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PATHOPHYSIOLOGY OF FETAL ASPHYXIA: FACTORS THAT INFLUENCE THE SEVERITY AND DISTRIBUTION OF NEURONAL DAMAGE

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ABSTRACT

Perinatal asphyxia is thought to be a major cause of subsequent neurological deficits. Pathological studies suggest that many of these events occur before birth. However, the relationship between specific prenatal events and neurological outcome is not clear. This thesis tested the hypothesis that certain fetal factors play a role in determining the severity and distribution of neuronal loss following in utero asphyxia.

Chronically instrumented fetal sheep at three different gestational ages; midgestation (90d), late-gestation (120-130d) or near term (>135d) were subjected to either a single or repeated insult. The insult consisted of an episode of either systemic asphyxia (umbilical cord occlusion) or cerebral ischaemia (carotid artery occlusion). The fetal parietal cortical electroencephalogram (EEG), cortical impedance (CI) indicating intracellular edema, blood pressure (MAP), electrocardiogram (ECG) and frequent fetal blood gases and metabolites were measured. Three days after the insult, histopathological analysis or immunohistochemistry was performed to determine neuronal loss and specific neurotransmitters respectively.

Transient (10min) occlusion of the umbilical cord in late-gestation fetuses, resulted in severe fetal asphyxia, hypotension (24±5mmHg, p<0.01), bradycardia (72±14bpm, p<0.001), depressed EEG activity (-17±2dB, p<0.001) and intracellular edema. The intracellular edema resolved within 27±6min, whereas the EEG activity was depressed for 5±2h, despite rapid recovery of pO₂. Neither seizures or infarction were observed. The degree of hypotension, increase in CI, lactate and recovery of post-asphyxial EEG intensity were more marked in 135d fetuses compared with the midgestation fetus (p<0.01). Neuronal loss, which was only observed in the older group, was predominantly in the hippocampus and associated with the severity of hypotension during occlusion.

Repeated episodes of cerebral ischaemia, altered the distribution of neuronal loss compared with single insults, inducing damage mainly in the striatum. The frequency of the insults determined the severity of the damage. Similarly, recurrent episodes of fetal asphyxia induced predominantly striatal neuronal loss. Each occlusion resulted in fetal hypoxia and bradycardia accompanied by increased T/QRS ratio as noted on the ECG. Progressively severe hypotension and lactic acidosis developed during successive occlusions. The EEG was depressed and CI increased with each occlusion. After the asphyxial episodes, blood pressure and heart rate returned to normal, while the T wave was inverted for 310±60min. The EEG remained depressed for 90±10min and intermittent seizures developed at 3.3±0.6h after the last occlusion. The extent of neuronal loss correlated with the degree of hypotension, increase in T/QRS ratio, duration of post-asphyxial EEG depression and number of seizures. Immunohistochemical analysis showed loss of striatal GABAergic projection neurons.

These findings demonstrate that certain prenatal factors, such as neurological maturation, pattern of the insult and cardiovascular instability can influence neuronal outcome following fetal asphyxia. An isolated brief episode of asphyxia can lead to selective hippocampal neuronal loss, while repeated insults induce predominantly striatal damage. These distributions of neuronal loss may be associated with postnatal sequelae such as learning disorders and cerebral palsy.
PAPERS:


REVIEWS/CHAPTERS:


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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>CaO₂</td>
<td>Oxygen content</td>
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<td>CI</td>
<td>Cortical impedance</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>Day</td>
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<td>Decibel</td>
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<td>EAA</td>
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<td>ECG</td>
<td>Electrocardiogram</td>
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<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
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<tr>
<td>EMG</td>
<td>Electromyogram</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
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<td>GM₁</td>
<td>Monosialoganglioside</td>
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<td>Hour</td>
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<td>Hb</td>
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<td>HR</td>
<td>Heart rate</td>
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<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth retardation</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
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<td>MK₈₀₁</td>
<td>(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-iminemaleate</td>
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<tr>
<td>NMDA</td>
<td>N-methyl d-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
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<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
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<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PVL</td>
<td>Periventricular leukomalacia</td>
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<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
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CHAPTER 1.
INTRODUCTION

Perinatal asphyxia is a major cause of subsequent neurological deficits. Pathological studies show that many of these events occur before birth and only some may be related to intrapartum asphyxial insults. However, the precise relationship between pathological cause and effect is not clear. Studies over the last decade have elucidated many of the cellular and molecular mechanisms involved in brain damage. It is now clear that, in association with an asphyxial event, neurons die in two phases. Primary cell death is associated with the insult itself. The initial insult induces a sequence of events that lead to secondary neuronal loss some hours to days later which may be accompanied by delayed seizures and swelling of the brain.

While the understanding of the general mechanisms of neuronal damage has recently advanced, additional factors may affect the outcome in the perinate. A wide range of neurological injuries are seen in children, such as cognitive, motor and sensory deficits. However, often the precise relationship between the nature of the insult and these different injuries is not known.

This thesis was designed to examine some of the prenatal factors that may influence different neurological outcomes. First, I briefly summarise fetal physiological responses to intra-uterine asphyxia and the general mechanisms of neuronal death. In chapter two I describe the methodology used and the subsequent chapters (3-7) are presented as independent studies. These studies describe how factors such as neuronal maturation and nature of the insult influence neuronal loss following either single or multiple in utero asphyxial events. The final discussion (Chapter 8) summarises the studies and provides some future research directions within this field.
1.1. Pathophysiology of fetal asphyxia

Central to fetal well-being is an appropriate constitution of the respiratory gases, oxygen and carbon dioxide. In utero, oxygen is transferred from the mother to the fetus and carbon dioxide removed from the fetal circulation, via the uterine-placental circulation. The transfer of oxygen to the fetus is accomplished by simple diffusion across the placenta, due to the maternal and fetal pO₂ difference (Carter, 1989). Placental oxygen transfer is facilitated by the high O₂-binding affinity (fetal haemoglobin) of fetal blood (Wilkening et al. 1988; Kitchen, Brett, 1974). Oxygen transport to the fetus is therefore determined by uterine and umbilical blood flows and the oxygen carrying capacity of the blood such as fetal haemoglobin concentration.

Asphyxia refers to an impairment in the exchange of oxygen and carbon dioxide.¹ During fetal life, asphyxia is predominantly a result of placental insufficiency or impaired uterine or umbilical blood flow, that may occur during cord compression or chronic utero-placental instability. Hypoxia only reduces oxygen supply to the fetus, reduction in uterine or umbilical blood flow also interferes with fetal carbon dioxide elimination as well as with energy substrate supply to the fetus. In most instances, asphyxia leads to systemic hypoxia, ischaemia, hypercapnia and metabolic acidosis. Under physiological conditions the fetus develops and functions well at low pO₂ compared with that postnatally. Furthermore, the fetus has several adaptive responses to mild asphyxia, including redistribution of blood flow and increased pO₂ extraction (Jensen et al. 1987; Yaffe et al. 1987; Itskovitz et al. 1983). However, further interference with oxygenation and/or circulation in-utero can lead to fetal compromise. The following sections will describe some effects asphyxia may have on the

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¹ Definitions: Asphyxia is defined as systemic hypoxia, hypercapnia and metabolic acidosis that may occur during umbilical cord occlusion. Cerebral ischaemia or hypoxia-ischaemia refers to insults that selectively interfere with the cerebral circulation. Hypoxia is defined as a reduction in pO₂ availability.
fetus, with particular emphasis on the effect of reduced umbilical blood flow.

1.1.1. Cardiac response to asphyxia

The major determinants of cardiac output are loading conditions of the ventricles, preload and afterload, myocardial contractility and heart rate. Studies in fetal sheep indicate that fetal ventricular performance is limited in its response to changes in pre- and afterload (Kirkpatrick et al. 1976; Gilbert, 1982; Thornburg, Morton, 1983). If the filling pressure is reduced below normal levels, ventricular output falls dramatically. In fact, during compression of the umbilical cord, there is a linear relationship between the change in combined ventricular output and the change in umbilical blood flow (Itskovitz et al. 1987). An increased afterload, such as increased arterial pressure in response to hypoxia, also results in reduced cardiac output (Hawkins et al. 1989).

Another determinant of cardiac output is heart rate, where an increase in heart rate increases cardiac output. However, in contrast to the adult, asphyxia in the near term fetus is always associated with a fall in heart rate. The bradycardia is likely to be mediated through both chemo- and baroreflexes (Rudolph, 1984). In addition, acute severe hypoxia has been suggested to cause bradycardia by direct myocardial depression (Itskovitz et al. 1982). Thus, umbilical cord occlusion invokes both reduced ventricular filling pressure and bradycardia, which leads to a fall in cardiac output.

1.1.2. The effect of asphyxia on fetal circulation

While the fetus has only limited ability to increase cardiac output in response to asphyxia, it has other adaptive responses, such as redistribution of blood flow. In general following hypoxia, the fetal circulation is redirected with preferential flow towards vital
organs such as the heart, brain and adrenals, at the expense of blood flow to other organs (Cohn et al. 1974; Sheldon et al. 1979; Peeters et al. 1979; Rudolph, 1984). The redistribution of cardiac output is accomplished by a reduction in vascular resistance in high priority organs, concomitantly with maintained or increased resistance in peripheral organs, such as muscle, kidney, intestines, and lung (Reid et al. 1991). The regulatory mechanisms triggering this complex response of the circulation are not fully understood, but involves activation of peripheral chemoreceptors and endocrine responses, including the secretion of arginine vasopressin (AVP) and catecholamines (Kjellmer, 1991; Espinoza et al. 1989; Iwamoto, 1993).

There are differences in the fetal vascular response depending on the cause of asphyxia. Thus, the cardiac output redistribution to different organs differs following umbilical cord occlusion (Itskovitz et al. 1987) compared with reduction of uterine blood flow (Jensen et al. 1991; Reid et al. 1991), maternal hypoxia (Cohn et al. 1974) or severe fetal haemorrhage (Meyers et al. 1991). With a 50% reduction in umbilical blood flow, increased blood flow was observed to the brain, heart, carcass, kidneys and gastrointestinal tract, whereas pulmonary blood flow fell. However, oxygen delivery, was only maintained to the brain and myocardium, but was reduced to peripheral, renal and gastrointestinal circulations (Itskovitz et al. 1987). Prolonged vasoconstriction to the peripheral organs often produces secondary problems, such as renal dysfunction, hyperperistalsis and passage of meconium (Perlman et al. 1989).

In addition to redistribution of cardiac output, preferential streaming of blood flow during fetal stress has been identified. For example, cord compression increases the proportion of umbilical venous blood passing through the ductus venosus and thus facilitates the delivery of the most highly oxygenated blood directly to the left ventricle and thus to the
brain, without prior passage through the hepatic circulation (Rudolph, 1984).

Cerebral and myocardial circulation is thus maintained during periods of mild to moderate asphyxia. However, as the asphyxia becomes more severe, the compensatory mechanisms fail and blood flow to the vital organs is reduced.

1.1.3. Cerebral blood flow response to asphyxia

Impaired cerebral blood flow is believed to be an important factor in the pathogenesis of cerebral injury (Vannucci et al. 1988). Total cerebral blood flow differs substantially in magnitude and regional distribution with advancing gestation and after birth (Jones et al. 1982; Szymonowicz et al. 1988). In the fetal sheep, brain blood flow increases during development, from approximately 30ml/min per 100 g of tissue in the 0.4 gestation fetus to approximately 130ml/min per 100 g in the term fetus (Rudolph, Heymann, 1970). In the fetus flow is highest to the caudate nucleus and the choroid plexus, followed by the brain stem, the cerebellum and the cortex. Cerebral blood flow is lowest in the white matter (Szymonowicz et al. 1988). In the newborn lamb, cerebral blood flow is higher in the cortex and cerebellum than the brainstem (Szymonowicz et al. 1988).

In response to hypoxia, the fetus shows cerebral vasodilation and subsequent increase in cerebral blood flow (Ashwal et al. 1984). Hypercapnia secondary to asphyxia causes further vasodilation of the cerebral vessels (Rosenberg et al. 1982; Ashwal et al. 1984; Habgood et al. 1991). During periods of hypoxia, cerebral blood flow is much greater to the brainstem than to the cortex and subcortex (Ashwal et al. 1984). However, under conditions of severe asphyxia such as uterine blood flow reductions to 25%, the augmentation of cerebral blood flow cannot be maintained (Yaffe et al. 1987).

Cerebral blood flow is kept relatively constant over a wide range of arterial pressures,
so called "autoregulation". However, during episodes of severe asphyxia, cerebral blood flow may become pressure passive and be directly related to arterial pressure. Therefore, severe asphyxial insults that cause a fall in arterial pressure lead to a decrease in the perfusion of the brain (Lou et al. 1979b; Lou et al. 1979a; Papile et al. 1985; Hohimer et al. 1991).

While moderate degrees of hypoxia and/or fluctuations in arterial pressure are likely to be well tolerated by the fetus, prolonged and severe hypoxia and hypotension are detrimental to the cerebral circulation (Richardson, 1989; Tweed et al. 1983).

1.1.4. The effects of asphyxia on cerebral oxygen consumption and metabolism

The maintenance of cerebral oxygen delivery is vital for the normal function and integrity of the brain. As described above, during periods of mild to moderate hypoxia, the fetal brain vasculature can vasodilate and increase blood flow in order to maintain oxygen supply. In addition, during sustained hypoxia, the fetal brain is able to increase extraction of oxygen (Richardson et al. 1989). For example, following umbilical cord compression, oxygen consumption of the brain is maintained by increased oxygen extraction until oxygen delivery is reduced by approximately 50% (Itskovitz et al. 1983) or arterial oxygen content remains above 1mM (Jones et al. 1977). However, with more severe reduction of umbilical blood flow, despite increased oxygen extraction to 75-80%, normal pO₂ consumption cannot be maintained (Itskovitz et al. 1987). Furthermore, when metabolic acidosis develops (fetal pH < 7.0), there is a rightward shift in the oxyhemoglobin dissociation curve, resulting in reduced fetal oxygen extraction (Richardson et al. 1993). Thus following reduction of cerebral blood flow, severe hypoxia or acidosis, cerebral oxygen consumption is reduced (Chao et al. 1989; Field et al. 1990; Richardson et al. 1989).

Cerebral energy metabolism is severely affected when oxygen delivery fails (Hohimer
et al. 1991). Oxidative phosphorylation cannot be maintained under these circumstances and cerebral energy production falls. During acute asphyxia in the near term fetal guinea pig and hypoxia-ischaemia in the immature and adult rat brain there is a progressive fall of high energy phosphates, such as, adenosine triphosphate (ATP), creatine-phosphate, glucose and fructose-1,6-diphosphate, whereas adenosine diphosphate, adenosine monophosphate and lactate concentrations increase (Berger et al. 1991; Palmer et al. 1990a; Crumrine, LaManna, 1991). Perturbation of cerebral cellular energy homeostasis results in cessation of cerebral electrical activity. Subsequently, there is loss of cerebral membrane ion homeostasis and failure of membrane function (see below).

1.2. Mechanisms of asphyxial brain damage

The remarkable resistance to hypoxia of the fetus and the newborn has been known for a long time (Dawes et al. 1959). However, severe asphyxia may have devastating effects on the developing brain. Mechanisms that lead to neuronal death are complex and interactive but it has recently become clear that cell death occurs in phases, one associated with the initial insult and the early reperfusion period and a second phase occurring several hours to days later. These phases have been described as primary and secondary neuronal death (Gluckman, Williams, 1992).

The precise processes involved are not fully understood, but a number of mechanisms have been implicated during these phases of neuronal death. Excessive release of excitatory neurotransmitters, intracellular sodium, water and calcium accumulation, and free radical production have all been suggested to play a role during the primary phase. Recent studies have clearly demonstrated that delayed neuronal death, identified as internucleosomal DNA fragmentation, develops several hours after cerebral ischaemia in adult gerbils and neonatal
rats (Okamoto et al. 1993; Beilharz et al. 1994). This delayed phase appears to be associated with changes in cerebral energy metabolism and increased intracellular calcium levels (Hashimoto et al. 1992). In addition, post-asphyxial excitotoxicity, seizures and secondary intracellular edema can occur during this time period (Williams et al. 1991; Williams et al. 1990). The role of some of these processes will be discussed below.

1.2.1. Intracellular edema

Neurons maintain plasma and intracellular membrane integrity by energy dependent processes. Under physiological conditions, up to 60% of total cerebral oxygen consumption is required to pump sodium out of the cell and potassium into the cell (Erecinska, Silver, 1989). Cerebral hypoxia-ischaemia, rapidly leads to depletion of high energy substrates and loss of ion homeostasis, resulting in influx of osmotic water and intracellular swelling, so called cytotoxic edema. Such, membrane instability may lead to cell lysis.

Although electrical activity of the brain ceases within seconds of ischaemia, disruption of the cell membrane integrity and intracellular swelling occurs only after some minutes (Astrup et al. 1981). Cessation of electrical activity itself does not appear to be a major determinant of acute brain damage, whereas intracellular edema and related perturbation of ion homeostasis and acid-base balance can lead to irreversible damage.

After severe cerebral ischaemia insults, acute cytotoxic edema does not recover immediately with reperfusion and is often followed by a secondary rise of edema (Williams et al. 1991). The exact role of the delayed brain swelling is not clear, but indicates a secondary phase of impaired ion homeostasis that may be associated with the delayed phase of neuronal loss. Following cerebral ischaemia, delayed cytotoxic edema correlates with poor neuronal outcome in fetal sheep (Williams et al. 1991). Secondary disruption of cerebral
oxidative metabolism has been observed in asphyxiated neonates over a similar time course (Wyatt et al. 1989) and may correspond with the secondary phase of cytotoxic edema seen in experimental animals (Williams et al. 1991).

1.2.2. Excitatory amino acids

Excitatory amino acids (EAA), such as glutamate and aspartate, may accumulate during fetal asphyxia and neonatal cerebral hypoxia-ischaemia (Hagberg et al. 1987; Andine et al. 1991; Silverstein et al. 1986a). Both increased release of presynaptic EAA and impaired neuronal and glial uptake mechanisms lead to accumulation of extracellular concentrations of EAA. Although, EAA activity plays a major role in normal cerebral function, there is considerable evidence that excessive amounts of extracellular EAA are involved in the pathogenesis of brain damage (Choi, 1992).

Glutamate exerts its effects through receptors that can be divided into three distinct subtypes; NMDA (N-methyl-D-aspartate), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate, the last two are referred to collectively as non-NMDA receptors. The non-NMDA receptors mediate fast excitatory transmission, passing mainly monovalent cations, while the NMDA receptor is associated with slower transmission and $\text{Ca}^{2+}$ permeability during membrane depolarisation (Collingridge, Lester, 1989; MacDermott et al. 1986).

In vitro studies have indicated that glutamate produces acute neuronal swelling by facilitating influx of sodium and chloride ions and osmotic water during hypoxia via the kainate receptor (Rothman, 1985; Rothman, Olney, 1986). Delayed neuronal death correlates with cellular uptake of calcium (Goldberg, Choi, 1993; Manev et al. 1989). Evidence exists that calcium ions enter the cell through the NMDA receptor (MacDermott et al. 1986). A
number of studies have shown neuroprotection by administering NMDA-antagonists either before or after cerebral hypoxia-ischaemia (Gill et al. 1988; McDonald et al. 1987; Hattori et al. 1989; Tan et al. 1992). Similarly, the selective AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)-quinoxaline (NBQX) improves neuronal outcome following cerebral ischaemia in the rat (Li, Buchan, 1993; Diemer et al. 1992; Nellgard, Wieloch, 1992). These studies support the concept of glutamate induced neuronal death.

1.2.3. Calcium

Under physiological conditions calcium plays an important role in multiple cellular reactions. However, a steep concentration gradient is maintained between intra- and extracellular compartments by energy dependent processes, with low cytosolic calcium concentrations.

