 2002) and activation of phospholipases and proteases, which have an alkaline pK_a (Mellgren 1987). Changes in pH_i also result in alterations of the delicate balance of pro- and anti-apoptotic triggers in mitochondria. Alkalinisation has been shown to: (i) activate Bax (a pro-apoptotic protein), inducing its mitochondrial translocation (Khaled et al 1999); (ii) inhibit the import of ADP into mitochondria, which impairs the synthesis of ATP (Khaled et al 2001) and (iii) transform the mitochondrial permeability transition pore into a high conductance state, a critical step leading to cell commitment to death (Lemasters et al 1997). Interestingly, however, in the current study rebound alkalosis is detected over the whole brain, not just the region of expected histological injury from previous studies (see chapter 3). This raises a number of questions about both the mechanism of rebound alkalosis and its role in neuronal injury. Of note in the current study, re-oxygenation was examined after 90 minutes of hypoxia during which there is a global bilateral acidosis and therefore the global rebound alkalosis may indeed be a rebound phenomenon. However, from the pseudocolour maps, it appears that alkalosis was most evident in regions of the brain away from the regions of greatest acidosis and expected tissue damage (ipsilateral dorsal cortex and hippocampus). Although previous studies have examined regional changes in pH_i following cardiac arrest, these studies examined pH_i changes after a global hypoxic-ischemic insult in contrast to the current study, which uses unilateral carotid occlusion and global hypoxia. In the human

neonatal studies, the ^{31}P MR spectra from which pH_i was calculated were acquired from a large voxel (at least 125cm^3), which encompassed both the right and left basal ganglia, as well as white matter (Robertson et al 2002). Here again, these measurements followed global hypoxia-ischemia. The identification of maximal rebound alkalosis away from the region of expected tissue damage may suggest that alkalosis, like acidosis is not a direct cause of neuronal injury. However it can be speculated that this alkalosis may have a direct impact of neuronal survival by a number of mechanisms. It is recognised that a rise in pH_i leads to an increase in neuronal excitability and increased susceptibility to epileptiform activity both *in vitro* and *in vivo* (Chesler 2003). Seizures in neonates following a hypoxic-ischemic event are associated with a worse prognosis, often distinguishing babies having mild and moderate encephalopathy (Sarnet et al 1976). It is of note, therefore, that in the human neonate the timing of onset of seizures is often delayed to 6-24 hours after the insult, and corresponds to the timing of secondary energy failure and rebound alkalosis (Penrice et al 1997). An alternative possibility is that the presence of a large infarct may disrupt the cellular processes that cause rebound alkalosis; hence, the pattern of alkalosis identified may be specific to this model and not be applicable to human neonatal encephalopathy where large infarctions are rare. It is of note that, in experimental models of middle cerebral artery occlusion, rebound alkalosis is detected in the penumbral region during reperfusion whilst the infarct core remains acidotic (Back et al 2000). Whilst, in the current study, the entire brain becomes alkalotic, this may only be of consequence where there are damaged neurones where, through the mechanisms described above, alkalosis may result in cell death whereas normal pH_i or even mild acidosis may be neuroprotective. The role of manipulation of pH_i following hypoxia-ischemia as a neuroprotective strategy will be considered further in chapter 7.

Conclusions

Clinical studies and animal models demonstrate progressive acidosis during hypoxia-ischemia and a rebound alkalosis in the reperfusion phase in both adult stroke and neonatal hypoxia-ischemia. Growing evidence suggests that alkalosis correlates with outcome. The current study demonstrates that these changes occur in a murine neonatal model of neonatal hypoxia-ischemia. Moreover alkalosis is most evident in regions of the brain away from the greatest acidosis and tissue damage. Previous studies of neonatal and adult hypoxic-ischemic brain injury have concentrated on the infarcted region and, in animal models, the penumbra. The current study may suggest that events in the remainder of the brain may have a direct influence on the fate of the injured region and, hence, therapeutic interventions aimed at altering / preventing rebound alkalosis in the uninjured regions of the brain may confer neuroprotection. Moreover, if this is true then the use of drugs that prevent rebound alkalosis may be effective in the post-injury phase as these regions of the brain have an intact normal blood supply and there is a therapeutic window in the early reperfusion phase prior to the onset of rebound alkalosis.

Chapter 7: *N*-methyl-isobutyl-amiloride Ameliorates Brain Injury when Commenced Prior to Hypoxia-Ischemia in Neonatal Mice.

Introduction

Over the last 20 years, studies using phosphorus (^{31}P) and (^1H) proton magnetic resonance spectroscopy (MRS) in both infants with neonatal encephalopathy (Hope et al 1984, Younkin et al 1984, Laptook et al 1989) and experimental models (Lorek et al 1994, Penrice et al 1997), have characterised the biphasic disruption of cerebral energetics that occurs in the hours following hypoxia-ischemia. These observations have led to the concept of a “therapeutic window” following hypoxia-ischemia, during which intervention may ameliorate the severity of brain injury. There has, until recently, been no intervention which has improved the outcome following perinatal hypoxia-ischemia. Recently, however, the results from the first randomised trials of mild hypothermia have been published (Gluckman et al 2005, Shankaran et al 2005, Eicher et al 2005). Considerable work is still required to optimise cooling strategies in the newborn. However there is a growing impression that optimum neuroprotection may involve the use of more than one therapy, targeting different molecular events that occur after hypoxia-ischemia.

A remarkable observation from the ^{31}P MRS studies in infants with neonatal encephalopathy was that during the second phase of energy decline that occurred from 8-24 hours following birth, brain intracellular pH (pH_i) was alkaline (pH_i 7.1-7.4). Under physiological conditions, brain pH_i is maintained at around 7.03; the Na^+/H^+ exchanger (NHE) tightly regulates both pH_i and cell volume by extruding protons from and taking sodium up into cells (Masereel et al 2003). Some evidence suggests that excessive activation of this transporter following hypoxia-ischemia is responsible for the observed rebound alkalosis (Wakabayashi et al 1992). Adult clinical studies have demonstrated brain alkalosis in areas of chronic infarction after stroke (Hugg et al 1992, Levine et al 1992) with a significant correlation between brain alkalosis and subsequent poor

clinical outcome (Welch et al 1990). A similar relationship between alkalosis and the severity of brain injury in term infants with neonatal encephalopathy has also been observed (Robertson et al 2002). Infants with the most alkaline brain pH_i had more severe changes on MRI within the first 2 weeks after birth, and the worst neurodevelopmental outcome at one year. Interestingly, this brain alkalosis was seen to persist for some months in those with the worst outcome (Robertson et al 1999).

These observations led to the hypothesis that prevention of rebound alkalosis would reduce the severity of brain injury observed after perinatal hypoxia-ischemia. Further evidence for this hypothesis comes from an *in vitro* tissue culture model (Vornov et al 1996) and *ex vivo* brain slices model (Robertson et al 2005). In these studies, prevention of rebound alkalosis using an inhibitor of the Na^+/H^+ exchanger resulted in reduced cell loss and preservation of brain energetics whilst the tissue remained alkalotic. If these findings can be replicated *in vivo*, blockade of the Na^+/H^+ exchanger may represent a therapeutic intervention to ameliorate brain injury in neonatal hypoxia-ischemia. To establish a proof of concept, amiloride, a drug known to block the action of the Na^+/H^+ exchanger, was administered prior to a hypoxic-ischemic insult and continued 8 hourly after hypoxia-ischemia until histological examination at 48 hours. *N*-methyl-isobutyl-amiloride (MIA) was used, as this analogue is known to cross the blood-brain barrier (Ferimer et al 1995).

