A STUDY OF THE EFFECTS OF GLUCOSE AND OXYGEN DEFICIENCY ON THE FUNCTION OF SYNAPTOSOMES: A MODEL FOR ISCHAEMIA IN THE IMMATURE AND ADULT RAT BRAIN

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Λ Thesis Submitted In Partial Fulfillment For The Degree Of Doctor Of Philosophy In The University Of London

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Abstract

Immature animals show a heightened resistance to episodes of ischaemia in comparison with fully matured animals. Although this phenomenon has been observed for a number of decades, the underlying physiological and biochemical mechanisms allowing this increased resistance remain obscure. This study has concentrated on comparing a number of biochemical responses of neonatal and adult nerve terminals to episodes of *in vitro* ischaemia and reperfusion.

Under such conditions of metabolic stress it was found that adult and neonatal isolated nerve terminals (synaptosomes) behaved very differently. Adult synaptosomes exhibited a decrease in adenosine triphosphate (ATP) levels and an increase in cytosolic calcium during ischaemia. Following a short recovery period there was a significant production of the free radical nitric oxide from adult synaptosomes. Increased calcium levels and increased free radicals have been implicated as causative factors in mitochondrial dysfunction. There was a significant decrease in the activities of two enzymes of the mitochondrial respiratory chain, namely complex II-III and complex IV after ischaemia in the adult synaptosomes.

Neonatal synaptosomes did not exhibit increased cytosolic calcium or nitric oxide production. Furthermore there was no significant decrease in the activity of any of the enzymes of the mitochondrial respiratory chain after ischaemia/reperfusion in these neonatal synaptosomes.

Oxidative stress of adult and neonatal synaptosomes was induced by the addition of exogenous peroxynitrite - a highly damaging reactive oxygen species which is widely implicated in oxidative damage. Adult synaptosomes exhibited the same pattern of mitochondrial dysfunction as that occurring after ischaemia/reperfusion. Furthermore, neonatal synaptosomes once again showed no such dysfunction. The total antioxidant status of adult and neonatal synaptosomes was found to be similar, however neonatal synaptosomes showed a two-fold greater concentration of the antioxidant peptide reduced glutathione. Interestingly, measured glutathione levels were also found to significantly increase in neonatal synaptosomes following ischaemia, but not in adult synaptosomes. It is concluded that oxidative damage to nerve terminals may have a role in the increased susceptibility of adult animals to episodes of ischaemia/reperfusion, and that glutathione could have an important role in protection.

List of Abbreviations

ATP	adenosine triphosphate
ADP	adenosine diphosphate
CVA	cerebrovascular accident
NO	nitric oxide
NOS	nitric oxide synthase
ONOO-	peroxynitrite
Ss	synaptosomes
RCR	respiratory control ratio
NAD(H)	nicotinamide dinucleotide (reduced)
TCA cycle	the citric acid cycle
IsM	isolation medium
PND	postnatal day
LDH	lactate dehydrogenase
КРВ	Krebs phosphate buffer
ANOVA	one-way analysis of variance
LSD	least square difference
GSH	reduced glutathione
GSSG	oxidised glutathione
[Ca ²⁺].	cytosolic calcium concentration
VACC	voltage activated calcium channels
PKC	protein kinase C
DAG	diacylglycerol
IP ₁	inositol 1,4,5 triphosphate
PTP	permeability transition pore
ER	endoplasmic reticulum
GABA	gamma-aminobutyric acid
EAA	excitatory amino acids
GDH	glutamate dehvdrogenase
AM	acetoxymethyl
DMSO	dimethyl sulphoxide
RO	respiratory quotient
PFK	phosphofructokinase
PDHC	pyruvate dehydrogenase complex
PPP	pentose phosphate pathway
EEG	electroencenhalogram
PCr	phosphocreatine
ROS	reactive oxygen species
XDH	xanthine dehvdrogenase
XO	xanthine oxidase
SOD	superoxide dismutase
GPx	superviside distinutise
GC	guantinone peroxiduse
RNOS	reactive nitrogen snecies
	reactive indication species
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To the one who always wanted to be married to a doctor......Pete your love has helped make this possible. At times we agonised and toiled over this thesis together, and by now you certainly know what a synaptosome is!! Thanks for helping with all those pretty diagrams and for everything that you have done to make sure I got to this stage.

DEDICATION

To Pete, and for the glory of God.