During hypoxia-ischaemia energy failure occurs and intracellular calcium increases rapidly via several mechanisms (Deshpande et al. 1987). The initial Na⁺/K⁺ flux leads to depolarisation of the cell membrane which opens voltage-sensitive calcium channels, resulting in Ca²⁺ influx. In addition, as mentioned above, calcium can enter the cell via NMDA-receptor operated channels (Andine et al. 1992; Randall, Thayer, 1992). Intracellular free calcium also increases due to release from mitochondria and endoplasmic reticulum. Pathological levels of intracellular calcium leads to activation of phospholipases, which can cause membrane breakdown and thus release of free fatty acids, leading to lysis and death of the cell. Furthermore, excessive intracellular calcium overactivates proteases leading to breakdown of the cytoskeleton and cell death (Siesjö, 1992).
1.2.4. Free radicals

Following transient asphyxia, cerebral blood flow is re-established and the brain is reoxygenated. During reperfusion oxygen free radicals are produced in the brain. It has been suggested that these compounds play an important role in neuronal death mainly through lipid peroxidation causing cellular membrane damage and edema.

Free radicals are molecules or atoms with one extra valence electron in its outer orbit. This makes these molecules very chemically reactive and permits them to initiate and perpetuate oxidative chain reactions. Under physiological conditions, endogenous scavenging systems exist, enzymes such as endoperoxidase and catalase or nonenzymatic scavengers (cholesterol, α-tocopherol, ascorbic acid and thiol containing compounds), to protect cellular components from the oxidising effects of free radicals. However, during cerebral hypoxia-ischaemia and in the period of reperfusion several processes take place that disrupt the balance between formation and scavenging of oxygen free radicals.

With energy failure, there is degradation of ATP to adenosine and to hypoxanthine. Thus an accumulation of hypoxanthine occurs during the insult. Under physiological conditions hypoxanthine is converted to xanthine by the enzyme xanthine dehydrogenase. However, during periods of hypoxia-ischaemia and reperfusion, xanthine dehydrogenase is converted to the enzyme xanthine oxidase, whose activation leads to the formation of oxygen free radicals (McCord, 1985). In fetal sheep, an increase in cerebral hypoxanthine has been observed following asphyxia (Kjellmer et al. 1989).

During hypoxia-ischaemia, free fatty acids, such as arachidonic acid, accumulate from increased turnover of membrane phospholipids. Arachidonic acid is the precursor of both prostaglandins and leucotrienes. Free radicals may be formed as a by-product of the synthesis of prostaglandins by the action of the enzymes cyclooxygenase and lipooxygenase.
Nitric oxide (NO) has recently been suggested to cause free radical damage (Dawson et al. 1992). NO is synthesized by the conversion of L-arginine to L-citrulline by the enzyme NO synthase (NOS) (Palmer et al. 1988). There are three isoforms of the enzyme NOS (Moncada et al. 1991; Garthwaite, 1991). Two forms are found in the vascular endothelium and neurons. These are constitutively expressed and are Ca$^{2+}$ and calmodulin dependent. The third isoform is inducible in macrophages and is independent of Ca$^{2+}$. An interaction between NO and glutamate toxicity has been suggested, whereby neuronal NO is produced in response to activation of NMDA receptors (Cazevieille et al. 1993). NO neurotoxicity appears to be mediated by NO reacting with superoxide ($O^2\cdot$) to produce peroxynitrite anion (ONOO$^-\cdot$) (Lipton et al. 1993).

Pre-treatment with allopurinol (Palmer et al. 1990b) or oxypurinol (Phillis, Sen, 1993), both xanthine oxidase inhibitors, protects against the formation of free radicals and neuronal loss following ischaemia/reperfusion in adult and neonatal rats. Recently, post-ischaemic (15min) treatment with allopurinol also proved neuroprotective in the neonatal rat (Palmer et al. 1993). Free radical scavengers, such as superoxide dismutase (SOD) have shown some neuronal protection following cerebral ischaemia in cats (Araki et al. 1992). Similarly, in vitro studies have shown inhibition of lipid peroxidation and in vivo neuronal protection by pre- or post insult administration of the antioxidant LY231617 in global ischaemia in rats (Clemens et al. 1993). Inhibition of NO synthesis, by N$^\omega$-nitro-L-arginine (NOARG), shows neuroprotection in neonatal rats, if given before but not after hypoxia-ischaemia (Hamada et al. 1994). In contrast, following focal ischaemia in rats, the NO synthase inhibitor N-nitro-L-arginine (L-NNA) was neuroprotective when given after the insult (Nagafuji et al. 1992).
1.2.5. Post-asphyxial depression and seizures

Asphyxia results in suppression of electrical activity, which may be caused by the release of inhibitory neuromodulators such as $\gamma$-aminobutyric acid (GABA), serotonin (Pappius, 1990) and adenosine (Lagercrantz et al. 1986). If the initial insult is mild the electrical activity returns to normal within a couple of hours. However, following severe events the neonate may be neurologically depressed and the electroencephalogram remains suppressed for several hours (Sarnat, Sarnat, 1976). Post-ischaemic depression for more than four hours is strongly correlated with poor neurological outcome in the fetal sheep (Williams et al. 1992).

Following severe insults and prolonged neurological depression, a period of hyperexcitability or seizures may occur. Post-asphyxial seizures are associated with poor neurological outcome in neonates (Mellits et al. 1982; Tudehope et al. 1988). In fetal sheep, post-ischaemic seizures worsen neuronal loss and treatment with the NMDA receptor antagonist and anticonvulsant MK-801 reduces damage (Tan et al. 1992).

The NMDA receptor appears to be involved in the generation of seizures, but exact mechanisms are not clear. Overstimulation of this receptor by excitatory amino acids may play a role (Stringer, Lothman, 1988; Morimoto, 1989). Alternatively, loss of inhibitory neuromodulators such as GABA has been suggested to cause seizures (Sloper et al. 1980). However, GABAergic anticonvulsants are often ineffective in suppressing neonatal seizures (Goldberg et al. 1986; Mizrahi, 1987).
1.3. Factors that influence outcome and regional distribution of damage

A wide range of neuropathological patterns of damage, subsequent to presumed perinatal asphyxial injury, are seen in the newborn. Some of these distributions of damage have been suggested to be due to factors such as the developmental stage of the brain and the nature of the insult, but there is no clear relationship between such factors and outcome (Wiklund et al. 1991; Sinha et al. 1990; Stewart, 1988).

1.3.1. CNS maturation

Neurodevelopment

The nervous system develops from the neural plate of the very early embryo. In general neurons are generated earlier than glia cells. Nervous system development consists of cell proliferation, migration, differentiation and myelination (for review see Rees, 1994).

Neuronal precursor cells first start to appear around the lumen of the neural tube in a zone called the ventricular zone. The neuronal cells remain mitotic over a number of cell divisions followed by a switch to postmitotic phase, the so called "birth date" of the neuron. Additional proliferative zones are also found in the subventricular zone, which generates the small neurons of the striatum, and the hilar zone of the dentate gyrus, which gives rise to the granule cells of the hippocampus. The main period of human neocortical neuronal proliferation takes place between 10 to 18 weeks of gestation (Dobbing, Sands, 1970).

As neurons terminate mitosis, most migrate away from the proliferative zone with the help of radial glia. The radial glia are early astrocyte-like cells that form a "scaffolding" from the ventricular zone through to the pial surface. In the cortex neurons migrate in an inside-to-outside fashion. Thus the earliest formed neurons are located in the deeper layers of the cortex while subsequently generated neurons are positioned closer to the cerebral
Once the neurons have reached their final destination differentiation begins, including development of dendrites, axons and synaptic specialisation. The dendritic tree expands in particular between 27 and 32 weeks gestation in the human (Purpura, 1975). The earliest synapses are found in the human cortical plate between 19 and 23 weeks gestation (Molliver et al. 1973).

The proliferation of glia cells, with the exception of radial glia, occurs after neuronal proliferation. In the human this process begins at midgestation and continues to after birth. Myelination occurs in different regions of the brain at different periods during development. While some sensory and motor systems are myelinated as early as 5 months gestation, myelination rates peak from birth to 1 year in the human (Yakolev, Lecours, 1967; McArdle et al. 1987).

Although neuronal development follows a similar sequence in the human and other mammalian species, the exact timing of specific events in relation to birth differs. The fetal sheep is precocial compared with the human, with many of the developmental stages described above occurring earlier in gestation (Cook et al. 1987). Thus, the neuronal proliferation occurs between 40-90 days (term 147 days) and glial proliferation between 95 and 135 days (McIntosh et al. 1979). Therefore, the near-term fetal sheep show similarities in neurodevelopment to the term human. The midgestation fetal sheep is at a neurodevelopmental stage prior to the onset of myelination and at the end of neuronal proliferation and thus resembles the 25 to 30 week gestation human fetus.

Rats are neurologically immature at birth. Therefore while the critical brain development period is before birth in many species, the most rapid brain development in the rat occurs during the first 21 postnatal days (PND). Most regions of the cerebrum are formed

**Neurotransmitters during development - selected aspects**

During development neurotransmitters are involved in a number of processes including regulation of neuronal survival, dendritic and axonal structure and synaptogenesis. Specific neurotransmitter systems develop at various time points, resulting in differences in their relative influence in the maturing brain. Certain transmitters appear early in gestation but disappear at later stages. Furthermore, immature neurons may change their neurotransmitters during development (Black et al. 1984). Several factors, including electrical activity, trophic peptides, glial cell interactions and neuronal target tissue, appear to be involved in this plasticity of the neurotransmitter systems during ontogeny (Landis, 1990). In general, neural development is thought to mature in a caudal to rostral sequence. Therefore brainstem neurons and their associated neurotransmitters tend to develop earlier than more rostral structures such as the cortex.

Ontogenic studies have revealed alterations in excitatory amino acids and their receptors. Transient increased binding to the NMDA receptor in the human frontal cortex has been observed between 1 and 2 years postnatal age (Kornhuber et al. 1988). Similar observations have been made in the neonatal rat hippocampus where glutamate binding sites overshoots at PND 9 followed by a decline to adult levels (Baudry et al. 1981). This has implications for the neurotoxicity of EAA during development. The mature nervous system is particularly vulnerable to kainate but this is relatively non-toxic in the immature brain. In contrast to the effect of kainate, NMDA toxicity peaks around PND 7 in rats (McDonald et al. 1988). The toxicity of NMDA at PND 7 is around 60 times greater than in the adult.
Therefore, the developmental susceptibility to excitotoxic damage may vary a great deal with age.

The inhibitory neurotransmitter GABA is present relatively early in gestation. In the fetal rat, GABA is first noted at 15 days gestation. At birth the endogenous levels of GABA are 60% of adult concentrations. Benzodiazepine receptors, which develop in parallel with GABA, are present by 68 days gestation in the fetal sheep, followed by a fourfold increase in binding between 100 and 120 days gestation at which time adult values are reached (Villiger et al. 1982). GABA can be released in the 85 day old fetal sheep in response to depolarisation (Penning et al. 1991). In the human fetus GABA levels are relatively low but detectable at 15-17 weeks of gestation (Gale, 1977). Glutamic acid decarboxylase (GAD) activity, the biosynthetic enzyme for GABA, begins to rise in the neocortex during the last trimester and reaches a peak during the first two postnatal years followed by a decline to adult concentrations (Diebler et al. 1979).

The relative vulnerability of GABAergic neurons to ischaemia during development is not clear. Both adult in vivo and in vitro studies have suggested that GABAergic neurons are relatively resistant to hypoxia-ischaemia and glutamate induced injury (Gonzales et al. 1992; Tecoma, Choi, 1989; Kreisman et al. 1991). However, others have shown selective loss of cortical GABAergic interneurons following hypoxia in the infant monkey (Sloper et al. 1980) and striatal GABAergic cells following forebrain ischaemia in adult rats (Francis, Pulsinelli, 1982). This could partially explain propensity to post-asphyxial hyperexcitability.

**Preterm vs term fetal brain damage**

The preterm infant is particularly vulnerable to lesions of the white matter and intraventricular haemorrhage (Volpe, 1992) White matter lesions or periventricular
leukomalacia (PVL) are often seen as bilateral infarctions adjacent to the lateral ventricles with secondary enlargement of the lateral ventricles (Banker, Larroche, 1962).

A limited number of investigations have studied the pathogenesis of brain damage in the immature brain. Unilateral hypoxia-ischaemia for up to 3.5 hours in the 7 day old rat produce severe cerebral injury (Rice et al. 1981). Although damage occurs in myelinating foci, widespread neuronal loss is also found in grey matter regions. In the midgestation fetal sheep, a combination of hypoxia and hypovolemia, results in severe necrosis of the entire cerebrum (Ting et al. 1983). Recently, prolonged hypoxia in the midgestation fetal sheep was shown to induce PVL in conjunction with cortical necrosis (Penning et al. 1994). Thus no experimental studies to date have been able to produce pure white matter damage.

In contrast to the preterm infant, white matter lesions in the term fetus or infant are less common (Low et al. 1989). Hypoxic-ischaemic cerebral injuries in the term fetus often involve diffuse infarctions, particularly in the parasagittal cortex (Allan, Riviello, 1992; Volpe, Pasternak, 1977). Similar pathological outcome is seen in late-gestation fetal sheep following prolonged cerebral ischaemia or partial asphyxia (uterine artery occlusion) (Williams et al. 1992; Gunn et al. 1992). Brief global asphyxia in the term monkey fetus has been shown to cause damage to deeper brain structures, including lower brain stem nuclei and cerebellum (Myers, 1972). This damage was suggested to be related to the higher metabolic rate in the injured regions (Myers, 1972). Other patterns of damage seen in the neonate involve the basal ganglia and thalamus (Burton et al. 1984; Hayashi et al. 1991). The aetiology of such injuries is not known.
1.3.2. Repeated insults

Cumulative effect

While the majority of studies have investigated the effect of a single insult on neuronal outcome, limited evidence in adult animals exists on the effect of repeated insults. In adult rats and gerbils, repeated ischaemic episodes appear to interact and aggravate neuronal loss when repeated at 1 hour intervals compared with a single episode of the same length (Kato et al. 1989; Nakano et al. 1989).

The mechanisms of the cumulative effect are not known. It has been suggested that additive neuronal loss is seen when insults are repeated during post-insult hypoperfusion (Masaoka et al. 1988). Cumulative edema and progressively more prolonged deficits in brain protein synthesis activity has been observed following repeated brain ischaemia in the gerbil (Nowak et al. 1990; Widmann et al. 1992). Treatment with the membrane stabiliser monosialoganglioside GM1 following repeated cerebral ischaemia in the fetal sheep, improves post-insult recovery of edema and reduces neuronal loss (Tan et al. 1994). Cumulative neuronal loss has also been associated with abnormal intracellular calcium accumulation (Araki et al. 1990). Although no acute cumulative effect on extracellular glutamate has been shown (Nakata et al. 1993), excitotoxic mechanisms have been suggested to play a role. Recently, repeated insults were shown to result in a delayed increase in the excitotoxic index (glutamate x glycine / GABA) which was not observed following single insults (Lin et al. 1992). Pre-treatment with the NMDA antagonist MK801 improves outcome following repeated ischaemia in the gerbil (Kato et al. 1990).

Ischaemic tolerance

The interval between repeated insults appear to be of crucial importance in
determining the neurological outcome. While insults repeated at 1 hour intervals worsen outcome, less frequent insults have been suggested to protect the brain from subsequent insults (Kitagawa et al. 1991). Therefore, a brief period of cerebral ischaemia, one to several days prior to a more prolonged insult reduces the extent of neuronal loss. The protective mechanism is thought to involve the induction of heat-shock proteins (Kirino et al. 1991; Nishi et al. 1993). Heat shock proteins may exert their protective effect by maintaining the tertiary structure of normal or partially denaturated proteins (Pelham, 1986). Ischaemic tolerance has been associated with early recovery of protein synthesis (Nakagomi et al. 1993) and accelerated heat shock gene expression (Aoki et al. 1993a; Aoki et al. 1993b).

The role of ischaemic tolerance in the immature brain is not known. However, induction of heat shock proteins has been demonstrated in the neonatal rat brain following unilateral hypoxia-ischaemia (Dwyer et al. 1989; Munell et al. 1994; Ferriero et al. 1990).

1.3.3. Growth retardation

Retarded growth during fetal life may affect subsequent development in the infant. Intra-uterine growth retardation (IUGR) in the human is associated with altered central nervous system (CNS) function (Kjellmer et al. 1992; Todorovich et al. 1987; Stanley et al. 1989). Studies in IUGR neonates and experimental animals have reported altered maturation of the CNS (Amiel-Tison, 1980; Cook et al. 1988). Histological studies have shown that following IUGR in the fetal sheep, there is a reduction in the growth of the neuropil in the cerebellum, motor and visual cortices and the hippocampus (Rees, Harding, 1988; Rees et al. 1988). In fetal guinea pigs, total and regional cerebral blood flow is lower in IUGR fetuses compared with controls (Detmer et al. 1991). Several studies have indicated that cerebral neurotransmitter levels are modified by IUGR (Thordstein et al. 1992; Represa et
A recent study, suggests that pregnancies complicated by IUGR and maternal hypertension are associated with subsequent poor neurological development in infants (Spinillo et al. 1993).

Although, growth retardation in newborns has been associated with an increased risk of subsequent neurological disabilities, it is not clear whether there is an increased vulnerability to asphyxia in IUGR. In IUGR guinea pigs, cerebral tolerance to hypoxia is reduced, as measured by somatosensory-evoked potentials (Thordstein, Kjellmer, 1988). In contrast, hypoxia-ischaemia in neonatal rats born with IUGR results in less brain damage than control litter mates (Trescher et al. 1990). Therefore, the relationship between IUGR and asphyxia remains uncertain.

1.4. Scope of the thesis

It has proven difficult to relate both acute encephalopathy and long term neurological disorders in the newborn to birth asphyxia. This thesis has used intrauterine experimental approaches to examine several prenatal factors that may influence the subsequent neuronal outcome. In particular, these studies describe how factors, such as neuronal maturation and pattern of the insult, influence the severity and distribution of neuronal loss following either single or multiple in utero asphyxial events. It is hoped that these findings may be useful in clarifying some of the cause and effect relationships between perinatal asphyxia and neuronal outcome.
The studies in this thesis employed the fetal sheep at three different gestational ages; midgestation (90 day), late-gestation (120-130 days) or near term (> 135 days, term=147 days). In each of the five studies (Chapters 3-7), the protocol consisted of applying either a single or repeated insult. The insult consisted of an episode of either systemic asphyxia or cerebral ischaemia. Experimental and control fetuses were randomly allocated to each treatment group to avoid potential experimental and seasonal biases. In all fetuses the fetal parietal cortical electroencephalogram (EEG), cortical impedance (CI), and mean arterial blood pressure (MAP) were measured, with other biophysical measurements made as appropriate for each protocol. Studies began 3-5 days after surgery, the biophysical data were recorded continuously on-line from 12 hours before the insult until sacrifice. Frequent fetal blood gas and metabolite measurements were made, at specified intervals, in the period surrounding the insult. On day 3 after the insult, the ewe was killed, the fetus quickly removed and the fetal brain perfusion-fixed for histopathological analysis. The primary outcome analyses compared the extent of histological damage within each treatment group of an experiment and examined the relationship between derived biophysical parameters and histological damage.

All studies in this thesis were approved by the Animal Ethics Committee of the University of Auckland.