Materials and Methods

Surgery

As described previously experiments were performed at postnatal day seven (P7) on C57/Bl6 mice (see Materials and Methods). Under isoflurane anaesthesia, the left carotid artery was permanently ligated and then the animals exposed to 8% oxygen in 92% nitrogen for 30 minutes (moderate insult) or 60 minutes (severe insult).

***N*-methyl-isobutyl amiloride administration**

10mg of *N*-methyl-isobutyl amiloride (MIA) was dissolved in 150 μ l 350mM acetic acid and then diluted to 40ml in sterile 0.9% saline to give a final concentration of 250 μ g/ml. Aliquots were stored at -80°C until required. Animals were sequentially allocated to receive intra-peritoneal injections of MIA (2.5 mg/kg, 10 μ l/g) or an equivalent volume of 0.9% saline every 8 hours, commencing 30 minutes before hypoxia.

Tissue Preparation

Animals were killed 48 hours after moderate and severe hypoxia-ischemia by intra-peritoneal injection of pentobarbitone, perfused, post-fixed and frozen as described previously (see Materials and Methods). 15 sections from each forebrain (200 μ m apart) were stained with cresyl violet. In the moderate insult group, five coronal sections per brain (600 μ m apart), were stained using immunohistochemistry for microglial and macrophage activation using the rat monoclonal 5C6 α M β 2 antibody (Serotec). Five further coronal sections per brain, with the same spacing, were stained using Terminal transferase mediated d-UTP Nick End-Labeling (TUNEL) and brain injury was quantified using infarct volume and brain injury scoring as described previously (see Materials and Methods).

Results

Severe Insult

Twenty animals underwent the severe insult protocol. Four animals died during the hypoxia (2 MIA, 2 saline). Four animals died in the subsequent 48 hours (1 MIA, 3 saline). Animals were weighed every 8 hours until they were sacrificed. There was no significant difference in weight between the two groups at the onset of treatment ($4.43\pm 0.30\text{g}$ in the saline treated group, $n=10$; $4.49\pm 0.34\text{g}$ in the MIA treated group $n=10$). Both groups of animals lost weight in the first 24 hours after hypoxia-ischemia, ($0.39\pm 0.24\text{g}$ in the saline treated group; $-0.34\pm 0.21\text{g}$ in the MIA treated group), and then plateaued. There was no significant difference in weight loss between the 2 groups during the 48 hour period (ANOVA).

The separate regions of the forebrain (cortex, hippocampus, striatum and thalamus) showed different sensitivity in their response to the carotid occlusion and 1 hour hypoxia ($F_{3,21}=47.7$ $p<0.05$, ANOVA). The effects were particularly severe in the hippocampus and much milder in the cortex, striatum and thalamus. No change was observed in the hypothalamus or brainstem. There was no significant difference between litters in their response to the hypoxia-ischemia.

Pretreatment with MIA resulted in an increase in surviving brain tissue, from 44% to 67% ($p<0.05$) in the affected forebrain regions, compared to the contralateral side (see Figure 7.1). Pretreatment with MIA resulted in increase in surviving brain tissue in the cortex from 50% to 76% ($p<0.05$), and an increase in the hippocampus from 10% to 19% ($p<0.05$). Treatment also resulted in an increase in tissue survival in the thalamus from 56% to 64% and an increase in the striatum from 34-47% but these did not reach statistical significance (see Figure 7.2). However, ANOVA common factor analysis revealed MIA to have a highly significant overall protective effect against carotid occlusion and 1 hour of 8% hypoxia across the different brain regions ($F_{1,21}=11.2$, $p<0.05$).

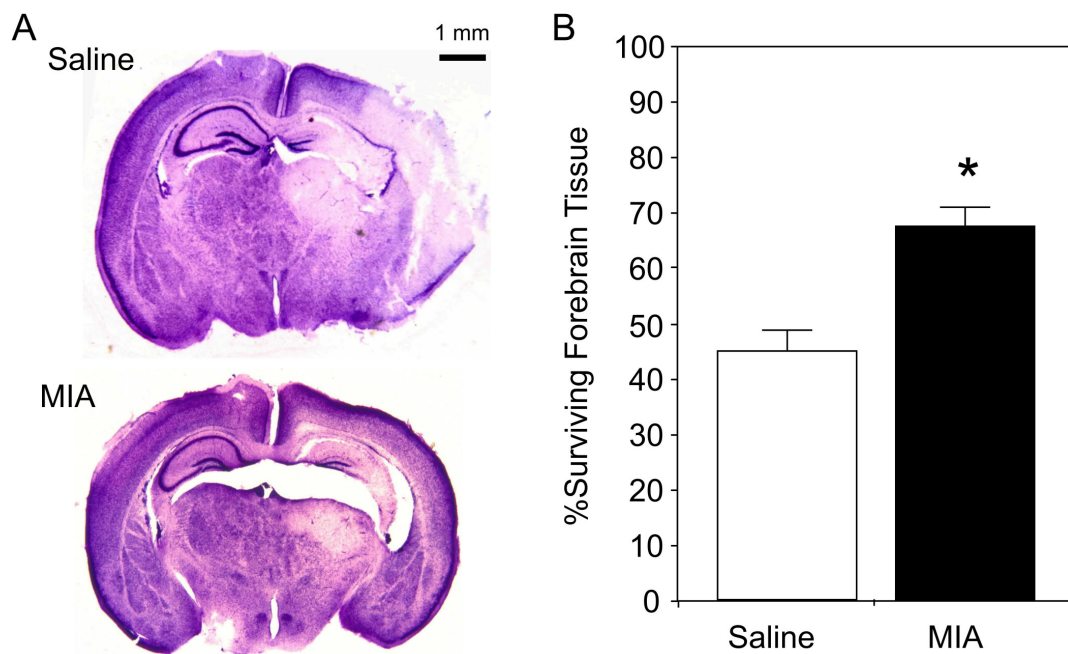


Figure 7.1: Effects of MIA following severe hypoxia ischemia: A. Cresyl violet stained coronal sections 48 hours following carotid occlusion and 1 hour hypoxia. Note the substantial loss of cortical tissue and the maintenance of the hippocampal dentate gyrus. Ventral cortex, amygdala and hypothalamus appear unaffected. B. Pretreatment with MIA resulted in a significant increase in surviving total forebrain hemispheric tissue expressed as percentage of the unaffected hemisphere (n=7 MIA / n=5 saline), (*p<0.05 by Student-t-test).