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"Before I came here I was confused about the subject. Having listened to your introduction I am still confused, but on a higher level"

Enrico. Ferni

<u>Chapter 1</u>

INTRODUCTION

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Introduction

CHAPTER 1

INTRODUCTION

<u>1.1</u> Brain Ischaemia

1.1.1 Introduction

This thesis consists of research which is directly relevant to the field of brain ischaemia. Ischaemia is defined as a decrease or abolition of tissue perfusion (partial or complete ischaemia) which leads to a decrease in substrate supply, decreased or abolished oxygen supply and accumulation of metabolic products.

The singular importance of the brain to life is exemplified by agreement on medical, legal and ethical grounds that a person dies when brain death occurs. Yet the brain is highly vulnerable to a variety of insults such as stroke, cardiac arrest, physical trauma, near-drowning, carbon monoxide poisoning etc. Clinically, the problem of brain injury and death is a large area of medicine which remains to be a source of much suffering and mortality. Although breakthroughs in prevention, palliative care and treatment are indeed occurring, there is currently no effective universal therapy for brain injury following an ischaemic episode. To enable a greater understanding of the extent of this clinical problem, we will now further explore the area of brain ischaemia and how and when it may occur.

<u>1.1.2</u> Stroke

1.1.2.a Stroke is a widespread problem

Stroke, also known as cerebrovascular accident (CVA) involves damage to the brain because of ischaemia. Someone has a stroke every five minutes somewhere in the United Kingdom. In any given year in Britain approximately 250,000 people suffer some form of stroke.

Over 20 percent of people aged over 45 will die from stroke. In the developed Western World stroke is the third-ranking cause of death after heart disease and cancer. Approximately one-fourth of the neurological patients in nursing homes are stroke victims. Of the patients admitted to hospital following a stroke, a fifth die within a month, and half of the survivors are left permanently disabled after six months. Many of the stroke episodes are minor and patients can survive, but the disabilities or disorders resulting from even relatively minor strokes can be quite devastating. Almost everyone in this nation knows someone who has suffered some form of stroke; it is a widespread problem¹.

1.1.2.b What exactly is a stroke?

The various precise definitions and intricacies of this disease have been discussed widely within medical circles for many years. However, stroke is a clinical syndrome (a collection of symptoms and signs) which has been defined by the World Health Organisation (WHO) as:-

"....rapidly developed clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin...."

The National Survey of Stroke in the U.S.A. used the following definition:-

"Stroke is a clinical syndrome consisting of a constellation of neurological findings, sudden or rapid in onset, which persists for more than 24 hours, and whose vascular origins are limited to:-

- a) thrombotic or embolic occlusion of a cerebral artery resulting in infarction (damage).
- b) spontaneous rupture of a vessel resulting in intracerebral or subarachnoid haemorrhage.

¹information acquired from the Stroke Association

Put more simply, stroke is the outward manifestation of a localised sudden interruption of the blood supply to some part of or all of the brain.

1.1.2.c Types of stroke

Occlusion

A stroke can be caused by narrowing or blockage of an artery by means of atherosclerosis, which is a progressive hardening of the arteries due to the formation of cholesterol plaques which build up on arterial walls. Hence blood passage through these vessels becomes reduced due to the obstruction caused by such fat derivatives. Another cause of stroke is blockage of the blood supply resulting from a blood clot or embolus. These kinds of stroke account for approximately 65 percent of all strokes.

Haemorrhage

Approximately 20 percent of all strokes are caused by cerebral haemorrhage. Haemorrhage is quite simply the escape of blood from any part of the cardiovascular system. Causes of spontaneous intracranial haemorrhage include hypertension (high blood pressure), aneurysm (thin walled enlargements of small portions of blood vessels), bleeding into a tumour, and a generalised bleeding tendency.

Whereas cerebral haemorrhage may cause ischaemia by inducing intense vasospasm; atherosclerosis, blood clot or embolus causes a similar effect by obstruction of cerebral blood vessels. Thus the end result of brain ischaemia is similar, but the initiating factors differ.

1.1.3 Cardiac arrest

As outlined previously, ischaemia due to stroke is a major cause of adult disability in western societies. Brain ischaemia due to cardiac arrest is also a widespread clinical problem. Heart disorders of many types including congenital and coronary can produce not only impaired blood supply to the heart tissue, but can also lead to brain ischaemia. Acute disorders of the blood supply from the heart, resulting from heart failure or myocardial infarction for example, can lead to a global brain ischaemia where the blood supply to the whole brain is impaired. In contrast, strokes are often given the descriptive term of *focal ischaemia* because only a small specific area of the brain becomes ischaemic.