2.1. General surgical procedures

Surgeries were performed under 2% halothane anaesthesia using strict aseptic techniques. The pregnant uterine horn was exposed through an abdominal midline incision.
The fetal head and forelimbs were exposed through a small hysterotomy incision. Polyvinyl catheters (i.d. 0.86mm) were inserted into each fetal brachial artery and into one brachial vein. An additional catheter was placed in the amniotic fluid. The fetal scalp overlying the parasagittal cortex was exposed and three pairs of bilateral holes were drilled through the skull but avoiding the dura at the following coordinates: anterior 5, 10 and 15 mm of bregma and 10mm lateral of midline. Two pairs of parietal EEG electrodes (AS633-5SSF, Cooner Wire Co, Chatswood, CA) were inserted via the 5 and 15mm anterior to bregma burr holes and placed bilaterally on the dura. To measure cortical impedance, one pair of current injection electrodes (AS633-3SSF, Cooner Wire Co, Chatswood, CA) was inserted through the burr holes between the EEG electrodes. Electrodes were secured to the skull with a small rubber disk glued with cyanoacrylate and skin flaps were glued back over the electrodes. A low impedance ground electrode was sewn into the posterior fetal scalp and a bipolar electromyogram (EMG) electrode sewn into the paraspinal nuchal muscle. The electrode leads and catheters were exteriorised to the maternal flank and uterine and abdominal walls closed. The arterial catheters were filled with heparin and sealed with stopcock caps. Gentamicin (80mg) was administered into the amniotic sac through the amniotic catheter. Warm 0.9% saline was added to the amniotic cavity through the amniotic sac to replace amniotic fluid lost at surgery and the amniotic catheter was sealed with stopcock caps. At the end of the surgery one catheter (i.d. 1.0mm) was inserted into the ewe’s femoral vein, filled with heparinised saline and sealed.

2.1.1. Fetal asphyxia

Fetal asphyxia was produced by transiently occluding the umbilical cord. The umbilical cord was exposed in surgery and an inflatable cuff (OC20HD, In Vivo Metric,
Healdsburg, CA) was placed around the cord close to the fetal body. The cuff lead was externalised to the maternal flank. Occlusion of the umbilical cord was achieved by inflating the cuff balloon with 3ml sterile saline that was withdrawn when the occlusion was released. Occlusion resulted in an isoelectric EEG (amplitude <5μV) within 1 minute.

2.1.2. Cerebral ischaemia

To induce cerebral ischaemia, an inflatable cuff was placed around each carotid artery at surgery and exteriorised to the maternal flank as previously described (Williams et al. 1990). To restrict cerebral vascular supply to the carotid arteries only, the vertebro-occipital anastomoses between the carotid arteries and vertebral arteries were ligated bilaterally. The vertebral arteries in the sheep do not supply the brain, but rejoin the common carotid arteries, while the basilar artery is supplied from the Circle of Willis (Baldwin, Bell, 1963). Thus, this procedure eliminates the collateral supply and reduce cortical blood flow to less than 5% of control levels during carotid artery occlusion (Gunn, 1993).

2.1.3. Postoperative management

Animals were housed at constant temperature (16°C) and humidity (50%) with free access to water and hay supplemented with concentrates. Post-surgical care included daily fetal (80mg gentamicin) and maternal (500mg penicillin + 500mg streptomycin (Streptopen™)) antibiotic administration. Experiments commenced 3-5 days after surgery. Only animals within the normal physiological range were studied (pO₂ > 17mmHg, lactate < 1.2 mM/l). Fetal arterial blood was sampled aseptically from the fetal artery, collected on ice and then analysed within 30 min for pO₂, pCO₂ and pH (Radiometer ABL 330, Copenhagen), and lactate and glucose (YSI 2300, Yellow Springs Instruments Co, Yellow
Springs, Ohio). Haemoglobin (Hb) and O₂ saturation were measured on a haemoximeter (Radiometer, OSM2, Copenhagen). Daily measurements were made and additional measurements as indicated in protocols.

2.2. Data acquisition and processing

2.2.1. Cardiovascular measurements

Fetal arterial and amniotic fluid pressures were measured continuously using Novatrans II (MX860, Medex Inc, Hilliard, Ohio) pressure transducers. Amniotic pressure was used as a reference to correct for maternal postural changes and was subtracted electronically from the arterial pressure signal. The pressure signal was low-pass filtered at 20Hz to reduce aliasing errors. Heart rate data was derived from the arterial blood pressure. Arterial lines were maintained patent by a slow (0.6ml/h) infusion of heparinised saline.

Bipolar electrocardiographic (ECG) electrodes (AS633-3SSF, Cooner Wire Co) were placed, in surgery, subcutaneously over the apex of the heart and on the opposite shoulder. The ECG signal was analog filtered with a first order highpass filter set at 0.05 Hz and an 8th order lowpass Bessel filter set at 80 Hz. During the experiment the signal was digitised at 256Hz (see data acquisition). This sampling rate provides a useful tradeoff between signal bandwidth and storage requirements.

For the ECG analysis a custom software that averages the ECG waveform (Viewdac Keithley Instruments, Inc, Taunton, MA 02780, USA) was used. The signal was highpass digitally filtered (FIR, 1.5Hz) and the complexes averaged with respect to the S wave peak over 1 minute intervals. This highpass filter minimises baseline wander but avoids significant distortion of the waveform. From these averaged waveforms measures such as ST/QRS and T/QRS ratios were extracted (Fig 2.1).
Figure 2.1. Diagram illustrating measurements of ST/QRS ratio and T/QRS ratio. The baseline (A) was defined 30 msec prior to the S wave peak. The amplitude of the QRS complex was measured between the baseline and the peak of the S wave. The ST segment (B) was defined as the interval between 20-40 msec after the S wave peak. The ST amplitude was measured between the baseline (A) and the ST peak. The T wave (C) was defined as the interval 75-150 msec after the S wave peak. The T wave amplitude was measured between the baseline (A) and the peak of the T wave. The ST/QRS ratio was calculated as the ratio between the amplitude of the ST segment and the QRS complex. The ratio between the amplitude of the T wave and the QRS was calculated as the T/QRS ratio.

2.2.2. Cortical EEG activity

Parietal EEG was recorded with a bipolar technique from electrodes placed to the right and left of midline as previously described (Williams et al. 1990). Movement artifacts and electrical interference were minimised by using low impedance EEG and ground electrodes, driven shield electrode leads and high common mode rejection headstage
amplifiers. Before the start of an experiment the EEG signal was checked to ensure that it did not contain significant movement artifacts, seizure activity or noise, otherwise the data collection was discontinued. The EEG signals were lowpass filtered by an eight-order Butterworth lowpass filter with the cut-off frequency set with the -3dB point at 30Hz then sampled at 256Hz. The EEG signal was calibrated by comparison with a 1μV²/Hz white noise signal recorded for 5 minutes prior to EEG recording. Averaged intensity (power) spectra were obtained by real-time spectral analysis (Williams et al. 1990).

For analysis, the EEG intensity was logarithmically transformed and normalised with respect to the 12 hour reference period prior to occlusion (Williams et al. 1990). Elimination of short term fluctuations was accomplished by smoothing with a digital Blackman filter as previously described (Williams et al. 1990). In Chapters 3, 4 and 5, the maximum depression of EEG during the occlusion was determined from data that was cut off at 0.4 cycle/point. Baseline, post-insult recovery period (until the EEG reached -5dB) and final 4 hour measurements were smoothed at 0.1 cycle/point.

In Chapters 3, 4 and 5 seizures were defined as EEG intensity greater than 5dB above the reference period (Williams et al. 1990). In Chapter 6, commercially available software (Monitor software, Stellate Systems, Quebec, Canada) was used off-line, to determine the time course of seizure and spike activity (Gotman, Gloor, 1994; Gotman, 1982). The seizure detection rate by the software is comparable to that detected by visual observation and this method allows detection of the time course of seizure activity.

2.2.3. Cortical impedance

A four-electrode system was used to record the CI signal as described previously (Williams et al. 1991). This approach prevents errors from changes in electrode contact
impedance that occur with 2-electrode systems. An isolated current source was used to inject a sinusoidal alternating current of ±0.2 μA at 200 Hz through the parasagittal cortex. The current density (< 0.03 μA/mm² at the electrode surface) was well below the level that can stimulate the cortex. The voltage recording electrodes were connected to a high-impedance EEG amplifier (10¹² ohm). The impedance signal was extracted using phase sensitive detector and lowpass filtered (Williams et al. 1991). Tissue impedance increases concomitantly with cytotoxic edema and persistent increases are associated with tissue necrosis (de Boer et al. 1989; Klein et al. 1993). At the beginning of the experiment, the impedance signal was calibrated at 0 and 200 ohms. For these studies the geometric factor for the electrodes used is not known but assumed to be constant for each study and similar between animals. Thus the percentage change, but not specific impedance, can be measured. The statistical comparisons were made on the percentage change with respect to pre-insult levels. In Chapters 3 and 4, the Maxwell equation was used to estimate the changes in extracellular space from the changes in impedance (Williams et al. 1991; Robillard, Poussart, 1979; Robillard, Poussart, 1977).

2.2.4. Data acquisition

Data acquisition was continuous during experiments. The signals were analog lowpass filtered and acquired at 256 Hz, using a 12 bit A/D with double buffered DMA, then processed on line and stored using custom signal processing software (Labview for Windows, National Instruments, Austin, TX). The design of the real time software was as previously published (Williams, Gluckman, 1990). Off-line analysis was done with the signal analysis program Viewdac (Keithley Instruments, Inc, Taunton, MA). A 12 hour baseline was recorded after signal calibration and before initiation of the insults; this was then followed
by 3 days of recording. Averages of physiological parameters MAP, HR, nuchal EMG activity, EEG intensity and CI were saved to disk at 1 minute intervals. Unprocessed EEG, averaged EEG spectra and unprocessed ECG were saved in separate files.

2.3. Histological and immunohistochemical procedures

2.3.1. Histology

Experiments were ended three days after the insult, by administration of intravenous sodium pentobarbital (3.5g) to the maternal venous catheter. The fetus was immediately removed and weighed. The fetal brain was perfused in situ through the carotid arteries with 500ml of saline, followed by 1000ml of 10% formalin (Chapters 3, 4 and 5) or 4% paraformaldehyde (Chapters 6 and 7). The brain was post-fixed for one week and dissected into 0.7cm thick coronal tissue blocks. Following gradual dehydration, tissue blocks were paraffin embedded and sectioned at 8μm. Every 40th section was mounted and stained. To determine histological neuronal loss a combined staining procedure (acid fuchsin/thionin) was used (Williams et al. 1992). This procedure stains the cytoplasm of the acidophilic neurons red. In Chapter 4 adjacent sections were also stained by a Luxol fast blue stain (Disbrey, Rack, 1970) to visualise myelin.

Histological neuronal loss was determined by examination under light microscopy at 100x and 400x magnification. Neurons showing ischaemic cell change with acidophilic (red) cytoplasm and contracted nuclei were considered dead (Fig 2.2). Analysis of neuronal death at 3 days is optimal for these studies since it is generally agreed that cell death has predominantly occurred by this time yet the dead neurons have not yet been phagocytosed (Perry, Gordon, 1988).
Chapter 2. General methodology

Figure 2.2. Photomicrographs illustrating neurons of the striatum in sham operated fetal sheep (upper photo) and selective neuronal loss following repeated episodes of umbilical cord occlusion (lower photo). Dead neurons show red cytoplasm (acidophilic) with a pyknotic nuclei. Acid fuchsin/thionin, 300x magnification.
Brains were assessed as previously described (Williams et al. 1992; Gunn et al. 1992; Gunn et al. 1994; Tan et al. 1994; Tan et al. 1993; Tan et al. 1992). Pre-determined cerebral regions were scored by two independent assessors, one whom was blind to the experiment. The correlation of neuronal loss scores between the two assessors was $r=0.92$. The following numbers in brackets refer to cerebral regions according to the stereotaxic atlas of the fetal sheep (Gluckman, Parsons, 1983). Examination for neuronal loss was performed in the following regions, striatum (29mm), the globus pallidus (26mm), the thalamus (20mm), the parasagittal cortex (29mm and 20mm), the substantia nigra reticulata (20mm) and dorsal and ventral hippocampus, including dentate gyrus and CA1-4 (13mm and 15mm). The proportion of dead neurons in each cerebral region was determined according to the following damage scale: $0=\text{no dead neurons}$, $5=0-10\%$, $30=10-50\%$, $70=50-90\%$, $95=90-<100\%$, $100=100\%$ of neurons dead. In Chapter 6, neuronal loss was determined according to the following damage scale: $0=\text{no neuronal loss}$, $1=<20\%$, $2=20-70\%$ and $3=70\%$ of neuronal loss.

2.3.2. Immunohistochemistry

The fixed brain was divided in the sagittal plane into two halves. Blocks of approximately 1cm thickness, comprising the striatum, globus pallidus and the substantia nigra were post fixed for 3-5 days in the perfusion fixative. Tissue from the left side was processed for paraffin histology as described above. The right half of the brain was processed for immunohistochemistry. Tissue comprising the basal ganglia was transferred to 25% sucrose in 0.1M phosphate buffered saline (PBS, pH 7.4) until the tissue sank (3-5 days). Sections were then cut on a freezing microtome at 50$\mu$m and stored overnight in PBS containing 0.1% sodium azide. Immunoreactivity for calbindin-D28k, somatostatin,
parvalbumin, enkephalin and substance P was localised on free floating sections using standard immunohistochemical methods as previously described (Waldvogel, Faull, 1993). Briefly, sections were washed in PBS with 0.2% triton (PBS-triton), incubated in 50% methanol with 1% H$_2$O$_2$ for 30 minutes, washed (3x15 min, PBS-triton) and incubated in primary antibody for 2-3 days. The primary antibodies were obtained from the following sources and used at the following dilutions: rabbit-anti-calbindin (Emson, Cambridge) (Cowan et al. 1990), 1:5000; rabbit-anti-somatostatin (Watpa, Auckland) 1:2000; mouse-anti-parvalbumin (Sigma) 1:10000; mouse-anti-leu-enkephalin (Seralab) 1:10000 and rabbit-anti-substance P (Watpa, Auckland) 1:10000. All antibodies were diluted in 1% goat serum in PBS. Sections were washed (3x15 min in PBS-triton), incubated overnight in goat anti-mouse or goat anti-rabbit biotinylated secondary antibody as appropriate, washed (3x15 min, PBS-triton) followed by incubation in ExtrAvidin™ (Sigma) 1:1000, for 4 hours. After thorough washing, the sections were reacted with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) solution and 0.01% H$_2$O$_2$ to produce a brown reaction product. To show non-specific binding, additional control sections were processed as above except that the primary antibody was omitted from the incubation solution. Sections were mounted on gelatinised glass slides, dried, dehydrated in alcohols and xylene and coverslipped.

The same regions of the basal ganglia which were analysed for neuronal loss were also examined using immunohistochemistry. The intensity of immunohistochemical staining was determined using an image analyser (Java, Jandel Scientific, CA94925, USA). Regions, encompassing the entire striatum (caudate/putamen complex), globus pallidus and substantia nigra pars reticulata, were digitised. Within each structure the percentage area of immunohistochemical staining that was of greater intensity than background regions, was calculated. The intensity of immunoreactivity of calbindin in the caudate/putamen complex,
of enkephalin in the globus pallidus and of substance P in the substantia nigra pars reticulata were evaluated and compared in both the control and asphyxiated animals.

Striatal somatostatin and parvalbumin immunopositive cells were analysed by light microscopy at 100x. Quantification was facilitated by superimposing a grid (10mm x 10mm) onto the brain section. The number of immunopositive cells within the grid was counted. Cell density (number immunopositive cells/unit area) was determined as the average from three full grid measurements per brain section.

2.4. Statistical analysis

Unless otherwise specified data are presented as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using the Stats+ (Statsoft Inc, USA) or Sigmastat (Jandel Scientific, USA) packages on an IBM AT compatible. Physiological and histological parameters were compared using analysis of variance (ANOVA). Several measurements over time or several different brain regions were treated as repeated measures. When overall differences were found, sub groups were subjected to Newman-Keul’s multiple comparison test. Measurements of intensity of immunohistochemical staining and cell densities were compared between control and experimental animals using independent t-test. Nonparametric data, such as histological scores, were compared using Friedman repeated measure analysis of variance on ranks and in Chapter 4, the presence or absence of neuronal loss in near term and midgestation fetal sheep were compared using Fisher exact test. Correlations were performed on rank transformed data.
CHAPTER 3

DOES TRANSIENT UMBILICAL CORD OCCLUSION CAUSE NEURONAL DAMAGE IN THE FETAL SHEEP?

3.1. Introduction

Perinatal asphyxia is a major cause of handicapping encephalopathy. However, considerable controversy remains as to the factors that determine the specific patterns of damage observed and indeed as to the timing of the insult. Increasing interest has focused on pre-partum rather than intra-partum insults (Bejar et al. 1988; Paul et al. 1986; Mann, 1986). Different clinical syndromes may reflect different aetiologies and gestational ages. Umbilical cord occlusion either due to fetal rotation or compression before or during labour has been frequently suggested as one cause of perinatal asphyxia (Mann, 1986; Hansen, Hillersborg, 1988). However, studies relating umbilical cord occlusion with neurological outcome are few and are difficult to interpret because of a variety of factors including the uncontrolled nature of the challenge, experiments being performed in exteriorised fetuses under the influence of anaesthetics and lack of long term recovery (Myers, 1972; Thiringer et al. 1987; Clapp et al. 1988). The purpose of this study was to examine the neurological response to a standardised period of cord occlusion in the chronically instrumented fetal sheep in late gestation.

3.2. Methods

Thirteen fetal sheep (120 - 127 days gestation) were operated on under halothane anaesthesia (2%) and instrumented with an umbilical cord occluder as described in Chapter 2. Three fetuses with poor blood gases (pH < 7.33, pO₂ < 13mmHg) were excluded from
analysis. All 3 fetuses died during the occlusion. After collection of basal data the umbilical cord was occluded for 10 minutes in 6 animals. Fetal arterial blood samples were collected prior to the occlusion and at 2 minute intervals during the occlusion. Following termination of the insult blood was sampled at 5 minutes, 1 hour, 2 hours and 72 hours. At 72 hours after the occlusion the sheep was sacrificed and the fetal brain was immediately perfusion fixed as described in Chapter 2. Four control animals were treated exactly the same as animals in the experimental group except the umbilical cord was not occluded, although the occluder had been placed around the umbilical cord at surgery.

3.3. Results

Control animals were not different from experimental animals in respect of any pre-insult parameter and showed no electrophysiological and metabolic abnormalities or histological damage at the end of the experiment.

The EEG activity was suppressed almost immediately (< 1 minute) following inflation of the occluder (Fig 3.1). During occlusion EEG intensity dropped by -17 ± 2 dB (p < 0.001). Following release of the occluder, the intensity gradually increased towards pre-insult values. At 5 ± 2 hours, the intensity had recovered to -5 dB. However, EEG intensity never fully returned to pre-occlusion values being -2.5 ± 0.6 dB at 72 hours (p < 0.01). Seizures were not observed.

Cortical impedance increased during the occlusion (Fig 3.2). At the end of the 10 minute asphyxia the impedance peaked at 120 ± 6% (p < 0.01) and the extracellular space was 85 ± 4% of pre-insult values. Cortical impedance then decreased and remained stable throughout the experiment (Fig 3.2).
Blood pressure showed a biphasic response to asphyxia with initial hypertension (80 ± 4 mmHg, p<0.001, Fig 3.2). Subsequently, blood pressure dropped so that at the end of the occlusion, the fetus was markedly hypotensive (24 ± 5 mmHg, p<0.01). The period of hypotension lasted for 4.7 ± 2 minutes. The magnitude of hypotension was correlated with neuronal loss in the CA3 region of the hippocampus (r = 0.78, p < 0.01). Following release of the cord cuff, blood pressure rose transiently above pre-asphyxia values and then recovered gradually towards control values.

An abrupt bradycardia occurred at the onset of occlusion, with a fall in heart rate from 178 ± 11 to 72 ± 14 bpm (p<0.001, Fig 3.2). During the asphyxia, several transient increases in heart rate were observed. After release of the cord occluder, heart rate recovered
immediately and a transient tachycardia was observed. In 3 out of 6 animals an episode of bradycardia occurred during the hour after release of the occluder.