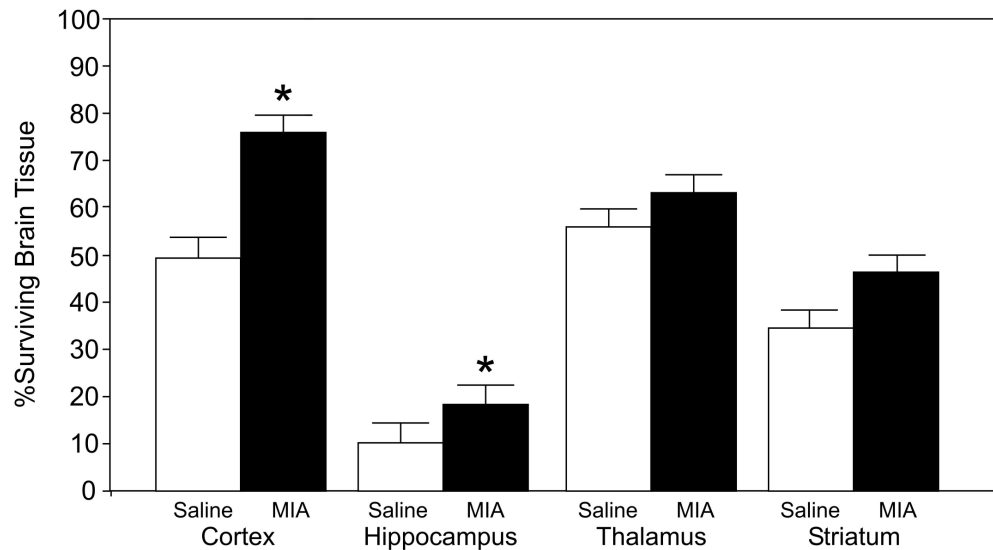


Figure 7.2 Effects of MIA following severe hypoxia ischemia on different forebrain regions: Treatment with MIA resulted in a significant increase in cortical ($p < 0.05$) and hippocampal ($p < 0.05$) tissue expressed as percentage of unaffected hemisphere but not in the thalamus and striatum.

Moderate Insult

Twenty animals underwent the moderate insult protocol. Three animals died during the hypoxia (2 MIA, 1 saline). There was no significant difference in weight between the two groups at the onset of treatment (3.62 ± 0.71 g in the saline treated group $n=10$; 3.77 ± 0.74 g in the MIA treated group $n=10$). Both groups of animals continued to gain weight over the 48 hours following hypoxia-ischemia and there was no significant difference in weight gain between the 2 groups at 48 hours ($+0.35 \pm 0.54$ g in the saline treated group, $+0.27 \pm 0.46$ g in the MIA treated group). Histological damage was only observed in the hippocampus with loss of neurons predominantly within CA1 and CA2. There was a relative preservation of neurons within the dentate gyrus. Immunohistochemistry for $\alpha M\beta 2$ integrin demonstrated both macrophage activation and the presence of deramified phagocytes in the same regions as histological damage (see Figure 7.3).

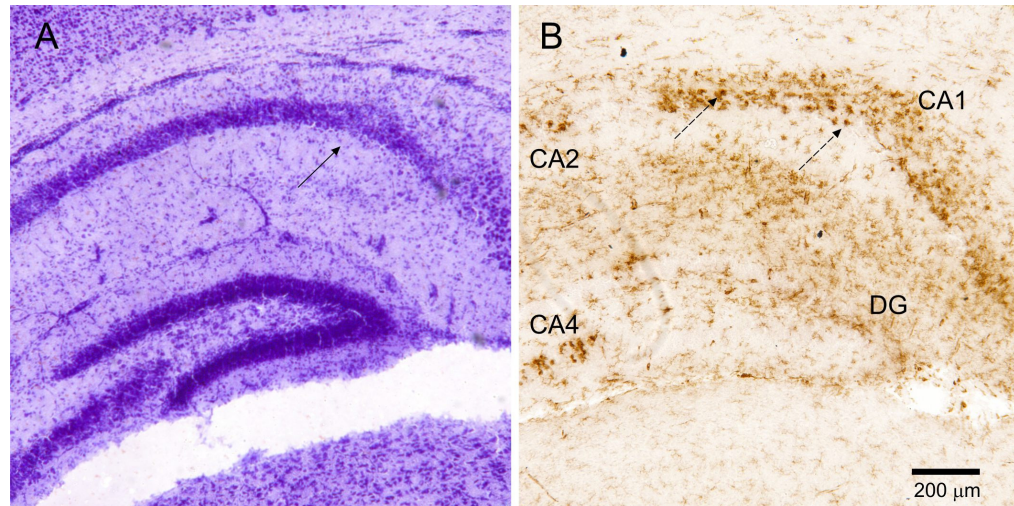


Figure 7.3: Sequential coronal sections stained with Nissl (A) and immunohistochemistry for $\alpha M\beta 2$ integrin (B) in the hippocampus following carotid occlusion and 30 minutes hypoxia. Mild neuronal loss is seen in CA1 (arrow). Immunohistochemistry reveals macrophage activation and the presence of deramified phagocytes (dashed arrows). Note the activation in CA1 and CA4 with maintenance of CA2 and dentate gyrus (DG).

Pretreatment with MIA resulted in a reduction in the mean injury score in the hippocampus, from 3.1 ± 1.8 to 0.7 ± 1.1 ($p < 0.05$) and a reduction in the average injury score across all affected forebrain regions from 1.0 ± 0.6 to 0.2 ± 0.3 ($p < 0.05$). Treatment also resulted in reduced injury scores in the cortex, thalamus and in the striatum but these did not reach statistical significance (see Figure 7.4).

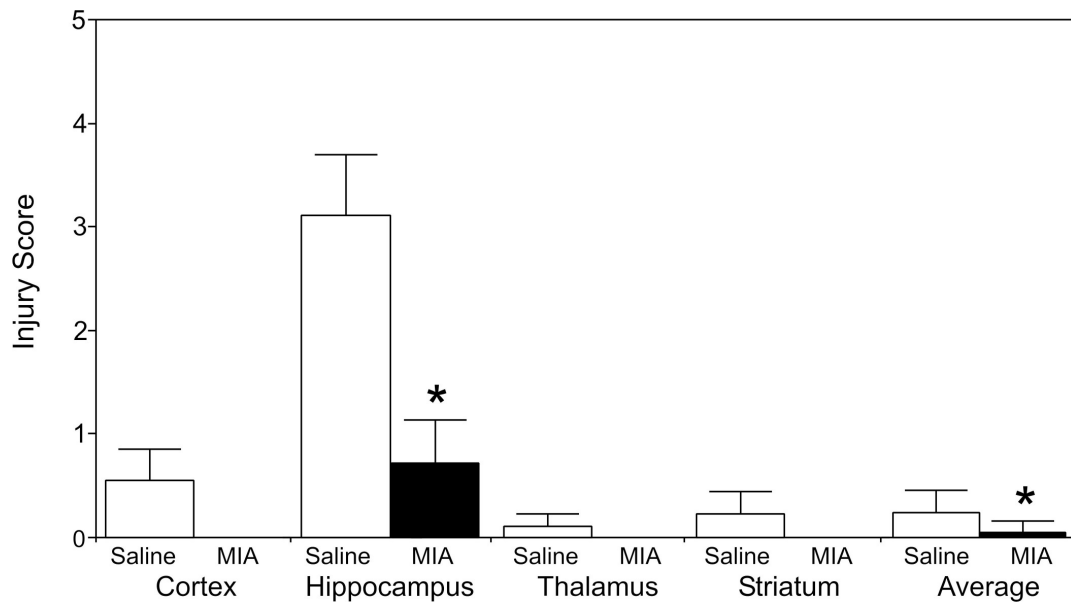


Figure 7.4: Effects of MIA following moderate hypoxia ischemia on different forebrain regions: Treatment with MIA (2.5mg/Kg) resulted in a significant decrease in average injury score ($p < 0.05$) and in the hippocampus ($p < 0.05$). A neuroprotective trend was seen in the cortex thalamus and striatum ($n = 9/7$; Student-t-test).