<u>1.1.4</u> Approaches to ischaemic therapy

Current therapeutic approaches for diminishing ischaemic brain damage resulting from stroke or cardiac arrest are mainly focused on preventative medicine. Such therapy aims to prevent the first or recurrent stroke or heart attack by reducing risk factors. Such preventative therapy is predominantly by the use of encouraging a change in patient lifestyle and by the prescription of drugs which lower blood pressure, reduce blood cholesterol levels, inhibit thrombus formation, inhibit platelet aggregation and which generally reduce the risk factors associated with these cerebral disorders. In addition to preventative medicine and also advances in the detection of cerebral disorders, there are now substantial research efforts being made to develop drugs that are 'neuroprotective', that is drugs that can be given to minimize the brain damage which follows an ischaemic episode. Such research is focused on the cellular pathological mechanisms which occur in response to an ischaemic incident and which ultimately lead to neuronal degeneration and brain cell death. Studies looking into the area of pathological brain physiology and cell metabolism are only a relatively recent area of medical research.

<u>1.2</u> Research into Ischaemic Brain Damage

The high incidence of stroke and cardiac arrest has promoted considerable research interest in brain ischemia. Early observations regarding the vulnerability of the brain to episodes of ischaemia (LeGallois, 1813) has been followed by a wealth of experimental literature on the subject.

During the last three to four decades, many experimental animal models of cerebral ischaemia have been developed to enable ongoing research into the response of the brain to ischaemia (Lowry *et al* **1964**{mouse};Levine and Payan, 1966 {gerbil}; Eklof and Siesjo, 1972 {rat}; Hossmann *et al.*, 1976 {cat}; Jackson and Dole, 1979

{gerbil}; Pulsinelli and Brierley, 1979 {rat}; Steen *et al.*, 1985 {primates}; D'Alecy *et al.*, 1986 {dog}; Globus *et al.*, 1988 {rat}, Mink *et al.*, 1990 {dog}). Such animal models have enabled increased understanding of some of the physiological and biochemical responses to brain ischaemia. To fully understand the pathophysiology <u>of</u> ischaemia in all its manifestations, the presence of vasculature is required, which means the use of animal models.

In vitro models are also useful tools for studying the cellular, biochemical and molecular mechanisms of ischaemic injury and for investigating pharmacological therapy. The studies undertaken in this thesis have all involved *in vitro* experimental models.

The realm of cerebral ischaemic research is a vast and at times complex one. Numerous researchers have investigated physiological, biochemical and molecular responses to ischaemia, using a wide variety of methods and experimental design. This thesis is relevant to the effects of glucose and oxygen lack on brain nerve terminal function alone. Therefore, the literature reviewed in this thesis will intentionally be limited to ensure that it is truly pertinent to the area of study presented here. I am fully aware that the literature concerned with ischaemia is expansive, and hence some studies will not, and indeed cannot, be covered in this thesis.

Studies during the last 20 years have provided evidence for many changes occurring in neurones during an ischaemic episode and the subsequent recirculation period. Although sensitive to injury, neurones also demonstrate a capacity for recovery. Astrup *et al.* (1981) have found that an ischaemic injury which occupies an extensive area and is associated with pronounced clinical symptoms may resolve to a strikingly small brain tissue defect with the death of only a few neurones. It is of paramount importance to study the biochemical mechanisms instigated by lack of oxygen and substrate and to further ascertain which of these changes are the critical ones leading to neuronal death.

Introduction

There is a rapidly growing body of literature on the biochemical and molecular effects of brain ischaemia in neurones. However, it would still be fair to say that many of the critical biochemical changes responsible for neuronal degeneration still remain poorly understood. What has not yet been fully elucidated is the exact sequence of events that lead to neuronal death and which changes are the most critical ones.

In spite of the fact that there are still major question concerning ischaemic damage that are unanswered, a number of the main biochemical mechanisms thought to be involved are identified. From the literature, energy metabolism and ATP, ion channels, membrane potential, Ca^{2+} , neurotransmitters and free radicals are some of the known major determinants of neuronal response and neuronal injury (Lowry *et al.*, 1964; Siesjö, 1978; Rehncrona *et al.*, 1979; Siesjö and Wieloch, 1985; Halliwell and Gutteridge, 1990; Choi, 1992; Haddad and Jiang, 1993; Phillis, 1994; Sims and Zaidan, 1995). Those areas most relevant to this thesis, including studies of changes in energy metabolism, calcium movements, neurotransmitter changes and free radical production are discussed in detail in later chapters.