During the occlusion severe respiratory acidosis, mild metabolic acidosis and hypoxemia developed (Fig 3.3). There was a transient elevation of glucose (p < 0.01) following release of the cord cuff and the acidemia recovered slowly with lactate levels only partially resolved at 2 hours (p < 0.01). Three days following occlusion all metabolic parameters were normal.
Histological examination showed neurological damage primarily in the hippocampal regions (Fig 3.4). The hippocampal region CA3 had more damage than CA1/2, CA4 and parasagittal cortex which were more damaged than striatum, dentate gyrus, thalamus, lateral cortex and amygdala \((p < 0.05)\). More neurons were lost in the dorsal horn of the hippocampus compared with the ventral horn \((p < 0.001)\). Selective neuronal loss found in the parasagittal cortex was predominantly in layers 3 and 4.
Figure 3.4. Photomicrographs of the hippocampus in control animals (a,c) and following 10 min of umbilical cord occlusion (b,d). The hippocampus appear normal in the sham operated animals (a) and pyramidal neurons from the CA1/2 region (between arrows) show no ischaemic cell change (c). Following cord occlusion there is marked neuronal death in the CA4, CA3 and CA1/2 regions of the hippocampus (b). Pyramidal neurons from the CA1/2 region (between arrows) show pyknotic nuclei with acidophilic cytoplasm (d). (e) Bar graph demonstrating the distribution of neuronal loss following cord occlusion. a,b = 20x; c,d = 270x
3.4. Discussion

The experimental approach used in the present study circumvents confounding factors present in previous studies of umbilical cord occlusion. Thus experiments were performed in chronically instrumented fetuses avoiding effects of anaesthesia and surgical trauma, they were highly reproducible and outcome was monitored both histologically and electrophysiologically. The few earlier experimental studies of neurological outcome following episodes of asphyxia in utero are difficult to interpret; they have not used standardised insults and have not followed outcome for more than a few hours after the primary insult (Myers, 1972; Thiringer et al. 1987; Clapp et al. 1988). This is important as it is now clear that post-asphyxial events may add to neuronal loss for many hours or even days after the insult is removed. For example fetuses subjected to 30 minutes of carotid artery occlusion developed secondary cerebral edema with intense seizures from 8 to 30 hours after the insult (Williams et al. 1990) and suppression of this epileptiform activity reduced the neuronal loss (Tan et al. 1992).

The almost exclusive hippocampal neuronal loss with this experimental approach contrasts with the distribution of neuronal loss seen in other approaches to cerebral asphyxia in the fetal sheep. Previously the effect of longer periods of uterine artery occlusion in the fetal sheep has been examined and shows neuronal loss predominantly in the parasagittal cortex (Gunn et al. 1992). Ten minutes of fetal carotid artery occlusion causes virtually no damage (Williams et al. 1992). However, in the adult rat and gerbil, vulnerability of hippocampal neurons have been reported (Smith et al. 1984; Kirino, 1982). This damage has been related to excitotoxins (Auer, Siesjö, 1988). Hippocampal damage is a recognised pattern of damage in man following cardiac arrest, which manifests itself clinically as memory or cognitive defects (Cummings et al. 1984). The distribution of neuronal loss
within the hippocampus supports earlier reports of loss of CA3 neurons in the immature brain rather than CA1 neurons as occurs in the adult (McDonald, Johnston, 1990).

The slight loss of EEG intensity observed three days following the insult is consistent with mild neuronal loss of the underlying parasagittal regions. It has previously been shown that there is a close relationship between changes in EEG and cortical neuronal loss following fetal ischaemia (Williams et al. 1992). The EEG intensity in this study recovered more slowly towards baseline levels than following 10 minutes of carotid artery occlusion in the fetal sheep (Williams et al. 1992). Thus 10 minutes of total asphyxia appears to have greater impact on the maintenance and recovery of neuronal function than 10 minutes of ischaemia, causing more neuronal loss.

In contrast to previous studies, in which only the blood supply to the fetal brain was occluded, cord occlusion causes systemic asphyxia. Metabolic factors may play a role in determining the severity and distribution of damage. Lactic acidosis has been suggested as a sensitising factor and whole blood lactate has been reported to well reflect cerebral lactate concentrations (Kastendieck et al. 1988). However, the role of lactic acidosis in the development of cerebral damage is still controversial (Yamaguchi, Myers, 1976). Indeed, in vitro studies have shown that lactic acid can protect hippocampal neurons from ischaemic injury (Tombaugh, Sapolsky, 1990). Only a mild lactic acidosis and a severe respiratory acidosis were observed in this study. In contrast, in prolonged partial asphyxia, associated with uterine artery clamping, lactate levels are high (> 14mmol/L) and the hippocampal neurons are relatively spared compared with the parasagittal cortex (Gunn et al. 1992). The presumed difference in cerebral lactate concentrations might be a partial explanation of the regional differences in neuronal loss between the two studies.

Haemodynamic factors are also believed to play a role in the development of cerebral
damage. In fetal sheep, autoregulation of cerebral blood flow is lost when mean arterial pressure falls below 30 mmHg (Ashwal et al. 1984) and cerebral perfusion is further decreased by combined hypotension and hypoxemia (Hohimer et al. 1991). The fetuses in the present study developed both severe hypotension and hypoxemia and the degree of hypotension was correlated with histological outcome in the CA3 region of the hippocampus. The initial rise in blood pressure and immediate fall in fetal heart rate in response to asphyxia has earlier been described as a baroreceptor response to hypoxia in the fetal sheep (Hanson, 1988). Cardiovascular parameters showed some instability for 40 minutes following the release of the occluder. This cardiovascular instability may be of central origin and may add to any reperfusion injury.

Attempts to correlate birth asphyxia with subsequent learning disorders and cognitive dysfunction have been relatively disappointing. It is increasingly recognised that many potentially damaging asphyxial episodes occur pre-partum (Mann, 1986). Hippocampal damage is known to effect memory in adult humans (Cummings et al. 1984). This study demonstrates that a brief period of fetal cord occlusion can cause hippocampal neuronal loss but may not be associated with clinical signs of perinatal asphyxia such as seizures.
ARE THERE DEVELOPMENTAL DIFFERENCES IN THE
RESPONSE TO FETAL ASPHYXIA?

4.1. Introduction

Neurological outcome following an episode of fetal asphyxia may depend on several factors. Two factors, gestational age and the nature of the insult have frequently been suggested as important determinants (Chapter 1). Epidemiological studies suggest that the mature fetus is more susceptible to grey matter lesions, whereas prematurity has been postulated as a risk factor for white matter disorders (Volpe, 1987; Volpe, 1992). While the aetiology of these deficits is not established, pathological studies indicate antenatal origins (Bejar et al. 1988; Scher et al. 1991).

Although, it is well known that the fetus is able to withstand periods of anoxia better than the adult, little is known about the ontogenic differences in susceptibility to asphyxia during pregnancy (Dawes et al. 1959). Early studies in fetal sheep indicated developmental differences in both cardiovascular and metabolic adaptations to asphyxia that may affect survival (Dawes et al. 1959). Furthermore, it has been suggested that immature neurons have lower energy requirements and are therefore more resistant to a period of hypoxia-ischaemia than those of the mature nervous system (Duffy et al. 1975). However, there is only limited experimental data directly investigating the effect of cerebral immaturity on neurological outcome following asphyxia (Ting et al. 1983; Rice et al. 1981).

The nature of the asphyxial insult may also influence the pattern and degree of neuropathology. Chapter 3 shows that 10 minutes of umbilical cord occlusion in the 120-127 day old fetal sheep can result in predominantly hippocampal damage, whereas pure cerebral
ischaemia of the same duration results in minimal damage (Williams et al. 1992). On the other hand, repeated short episodes of cerebral ischaemia in the fetal sheep induce severe damage of a different distribution than that seen after a single prolonged episode, with the damage becoming primarily striatal rather than cortical (Chapter 5).

The objective of this study was to compare the neurological, metabolic and cardiovascular responses to 10 minutes of total umbilical cord occlusion in midgestation (90 day) fetal sheep, and near term gestation (>135 day) fetal sheep.

4.2. Methods

Fetal surgeries were performed as described in Chapter 2. Ten fetal sheep of 130-132 days of gestation and nine fetal sheep of 86-88 days of gestation were instrumented. In the 86-88 day fetuses, a catheter was inserted into the right carotid artery as the small size of the brachial artery precluded its catheterisation. In all other respects the instrumentation was identical in both groups of fetuses. One umbilical occluder was placed around the umbilical cord. Experiments began 3-5 days after surgery. Thus the midgestation fetal sheep were of an gestational age of 90-92 days and the near term fetal sheep were 135-136 days of gestation. After collection of baseline data, the umbilical cord was transiently occluded by inflating the cuff for 10 minutes. Fetal arterial blood samples were collected on ice at 30 minutes before and immediately before the occlusion and at 2-minute intervals during the occlusion. After termination of the insult, blood was sampled at 5 minutes, 1 hour, 2 hours and 72 hours. Experiments were terminated 72 hours after the occlusion and the fetal brain prepared for histological analysis as described in Chapter 2.
4.3. Results

The umbilical cuff failed to inflate in two fetal sheep (one in each age group), which then showed no electrophysiologic or metabolic abnormalities or histological damage at the end of the experiment. These two fetuses have been excluded from analysis.

There were no significant differences in pre-insult blood gases, pH, lactate and glucose measurements in the midgestation and near term fetal sheep (Fig 4.1). All 90-92 day fetuses survived the umbilical occlusion, whereas 2/9 near term fetal sheep did not recover following release of the umbilical cord cuff. There were no differences in pre-insult blood gas and metabolic values between the fetuses that died during the occlusion compared with the rest of the study group. One near term fetus delivered alive but prematurely 32 hours after occlusion. These three animals were excluded from histological analysis. Occlusion of the umbilical cord resulted in severe hypoxia (90-92 day: $\text{pO}_2$ 8.1±1mmHg; 135-136 day: $\text{pO}_2$ 9.8±0.9mmHg) and acidosis (90-92 day: pH 6.94±0.01, lactate 5.5±0.2mmol/L; 135-136 day: pH 6.89±0.01 lactate 6.8±0.3mmol/L) at both gestational ages (Fig 4.1). Whereas the 90-92 day fetuses became and remained hypoglycaemic during the asphyxia, the 135-136 day fetal sheep showed a transient elevation of glucose levels during the last four minutes of the occlusion ($P<0.001$). The glucose measurements were highest in the two animals that did not survive the occlusion. Following the occlusion, both lactate and pH values recovered more slowly in the near term fetal sheep ($P<0.001$).

Resting arterial blood pressure was lower ($P<0.05$) and heart rate higher ($P<0.05$) in the midgestation fetal sheep. Cardiovascular changes were rapid during the umbilical cord occlusion (Fig 4.2). Severe bradycardia developed immediately when the cord was occluded, followed by a transient tachycardia on the release of the occlusion. The degree of bradycardia was similar in midgestation and near term animals during the occlusion: however, the post-
Figure 4.1. Systemic blood gas and metabolic changes in 135 day old fetuses (black bars) and 90 day old fetuses (white bars) before, during and following 10 minutes of umbilical cord occlusion. Comparisons are made between the two groups of animals. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
occlusion tachycardia was more marked in the older fetuses (79±12 bpm above baseline compared to 17±5 bpm above baseline). Both groups of animals responded to umbilical cord occlusion with an initial elevation of arterial pressure. There was a trend (p<0.06) for the near term fetal sheep to have a more pronounced increase (57.5±9%) compared with midgestation fetal sheep (37.3±4%). Following the initial elevation, blood pressure dropped by 67.0 ± 6% in near term fetal sheep and by 32.0 ± 2% in immature fetal sheep at the end of the occlusion (p<0.001). The time course of recovery was similar in both groups.

![Graph showing changes in MAP and HR during and following 10 minutes of umbilical cord occlusion.](image)

**Figure 4.2.** Changes in blood pressure (upper panel) and heart rate (lower panel) during and following 10 minutes of umbilical cord occlusion in 135 day old fetuses (filled symbols) and 90 day old fetuses (open symbols). All parameters were normal within 20 minutes of release of the umbilical cuff.
The EEG became suppressed almost immediately (< 1 minute) following occlusion of the umbilical cord (Fig 4.3). After release of the umbilical cuff there was a gradual increase in EEG intensity towards pre-insult values. The near term fetal sheep recovered more slowly than midgestation fetuses (3.1±0.8 vs 0.9±0.1 hours, p<0.001). At the end of the experiment the older animals had reduced EEG activity with respect to baseline (p<0.05), which was not observed in the younger fetuses. However, there was no difference in the loss of EEG intensity between the two groups of animals at 72 hours.

![EEG intensity graph](image)

**Figure 4.3.** Changes in total electroencephalographic (EEG) intensity during (time zero) and for 72 hours following 10 minutes of umbilical cord occlusion in 135 day old fetal sheep (filled circles) and 90 day old fetal sheep (open circles). The older fetuses showed a reduction of EEG intensity during the last 4 hours with respect to pre-insult baseline (p<0.05), but there was no difference between the two groups of animals.

Cortical cytotoxic edema, detected as an increase in cortical impedance developed during the occlusion (Fig 4.4). The increase in impedance was very slight in the younger
animals and was markedly higher in the near term fetal sheep (p < 0.001). There were no delayed onset of secondary increases in impedance in either group of fetuses.

The histological outcome was different between the two groups (p < 0.001). Mid-gestation fetal sheep showed no detectable neuronal loss or injury to white matter regions. Furthermore no periventricular haemorrhage was detected. In contrast, neuronal loss was found in brains from all the near term fetal sheep. The neuronal injury was most prominent in the hippocampus (Figs 4.5, 4.6). In particular, the CA3 and CA1/2 region of the dorsal hippocampus showed marked neuronal loss. Mild selective neuronal loss (< 10%) was also found in the middle layers of the parasagittal cortex. One near term fetus showed a focal unilateral infarct in one sulcus of the parasagittal cortex. Damage to myelin was not detected in older fetuses. In late-gestation fetal sheep, the percentage fall in arterial pressure at the

**Figure 4.4.** Cytotoxic edema, detected as an increase in cortical impedance (CI), during and following 10 minutes of umbilical cord occlusion in the 135 day old fetal sheep (filled circles) and 90 day old fetal sheep (open circles). Cytotoxic edema was markedly increased in the late-gestation fetuses compared to mid-gestation fetuses during the umbilical cord occlusion. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 4.5. Distribution of neuronal loss after 10 min of umbilical cord occlusion in near term fetal sheep. The neuronal damage was most prominent in the CA1/2, CA3 and CA4 region of the hippocampus. No neuronal loss was found in midgestation fetuses. PsCx, parasagittal cortex; Ltx, lateral cortex; Str, striatum; Dg, dentate gyrus; Thal, thalamus; Amg, amygdala.

Figure 4.6. Representative photomicrographs of the dorsal hippocampus in midgestation fetal sheep (a,b) and near term fetal sheep (c,d) subjected to 10 minutes of umbilical cord occlusion. Pyramidal neurons in the CA1 subfield (between the arrows) are shown under higher magnification (b,d). Neuronal loss was only observed in the brains of near term fetuses. Thionin and acid fuchsin stain. a,c=20X; b,d=270X.
end of the occlusion was strongly correlated with neuronal loss in both the CA3 \((r=0.876)\) and CA1/2 \((r=0.922)\) regions of the hippocampus (Table 4.1). There was no correlation between plasma lactate or glucose levels and histological outcome in the older fetuses (Table 4.1).

<table>
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<tr>
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<th>correlation coefficient</th>
<th>p</th>
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<tr>
<td>CA3 neuronal loss vs % blood pressure drop</td>
<td>0.876</td>
<td>0.02</td>
</tr>
<tr>
<td>CA1/2 neuronal loss vs % blood pressure drop</td>
<td>0.922</td>
<td>0.01</td>
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<td>CA3 neuronal loss vs Lactate 10min insult</td>
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<td>NS</td>
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<tr>
<td>CA3 neuronal loss vs Lactate 1hr post insult</td>
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<td>NS</td>
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<tr>
<td>CA3 neuronal loss vs Glucose 10min insult</td>
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4.4. Discussion

Pathological studies suggest that neuronal injury in the newborn is often of antenatal origin (Bejar et al. 1988; Scher et al. 1991). The majority of previous studies investigating the fetal response to asphyxia have been conducted in fetal sheep after 120 days of gestation (Raju, 1992). However, fetal sheep are precocial compared to man, and brain growth peaks around 125 days \((\text{term } = 147 \text{ days})\) in sheep, while human brain growth peaks at term (McIntosh et al. 1979). The present study compared neuronal outcome following umbilical
cord occlusion in 90-92 day fetal sheep with that of 135-136 day fetal sheep. The 90-92 day fetal sheep are at a neurodevelopmental age where myelination has not yet begun and are therefore more similar to the pre-term human infant (McArdle et al. 1987). I found marked differences in the neurological outcome, following a single brief episode of in utero asphyxia, between midgestation and near term fetal sheep. While the younger fetuses did not develop neuronal damage, the older group did.

Necrotic neurons, predominantly in the dorsal hippocampus, were found 72 hours after cord occlusion in the near term fetuses. This distribution and degree of damage is similar to the 120-127 day old fetus (Chapter 3). Clamping of the umbilical cord in prenatal rats also results in degenerative changes in the hippocampus (Shen et al. 1991). In contrast, no neuronal loss was detected in the immature fetal sheep. Widespread neuronal loss was found in the only previous study of asphyxia in midgestation fetal sheep which used a complex insult of combined hypoxemia and hypovolemia (Ting et al. 1983). The discrepancy in outcome between the present study and the previous investigation of midgestation fetal sheep may be due to differences in duration and severity of the insult.

Periventricular leukomalacia is a common lesion in premature human neonates (Volpe, 1992). In the present study, damage to the white matter was not observed in either of the gestational ages studied. It is unlikely that the failure to detect white matter injury was due to the early histological examination as white matter damage has been detected as early as 3 days after insult in both midgestation fetal sheep as well as in immature rats (Rice et al. 1981; Ting et al. 1983).

Cytotoxic edema, which can be detected as an increase in cortical impedance occurs during acute hypoxic-ischaemic injury and indicates compromised cellular membrane function (Matsuoka, Hossmann, 1982). Persistent increases in tissue impedance are associated with
tissue damage in the brain (de Boer et al. 1989; Klein et al. 1993). In this study, near term fetal sheep developed transient cytotoxic edema during umbilical cord occlusion suggesting acute injury. This is in agreement with previous studies in fetal sheep, which indicate that, following a brief transient cerebral ischaemia, cytotoxic edema is reversible and only mild neuronal injury is seen (Williams et al. 1991). Whereas after more severe insults, there is a persistent increase followed by a further increase some hours after the initial insult, which is associated with development of cortical infarction (Williams et al. 1991). Cytotoxic edema was not observed in the midgestation fetal sheep. The immature brain has a relatively high water content and this may have reduced the ability to detect changes in cytotoxic edema. However, in immature rats, the increased resistance to anoxia is related to their enhanced ability to keep near normal potassium gradients across the cellular membranes (Hansen, 1977). Hence, the lack of brain injury in the midgestation fetal sheep in the present study may be due to its ability to maintain cellular membrane function.

The EEG became iso-electric during occlusion of the umbilical cord in both midgestation and near term fetal sheep. However, the immature fetuses recovered more quickly towards basal levels than the older fetal sheep. The rate of recovery period following hypoxic-ischaemic insults in fetal sheep has previously been related to the severity of the injury (Williams et al. 1992). Certainly in this study, a rapid recovery of the EEG was predictive of an absence of histological abnormalities.