Following the moderate insult, TUNEL staining only showed significant DNA fragmentation in CA1 and CA2 of the hippocampus in the injured hemisphere. Occasional TUNEL positive cells were seen in other regions such as the dentate gyrus and cerebral cortex; however, no difference was observed between the injured and contralateral hemisphere or between treatment groups in other brain regions. Pretreatment with MIA resulted in a reduction in the average number of TUNEL positive within the hippocampus, from 241 ± 262 cells/mm² to 21 ± 55 cells/mm² ($p < 0.05$) (see Figure 7.5).

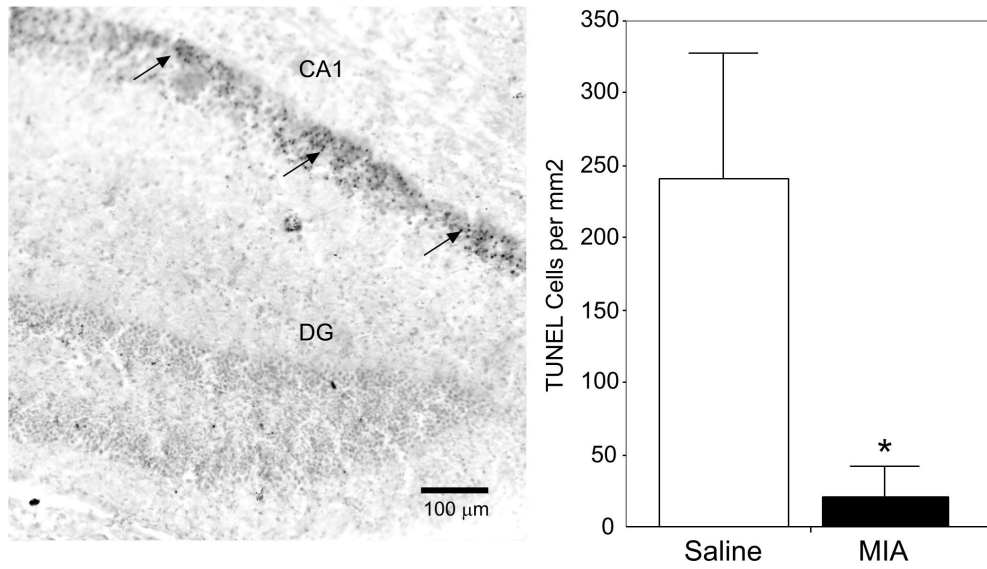


Figure 7.5: Effects of MIA on TUNEL positive cells within the Hippocampus. A. TUNEL stained cells in the CA1 of the hippocampus (arrows); note occasional TUNEL positive cells within the dentate gyrus (DG). B. Pretreatment with MIA results in a significant reduction in TUNEL positive cells, (n=9/7 * p<0.05 Student t-test).

Discussion

Treatment with MIA commenced prior to hypoxia-ischemia ameliorated histological brain injury at 48 hours following hypoxia-ischemia in a neonatal mouse model. Importantly, neuroprotection was seen following hypoxia-ischemia of varying severity. The results are consistent with the increasing evidence that NHE blockade may be beneficial to cell survival after hypoxia-ischemia. Blockade of the NHE has previously been shown to confer neuroprotection in adult rats when administered immediately after focal cerebral ischemia (Horikawa et al 2001). In a tissue culture model of cerebral ischemia, the prevention of rebound alkalosis using amiloride after *in vitro* hypoxia-ischemia delayed the onset of injury for as long as the cells remained acidotic (Vornov et al 1996). Recent studies in an *ex vivo* model of neonatal rat brain slices demonstrated a preservation of brain slice energetics over several hours in those brain slices exposed to amiloride. Of note, this preservation of brain slice energetics with amiloride was similar to that observed in brain slices maintained under hypothermic conditions (Robertson et al 2005). Further *in vivo* studies in neonatal models are required to demonstrate whether amiloride retains its neuroprotective effect when treatment is commenced after hypoxia-ischemia, and whether its neuroprotective effect is enhanced in combination with other strategies, such as hypothermia.

In this study, the dose of 2.5mg/kg MIA was selected as a similar dose in adult rats has been shown to cross the blood-brain barrier and to be a potent inhibitor of the NHE (Ferimer et al 1995). The absence of enhanced weight loss in MIA treated animals, suggests that, at this dose, the drug is non-toxic and well tolerated. As amiloride is currently used on occasion in the clinical setting as a potassium sparing diuretic and is not teratogenic or toxic, it may be possible in the future to use amiloride analogues clinically, especially if they retain their neuroprotective effects when given after hypoxia-ischemia. However, as amiloride crosses the placenta, it also may be possible to administer it to high-risk mothers prior to delivery to provide protection from brain injury during reperfusion.

One of the mechanisms by which amiloride is neuroprotective may be by preventing rebound alkalosis through blockade of the NHE. Inhibition of the NHE has been shown to block phospholipase activation in the ischemic / reperfused rat cerebral cortex as a consequence of its stabilising effect on acidotic pH_i and prevention of alkalosis (Phillis et al 2000). As described in the previous chapter, mild acidosis (both extra and intracellular) during reperfusion after transient hypoxia-ischemia has previously been shown to protect the brain (Simon et al 1993, Vannucci et al 2001). Additionally, an alkaline pH_i may exacerbate injury during reperfusion by various mechanisms, including increased *N*-methyl-D-aspartate activation (Traynelis et al 1990, Giffard et al 2002) activation of phospholipases and proteases, which have an alkaline pK_a (Mellgren 1987) and changes the balance of pro- and anti-apoptotic triggers in mitochondria. This mechanism of increased apoptosis in the alkaline state would be in keeping with the finding of the current study where a significant reduction in TUNEL positive cells was noted in the MIA treated group following mild hypoxia-ischemia.

Further mechanisms of neuroprotection by amiloride may relate to a reduction in energy consumption and calcium influx into the cell. As well as influencing pH_i , the excessive action of the NHE after hypoxia-ischemia leads to an increase in intracellular Na^+ concentration and subsequent activation of the Na^+/K^+ ATPase, consuming cellular energy. The high intracellular Na^+ level also activates the sarcolemmal $\text{Na}^+/\text{Ca}^{++}$ antiporter leading to a rise in intracellular Ca^{++} (Manev et al 1990, Masreel et al 2003). This overload of intracellular calcium leads to a variety of deleterious effects as part of the neurotoxic cascade seen following hypoxia-ischemia including mitochondrial accumulation of calcium, activation of proteolytic enzymes, free radical production and lipolysis.