The literature which is relevant to ischaemia often encompasses studies of the effects of hypoxia or anoxia (decreased or abolished oxygen supply) on brain function. Some workers have reported differences in the response of neurones to ischaemia or hypoxia/anoxia (Boakye *et al.*, 1991; Bachelard *et al.*, 1993; Haddad and Jiang, 1993). This thesis is primarily concerned with neuronal response to combined glucose and oxygen lack, however at times oxygen lack alone has been investigated so that the effects of the hypoxic component of ischaemia could be investigated and compared with current literature.

Hypoxic and ischaemic damage in neonates and during early development is of considerable clinical interest and has been the subject of a number of studies (see Volpe, 1987, 1990; Choi, 1990, Palmer and Vannucci, 1993). There are considerable differences in the resultant neuronal damage compared with that in adults (Palmer and Vannucci, 1993).

The greater resistance of neonatal as compared with adult mammals to episodes of hypoxia and ischaemia was observed many decades ago (LeGallois, 1813) and commented on a little more recently (Kabat, 1970). The extent of this resistance is striking, illustrated in one study by a survival time of 25 minutes for newborn rat pups in 100% nitrogen (Duffy et al., 1975). Following on from such observations, many workers have studied the biochemical mechanisms in neonatal brain instigated by lack of oxygen and substrate (Vannucci and Vannucci, 1978; Cady et al., 1987; Hagberg et al., 1987; Hope et al., 1987; Ikonomidou et al, 1989; Palmer et al., 1990; Raju, 1992; Powers, 1993; Sundvall et al., 1994). Relatively few studies have looked at direct comparisons of events occurring in neonatal as compared with adult brain during an ischaemic episode and the subsequent recirculation period. Such parallel studies could provide insight into what lies behind the resistance of neonatal brain to oxygen and fuel lack if due to inherently different biochemical properties. In particular, very few studies have focused on investigation into the fundamental differences between post-ischaemic biochemical changes in neonatal as compared with adult synapses (Haddad and Jiang, 1993). The studies I have undertaken were aimed at directly comparing and characterising some changes in synaptic processes and metabolism in ischaemic adult and neonatal nerve terminals. This comparison was intended to highlight any differences in response between the mature and immature synapses which may contribute to the vulnerability of adult neurones and the relative resistance of immature neurones to ischaemia.

Rat brain is a widely used model for ischaemic research, and has been used for the studies presented here. Although not ideal, it is a good scientific model for investigating ischaemia primarily because the developmental profile and mature properties of rat and human brain are similar (Nyakas *et al.*, 1996). We will now turn briefly to the human brain.

<u>1.3</u> The Human Brain

The large size and distinctive organisation of the human brain confer upon human beings the ability to communicate, to think abstractly and to create - abilities that are unmatched by any other animal. The average human brain at birth weighs 390g and its average maximum weight, reached at approximately age 15 years, is 1.315 Kg. The studies in this thesis are primarily concerned with the cellular response of the brain to ischaemia, and more specifically the mechanisms which occur in the nerve terminal. Although brain ischaemia can have profound effects on macrostructure and often leads to widespread tissue necrosis, the information detailed in this thesis will be restricted to the cellular level.

<u>1.3.1</u> Microscopic brain structure.

Neural tissue is composed of nerve cells (or *neurones*), glial cells, and blood vessels. There are perhaps ten million million of the microscopic neurones and glial cells in the brain. One heroic quantification of the cells in a single milligram of monkey nervous tissue revealed 120,000 neurones, 60,000 glial cells and approximately 280,000,000 synapses! (Nicholls, 1994)

1.3.1.a The neurone

The basic building block of the brain is the neurone. It is a cell that is specialised for the transmission of information into, within, and out of the animal. Although the size of neurones may be difficult to comprehend, we can begin to understand their organisational style if we draw the analogy of them being a living, dynamic community. They are separate; they communicate; they come in many different shapes and sizes; they live in clusters (called nuclei and ganglia); they adapt, learn, forget; and they need constant oxygen and nutrition.

Structure and function

There are several hundred types of neurones (Nicholls, 1994), with different neuronal configurations depending on their particular role and function. For example a sensory neurone, which receives sensory information from the periphery and

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transmits it to the central nervous system, has a different structure to a motor neurone, whose role is to innervate skeletal muscle. However, there are a number of certain features that neurones have in common. These characteristic components of a neurone are illustrated in figure 1.1. The neuronal cytoskeleton, consisting of microtubules, microfilaments and associated proteins, controls the complex shape of the neurone.