The observations with respect to baseline cardiovascular parameters are in agreement with previous studies in 84-99 days of gestation fetal sheep (Iwamoto et al. 1989). Thus, the immature animals had a lower arterial pressure and an elevated heart rate compared with near term fetal sheep. Lower systemic vascular resistance and lower vagal input respectively have been suggested to explain these phenomena (Bell et al. 1986).
Heart rate fell immediately, in both immature and near term fetal sheep, in response to asphyxia. Both the initial hypertension and the immediate hypoxia have been suggested to lead to vagal stimulation mediated via baro- and chemoreceptors resulting in bradycardia (Rudolph, 1984). The bradycardia was similar in both groups of animals and there was no correlation with neurological outcome. However, the rebound tachycardia was more marked in the older group.

In agreement with results in Chapter 3, occlusion of the umbilical cord resulted in an initial rise in blood pressure followed by hypotension. There was a trend \( p=0.06 \) towards a smaller rise in arterial pressure in the younger fetuses, which might be explained by a lesser adrenergic response to hypoxia in midgestation fetal sheep (Matsuda et al. 1992). A similar curtailed rise in arterial pressure has been observed in midgestation fetal sheep in response to acute asphyxia caused by arrest of uterine blood flow (Jensen, Berger, 1991). These authors, concluded that this diminished ability of the younger fetuses to mobilise a stress response may result in greater mortality in these animals compared to older fetuses. However, this was not observed in the present study. In contrast, mortality rates were higher in the near term fetal sheep.

Previous studies have shown that the magnitude of the fall in arterial pressure during both total and partial asphyxia in near term fetuses is related to neurological outcome (Gunn et al. 1992) and Chapter 3. Similarly, in this study the magnitude of hypotension during asphyxia in the older fetuses was strongly related to the degree of neuronal loss in both the CA3 and CA1/2 regions of the hippocampus. Several authors have found that low arterial blood pressure leads to loss of autoregulation of cerebral blood flow and under such conditions cerebral blood flow becomes pressure passive (Papile et al. 1985). Both animal studies and clinical observations have related cerebral blood flow fluctuations with injury to
the immature brain (Ting et al. 1983; Volpe, 1992). In this study midgestation fetal sheep maintained arterial pressure better than late gestation fetuses. Similar results to these were found in earlier studies in exteriorised midgestation fetal sheep (Dawes et al. 1959), where it was concluded that blood pressure was maintained during periods of anoxia by the higher levels of cardiac glycogen found in the immature fetuses. Possibly the maintenance of arterial pressure during asphyxia in the immature fetuses in this study protected them, in part, from neuronal loss. Lower cardiac glycogen levels in late-gestation fetal sheep may have resulted in earlier cardiac failure and thus the higher mortality rate in the older group.

While both groups of animals developed metabolic acidosis during the asphyxia period, the immature fetuses recovered more quickly than near term fetuses. In addition, there was an increase in plasma glucose during the umbilical cord occlusion, which was only seen in the older fetuses. The significance of the prolonged period of lactic acidosis and brief period of hyperglycaemia in the older fetuses is not clear. Early studies in juvenile monkeys suggested that hyperglycaemia prior to asphyxia caused a greater accumulation of lactic acid through the anaerobic pathway and markedly augmented the severity of brain injury (Myers, Yamaguchi, 1977). In contrast, in immature rats hyperglycaemia superimposed on cerebral hypoxic-ischaemic insults, does not seem to be related to increased neuronal damage and may in fact improve neuronal survival (Vannucci, Muisce, 1992). Furthermore, in immature rat brains, high levels of lactate appear to be dissociated from changes in cerebral pH (Hida et al. 1991). In the present study, there was no correlation between neuronal loss and moderate systemic blood lactate levels during either the occlusion or the recovery period. In addition, there was no correlation between moderate plasma glucose and neuronal injury and it is likely that the brief period of hyperglycaemia indicated a more mature systemic stress response in the older fetuses which did not influence neuronal outcome.
This study has shown an increased resistance to neuronal damage in the immature fetal sheep compared with near term fetuses. This may be accomplished by the greater ability of the immature fetal sheep to maintain arterial blood pressure and to conserve cerebral cell membrane function during asphyxia.
CHAPTER 5.

WHAT IS THE EFFECT OF REPEATED BRIEF EPISODES OF CEREBRAL ISCHAEMIA ON NEURONAL LOSS?

5.1. Introduction

Epidemiological studies have often failed to find a clear association between isolated episodes of fetal asphyxia and neurological outcome in the newborn (Mann, 1986; Paneth, Stark, 1983). This discrepancy may be due to, in part, multiple brief episodes of in utero asphyxia being unrecognised but more common than major and identifiable single events. However, few experimental studies have considered the neurological consequences of multiple insults. Limited evidence from studies in the adult rat and gerbil suggest that brief episodes of ischaemia repeated during the period of post-ischaemic hypoperfusion may produce additive degrees of neuronal damage in the cortex, hippocampus and caudate nucleus and vasogenic edema (Tomida et al. 1987; Nakano et al. 1989). It has previously been shown that the parasagittal cortex and hippocampus are particularly vulnerable to both brief (10 minutes) and prolonged (30-40 minutes) single episodes of cerebral ischaemia or global asphyxia in the fetal sheep (Williams et al. 1992) and Chapter 3. However despite frequent clinical observations of striatal damage with perinatal encephalopathy (Spiegel, Baird, 1968), neither cerebral ischaemia or global asphyxia induced predominantly striatal patterns of injury.

The objective of this study was to determine whether multiple brief episodes of ischaemia sensitise the fetal brain to injury or alter the pattern of damage compared with isolated insults and whether the length of the interval between insults is of importance. An experimental preparation of transient cerebral ischaemia in the chronically instrumented fetal sheep has previously been described that induces encephalopathy similar to hypoxic-ischaemic
brain damage observed in some asphyxiated term infants (Williams et al. 1990). Therefore the electrophysiologic and histologic consequences of isolated and brief episodes of reversible cerebral ischaemia repeated at either 1 hour or 5 hours were investigated.

5.2. Methods

Thirty-four pregnant ewes 119-127 days of gestation were operated on under 2% halothane/oxygen general anaesthesia as described in Chapter 2. One pair of inflatable cuffs were placed around the carotid arteries. Fetal blood samples were drawn immediately before and after each occlusion and at post mortem, and analysed for pH, CaO₂, pCO₂, lactate and glucose.

Four protocols were followed; in the first protocol, three 10 minute carotid artery occlusions were performed at 1 hour intervals (3x10min, 1h), whereas in the second protocol, three 10 minute occlusions were repeated at 5 hour intervals (3x10min, 5h). In the third protocol a single 10 minute (10 min) and in the fourth protocol a single 30 minute (30 min) ischaemia was induced. Data from the single 10 min and 30 min occlusions have been reported elsewhere (Williams et al. 1992). In this study only histological outcome from these latter fetuses was considered as the configuration of electronic processing units previously used do not allow a valid direct comparison. Eight fetuses underwent 3x10min, 1h, of which three died 60-65 h following the last occlusion due to proven infection. These three fetuses were excluded from final EEG measurements and histological analysis. Five further fetuses were subjected to 3x10min, 5h, four fetuses to 10 min and seventeen fetuses to 30 min. Three days following the last occlusion fetuses were prepared for histological analysis as described in Chapter 2.
5.3. Results

3x10 minute ischaemia, separated by 1 hour

During each occlusion the EEG became iso-electric and EEG activity remained suppressed between occlusions (Fig 5.1). EEG suppression lasted for $5 \pm 0.7$ hours following the initial occlusion. Hyperexcitability developed $9 \pm 3$ hours after the initial occlusion and lasted for $29 \pm 8$ hours. Two animals developed continuous seizure activity, while the other six showed discrete episodes of hyperexcitability. By three days EEG intensity was $-4 \pm 2$ dB, which was not different from baseline.

![Figure 5.1. Typical examples of the EEG intensity changes during and following 3 x 10 minutes of ischaemia repeated at either 1 hour (upper panel) or 5 hour intervals (lower panel). The first occlusion is at time zero in both experiments.](image)

Cortical impedance increased during each occlusion (Fig 5.2). Following recirculation, the impedance recovered towards pre-insult values, but did not fully resolve prior to the second and third occlusion. Thus there was an increase in impedance with time,
each occlusion resulting in a further increase, which was also reflected in loss of estimated extracellular space ($p < 0.01$, Fig 5.3). Cortical impedance returned to baseline following the third occlusion, after which there were no changes except in the two animals with continuous seizure activity which were associated with a secondary rise in impedance.

Figure 5.2. Changes in cortical impedance (CI) relative to baseline (100%) following 3x10 minutes of transient cerebral ischaemia repeated at 1 hour (upper panel) or 5 hour (lower panel) intervals. Time zero indicates the first occlusion.

Figure 5.3. Maximum decrease of estimated extracellular space during each occlusion relative to baseline (100%). Changes in estimated extracellular space are derived from cortical impedance measurements. □ = 3x10 min insults, 1 h apart
□ = 3x10 min insults, 5 h apart. **$p < 0.01$ compared with the first insult.
A progressive metabolic acidosis developed with the occlusions (Table 5.1). Glucose levels increased following the first occlusion and then stabilised at that level during occlusion two and three (Table 5.2). No changes were observed in CaO₂ or pCO₂ during the experiment.

### Table 5.1. Fetal arterial lactate levels (mmol/L)

<table>
<thead>
<tr>
<th></th>
<th>Pre-insult 1</th>
<th>Pre-insult 2</th>
<th>Pre-insult 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>3x10 minutes, 1 hour apart</td>
<td>0.9 ± 0.06</td>
<td>2.0 ± 0.2 **</td>
<td>2.6 ± 0.4 **</td>
</tr>
<tr>
<td>3x10 minutes, 5 hours apart</td>
<td>0.8 ± 0.09</td>
<td>0.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

** = p<0.01 compared with the first insult

### Table 5.2. Fetal arterial glucose levels (mmol/L)

<table>
<thead>
<tr>
<th></th>
<th>Pre-insult 1</th>
<th>Pre-insult 2</th>
<th>Pre-insult 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>3x10 minutes, 1 hour apart</td>
<td>1.0 ± 0.09</td>
<td>1.5 ± 0.1 **</td>
<td>1.5 ± 0.1 **</td>
</tr>
<tr>
<td>3x10 minutes, 5 hours apart</td>
<td>0.9 ± 0.07</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

** = p<0.01 compared with the first insult

The distribution of neuronal loss is shown in figure 5.4. Overall neuronal loss was similar to fetuses that were subjected to a single 30 minute ischaemia (p=0.39), but the distribution of damage differed (p<0.001, Fig 5.5). Striatal damage was significantly increased (p<0.01) and parasagittal cortical damage significantly decreased (p<0.01) compared with a single 30 minute ischaemia.
Chapter 5. The effect of repeated cerebral ischaemia

Figure 5.4. Distribution of histological damage in fetuses subjected to 3 x 10 min insults at 1 hour (top panel) or 5 hour (low panel) intervals. Animals were sacrificed 72 h following the last insult. PsCx = parasagittal cortex, LtCx = lateral cortex, Str = striatum, Dg = dentate gyrus, Ca4 = Ca4 region of the hippocampus, Ca3 = Ca3 region of the hippocampus, Ca1/2 = Ca1 and Ca2 regions of the hippocampus, Thal = thalamus, Amg = amygdala
Figure 5.5. Histological outcome in selective regions three days after transient cerebral ischaemia. ■ = 1x30 min □ = 3x10 min, 1 h apart ■■ = 1x10 min ■■■ = 3x10 min, 5 h apart. ** = p<0.01 compared with a single episode of 30 minute ischaemia.

3x10 minute ischaemia, separated by 5 hours

Following an iso-electric EEG during each occlusion, EEG returned to baseline between insults (Fig 5.1). In 4/5 animals, discrete episodes of hyperexcitability, similar to animals in 3x10 min, 1h, developed 8 ± 2 hours following the first occlusion with a duration of 44 ± 9 hours. Final EEG activity (-0.3 ± 1 dB) was not different from baseline or 3x10min, 1h.

There was an increase in cortical impedance during each occlusion, all three insults resulting in similar increases (Fig 5.2). Following the third occlusion, cortical impedance returned to baseline and there was no change in impedance throughout the rest of the experiment.

No changes in metabolic parameters were observed (Table 5.1, 5.2).

Overall neuronal loss was slight (Fig 5.4) and similar to a single 10 min ischaemia
(p=0.48, Fig 5.5), except for marked striatal damage. Neuronal loss following 3x10min,5h was less severe than following 3x10, 1h (p<0.01), but showed a similar distribution with striatal damage being predominant in both protocols.

**1x10 minute ischaemia and 1x30 minute ischaemia**

The electrophysiological and histological data are reported in detail elsewhere (Williams et al. 1992). The distribution of neuronal loss is shown in figure 5.5.

### 5.4. Discussion

Choreoathetosis secondary to striatal injury is one of the classical syndromes of cerebral palsy and is presumed to be a result of striatal injury in the perinatal period (Spiegel, Baird, 1968). However, despite its clinical importance, little is known about the aetiology of such perinatal injury (Stanley, 1984). Attempts to correlate pregnancy risk factors with the various patterns of cerebral palsy have not been successful (Nelson, Ellenberg, 1986). Similarly, attempts to relate immediate perinatal events to the different clinical outcomes have not been elucidatory.

Following repeated short episodes of ischaemia, irrespective of the interval between insults, striatal damage was a dominant histological finding. In contrast with these results, previous studies show that a single 10 minute ischaemia in the fetal sheep causes trivial cerebral damage, while an isolated more prolonged ischaemia (30 minutes) results in severe neuronal loss in the hippocampus and infarction of the parasagittal cortex, but relatively little damage in the striatum (Williams et al. 1992; Tan et al. 1992). Although these previous studies were done to the same protocol it is possible that conditions were not identical, which may have influenced the outcome. In other experimental approaches to isolated episodes of in utero asphyxia, such as umbilical cord occlusion, particular vulnerability to the
hippocampus has been demonstrated (Chapter 3). Therefore it appears that the distribution of damage may be dependent on the temporal sequencing as well as the specific nature of the insult. Marked striatal damage may be a feature of multiple but not of isolated in utero insults.

A number of mechanisms have been implicated in hypoxic-ischaemic cell loss, including intracellular sodium and water accumulation leading to lysis, intracellular calcium accumulation and toxic effects of excitatory amino acids (Gluckman, Williams, 1992). Injury to hippocampal neurons following ischaemic insults has been associated with excessive release of glutamate (Choi, 1990). However, whether similar mechanisms apply to striatal damage following ischaemia is not clear. Striatal neurons, particularly small-medium sized, have been shown to attain ischaemic cell change more rapidly than cortical and hippocampal neurons (Pulsinelli et al. 1982). This early degeneration of small to medium sized striatal neurons has been related to changes in striatal dopamine neurohistochemistry (Nemeth et al. 1991). It may be that repeated insults enhances these early ischaemic cell changes sensitising striatal neurons in particular.

The magnitude of neuronal loss following multiple episodes of ischaemia in the present study was related to the interval between occlusions: cerebral damage being greater following more frequent insults. Three 10 minute insults repeated at 1 hour intervals showed a similar amount of overall damage as a single 30 minute ischaemia, whereas three 10 minute insults 5 hour apart resembled a single 10 minute ischaemia. These results are similar to studies in adult rats demonstrating more damage in the hippocampus following three 3 minute 4-vessel occlusions delivered hourly than after a single 9 minute occlusion (Nakano et al. 1989). Similarly, in the adult gerbil repeated cerebral ischaemia causes greater neuronal damage following 3 episodes of 5 minute ischaemia at 1 hour intervals than 15 minute of
ischaemia produced as a single insult (Tomida et al. 1987). The increased damage in those experiments was suggested to result from repeated insults coinciding with the time of post-ischaemic cerebral hypoperfusion (Tomida et al. 1987), although other mechanisms must be considered. For example, the cytotoxic milieu as a result of free radical production, excitatory amino acid and Ca$^{2+}$ release and changes in extracellular volume may place the neuron in a more vulnerable state when episodes of ischaemia are repeated more frequently. Conversely, homeostatic mechanisms including the release of inhibitory neuromodulators such as adenosine and the gradual resolution of cytotoxin accumulation may mean that greater protection is offered by less frequent insults. Indeed in the adult gerbil infrequent episodes of ischaemia has been shown to be neuroprotective (Kirino et al. 1991).

Post-ischaemic hypoperfusion has been related to cerebral edema in the adult rat (Schurer et al. 1990) and vasogenic edema was found 24 hours following ischaemic insults repeated at 1 hour intervals in gerbils (Tomida et al. 1987). However, these studies did not measure the acute cytotoxic edema formation during insults. In the fetal sheep, it has previously been reported that transient loss of extracellular space is due to cytotoxic edema during ischaemic insults (Williams et al. 1991). The intracellular swelling is understood to result from increased Na$^+$ and Cl$^-$ influx and uptake of osmotic water (Randall, Hoff, 1990). In vitro studies of hippocampal cells have suggested that cellular swelling plays a role in neuronal death (Rothman, 1985). Data in this study shows that frequently repeated insults lead to progressively worse cytotoxic edema during the insult period. The exact neuronal consequences of this phenomenon are unclear but are likely to reflect longer lasting defects in cellular integrity. This incomplete restoration of membrane function could leave the neuron more sensitive to the ion and water fluxes associated with the frequently repeated insults.

There was a prolonged depression of EEG activity following 1 hour apart occlusions,
whereas EEG activity recovered between each insult when they were separated by 5 hours. Following isolated transient ischaemic insults in the fetal sheep, prolonged EEG depression is associated with cortical injury (Williams et al. 1992). While the underlying mechanisms are not well understood, depression of EEG activity reflects loss of electrical activity. Electrical activity and trophic factors such as nerve growth factor (NGF) and interleukin one (IL-1) has been suggested to regulate neuronal survival in vitro (Koike et al. 1989; Brenneman et al. 1992). Recently other neurotrophic factors, such as insulin-like growth factor 1 (IGF-1) have been shown to be induced following hypoxic-ischaemic injury in the rat brain and that post-asphyxial central administration of IGF-1 is neuroprotective (Gluckman et al. 1992). Possibly neuronal loss in the present study was less following less frequent insults because the EEG activity recovered between insults allowing for induction of neurotrophic action.

Continuous seizure activity following 30 minutes of ischaemia in the fetal sheep is associated with infarction of the underlying parasagittal cortex (Williams et al. 1990) and that intermittent hyperexcitability develops following uterine artery occlusion only when mild to moderate neuronal loss is found in the parasagittal cortex (Gunn et al. 1992). However, suppression of these seizures with an NMDA receptor antagonist MK-801 improves neuronal outcome in the lateral cortex and the hippocampus, but not in the parasagittal cortex (Tan et al. 1992). Similarly, in this study discrete episodes of hyperexcitability were associated with mild to moderate degrees of cortical neuronal loss following repeated insults.

Metabolic factors have long been considered to have some affect on outcome of cerebral hypoxic-ischaemic insults. Systemic hyperglycaemia prior to ischaemia is known to worsen outcome in adult rats (Warner et al. 1987), but may be neuroprotective in the immature (Voorhies et al. 1986). Similarly, elevated systemic lactate has long been thought
to play a role in the development of ischaemic brain damage in monkeys (Yamaguchi, Myers, 1976). Increased plasma lactate and glucose levels following the initial occlusion were observed and these changes were more pronounced in the 1 hour apart protocol. However, the peripheral acidosis and hyperglycaemia observed, presumed to reflect cerebral concentrations, were only mild and it is doubtful whether such small changes could have a central role in the differences in outcome found in this study.

The present study shows that brief episodes of ischaemia, repeated before full recovery of EEG and intracellular edema, sensitize neurons to damage. Repeated insults, independent of the interval between the insults, alter the pattern of brain injury. This implies that the patterning rather than the nature of the insult may be important in certain neuropathologies. In particular, striatal damage appears to be a feature of multiple but not single insults. These observations offer at least a partial explanation of epidemiological difficulties linking perinatal events with subsequent outcome.
CHAPTER 6.