In addition to these direct effects on neuronal pH_i and ion transport, it is also possible that the neuroprotective effects of amiloride may be mediated through interaction with

other cell types, including blood vessel endothelia, astrocytes, and neutrophil / granulocytes. An amelioration of endothelial cell dysfunction after transient ischemia has been observed with NHE inhibition (Horikawa et al 2001). NHE inhibition was also associated with reduced blood-brain barrier disruption, improved post-ischemic perfusion, and neuroprotection (Horikawa et al 2001). In astrocyte cultures, oxygen and glucose deprivation resulted in a large increase in intracellular sodium and an increase in cell volume (Masreel et al 2003). NHE inhibition or genetic ablation of NHE1 reduced the increase in intracellular sodium and astrocyte swelling (Kintner et al 2004). Finally, NHE inhibition influences neutrophil granulocytes, which are known to invade central nervous system tissue after direct trauma or ischemia (Hudome et al 1997). Neutrophil chemotaxis is associated with cytoplasmic alkalinisation; NHE disruption or amiloride (Simchowicz et al 1986, Fukushima et al 1996) inhibits such chemotactic behavior. Following phagocytosis, neutrophils undergo an NHE-mediated rebound alkalosis that is associated with an increase in the generation of superoxide radicals (Fukushima et al 1996, Simchowicz 1985). The precise neutrophil pH_i after phagocytosis is believed to play a pivotal role in the regulation of neutrophil apoptosis (Coakley et al 2002). The role of neutrophils in the generation of brain injury is further emphasised by a study of neonatal rats that were rendered neutropenic with an anti-neutrophil serum and that displayed a 70% reduction in brain swelling after hypoxic-ischemic injury (Hudome et al 1997). It is still unclear, however, whether this is the direct effect of amiloride or secondary to brain injury amelioration. Nevertheless, all three-cell types (endothelia, astrocytes, and the newly recruited blood-borne neutrophil granulocytes) may provide additional non-neuronal targets for a neuroprotective action of NHE inhibitor drugs such as amiloride.

Summary

The current study supports the increasing *in vitro*, *ex vivo* and *in vitro* evidence that NHE blockade, with resultant delay in the rapid return of pH_i and prevention of rebound alkalosis, may be beneficial to cell survival after hypoxia-ischemia. Drugs that cross the blood-brain barrier and result in NHE blockade may therefore represent a useful therapeutic intervention following neonatal hypoxia-ischemia. If changes in brain pH_i account for the observed neuroprotective effect of MIA, further clinical implications include neonatal resuscitation protocols which do not currently aim to achieve a slow return of pH_i to baseline or prevent rebound alkalosis.

Chapter 8: General Discussion

In addition to the discussion and conclusions drawn in the individual chapters, a number of further conclusions can be made when this study is considered in its entirety.

The Neonatal Mouse Model of Hypoxia-ischemia

Taken in the context of previous studies the current study demonstrates changes in both the white and grey matter of the brain following significant hypoxia-ischemia. These findings underline the role of this model in the study of neonatal hypoxic-ischemic injury, which also affects both the white matter and the deep grey matter of the basal ganglia. The presence of amorphous debris on electron microscopy of microglia within the white matter following hypoxia-ischemia is of particular interest and in need of further study to help unlock the question of whether white matter injury is predominantly an axonopathy or as a result of oligodendrocyte damage. Whilst a number of cellular processes such as the neurotoxic cascade, changes in cerebral energetics, changes in intracellular brain pH and the release of cytokines have been demonstrated in the hours after a hypoxic-ischemic insult, much work is still required to identify how these different processes are interrelated, and therefore how interventions may modify the neurological outcome. It is of note, however, that the current study demonstrates that in this model, prolonged hypoxia-ischemia has global effects on the brain at least in changes in intracellular pH, and it is only in these circumstances that large cerebral infarcts occur. Whilst neither this nor previous studies have demonstrated significant histological injury in the hemisphere contralateral to the carotid occlusion, both hemispheres of the brain are affected by the insult given. This would, however, tend towards underestimating injury when comparing occluded and non-occluded hemispheres and, therefore, tend towards non significant results.

Inflammatory Sensitisation of the Developing Brain to Hypoxia-ischemia

It is clear from retrospective studies that the majority of children who develop brain injury such as cerebral palsy thought to arise in the perinatal period, have not been exposed to hypoxia-ischemia of sufficient severity to explain their neurodevelopmental outcome. The possibility of modulating factors that sensitise the brain to subsequent mild or moderate levels of hypoxia-ischemia, which may be part of a normal labour, has been suggested from both epidemiological studies (Nelson and Grether 1998) and animal models (Eklind et al 2001). Infection and the resultant fetal inflammatory response has remained the single most identifiable factor which may alter cellular and humeral responses to hypoxia-ischemia and may, therefore, affect the resultant neurodevelopmental outcome. Previous studies in adult and neonatal models of brain injury have shown apparently paradoxical sensitising and pre-conditioning effects of endotoxin pretreatment prior to hypoxia-ischemia. From these studies, it appears the effect is dependent on a number of factors including the age of the animal, the time interval between inflammatory and hypoxic-ischemic insults and the severity of hypoxia-ischemia (Ahmed et al 200, Eklind et al 2001, Eklind et al 2005). This intriguing observation forms the basis of the current study which attempts to identify some of the cellular and humeral events that occur following an inflammatory stimulus, which, in turn, may be crucial in the mechanism of perinatal brain injury. The current study identifies a specific time window of 4-12 hours after a single injection of endotoxin during which the brain is sensitised to mild hypoxia-ischemia. This finding is similar to that of other authors who have described a phase of sensitisation in the neonatal rat occurring between 4 and 6 hours after endotoxin but not at 2 or 24 hours (Eklind et al 2001, Eklind et al 2005). Additionally, the current study demonstrates that the peak sensitisation occurs around 12 hours after endotoxin injection. Furthermore, the recognition from this and previous studies that sensitisation is not instantaneous, possibly taking more than 2 hours, suggests that endotoxin injection results in a number of events including the up-regulation over 1500 genes (Eklind 2006), some of which may form the basis of the observed sensitisation. In the current study, a number

of changes were detectable in expression of adhesion molecules on vascular endothelium and microglia, within the neonatal brain, following low dose endotoxin administration and during the sensitisation window to subsequent hypoxia-ischemia. It is interesting to speculate on how the effects of endotoxin identified in the current and previous studies may be linked in an attempt to identify the mechanism by which endotoxin-mediated sensitisation occurs. Endotoxin is known to bind to Toll-like receptors (TLR) on macrophages (including microglia) as part of the innate immune response, and triggers intracellular signaling mechanisms which result in the production of pro-inflammatory cytokines (Miyake 2004, Gay et al 2007) (see Figure 8.1). Furthermore, it appears that it is through this TLR signaling that endotoxin sensitises the neonatal rodent brain to subsequent hypoxia-ischemia with no sensitisation seen in TLR deficient mice (Lehnhardt et al 2003). Whilst endotoxin may itself cause neuronal damage in cell culture, this effect is dependent on the presence of microglia (Lehnhardt et al 2003), again suggesting that it is the activation of the innate immune system through TLR4 that is critical in endotoxin mediated injury. However in the current model, low dose endotoxin did not result in neuronal loss *in vivo* or histological brain injury. Whilst it is clear from clinical observations that severe infections, particularly meningitis, may result in permanent brain injury, this form of brain injury was not investigated as part of the current study. In contrast, oxygen and glucose deprivation can directly result in neuronal loss in pure neuronal cell culture; however, the addition of microglia to the culture confers significant neuronal protection to oxygen-glucose deprivation, associated with microglial migration and close cell to cell contact with neurons (Neumann et al 2006). Interestingly this neuroprotective effect of microglia is lost if they are pretreated with endotoxin. In the animal model used in the current study, it has not been proven that endotoxin exerts its effects directly within the central nervous system. Alternatively, the changes within the central nervous system seen after systemic endotoxin may result from the systemic inflammatory response, mediators of which may cross the blood-brain barrier and in turn sensitise the developing brain to subsequent hypoxia-ischemia (Hagberg and Mallard 2005).

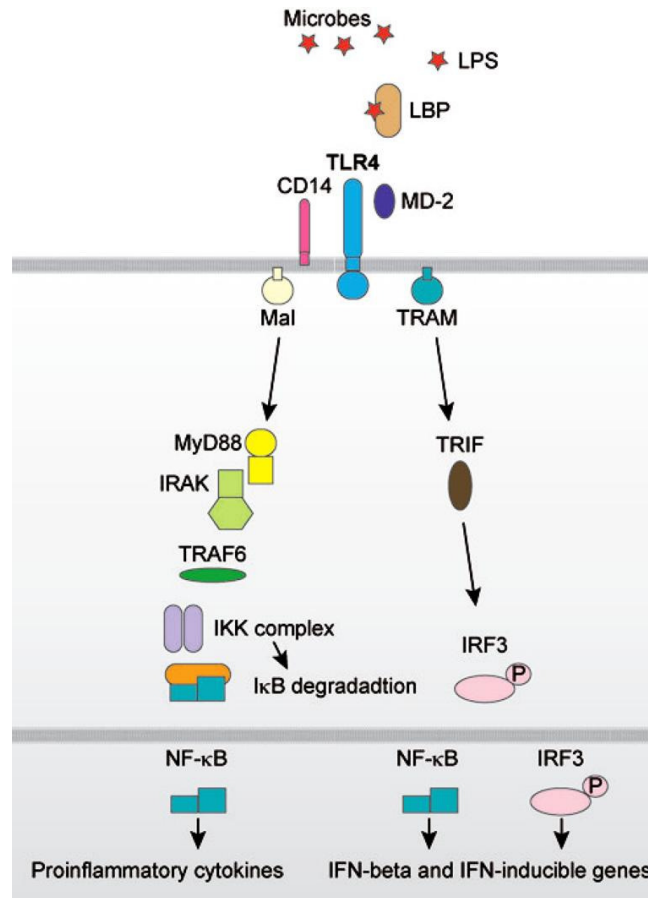


Figure 8.1: Lipopolysaccharide signaling via Toll-like receptor 4. (IKK - I κ B kinase, IRAK - interleukin-1 receptor-associated kinase, IRF3 - interferon response factor 3, LBP – LPS binding protein, LPS – lipopolysaccharide, Mal - MyD88 adaptor-like, MD2 - co-receptor of TLR4, MyD88 - myeloid differentiation primary response protein 88, NF- κ B, nuclear factor κ B, TLR4 – Toll like receptor 4, TRAF6 - TNF receptor-associated factor 6, TRAM - TRIF-related adaptor molecule, TRIF - TIR domain-containing adaptor protein inducing interferon- β). From Gay et al 2007, reproduced with permission.

In the current study, systemic endotoxin has been demonstrated to result in changes in both microglia and vascular endothelium within the window of sensitisation along with up-regulation of genes for pro-inflammatory cytokines including TNF α (Eklind et al 2006). TNF α has been previously shown to up-regulate vascular endothelial adhesion molecules such as ICAM1 and P-selectin, resulting in leukocyte adhesion, rolling and

migration into the central nervous system (Bohatschek et al 2001, Zhou et al 2006). In the current study deletion of the entire TNF cluster (TNF α , lymphotoxin α and lymphotoxin β) abolishes both sensitisation to subsequent hypoxia-ischemia and the effects of endotoxin on both microglia and vascular endothelium. Whilst the current study does not demonstrate that abolition of endotoxin-mediated ICAM1 up-regulation in animals with deletion of the TNF cluster is directly related to the loss of endotoxin-mediated sensitisation, it is possible that ICAM1 has a specific role. In the current study migration of blood borne macrophages into the CNS was not seen with low dose endotoxin (0.3 μ g/g) but this was suggested in the pilot studies using high dose (10 μ g/g) (see Chapter 3). However, it is possible that the ICAM1 expression on the vascular endothelium seen following low dose endotoxin facilitates increased migration of leukocytes into the CNS if the animal is subsequently exposed to hypoxia-ischemia. The presence of increased numbers of leukocytes may, in turn, result in an increase in neuronal loss.

Similarly, the current study, whilst demonstrating changes in integrin expression on microglia following low dose endotoxin, does not directly demonstrate their role in endotoxin-mediated sensitisation of the developing brain. As described previously, microglia have specific roles within the CNS, including immune surveillance, cytokine production and phagocytosis. Integrins are a large family of heterodimeric glycoproteins that play a crucial role in cell adhesion during development, inflammation, and tissue repair (see Figure 8.2).

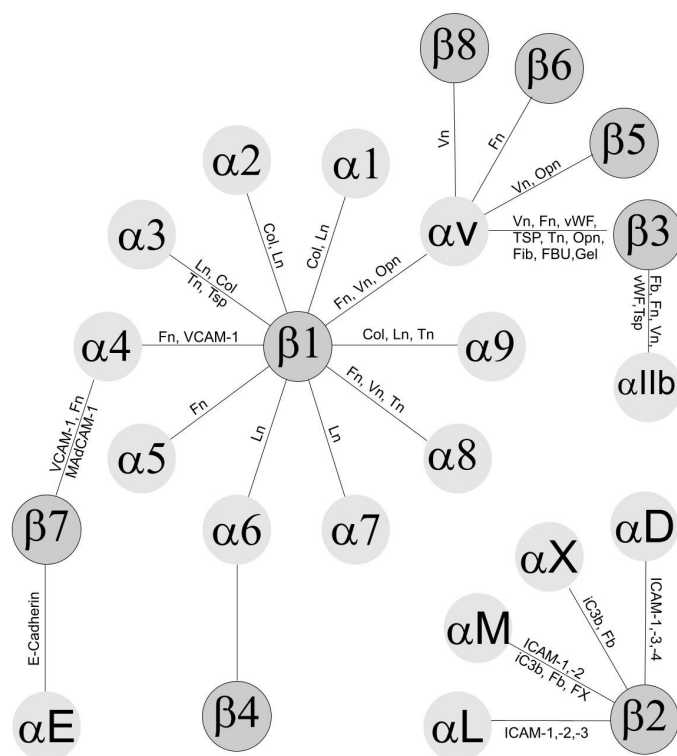


Figure 8.2: Integrin subunit association. Integrin subunits, subunit combinations and ligands of the known $\alpha\beta$ -heterodimers (adapted from Kloss et al 1999 reproduced with permission). Col, collagen; Fb, fibrinogen; Fbu, fibulin; Fib, fibrillin; Fn, fibronectin; FX, factor X; Gel, gelatin; iC3b, inactivated complement factor 3b; ICAM, intercellular adhesion molecule; Ln, laminin; MAdCAM, mucosal addressing cell adhesion molecule; Opn, osteopontin; Tn, tenascin; sp, thrombospondin; VCAM, vascular cell adhesion molecule; Vn, vitronectin; vWF, vonWillebrand factor.