DO RECURRENT EPISODES OF FETAL ASPHYXIA SENSITISE THE BRAIN AND THE HEART TO DAMAGE?

6.1. Introduction

Prenatal asphyxia is thought to be a cause of intrauterine death or impaired postnatal neurological development in survivors. However, specific cause and effect relationships have been difficult to establish in the human fetus because of inadequate means of monitoring fetal events. Studies in Chapter 5 show that repeated episodes of cerebral ischaemia in the fetal sheep alter the degree and distribution of brain injury compared with single insults. The most striking effect of the repeated insults being the loss of striatal neurons.

Intermittent partial umbilical cord occlusion has been reported to cause cerebral white matter injury in fetal sheep (Clapp et al. 1988). Others have shown increasing acidosis and electrocardiographic evidence of asphyxia during repeated umbilical cord occlusions (Watanabe et al. 1992). However, the relationship between cardiac function, cerebral function and neuronal loss following recurrent episodes of fetal asphyxia is not clear. Results in Chapter 3 show that systemic asphyxia induced by umbilical cord occlusion in fetal sheep results in hippocampal injury, the severity of which is related to the degree of associated arterial hypotension. It is likely that when the human fetus is compromised, multiple episodes of asphyxia occur rather than a single prolonged event. Interactions between asphyxial episodes may be an important determinant of both the extent and the localisation of neuronal loss. The purpose of the present study was to examine cerebral and cardiac function during and after repeated umbilical cord occlusions in the sheep fetus and to determine their relationship to neuropathological outcome.
6.2. Methods

Eleven fetal sheep (118-126 days of gestation) were operated on under halothane anaesthesia (2%) with sterile techniques as described in Chapter 2. A reversible inflatable umbilical cuff was placed around the umbilical cord and one pair of ECG electrodes was placed subcutaneously, one over the apex of the heart and one on the opposite shoulder. After collection of baseline data, the umbilical cord was transiently occluded for 5 minutes and then released. This procedure was repeated four times, separated by 30 minute intervals. Fetal arterial blood gases were sampled at the following time points: 30 minutes before the first occlusion, immediately before and at the end of each occlusion, and at 30, 60, 120, 180, 240 minutes, 24, 48, and 72 hours following the last occlusion. Three days after the last occlusion, sheep were killed and the fetal brain was prepared for histologic analysis as described in Chapter 2.

6.3. Results

Three animals died on recirculation from occlusion due to cardiac fibrillation, two immediately after the third occlusion and one after the fourth occlusion. These animals have been excluded from analysis.

The first cord occlusion resulted in transient severe asphyxia, including hypoxia (pO₂ 5.1±1mmHg), hypercarbia (pCO₂ 97.0±4) and acidosis (lactate 3.81±0.2, pH 7.08±0.01) (Fig 6.1). The majority of blood gas and metabolic measurements showed similar changes with each successive occlusion. The exception was arterial lactate, which showed a progressive increase in lactate with each occlusion and following each recirculation lactate remained high. Following the last occlusion, arterial lactate was increased for 240 minutes, but had returned to baseline by 24 hours.
Figure 6.1. Fetal arterial blood gas and metabolic measurements during and following asphyxia. Lactate levels progressively increased with successive occlusions and was elevated by 240 minutes after the last occlusion but had returned to baseline by 24 hours. * = p < 0.05 compared with baseline.
Arterial blood pressure showed variable changes during the occlusions (Figs 6.2 and 6.3). The first occlusion resulted in arterial hypertension throughout the occlusion, after which there was a progressive hypotension at the end of each occlusion. Post-asphyxial blood pressure was normal. Bradycardia developed during each occlusion of similar severity (Figs 6.2 and 6.3) and upon recirculation heart rate returned to normal. There were no secondary changes in heart rate after the insults.

**Figure 6.2.** Heart rate (HR), mean arterial pressure (MAP), electrocorticogram (EEG) and cortical impedance (CI) during and following repeated umbilical cord occlusion. Arterial blood pressure became increasingly hypotensive with each occlusion. * = p < 0.05 compared with baseline.
Figure 6.3. Typical example of heart rate (HR), mean arterial pressure (MAP), electrocorticogram (EEG) and cortical impedance (CI) during and following repeated umbilical cord occlusions. Bradycardia developed with each occlusion and the arterial blood pressure became progressively hypotensive with successive insults. EEG became severely depressed during occlusion and remained depressed between occlusions. A similar increase in CI was observed with each insult.
ECG waveform analysis revealed large positive and negative deviations in the ST segment during the occlusions with no consistent pattern. Following the last occlusion the ST segment was depressed for 270±40 minutes. The T/QRS ratio increased during each occlusion (Fig 6.4). Following the last occlusion the T wave became inverted and the T/QRS ratio decreased below preinsult values for 310±60 minutes.

Figure 6.4. Changes in the T/QRS ratio during and following repeated umbilical cord occlusion. Upper panel showing typical example of the electrocardiographic changes. Each occlusion results in an increase in the T/QRS ratio. Following the last occlusion the T/QRS ratio decreased below pre-insult values and the T wave became inverted. *=p<0.05 compared with baseline.
Each occlusion resulted in severely reduced EEG intensity (p<0.05, Figs 6.2 and 6.3). Depression of EEG intensity was more severe during the second, third and fourth occlusion compared with the first insult (p<0.05). There was no difference in intensity between the last three occlusions. Following the fourth occlusion, EEG intensity was suppressed for a further 90±10 minutes then recovered to pre-insult levels. Following the last insult the EEG spectral edge frequency recovered from 9.1±0.7 Hz to pre-insult levels and remained unchanged throughout the experiment.

One animal was excluded from the seizure detection and two animals from spike detection analysis due to technical difficulties. Seizures occurred in 5/7 animals, starting at 3.3±0.6 hours (2.0-5.6 hours), with most seizures occurring between 11-15 hours (Fig 6.5). All animals demonstrated spike activity. The amplitude of the spike waves were 18-450μV, starting immediately after the first occlusion and with the highest rate at 11-15 hours after the last occlusion (Fig 6.5).

**Figure 6.5.** Number of spikes/hour and number of seizures following repeated umbilical cord occlusion. The highest number of seizures and spikes occurred 11-15 hours following the last occlusion. *=p<0.05 compared with pre-insult.
Cortical impedance increased during each occlusion (Figs 6.2 and 6.3). Each occlusion resulted in a similar increase. Following each recirculation cortical impedance returned towards pre-insult values. At 30-35 hours post-insult there was a small increase (104 ± 1%) in cortical impedance compared with pre-insult which persisted until the end of the experiment (p < 0.05).

Histological neuronal loss in the striatum was found in all eight surviving animals (Fig 6.6). Dead neurons appeared shrunken with pyknotic nuclei and red cytoplasm. Three animals showed severe injury throughout the striatum. In animals with mild striatal damage, the neuronal loss was in the putamen. Mild to moderate neuronal loss was observed in the globus pallidus. Mild injury was found in the hippocampus and cortex. No neuronal loss was found in the thalamus.

![Graph](image)

**Figure 6.6.** Average histological neuronal loss scores following repeated umbilical cord occlusion. The striatum showed significantly more damage than any other region. Scores: 0 = no neuronal loss, 1 = < 20% neuronal loss, 2 = 20-70% neuronal loss, 3 = > 70% neuronal loss. Str: Striatum, Gp: Globus pallidus, Hc: Hippocampus, Cx: Parasagittal cortex, Thal: Thalamus. *p < 0.05.
The degree of blood pressure reduction and the amplitude of the T/QRS ratio during the last occlusion were positively correlated with the extent of total neuronal loss (Table 6.1). There was no correlation between the extent of total neuronal loss and peak arterial lactate, glucose, pO₂, pH and the degree of bradycardia (Table 6.1). Post-insult, the duration of EEG depression and the number of seizures were correlated with the degree of total neuronal loss (Table 6.1).

<table>
<thead>
<tr>
<th>Neuronal loss</th>
<th>HR (% of baseline)</th>
<th>MAP (% of baseline)</th>
<th>T/QRS ratio</th>
<th>pO₂ (mmHg)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r=0.280</td>
<td>r=0.857</td>
<td>r=0.749</td>
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<tr>
<td></td>
<td>p=0.543</td>
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</table>

<table>
<thead>
<tr>
<th>Neuronal loss</th>
<th>lactate (mM/l)</th>
<th>glucose (mM/l)</th>
<th>EEG depression (min)</th>
<th>number of seizures</th>
</tr>
</thead>
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<tr>
<td></td>
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<td></td>
<td>p=0.263</td>
<td>p=0.780</td>
<td>p=0.024</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

Table 6.1. Correlations between total neuronal loss score and changes in heart rate (HR), mean arterial blood pressure (MAP), T/QRS ratio, arterial lactate, glucose and pO₂ during the 4th umbilical cord occlusion or between total neuronal loss and duration of EEG depression and number of seizures after the 4th occlusion.

6.4. Discussion

The present study shows that four repeated episodes of asphyxia, each of 5 minute duration, in the late gestation fetal sheep result in neuronal loss predominantly within the striatum. In contrast, an isolated 10 minute episode of umbilical cord occlusion in fetal sheep results in hippocampal damage (Chapter 3). A similar change in distribution of injury
was seen following repeated cerebral ischaemia insults in fetal sheep. While a single episode of cerebral ischaemia causes cortical and hippocampal injury, repeated brief episodes of cerebral ischaemia sensitise the striatum to damage (Chapter 5). Thus it appears that preferential striatal injury is dependent on the temporal sequencing of the asphyxial/ischaemic insult and not the nature of the insult.

Fetal arterial blood gas measurements showed evidence of severe asphyxia during each occlusion. Only arterial lactate demonstrated a cumulative effect, with each occlusion. However, only modest increases in arterial lactate levels were reached and there was no relationship between peak lactate level and neuronal loss. The role of elevated lactate levels in perinatal brain injury, remains a controversial issue (Vannucci, Yager, 1992). In the present study moderate lactic acidemia does not appear to be related to neuronal outcome.

There was a progressive fall in arterial blood pressure with each cord occlusion suggesting a cumulative effect on cardiovascular function. In agreement with previous studies the degree of hypotension, but not hypoxia, during the last umbilical cord occlusion was correlated with poor neuronal outcome Chapter 3 and (Gunn et al. 1992). During moderate asphyxia arterial blood pressure and peripheral vascular resistance are increased and blood flow to vital organs such as heart, brain and adrenals is increased (Rudolph, 1984). However, during severe asphyxia, peripheral vascular resistance may decrease and circulatory decentralisation may occur resulting in a reduction in blood flow to the heart and brain (Jensen, Berger, 1991). Hypotension during asphyxia can lead to compromised cerebral autoregulation (Papile et al. 1985). Under these conditions, cerebral blood flow becomes pressure passive and falls with the arterial pressure. Hence, in the present study, it is hypotension combined with hypoxia, induced by asphyxia, which results in cerebral hypoxia-ischaemia. Thus it is to be anticipated that in this study it is the degree and duration of
hypothesis that is predictive of outcome.

Although, severe bradycardia developed during each occlusion there was not an additive effect with repeated insults and the severity of bradycardia did not correlate with outcome. During bradycardia, there were variable changes in the ECG waveforms. Both the ST segment and the T wave are generated by repolarisation of the myocardium. The ST segment corresponds to the early slower phase of repolarisation and the T wave corresponds to the more rapid terminal portion of the repolarisation. While the ST segment during normal ventricular activation is predominantly generated by transmural potential differences, acute myocardial ischaemia can cause large transventricular potential gradients, resulting in ST vectors deviating in either direction (Mirvis, 1992). Both large positive and negative deflections in the ST segment during each occlusion were noted in the present study, thus suggesting acute episodes of myocardial hypoxia-ischaemia during umbilical cord occlusions. However, ST segment changes, derived from bipolar recordings, were too variable to be of predictive value.

Several previous clinical and experimental studies have observed increases in the T wave during acute fetal hypoxia and asphyxia (Widmark et al. 1989; Westgate et al. 1992). These changes have been attributed to anaerobic myocardial metabolism (Rosen et al. 1984). Following repeated umbilical cord occlusion in fetal sheep, an increase in T/QRS ratio correlated with fetal acidemia and duration of elevated blood pressure during occlusions (Watanabe et al. 1992). In the present study a correlation between the severity of neuronal loss and both the degree of hypotension and the increase in T/QRS ratio was found. These correlations suggest a functional relationship between compromised cardiac function and cerebral injury.

Three animals died due to ventricular fibrillation on reperfusion following the third
and fourth episode of umbilical cord occlusion. Repeated myocardial ischaemia cause impaired recovery of cardiac function in dogs (Gall et al. 1993). Reperfusion injury due to the formation of oxygen free radicals can occur following transient myocardial ischaemia (Otani et al. 1987). Free radical activity may cause ventricular fibrillation. Repeated reperfusion in the present study may have caused cumulative damage, resulting in ventricular fibrillation and fetal death.

Electrocortical activity, as indicated by EEG intensity function, was severely depressed during and between the periods of umbilical cord occlusion, but recovered within 90±10 minutes following the last occlusion. In the present study, duration of recovery of EEG intensity was correlated with the degree of total neuronal loss. A previous study found that prolonged (>4 hours) post-insult EEG depression was associated with severe cortical neuronal loss following cerebral ischaemia in fetal sheep (Williams et al. 1992). The more rapid recovery of EEG intensity in this study is compatible with the observation of none or minimal cortical damage.

Continuous cortical seizure activity following 30 minutes of cerebral ischaemia in fetal sheep is associated with infarction of the underlying cortex (Williams et al. 1992). In contrast, intermittent seizures develop when mild to moderate degrees of cortical injury is observed following uterine artery occlusion in fetal sheep (Gunn et al. 1992). In the majority of animals, episodes of spike discharges and intermittent seizure activity developed, superimposed on normal activity, following repeated episodes of systemic asphyxia. The number of seizures was strongly related to the degree of total neuronal loss. Only minimal cortical injury was observed in four animals in the present study, while deeper structures such as the striatum were frequently severely damaged. Thus the mechanisms of the initiation of the seizure activity seen in the present study may be different compared with the previous
Chapter 6. Repeated umbilical cord occlusion

fetal sheep studies. Chapter 7 shows, using immunohistochemical techniques, that GABAergic neurons of the basal ganglia are affected by repeated umbilical cord occlusion. Injury to GABAergic neurons have been indicated in seizure disorders (Houser, 1991). Similarly, injury to the substantia nigra reticulata (GABAergic innervation) precedes seizure induction in adult rats (Smith et al. 1988). Possibly loss of the striatal GABAergic inhibition is associated with the intermittent cortical seizures in the present study. Alternatively, it is possible that the seizures were initiated in the cortex causing distal neuronal loss in the striatum by overstimulating the glutamatergic input into the striatum. Prolonged status epilepticus (> 30 minutes) in the cortex can cause distal injury in the hippocampus and lateral cortex (Tan et al. 1992). However, it is unlikely that the intermittent seizures in the present study caused the striatal neuronal loss observed since they were of much shorter duration compared with previous studies.

A cumulative effect of repeated insults on post-insult brain edema in adult rats and gerbils following hypoxia-ischaemia has been suggested (Nowak et al. 1990). Chapter 5 shows an additive effect of frequently repeated insults on cytotoxic edema during cerebral ischaemia in fetal sheep. Treatment with the membrane stabiliser ganglioside GM1 improved recovery of primary edema and markedly protected against neuronal loss following repeated insults (Tan et al. 1994). No cumulative effect of repeated insults on cortical cytotoxic edema was observed in the present study. This may be a reflection of where impedance was recorded. Impedance was measured in the parietal cortex region which was not significantly damaged in the present study. A previous study, measuring striatal tissue impedance following repeated brain ischaemia in adult rats, found irreversible changes in impedance following more than two episodes of ischaemia, indicating cumulative plasma membrane dysfunction (Klein et al. 1993).
In summary, this study shows that recurrent episodes of fetal systemic asphyxia sensitise the striatum to neuronal loss in a similar fashion to repeated cerebral ischaemia. Both the progressive hypotension and the increases in the T/QRS ratio that developed with successive insults were related to the degree of neuronal loss. Several post-insult measures, including the duration of recovery of the EEG intensity and the number of seizures were associated with the degree of neuronal loss. It may be feasible to use these measures clinically to predict outcome. These findings indicate that recurrent episodes of fetal asphyxia interact and compromise the cardiovascular response to asphyxia, causing intrauterine death or a striatal pattern of brain injury in survivors.
**Chapter 7. Vulnerability of striatal neurons to repeated episodes of asphyxia**

**CHAPTER 7.**

**WHICH STRIATAL NEURONS ARE VULNERABLE TO REPEATED EPISODES OF FETAL ASPHYXIA?**

**7.1. Introduction**

Many types of neurological handicap may result from asphyxia during the perinatal period. However, the precise relationship between specific neurological injuries and defects are unclear. Chapters 5 and 6 show that intermittent episodes of cerebral ischaemia and global asphyxia in fetal sheep induce focal injury in the brain which is mainly localised to the striatal complex of the basal ganglia. The striatum plays a crucial role in motor control and some movement disorders, such as choreoathetoid cerebral palsy, have been associated with striatal dysfunction (Hayashi *et al.* 1991). However, little is known of the specific neural basis of these handicaps.

The major neuronal population in the striatum are the medium-sized spiny GABAergic projection neurons. These cells can be distinguished into two types on the basis of their connections and neurotransmitters. It is now well established that striatal efferent GABAergic/enkephalin neurons innervate the external segment of the globus pallidus, while neurons containing GABA and substance P, project directly to the internal segment of the globus pallidus and to the substantia nigra pars reticulata (DeLong, 1990). Most other cells within the striatum are considered to be aspiny interneurons based on both morphological criteria and the observation that none have been shown to project out of the striatum. Specific populations of striatal interneurons have been identified containing GABA/parvalbumin, (Gerfen *et al.* 1985) somatostatin and neuropeptide Y (Smith, Parent, 1986), and acetylcholine (Lehmann, Langer, 1983).
Studies in postnatal animals indicate that there may be differences in vulnerability of specific neuronal subtypes within the striatum to hypoxia-ischaemia. Cholinergic and somatostatin interneurons were found to be resistant to hypoxia-ischaemia in the neonatal rat and adult gerbil (Burke, Kenyon, 1991; Uemura et al. 1990). Similarly, GABAergic interneurons, as revealed by parvalbumin immunoreactivity were relatively resistant to hypoxia-ischaemia in the adult gerbil (Gonzales et al. 1992). The effect of hypoxia-ischaemia on calbindin-positive cells, which comprises most of the medium-sized spiny neurons in the striatal matrix compartment (Gerfen et al. 1985), is not clear. While, a recent study indicates that these neurons are resistant to hypoxia-ischaemia in the neonatal rat (Burke, Baimbridge, 1993), others found that there was a loss of calbindin-positive neurons following forebrain ischaemia in adult rats (Freund et al. 1990).

In human neurodegenerative disorders such as Huntington’s disease, there is a major loss of both GABA/enkephalin and GABA/Substance P striatal projection neurons, while somatostatin/neuropeptide Y interneurons are spared (Storey, Beal, 1993; Faull et al. 1993). Recent studies have suggested that a major factor in this neuronal loss is due to an excitotoxic mechanism and it has been demonstrated that injections of quinolinic acid into the striatum mimic many of the neurochemical changes seen in Huntington’s disease (Beal et al. 1991; Faull et al. 1993; Faull et al. 1994).