Previous studies have described the detection of a set of integrins on microglia following neuronal injury with different temporal profiles (Kloss et al 1999). It appears that the patterns of integrin expression coincide with early activation ($\alpha M\beta 2$), homing and adhesion ($\alpha 5\beta 1$, $\alpha 6\beta 1$), or bystander activation ($\alpha 4\beta 1$, $\alpha M\beta 2$). In the current study, low dose endotoxin appears to have its effects predominantly on $\alpha M\beta 2$, suggesting an early activation. Whilst a significant rise in $\alpha 6$ was detected, this was histologically in a pattern consistent with vascular endothelium. Here again, it is interesting to note the loss of $\alpha M\beta 2$ integrin up-regulation in animals with a deletion of the TNF cluster. It is also interesting that, in the current study, the brain injury score used following hypoxia-

ischemia, which includes an assessment of both α M β 2 integrin immunoreactivity and microglial morphology, correlates well with quantitative injury measures such as infarct volume. It can therefore be speculated that endotoxin pretreatment begins the process of microglial activation which, without a second insult, has no implication for the developing brain. If, however, whilst the microglia are activated there is a period of hypoxia-ischemia the resultant brain injury is much greater than would be the case if the microglia were in a resting state.

In summary, this and previous studies have demonstrated that, in the hours following administration of systemic endotoxin, there is a window of sensitisation during which the neonatal brain is sensitised to hypoxia-ischemia. It appears that the toll like receptor TLR4 and the TNF family of cytokines are critical components in this sensitisation phenomenon. As endotoxin binds to TLR4 on microglia and results in the release of pro-inflammatory cytokines, including TNF α , and the current study demonstrates increased levels of the mRNA for these cytokines within the brain following endotoxin administration, it can be postulated that this sequence is at least a part of the events that trigger the sensitisation of the brain to subsequent hypoxia-ischemia. Furthermore, it is likely that microglia play a key role in this sequence of events either through direct activation by endotoxin, or by indirect activation from the systemic inflammatory response that occurs after endotoxin administration. Once activated to an 'alert' phenotype, competing theories for the downstream mechanism of sensitisation to subsequent hypoxia-ischemia include loss of normal microglial neuronal support and, hence, reduced neuroprotection (Neumann et al 2006) or increased brain injury resulting from the production of further mediators, including oxygen free radicals (Hagberg and Mallard 2005). It is, of course, entirely possible that both these mechanisms play a role in the observed effects.

This sensitisation of the brain may account for some of the children that develop brain injury of perinatal origin without evidence of a significant hypoxic-ischemic insult. It can

be speculated that, if the presence of a sub-clinical inflammatory response could be identified, for example by analysis of the maternal cytokine profile in early labour, this could be used to guide the clinician in decisions regarding intervention. Specifically, if it could be identified that a neonate was at low risk for hypoxic-ischemic injury, moderate changes in the fetal heart rate or fetal scalp blood gas profile may be acceptable. Conversely, in the presence of a significant inflammatory response, it may be preferable to proceed to operative delivery in an attempt to reduce the risk of permanent brain injury, even in early labour prior to the onset of clinical findings suggestive of hypoxia. Such stratification in the assessment of women during labour has the potential to both reduce unnecessary medical intervention, and to reduce the incidence of perinatal brain injury. Furthermore, additional information may come from assessment of differences in individual inflammatory responses caused, for example, by specific cytokine genetic polymorphisms which may put individual neonates at increased risk of injury from a variety of insults. Such assessment may, in the future, be possible prior to labour and allow informed discussion regarding method of delivery dependent on the progress of the pregnancy and labour. In addition to guiding the clinician and woman regarding method of delivery, the identification of the TNF family of cytokines as key mediators in perinatal brain injury raises the prospect of therapeutic strategies aimed at these molecule using clinical preparations already available, such as anti-tumour necrosis factor. It is of note that recent clinical observations in women with rheumatoid arthritis and inflammatory bowel disease suggest that anti-tumour necrosis factor may be safe to administer throughout pregnancy (Roux et al 2007, Rosner et al 2007). Finally, in neonates with clinical evidence of perinatal brain injury, such as neonatal encephalopathy, identification of infection as part of their neuropathology may present further neuroprotective strategies in the hours following birth by modulating the neonatal inflammatory response away from a pro-inflammatory state towards an anti-inflammatory state, hence, theoretically, reducing the amount of neuronal loss and maximising neurodevelopmental outcome.

The Role of pH Changes in the Developing Brain Following Hypoxia-ischemia

The current study has demonstrated the changes in intracellular pH that occur within the neonatal mouse brain during hypoxia and in the hours following reoxygenation. This has confirmed the finding from human and other animal studies which demonstrate acidosis during hypoxia due to anaerobic respiration and the accumulation of lactic acid. During the hours after reoxygenation, there is a transient period during which the intracellular pH of the brain is relatively alkalotic compared to baseline. Previous authors have suggested that this alkalosis is mediated by the Na^+/H^+ exchanger (LaManna 1996) and that the magnitude and duration of alkalosis correlates to the severity of neurodevelopmental outcome in the human neonate (Robertson et al 2002). In this context, it is interesting that the current study demonstrates that blockade of the the Na^+/H^+ exchanger using amiloride is able to confer significant neuroprotection *in vivo* which is in keeping with previous *ex vivo* brain slice data (Robertson et al 2005). It is possible, therefore, to speculate that the effects of amiloride seen in the current study are a result of a modulation of the pH changes that occur following hypoxia-ischemia, and that alkalosis may indeed result in a worse histological outcome in areas of brain tissue damaged by hypoxia-ischemia. This has both implications for specific therapeutic intervention in the neonatal period and in best practice for the care of neonates affected by hypoxic-ischemic encephalopathy. These will be considered further in Chapter 8: Further Work.

Chapter 8: Further Work

The current study suggests a number of interesting areas potentially requiring further investigation.

Primary Prevention

A growing body of evidence now supports that infection / inflammation acts as a sensitising agent for subsequent hypoxia-ischemia and the hypothesis of a continuum of worsening inflammation resulting in permanent brain injury from minimal hypoxia such as may occur in a normal labour (Peebles et al 2002) is intriguing. The challenge from a primary prevention perspective is the identification of such mild inflammatory responses in the mother and / or fetus so as to alert the obstetrician to avoid even minimal intrapartum hypoxia. This and previous studies have used endotoxin from E.Coli as the basis for an inflammatory stimulus. In the clinical context of obstetrics, E.Coli bacteraemia is very rare, and unlikely to be the cause of any endotoxaemia. Intriguingly, a number of organisms which make up the perioral flora in humans can produce endotoxin, which raises the possibility of a transient sub clinical bacteraemia / endotoxaemia following oral instrumentation, for example teeth scaling. The effect of these endotoxins in sensitising the developing brain to hypoxia-ischemia has yet to be studied.

Mechanisms of Injury

The current study shows a window of sensitisation between 4 and 12 hours following endotoxin administration. Other studies suggest that there may be a biphasic response to endotoxin with sensitisation seen 6 hours and 72 hours but not 24 hours after endotoxin when animals are exposed to mild hypoxia-ischemia. Interestingly, in this study, when animals were exposed to a more severe hypoxic-ischemic insult, sensitisation was seen at 6 hours after endotoxin, neuroprotection was seen at 24 hours after endotoxin and no effect was seen when endotoxin was given 72 hours prior to hypoxia-ischemia (Eklind et al 2005). This biphasic response warrants further investigations, specifically to identify the cellular and humeral events which mimic this biphasic response suggesting their role in a sensitising or neuroprotective effect. The possibility that, in certain circumstances, endotoxin can be neuroprotective may help unlock the critical events of sensitisation and may, therefore, allow therapeutic interventions in the future.

The identification of ICAM1 upregulation as the earliest detectable central nervous system change following endotoxin administration raises the question of the role of this adhesion molecule in inflammatory sensitisation. ICAM1-deficient mice have previously been shown to have impaired influx of granulocytes into the brain following systemic endotoxin in adult animals (Bohatschek et al 2001). Repeating the sensitisation experiments in ICAM1-deficient animals would help unlock the role of this molecule in the observed sensitisation phenomenon.

The current study examining the role of the TNF family of cytokines raises the potential that one or more of these cytokines ($TNF\alpha$ / $LT\alpha$ / $LT\beta$) may be the critical mediator in sensitising the developing brain to subsequent hypoxia-ischemia. An examination of animals with single cytokine knockouts which could then be cross-bred to produce double deletions and triple deletion would help to identify the role of the individual cytokines. Furthermore, the use of conditional knockouts, for example using the Cre-lox

system, would also allow the identification of the cell types involved in the sensitisation pathway. Unfortunately, at present, it is not possible to identify whether resident macrophage in the brain or recruited circulating phagocytes are involved. Under other circumstances, the use of bone marrow chimera animals can answer such questions, a technique which is not possible in the study of neonatal animals. If, as may be suspected from the body of evidence implicating TNF as the major cytokine involved, the possibility of therapeutic intervention is interesting. As mentioned previously, the use of anti-TNF monoclonal antibodies during pregnancy has been reported in the literature (Rosener et al 2007, Roux et al 2007). The potential for anti-TNF monoclonal therapy or soluble TNF receptor could be further explored in the neonatal mouse model of both hypoxia-ischemia and combined endotoxin hypoxia-ischemia as the first stage of this investigation.

pH Studies

The identification of both rebound alkalosis in the neonatal model of hypoxia-ischemia and the development of new method to visualise regional brain pH raises a number of exciting possibilities for further investigation. In the current study, the changes in pH_i during reoxygenation were studied after a prolonged period of hypoxia-ischemia, which was demonstrated to produce a global brain intracellular acidosis. Further investigation should examine the changes in pH_i following shorter periods of hypoxia-ischemia, similar to those in adult animals (Chopp et al 1990). Furthermore, in an attempt to understand the mechanism of rebound alkalosis, a number of investigations should be undertaken. The amiloride studies suggest that the Na^+ / H^+ exchanger (NHE) is critical in this mechanism. Investigating the timecourse of pH_i changes in animals with specific mutations of NHE subtypes would be exceptionally valuable in understanding the role of this transporter in both rebound alkalosis and in perinatal hypoxic-ischemic brain injury. From a potential therapeutic angle further studies of amiloride and NHE blockade are essential. Firstly using the techniques described in this study to investigate the changes in pH_i after hypoxia-ischemia in animals pretreated with of *N*-

methyl-isobutyl amiloride (MIA) would help to demonstrate the mechanism by which this drug confers neuroprotection. Furthermore, the use of other agents with differential blockade of different NHE subtypes may allow the realisation of maximal neuroprotection from preventing rebound alkalosis. The techniques described in this study would also allow the mechanism of cell signaling that underlies NHE activation. For example, the role of MEK signaling may be unlocked by examining changes in pH_i after hypoxia-ischemia in animals with MEK blockade, such as animals with a dominant negative construct.

Identification of the association between changes in intracellular pH and severity of brain injury and the sensitising effect of endotoxin on the developing brain to subsequent hypoxia-ischemia suggests that examining changes in pH_i after endotoxin and combined endotoxin / hypoxia-ischemia would be of interest. This association of pH_i changes and outcome raises a number of questions about the clinical practice of intravenous sodium bicarbonate to correct systemic metabolic acidosis in the hours after hypoxia-ischemia. Investigating the changes in intracellular brain pH, especially during the early re-oxygenation phase, in animals treated with sodium bicarbonate or saline immediately after hypoxia-ischemia may give some information about the effect of current clinical practice.

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Chapter 11 Appendix:

0.5 M Phosphate Buffer (PB)

40 ml NaOH, 142 g NaH_2PO_4 , made up to 2 litres with double distilled H_2O pH adjusted to 7.4-7.5 with H_3PO_4

0.1 M PB

200 ml 0.5 M PB made up to 1 litre with double distilled H_2O

10 mM PB

50ml 0.1M PB made up to 500 ml with double distilled H_2O

Phosphate buffered saline (PBS):

8.5 g NaCl, 20 mls 0.5 M PB made up to 1 litre with double distilled H_2O pH adjusted to 7.4 with H_3PO_4

20% Paraformaldehyde (PFA)

400 mg NaOH, 200 ml PFA made up to 1 litre with double distilled H_2O pH adjusted to 7.4 with H_3PO_4 . Stored at 4°C.

4% PFA (for perfusion)

200 ml 20% PFA 8.5 g NaCl, 20 mls 0.5 M PB made up to 1 litre with double distilled H_2O . Stored at 4°C, but freshly made each time.

4% PFA (for immunohistochemistry and Nissl)

2 ml 37% formaldehyde (BDH), made up to 50 ml with 0.1M PB

30% Sucrose

30 g sucrose (Fluka) 2 mls 0.5 M PB made up to 100ml with double distilled H₂O.
Stored at 4°C but freshly made each time.

0.1% Bovine Serum Albumin in PB (PB/BSA)

500 mg BSA, made up to 500 ml with 0.1 M PB stored at 4°C and used within 48 hours.

5% goat serum

50 µL goat serum, 1 ml 0.1M PB

'ABC' solution (Vector Labs)

50 µL A, 50µ L B, 5 ml 0.1M PB. Left to incubate at room temperature for at least 30 minutes before use.

0.05% diaminobenzidine (DAB) in the presence of 0.01% hydrogen peroxide

25 mg DAB made up to 50ml with 10mM PB filtered. 16.7 µl 30% hydrogen peroxide added prior to use

0.05% diaminobenzidine (DAB) in the presence of 0.01% hydrogen peroxide with cobalt nickel enhancement

25 mg DAB, 0.5 ml cobalt sulphate, 0.5 ml nickel chloride made up to 50 ml with 10 mM PB and filtered.16.7 µl 30% hydrogen peroxide

Cacodylate Buffer

3.64 g TRIS in 80 ml double distilled H₂O, 29.96 g Cacodylate. pH adjusted to 7.5 with HCl. 0.24 g cobalt chloride added pH adjusted to 7.2 with HCl made up to 100 ml with double distilled H₂O. Stored at 4°C

TdT solution

1 µL TdT (Roche), 1.5 µL dUTP-biotinylated (Roche), 100 µL cacodylate buffer, 897.5 µL double distilled H₂O kept on ice and used immediately.

TUNEL stop solution

300 mM NaCl (1753.2 mg / 100 ml double distilled H₂O) 30 mM sodium citrate (882.3 mg/100 ml double distilled H₂O).

Cresyl Violet (BHD)

4 g cresyl violet dissolved in 40 mls 100% ethanol in a closed 50 ml falcon tube for 15 minutes. Add to 360mls warm double distilled H₂O and mixed on a warming stirrer plate for 20 minutes. Filter and use immediately.

Biotinylated Tyramide solution

0.1% biotinylated tyramide, 0.001% hydrogen peroxide in 10 mM PB