Chapters 5 and 6 show that repeated cerebral ischaemia and asphyxia cause neuronal loss predominantly in the striatum. It is however not yet clear how specific sub-populations of striatal neurons are affected by perinatal asphyxia. Therefore, in this study the chemical and anatomical changes in the basal ganglia (striatum, globus pallidus and substantia nigra) of the fetal sheep brain were examined following repeated asphyxial episodes resulting from occlusion of the umbilical cord. In particular, the effect of repeated fetal asphyxia on specific
neuronal sub-populations within the basal ganglia was investigated using a variety of immunohistochemical markers (calbindin, somatostatin, parvalbumin, substance P and enkephalin) to identify known subpopulations of neurons in the striatum.

7.2. Methods

Ten fetal sheep (118-126 days of gestation) were operated on under halothane anaesthesia (2%) with sterile techniques as described in Chapter 2. A reversible inflatable umbilical cuff was placed around the umbilical cord. The umbilical cord was transiently occluded for 5 minutes followed by reperfusion. This procedure was repeated 4 times, separated by 30 minute intervals. Three fetuses died due to the umbilical cord occlusions. These were excluded from histological and immunohistochemical analysis. Three days after the umbilical occlusions the ewe was killed and the fetal brain was immediately perfused and prepared for histology and immunohistochemistry as described in Chapter 2. In addition to the experimental brains, five gestational age-matched normal fetuses were perfused in an identical manner and used as controls.

7.3. Results

Description of the histological outcome following repeated umbilical cord occlusions was described in Chapter 6. Briefly, all surviving animals (n=7) showed neuronal loss in the striatum (caudate/putamen). The damage was particularly severe in the lateral regions. In addition, 4 animals showed some histological damage to the globus pallidus. There was no observable neuronal death in the substantia nigra. Ischaemic cell changes were not observed in the control brains.
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Immunohistochemistry - caudate/putamen

Calbindin:

In control brains calbindin immunoreactivity was found throughout the caudate/putamen complex (Fig 7.1a). The most anterior regions of the caudate nucleus were uniformly densely labelled; whereas more caudally staining was most dense ventrally and medially with gradually decreasing densities of staining in the dorso-lateral two-thirds of the caudate/putamen complex (Fig 7.1a). Within the region of dense calbindin staining there were patches which were devoid of staining. These patches of low immunoreactivity were irregularly shaped, but often elongated and convoluted. Calbindin immunoreactivity was revealed as a dense neuropil staining containing densely packed medium sized cell bodies (Fig 7.1c). These cell bodies were rounded or triangular in shape with a few emanating dendrites.

In the asphyxiated animals there was a marked loss of calbindin staining (Figs 7.1b and 7.1d). Loss of staining was most prominent in the dorsolateral two-thirds of the caudate/putamen complex especially in regions anterior to the globus pallidus. This pattern of loss of immunostaining corresponded to regions of neuronal loss as observed with acid fuchsin staining. High magnification light microscopy revealed a reduction in calbindin staining due to reduced numbers of immunoreactive cells (Fig 7.1d), and a reduction in staining of the neuropil. Quantitative analysis demonstrated a significant overall decrease of calbindin immunoreactivity in the caudate/putamen from 74.0±3 to 43.2±6 %, (p<0.05, Fig 7.2).
Figure 7.1. Low (a,b) and high (c,d) magnification photomicrographs showing the distribution of calbindin immunoreactivity in the anterior region of the caudate/putamen complex of the control (a,c) and asphyxiated (b,d) fetal sheep brains. Note the marked loss of calbindin immunoreactivity in the dorsal two-thirds of the caudate/putamen in (b) following asphyxia in comparison with the normal caudate/putamen in (a). (c) and (d) are high magnification photomicrographs from regions in the caudate nucleus indicated by the arrows in (a) and (b) respectively and show a loss of calbindin immunoreactive cells and neuropil staining following asphyxia. CN caudate nucleus; P putamen. Scale bars: a,b = 5mm ; c,d = 50μm.
Figure 7.2. Densitometry measurements of calbindin (Calb) immunoreactivity in the caudate/putamen, enkephalin (Enk) immunoreactivity in the globus pallidus and substance P (SP) immunoreactivity in the substantia nigra pars reticulata in control and asphyxiated brains. Calbindin and enkephalin immunoreactivity were markedly reduced in asphyxiated brains, while there was a smaller decrease in substance P immunoreactive staining. Open bars = normal brains, solid bars = asphyxiated brains. * = p < 0.05.

Parvalbumin:

In control brains parvalbumin immunoreactive cells were located in the dorsolateral region of the caudate and putamen in a more or less complementary pattern to that of calbindin. The density of these cells was much lower than that of calbindin. These cells had medium sized soma, long slender aspiny dendrites and also some long beaded processes which appeared to be axons (Fig 7.3a).

In the asphyxiated animals analysis of cell densities showed that there was a significant reduction in the number of parvalbumin immunoreactive cells (34.4 ± 3 to 25.6 ± 2
Figure 7.3. Photomicrographs showing parvalbumin immunoreactive neurons (a,b) and somatostatin immunoreactive neurons (c,d) from similar regions of the caudate/putamen of control (a,c) and asphyxiated (b,d) fetal sheep brains. Note the loss of parvalbumin immunoreactive neurons following asphyxia, whereas there is little change in the number of somatostatin immunoreactive cells after asphyxia. Scale bar a-d = 100μm.
neurons/10mm² square) in the region of acidophilic neuronal loss in the striatum (Fig 7.4). Also the parvalbumin positive cells showed a marked reduction in their dendritic and axonal processes as compared to controls (Fig 7.3b).

![Bar graph](image.png)

**Figure 7.4. Number of somatostatin (Smt) and parvalbumin (Parv) immunoreactive cells/cm² in the caudate/putamen in control and asphyxiated brains.** There was a loss of parvalbumin immunoreactive cells following asphyxia, while the number of somatostatin immunoreactive cells showed no significant change. Open bars = control brains; solid bars = asphyxiated brains. * = p < 0.05.

**Somatostatin:**

Somatostatin cells were scattered throughout the entire caudate/putamen. These cells were mainly bipolar or tripolar, medium in size and had smooth dendrites (Fig 7.3c). There was also a dense immunoreactive axonal network which presumably emanated from these cells.

Analysis of somatostatin positive cells showed that there was no significant change in their numbers or morphology after asphyxia (Fig 7.3d).
Enkephalin immunoreactivity - globus pallidus

In control animals immunoreactivity for enkephalin was found throughout the globus pallidus (Fig 7.5a). The staining consisted of a dense network of immunoreactivity which when viewed with high power light microscopy was observed to be terminations of axons on pallidal dendrites; these have previously been described as woolly fibres (Haber, Nauta, 1983). Staining was particularly dense in medial and ventro-lateral regions, with slightly decreased staining in the dorsal region.

Asphyxiated animals showed marked reduction of enkephalin throughout most of the globus pallidus, with only the most ventral area showing significant staining (Fig. 5b). Similarly, morphometric analysis supported these findings showing a marked reduction in the enkephalin immunoreactivity in the asphyxiated brains from 63.0±8 to 33.6±4 % (p<0.05, Fig 7.2).

Substance P immunoreactivity - substantia nigra pars reticulata

The substantia nigra in control brains demonstrated extremely high levels of substance P reactivity. The substance P immunoreactivity extended throughout the full extension of the nucleus, although there was an area in the central one third of the complex which showed slightly less dense staining (Fig 7.5c). By contrast, in asphyxiated animals, there was virtually a total loss of immunoreactivity in the central one third of the substantia nigra (Fig 7.5d). Densitometry analysis confirmed that there was an overall reduction of immunoreactivity in the substantia nigra pars reticulata from 95.5±1 to 78.7±2 % (p<0.05, Fig 7.2). Visual examination of the sections indicate this reduction to be mainly localised to the central third of the substantia nigra pars reticulata.
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Figure 7.5. Photomicrographs showing the distribution of enkephalin immunoreactivity in the basal ganglia (a,b) and substance P immunoreactivity in the substantia nigra pars reticulata (c,d) in the control (a,c) and asphyxiated (b,d) fetal sheep brains. Note: (i) the decrease in enkephalin immunoreactivity in the caudate nucleus, putamen and especially in the globus pallidus following asphyxia, and (ii) the loss of substance P immunoreactivity in the substantia nigra pars reticulata after asphyxia. CN caudate nucleus; GP globus pallidus; P putamen; SNr substantia nigra pars reticulata. Scalebars a-d = 5mm.
7.4. Discussion

Striatal damage is a frequent neuropathological finding in many neurological disorders such as choreoathetoid cerebral palsy and Huntington’s disease (Storey, Beal, 1993; Carpenter, 1950). Chapters 5 and 6 show that repeated episodes of cerebral ischaemia and intermittent periods of global asphyxia in fetal sheep result in predominantly striatal neuronal loss. In contrast, single episodes of cerebral ischaemia or global asphyxia in fetal sheep cause predominantly cortical and hippocampal neuronal loss, with little or no striatal damage (Williams et al. 1992) and Chapter 3. Similarly, in adult rats, multiple ischaemic insults were found to accentuate injury in the striatum compared with single insults (Lin et al. 1992). Thus repetitive ischaemic insults to the brain appear to induce striatal injury. However, little is known about the specific cell populations involved in this pattern of injury.

The different cell types identified immunohistochemically in this study agree with reports from other animal studies using similar methods. The calbindin staining shown in this study on the normal fetal sheep brain is essentially the same as that seen in the striatum in other mammals (Cowan et al. 1990; Gerfen et al. 1985; Celio, 1990; DiFiglia et al. 1989; Cote et al. 1991) where calbindin distribution shows a decreasing gradient from ventral to dorsolateral regions. Calbindin staining is also used as a label to delineate the striatal matrix compartment, leaving the patches or striosomes unstained (Cowan et al. 1990). The calbindin positive cells have been identified as the GABAergic medium spiny neurons projecting to substantia nigra and globus pallidus (DiFiglia et al. 1989; Gerfen et al. 1985).

In all asphyxiated brains there was a marked loss of calbindin immunoreactivity in the neostriatum. The loss of staining occurred primarily in the lateral two-thirds of the caudate/putamen complex. The reduction in immunostaining was due to both a reduction in cell numbers and loss of neuropil staining. Similarly, neuronal injury in the striatum was
associated with a reduction in calbindin-immunopositive cells following hypoxia-ischaemia in adult rats (Freund et al. 1990). In contrast, others have found no loss of calbindin-immunopositive neurons following an isolated hypoxic-ischaemic striatal injury in the neonatal rat (Burke, Baimbridge, 1993). In human neurodegenerative diseases, such as Huntington’s disease, which are associated with neuronal loss in the striatum, calbindin-immunopositive cells are diminished (Kiyama et al. 1990; Seto-Ohshima et al. 1988). Thus these results resemble findings in adult rats, but not neonatal rats, following a single insult, and also certain human neurological disorders. The reasons for these discrepancies are not clear; possible factors involved may include species and/or age differences, or severity or nature of the neuronal injury.

The parvalbumin cells labelled in this study on the fetal sheep are very similar in morphology and distribution in the striatum to parvalbumin immunopositive cells reported in the striatum of other animals (Celio, 1990; Kita et al. 1990; Cowan et al. 1990; Cote et al. 1991; Gerfen et al. 1985). Parvalbumin-containing striatal interneurons have previously been reported to be relatively resistant to ischaemia-induced lesions in adult gerbils (Gonzales et al. 1992). Previous studies have shown that parvalbumin-immunopositive cells are particularly sensitive to kainic acid, suggesting a non-NMDA excitotoxic action on these cells (Waldvogel et al. 1991). By contrast, in the present study, a small decrease in the number of parvalbumin immunoreactive neurons in the caudate/putamen was found, showing that parvalbumin cells in the striatum are less sensitive to asphyxia than the calbindin cells.

The somatostatin cells identified in this study are very similar in all respects to somatostatin immunoreactive cells which have been shown in the striatum of other species (Smith, Parent, 1986; Takagi et al. 1983). In other studies on the mammalian striatum somatostatin has been shown to be colocalised in striatal interneurons with neuropeptide Y,
avian pancreatic polypeptide (APP) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) diaphorase (Sandell et al. 1986). In contrast to the calbindin and parvalbumin cells, somatostatin containing interneurons were relatively spared in the present study. This is in agreement with several previous studies, which found somatostatin positive cells to be resistant to ischaemic damage in both neonatal and adult animals (Uemura et al. 1990; Chesselet et al. 1990; Ferriero et al. 1988). Selective sparing of somatostatin neurons have been suggested to be due to fewer NMDA receptors on these neurons and it has recently been shown that somatostatin positive neurons show low amounts of mRNA for the NMDA receptor subunit NR1 which is essential for functional NMDA receptors (Augood et al. 1994).

Marked loss of enkephalin immunopositive fibres in the globus pallidus and substance P immunoreactivity in the substantia nigra pars reticulata was observed. It is now well established that striatal efferent GABAergic/enkephalin neurons innervate the globus pallidus, while neurons containing GABA and substance P innervate the substantia nigra pars reticulata (DeLong, 1990). Thus, the loss of enkephalin staining in the globus pallidus observed here following asphyxia suggests involvement of the striatopallidal GABAergic/enkephalin projection neurons. Similarly, the reduction of substance P immuno-staining in the substantia nigra suggests involvement of striatonigral GABAergic/substance P projection neurons. These findings are consistent with the loss of calbindin immunoreactivity in the striatum since calbindin is a marker for both the GABAergic striatopallidal and striatonigral projection neurons. These results and these conclusions are therefore in agreement with previous studies showing preferential loss of striatal GABAergic cells, as measured by GAD activity, following forebrain ischaemia in adult rats (Francis, Pulsinelli, 1982).

A number of mechanisms have been implicated in hypoxic-ischaemic injury, including
toxic effects of excitatory amino acids leading to excessive intracellular calcium accumulation and DNA degradation (Choi, Hartley, 1993; Kure et al. 1991). In the adult rat, repeated episodes of ischaemia result in a greater release of glutamate into the striatal extracellular space compared with a single insult (Lin et al. 1992). A previous study has shown that treatment with the ganglioside GM1 markedly protects against neuronal loss following repeated episodes of cerebral ischaemia in fetal sheep (Tan et al. 1994). GM1 has been shown to protect against excitotoxic injury in the immature rat (Lipartiti et al. 1991). The present study showed a marked loss of striatal projection neurons, while somatostatin positive interneurons were spared. The major cortical glutamatergic input to the striatum is onto the projection neurons, while somatostatin immunoreactive cells contain few NMDA receptors. Thus a significant component of the striatal damage observed in the present study may result from excitotoxicity.

Calbindin and parvalbumin are calcium binding proteins and have been suggested to act as a cytosolic calcium buffer (Scharfman, Schwartzkroin, 1989). Therefore, both in vivo and in vitro studies have indicated that calbindin containing neurons may be protected from excitotoxic injury (Iacopino et al. 1992; Weiss et al. 1993). However, there was no relationship in the present study between calcium binding protein containing neurons and protection to striatal injury. In contrast, both calbindin and parvalbumin immunoreactive cells appeared to be the most vulnerable. Similarly, following hypoxia-ischaemia in adult rats, there was no association between neuronal injury and calbindin and parvalbumin immunoreactivity (Freund et al. 1990). In addition, in neurodegenerative diseases, such as Huntington’s chorea, that have been associated with excitotoxicity and excessive calcium accumulation (Coyle, Puttfarcken, 1993), loss of striatal calbindin-positive cells is a prominent feature (Kiyama et al. 1990; Seto-Ohshima et al. 1988).
Whilst caution is necessary when making direct comparisons between experimental studies and neurological diseases in the human, the present study may imply some functional consequences of repeated episodes of prenatal asphyxia. It is now clear that cerebral palsy originates more often from prenatal events than from postnatal events (Eicher, Batshaw, 1993; Hagberg et al. 1993). The striatum plays a central role in motor function and injury to the striatum is often associated with choreoathetoid cerebral palsy. However, little is known of the specific neuronal basis of this disorder. In the present study, it was found that striatal injury was associated with loss of GABAergic projection neurons to the globus pallidus and the substantia nigra. In adult patients with Huntington’s disease loss of striatal GABAergic projection neurons is associated with motor impairment such as chorea and/or rigidity (Albin et al. 1990; Storey, Beal, 1993). Thus, if these observations can be extrapolated to the human, they suggest that repeated episodes of prenatal asphyxia may lead to selective striatal injury resulting in choreoathetoid movement defects.

In summary, this study shows that there is selective vulnerability within subpopulations of striatal neurons to multiple episodes of asphyxia in the late gestation fetus. Whilst, striatal somatostatin neurons are preserved, GABAergic projection neurons to both the globus pallidus and the substantia nigra appear to be severely affected suggesting that no protective effect is provided by calcium-binding proteins against neuronal injury. Repetitive asphyxial injuries to these major striatal pathways may lead to neurological deficits such as choreoathetoid cerebral palsy.
CHAPTER 8.

FINAL DISCUSSION

Studies presented in this thesis have examined the relationship between specific asphyxial events and neuronal loss in the fetal sheep. The pattern and the timing of the insult were shown to play important roles in determining the degree and distribution of neuronal loss. This section will discuss several important issues in relation to these findings. Firstly I will address the relevance of the experimental design. Secondly, I will discuss possible factors that may sensitise the immature brain to injury and the clinical relevance of the neuropathological outcomes observed in these studies. The results will be summarised in the conclusion and areas that deserve further study will be identified.

8.1. The relevance of the experimental design

8.1.1. Fetal sheep preparation

Neuropathological and brain imaging studies have clearly shown that a significant number of brain injuries in the newborn are of prenatal origin (Bejar et al. 1988). The majority of experimental studies of perinatal brain damage, to date, have been performed in the neonatal rat. However, the use of postnatal animals, such as the neonatal rat may not reflect the true nature of the in-utero situation. For example, the thermal environment plays an important role in the pathogenesis of brain damage. While hyperthermia either during or immediately after an asphyxial insult leads to greater brain injury, cooling of the brain can protect against neuronal loss (Dietrich et al. 1993; Ginsberg et al. 1993; Dietrich, 1992). Thus to adequately mimic in utero asphyxia and to evaluate subsequent brain damage requires maintenance of both the environmental and brain temperature at intrauterine levels.

The majority of previous studies examining the effect of asphyxia on the fetal brain,
have used the acute exteriorised fetal sheep preparation (Thiringer et al. 1987; Hagberg et al. 1987; Myers, 1972). There is evidence that surgical trauma and anaesthesia may affect physiological parameters such as heart rate, blood pressure, fetal respiratory movements and endocrine responses (Robinson et al. 1977; Boddy et al. 1974). Furthermore, the duration of such studies is often brief. It is now clear that many pathological events take place several hours to days after the initial asphyxial event (Gluckman, Williams, 1992). Thus, long term recovery is necessary to accurately evaluate factors that may influence neuronal outcome.

The chronically instrumented fetal sheep preparations used in this thesis allow for an experimental approach without confounding factors such as surgical stress, anaesthesia and thermal instability. Because of the relatively large size of the fetus, comprehensive instrumentation of electrodes and indwelling catheters is possible. This allows for continuous measurements of biochemical and biophysical parameters during periods of development relevant to the mid and third trimester human fetus as described in Chapter 1.

While caution is necessary when comparing experimental studies in animals with human situations, the chronically instrumented fetal sheep is useful as an experimental approach to address questions related to perinatal asphyxial brain damage.

8.1.2. Insult paradigm: repeated cerebral ischaemia and systemic asphyxia

The great majority of previous studies of fetal asphyxia have not examined neuronal outcome (Raju, 1992). In this thesis, two experimental approaches were developed to induce brain damage in the fetus, repeated cerebral ischaemia and umbilical cord occlusion. The cerebral ischaemia model induces selective repetitive cerebral hypoperfusion without confounding factors such as cardiac effects. This approach has limitations in its relevance to spontaneous in utero events that are likely to be of a more complex nature including systemic
asphyxia. Nonetheless, it induces a highly reproducible degree of neuronal loss and the distribution of neuronal loss, i.e. predominantly striatal damage shows neuropathological outcome similar to some clinical syndromes.

Oclusion of the umbilical cord results in systemic asphyxia and may reflect asphyxial intrauterine events more closely than the cerebral ischaemia model. In contrast to previous studies of systemic asphyxia in the fetus (Myers, 1972), both the duration and severity of the insults were standardised. Furthermore, outcome was reproducible and mortality rates low. Other models of systemic fetal asphyxia, such as combined hypovolemia and hypoxia, were associated with high mortality rates (Ting et al. 1983). A common experimental approach is partial clamping of the uterine artery. However, these experiments have proven to be of limited value because of variable outcome and limited degrees of neuronal loss. The long duration of the insult necessary to induce damage results in non standardised insults (Gunn et al. 1992). Maternal hypoxia has often been used to induce fetal asphyxia and may mimic some clinical situations such as chronic hypoxia associated with growth retardation. However, similarly to the uterine artery occlusion model, very prolonged insults are necessary to induce brain damage and the outcome is highly variable (Penning et al. 1994). Thus, although the experimental approaches described above show similarities to some clinical situations, the great variability makes them difficult to use for determining precise cause and effect relationships in perinatal asphyxia.

In summary, both the repeated cerebral ischaemia and the umbilical cord occlusion approaches provide experimental paradigms that show highly reproducible outcomes, low mortality rates and standardised insults. Although direct comparisons cannot be made with the human, neuronal outcome shows similarities to some clinical syndromes.
8.1.3. Evaluation of neuronal loss

Evidence now exists that neuronal death evolves over several days (Pulsinelli W et al. 1982). In both adult and immature animals, viability of the neuron is lost from two hours up to 72 hours after the initial insult depending on the severity of the insult and susceptibility of specific neuronal populations (Kirino, 1982; Pulsinelli W et al. 1982; Massarweh et al. 1987). Similarly, severely asphyxiated infants often show a delayed deterioration of neurological function (Lupton et al. 1988). Thus, neuronal death cannot be considered as a static event but as a dynamic process.

Ultrastructurally, changes such as mitochondrial swelling can be seen within hours of hypoxia-ischaemia (Brown, Brierley, 1972). Such changes may be transient and are not necessarily a sign of death of the neuron (Petito, Pulsinelli, 1984). In contrast, dying neurons have been described as showing "ischaemic cell change" (Brown, Brierley, 1972). These neurons become acidophilic due to accumulation of coagulated proteins in the cytoplasm concurrent with loss of basophilic endoplasmic reticular formation (Smith et al. 1984). The combined staining technique used in the present experiments stain such dying neurons as intensely red-staining cytoplasm and dark blue pyknotic nuclei (Auer et al. 1984). Acidophilic neurons have been well established as dying neurons by their disappearance and removal from the brain in experiments allowing two weeks and longer survival (Auer et al. 1984).

Therefore, the timing (72 hours after insult) and interpretation of neuronal death in the present experiments conforms well with previous studies. However, neurological dysfunction in the newborn is likely to involve more complex interactions than simply neuronal death. Subtle imbalances in neurotransmitters or neuronal connections may occur and such changes cannot be recognised by staining procedures for neuronal death. These
questions were briefly addressed in Chapter 7 using immunohistochemical techniques to investigate the effect of repeated episodes of asphyxia on neuronal connections in the basal ganglia. As suggested above, loss of specific neurotransmitter markers were identified in regions where no neuronal death was detected. Furthermore, these techniques recognised specific subpopulations of injured neurons that may be important in a better understanding of the underlying pathogenesis.

An additional aspect of evaluation of brain damage, which has not been addressed in these studies, is the effect of fetal asphyxia on myelination. Myelination disorders, such as delayed myelination, are seen particularly in some preterm infants (De Vries et al. 1987; De Vries et al. 1989). Adequate examination of such pathologies requires longer recovery periods than used in these studies. Such experiments could be designed in the fetal sheep by induction of fetal asphyxia prior to the onset of myelination (90 day fetus). Subsequent neuropathological examination at the peak of myelinisation (125 day gestation) may reveal impairment in myelination.

8.2. Factors that sensitise the brain to intrauterine asphyxia

8.2.1. Systemic responses to asphyxia

Results in Chapters 3, 4 and 6, consistently demonstrated a correlation between the degree of hypotension during asphyxia with the extent of neuronal loss. On the other hand, neither the degree of hypoxia, metabolic acidosis nor bradycardia was associated with outcome. These results agree with previous observations suggesting that arterial pressure changes are important in the pathogenesis of perinatal brain damage. Both animal experiments and observations in distressed neonates demonstrate that cerebral autoregulation is compromised during large fluctuations in arterial blood pressure (Lou et al. 1979b; Lou
et al. 1979a; Lou, 1988). The consequences of unstable blood pressure are risk of haemorrhage when blood pressure is elevated and vulnerability of cerebral ischaemia when blood pressure falls. Although, cerebral blood flow was not measured in any of the studies presented in this thesis, it is likely that the observed fall in arterial blood pressure was associated with impaired cerebral blood flow resulting in cerebral ischaemia.

Clinically, loss of cerebral autoregulation is particularly common in the preterm distressed infant, often in association with recurrent episodes of apnoea and severe bradycardia (Perlman, Volpe, 1985). It was of interest to note that the midgestation fetus compared with the near term fetal sheep showed less change in arterial pressure in response to 10 minutes of umbilical cord occlusion (Chapter 4). The reasons for this are not clear. However, as discussed in Chapter 4, animal studies suggest that the midgestation fetal sheep can maintain arterial blood pressure during periods of anoxia because of its higher levels of cardiac glycogen compared with the late-gestation fetus (Dawes et al. 1959). Given the probable intermittent nature of the insults in the preterm neonate and the interaction between repeated insults demonstrated in the late-gestation fetus, further studies are needed to elucidate the effect of recurrent episodes of asphyxia in the midgestation fetus.

8.2.2. Cardiac responses to asphyxia

Electronic fetal heart rate monitoring has often been used as a measure of fetal distress in the human with the hope that clinical intervention would avoid the risk of perinatal death or neurological damage (Knopf et al. 1990). However, recent studies recognise the limitations of fetal heart rate monitoring and suggest that, at best, it may identify fetuses at risk during parturition but not predict outcome (Rosen, Dickinson, 1993; Henderson Smart, 1991). Such findings are compatible with the results in this thesis where no relationship
between the degree of bradycardia and extent of neuronal loss was found.

In contrast to fetal heart rate changes, waveform changes in the ECG, such as increase in T/QRS ratio, during bradycardia could be related to outcome (Chapter 6). Such T wave changes have previously been associated with depletion of myocardial glycogen stores and development of metabolic acidosis (Hökegård et al. 1981). Similarly, clinical observations suggest a predictive value of T/QRS ratio (Greene, Westgate, 1992; Westgate et al. 1993). Thus, ECG waveform changes may reflect substantial fetal distress and deserve further study. For example, it has been suggested that the less mature (< 126d) fetal sheep show less evidence of T/QRS increase compared to the 129-141 day old fetus (Widmark et al. 1989). The ECG response in the midgestation fetus in response to asphyxia is not known and should be investigated. Furthermore, an inverted T wave was noted on the ECG following repeated episodes of asphyxia (Chapter 6). Such T wave inversions are often associated clinically with myocardial pathology in the adult. Therefore histological examination of hearts following repeated episodes of fetal asphyxia may identify myocardial pathology.

In summary, the results in this thesis suggest that there is a relationship between cardiovascular failure, such as hypotension and increases in the T/QRS ratio, and neuronal loss. In contrast, other clinical measurements of asphyxia, such as hypoxia and acidosis, were not related to outcome.

8.2.3. Brain maturation

The sensitivity of the developing brain to asphyxial insults may change with advancing gestation as discussed in Chapter 1. These differences correspond both to the degree of neurological maturation and cerebral vascular development. The developing brain has been
postulated to be particularly sensitive to white matter lesions while immature neurons, due to their lower energy requirements, are less susceptible (Volpe, 1992).

The vulnerability of the preterm infant to white matter damage has been attributed to, in part, the vascular supply of the periventricular brain region during development. The lesions have been located at the boundary between ventricular fugal (long medullary penetrating arteries) and ventriculopetal (recurrent collaterals or transventricular) arteries (Dereuck et al. 1972). Thus during a decrease in overall cerebral perfusion, this area would be the most affected. Similarly, the relative sparing of the cortex during this developmental stage is thought to be due to the presence of leptomeningeal anastomoses between the anterior, middle and posterior cerebral arteries (Vander Eecken, 1959). However, the presence of these vessels have recently been questioned (Nelson et al. 1991; Mayer, Kier, 1991).

Alternatively, increased vulnerability to the actively differentiating periventricular glia cells during development may also play an important role in the pathogenesis of preterm brain damage. Experimental studies in neonatal animals suggest that when overall cerebral blood flow is reduced the white matter becomes relatively underperfused (Cavazzuti, Duffy, 1982; Young et al. 1982). Recent in-vitro studies have shown that differentiating oligodendroglia are exceptionally vulnerable to glutamate toxicity (Oka et al. 1993). This may provide an alternative explanation of the susceptibility of the preterm to white matter lesions.

The study in Chapter 4 shows that neurons at late gestation are more vulnerable to a brief period of in utero asphyxia compared with the midgestation fetus, while white matter was not affected in either group (Chapter 4). These findings support the concept that immature neurons are more resistant to an insult. Furthermore, the finding that there was
minimal increase in cerebral impedance during asphyxia in the 90 day old fetuses suggest that
depolarisation of the immature cells is delayed. The resistance of the white matter may be
related to the relatively mild insult. Recent studies of very prolonged hypoxia insults (8
hours) have shown white matter lesions at a similar age in the fetal sheep (Penning 1994).
These results suggest further studies are needed to evaluate the effect of longer periods of
in utero asphyxia on the developing brain.

8.2.4. Recurrent episodes of asphyxia

Several situations may expose the fetus or the newborn to recurrent episodes of
asphyxia. Intermittent cord compression has been postulated as one such cause of asphyxia.
This is likely to be more common in patients with a short or long cord, cord entanglements,
knots in the cord or significant oligohydramnios (Naeye, 1985; Shields, Schifrin, 1988).
Similarly, chronic utero-placental instability could lead to repeated hypotensive episodes
when oxygen delivery to the developing brain is critically impaired. During birth, prolonged
labour may cause recurrent episodes of cord compression. In the premature neonate,
situations of recurrent apnoea and cardiovascular instability may occur. Thus, a number of
situations are possible where the developing brain is exposed to intermittent episodes of
asphyxia.

Following repeated cerebral ischaemia or systemic asphyxia in the fetal sheep, three
main observations were made:

- Repeated insults alter the distribution of neuronal loss compared with single
  insults, inducing loss of striatal GABAergic neurons.
- The frequency of the insults determine the severity of the neuronal damage.
- Recurrent episodes of asphyxia compromise the cardiovascular response to further
insults and the extent of neuronal loss is related to the degree of cardiovascular compromise.

These results raise several questions:

First, why are striatal GABAergic neurons particularly sensitive to repeated insults? A number of mechanisms have been implicated following isolated hypoxic-ischaemic insults, including intracellular sodium and water accumulation leading to lysis, intracellular calcium accumulation and toxic effects of excitatory amino acids (Chapter 1). However, whether similar mechanisms apply to striatal damage following repeated ischaemia is not clear. In developing rats the striatum and hippocampus contains high density of post-synaptic glutamate receptors (Silverstein et al. 1986b). Immediately following severe global asphyxia in exteriorised fetal sheep the concentration of extracellular excitatory amino acids increases markedly, particularly in the striatum (Hagberg et al. 1987). Interactions between corticostriatal glutamatergic pathways and nigrostriatal dopaminergic afferents have also been suggested to play a role in post-ischaemic striatal damage (Globus et al. 1988). By lesioning the substantia nigra, the marked post-asphyxial increase in striatal dopamine was prevented and striatal neuronal loss attenuated (Globus et al. 1987). Cortical glutamatergic input is particularly dense on striatal GABAergic neurons. Thus the results in Chapter 7 showing preferential loss of striatal GABAergic neurons support the role of excitotoxic mechanisms in striatal neuronal loss.

Secondly, why is the interval between insults important? Similar to results in this thesis, more frequent insults increase the severity of neuronal loss following cerebral ischaemia in the adult gerbil (Tomida et al. 1987). Sensitisation is likely to occur if critical components of the neural response to the insult have not fully recovered at the time of the next insult. Factors including persistent membrane instability, prolonged EEG suppression,
regional cerebral blood flow or metabolism may all play an important role in the pathogenesis. Conversely, mechanisms such as heat-shock protein release may result in greater protection by less frequent insults (Kirino et al. 1991).

In summary, these results have introduced the concept that repeated insults irrespective of the nature of the insult result in striatal damage. Repeated episodes of asphyxia can sensitise the heart as well as the brain and the frequency of the insults may determine the severity of the injury.

8.3. Are there clear cause and effect relationships between perinatal asphyxia and neurological outcome?

A variety of brain injuries are seen in the newborn, ranging from severe cerebral palsy with major motor and sensory deficits to more subtle developmental delays and learning disorders. Although birth asphyxia has been related to some of these childhood handicaps there is little consensus as to how common such instances are. Epidemiological studies have often failed to identify a common cause of brain damage in the newborn (Nelson, Ellenberg, 1986). Among children with cerebral palsy, at least 50% of all cases have no association with birth asphyxia and no more than 15% of severe mental retardation can be attributed to perinatal events (Paneth, Stark, 1983). More recently, neuropathological and imaging studies have indicated that many injuries are sustained before birth (Bejar et al. 1988). However, very little is known about the etiology of such intrauterine events. Studies presented in this thesis have related specific types of in utero situations with distinct distributions of neuronal loss thus indicating possible antecedents of certain neuropathologies.
8.3.1. Basal ganglia damage

Morphological abnormalities in the basal ganglia have been associated with clinical syndromes such as choreoathetoid cerebral palsy and dystonia in childhood (Foley, 1983; Burton et al. 1984; Rutherford et al. 1992). The pathogenesis of choreoathetosis is not well understood (Foley, 1992). The syndrome is often associated with patients born at term and many appear normal for the first year of life. Pathological studies typically show damage of the basal ganglia and thalamus, but with preservation of cortex. This is consistent with observations that these children often demonstrate relatively normal cognitive functions but manifest severe motor handicap.

Repeated insults, irrespective of whether they were induced by cerebral ischaemia or systemic asphyxia, consistently caused predominant damage in the basal ganglia. These results suggest that the temporal sequencing of the insult may be important in the pathogenesis of injury to the basal ganglia. Whether, such injury can be related to syndromes such as choreoathetoid cerebral palsy remains to be proven. However, immunohistochemical abnormalities observed in these studies show great similarities to neurochemical changes observed in the adult with dyskinetic disorders such as Huntington’s Chorea (Kiyama et al. 1990; Seto-Ohshima et al. 1988). Thus, it can be speculated that prenatal recurrent episodes of asphyxia may result in such movement disorders in the child.

8.3.2. Hippocampal neuronal loss

Hippocampal damage can occur in the adult human following cardiac arrest (Petito et al. 1987). Clinical manifestation includes memory or cognitive defects (Cummings et al. 1984). In both adult and neonatal animals, selective neuronal loss in the hippocampus often develops after hypoxia-ischaemia (Katoh et al. 1992; Zola Morgan et al. 1992; Dell’Anna
et al. 1991; Kiyota et al. 1991). These animals show deficits on various learning and memory tasks. Thus evidence exists that the hippocampal region is involved in memory processing and the hippocampus is often damaged following asphyxia. Epidemiological studies have suggested a correlation between mental retardation and perinatal asphyxia although it has been difficult to assign an exact etiology (Paneth, Stark, 1983).

Observations in this thesis show that a brief intrauterine asphyxial event can cause severe hippocampal neuronal loss (Chapters 3 and 4). This insult was well removed from the timing of birth and any clinical signs of asphyxia such as metabolic acidosis or depressed EEG quickly returned to normal once the insult was removed. Hence, these studies strongly suggest that brief in utero events can cause brain damage without subsequent clinical signs of asphyxia. This may, at least partially, explain difficulties in relating birth events with subsequent behaviour.

8.3.3. Cortical neuronal loss

As discussed in Chapter 1, cortical neuronal loss or infarction is more often seen in the asphyxiated term infant compared with the preterm. The damage may be diffuse, focal or follow the horizontal laminae of the cortex. Often the injury is in the parasagittal region of the cortex (Volpe et al. 1985). This distribution of damage appear to correlate with the border zone between the major cerebral arteries (Volpe et al. 1985). Experimental studies suggest that parasagittal cortical damage develops following prolonged cerebral hypoperfusion (Williams et al. 1992; Gunn et al. 1992). This may explain why the cortex was relatively spared in the present studies, which induced brief episodes of asphyxia or cerebral ischaemia.
8.4. Future studies

These studies have delineated some of the possible causes underlying clinical syndromes following intrauterine insults. Clearly, many more questions need to be answered before a better understanding of the pathophysiological and temporal processes surrounding asphyxial injury to the fetal brain can be achieved. The following hypotheses could be addressed in future studies:

**How does the severity of the insult affect injury of the immature fetal brain?**

A major clinical concern is the preterm neonate and their associated neurological dysfunctions. No neuronal loss could be detected following 10 minutes of umbilical cord occlusion in the midgestation fetal sheep (Chapter 4). The reasons for this are not clear. Further studies could be performed where longer periods of systemic asphyxia or cerebral ischaemia were induced. Comparisons with late-gestation fetuses may identify developmental changes in the response to the insult and there may be differences in the distribution of the damage. Preliminary studies have shown that the midgestation fetus can withstand at least 20 minutes of total umbilical cord occlusion. This insult appears to be related to activation of glia cells, in particular in the white matter tracts (Mallard et al., unpublished).

**How do repeated episodes of asphyxia affect injury of the immature fetal brain?**

Recurrent apnoea associated with severe episodes of bradycardia is common in the preterm human and has been suggested to cause brain damage, although this has never been systematically studied. The effect of repeated episodes of asphyxia in the midgestation fetal sheep could partially address this question. These studies could also examine the interaction between the cardiac and the cerebral response to repeated asphyxia at midgestation.
How does asphyxia at midgestation affect subsequent myelination?

Delayed myelination is observed in some preterm human infants. To test the hypothesis that prenatal asphyxial injury causes delayed myelination, insults could be induced in the midgestation fetus, prior to myelination. Neurological outcome may be assessed several weeks later after myelination is supposed to have occurred.

What are the mechanisms of striatal injury following repeated episodes of asphyxia?

Although several mechanisms have been postulated to play a role in repeated insults, clear evidence does not exist. The role of excitotoxins in striatal damage may be evaluated by implantation of microdialysis probes into the striatum and the cortex. While the striatum shows severe neuronal loss, little or no damage is found in the cortex. By comparing the release of extracellular amino acids in the two regions, striatal sensitisation may be associated with specific responses.

8.5. Conclusion

The developing fetal brain may be injured by several types of insults including maternal infection, teratogenic substances or episodes of asphyxia. Prenatal asphyxia may be more common than previously thought and may be associated with intrauterine death or postnatal neurological sequelae. These injuries if sufficiently severe can lead to a variety of cognitive, motor and sensory deficits in childhood and beyond. The understanding of the general mechanisms of neuronal damage have recently advanced and provided opportunities for therapeutic intervention. However, to safely employ such neonatal management strategies, a clear understanding of all factors that may influence in utero asphyxia is required.

This thesis has addressed aspects of this problem by examining the effects of brief
intrauterine asphyxia at different developmental stages and by investigating the role of repeated episodes of asphyxia in brain damage. The results have highlighted several factors that sensitise the developing brain to injury and have delineated some of the possible aetiologies of clinical syndromes such as choreoathetoid cerebral palsy and learning disabilities.
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