A neurone has three main regions: -

[1] The *cell body*, also called the *soma* or *perikaryon*. Cell bodies can vary in diameter from 5 to 60 micrometers. The cell bodies do not in any way contribute to these cells being atypical. The other two regions of the neurone make them unique.

[2] *Dendrites* extend out of the cell body and have cytoplasmic continuity with the cell body. These extensions are the means of communication within the brain - effectively being the 'telephone wires' of the nervous system. Dendrites receive incoming (or afferent) information from other neurones and transmit it back to the cell body (efferent information).

[3] The third region of the neurone - the *axon* - also extends out of the cell body. In contrast to dendrites there is usually only one axon per cell. Axons transmit information (efferent information) from the cell body - to which they are attached at a projection called the *axon hillock* - to other neurones, rather than receive it.

Long axon fibres are usually covered with a myelin sheath which acts like electrical insulation on a conducting wire and greatly extends the efficiency of the conduction of electrical discharges along the axon. At the end of each axon branch is a nerve terminal, or *synapse*. The term *synapse* was first coined by a famous British neuroscientist, Sir Charles Sherrington. It means "to clasp tightly". The synapse is the point of closest affinity and functional connection between neurones. The synapses consist of a pre-synaptic nerve terminal, a post-synaptic terminal and a synaptic cleft in the intervening gap, which is approximately 200 angstroms wide.



Figure 1.1 Characteristic components of a neurone

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The release of neurotransmitters in response to the arrival of an electrical signal from the axon occurs from the synapse. The release of neurotransmitters into the synaptic cleft enables a response in the post-synaptic cell once transmitters interact with post-synaptic receptors. Receptors on the post-synaptic membrane translate stimulation by neurotransmitters into rates of ionic fluxes, and therefore into depolarising or hyperpolarising responses in the cell body. Stimulation is terminated by an array of mechanisms which eliminate neurotransmitter from the synaptic cleft.

The way that neurones communicate is efficient. Neurones can respond with new action potentials as frequently as one thousand times per second. However, the communication possibilities between neurones are greater than their numbers suggest. With the abundance of cell dendrites, each neurone may receive unique messages from up to one thousand neighbouring neurones and send messages to another thousand. The complexity of communication is further enhanced by the large numbers of different chemical combinations of neurotransmitters in the brain. The communication possibilities are hard to analyse and analogies to the most powerful computers are somewhat of an underestimation.

1.3.1.b Glia

Neurones share the brain with glial cells. There are actually more glial cells in the brain than neurones, with glia being some ten to fifty times more numerous. Glial cells generally support the work of the neurones and occasionally act as microscopic rubbish collectors. Glia repair nerve cells, maintain the proper chemical balance in the extracellular fluid and provide structural support. It is the role as structural supporters which first led to the naming of this type of brain cell. In 1846, Virchow came across a tissue that was not neuronal in nature, and which occupied all of the remaining space in the brain which was not already taken up by nerve cells. He named it "glia" which means "glue", pertaining to its first assigned function - to hold the neuronal network together. Glia also pay an important part in the formation and maintenance of the blood-brain barrier - a semi-permeable barrier of brain endothelial cells which enable the correct chemical composition of the brain extracellular fluid to

be maintained so that brain cells can function appropriately. Despite the extent of glial numbers, their exact functions remain uncertain but they appear to have several roles from protection of the brain against electrical/ionic environment changes to the uptake and metabolism of released neurotransmitters. In addition, they participate in CNS repair, and are seen to proliferate subsequent to trauma. Glia can be further divided into different types of basic cell, namely *astrocytes, oligodendrocytes and microglia*.

<u>1.3.2</u> Ischaemic microscopic brain damage

Pathological studies in rat brain have identified that there are neurones in certain areas of the brain which are particularly susceptible to short term ischaemia if the blood supply to the whole brain is restricted (i.e. global ischaemia) (Brierley, 1976; Pulsinelli et al., 1982). By contrast focal ischaemia, in which there is a blockage in or damage to a blood vessel in the brain results in the loss of all cells within the affected area (cerebral infarction) (Petito et al., 1982; Ginsberg and Busto, 1989; Pulsinelli, 1992; Siesjö, 1992). The majority of brain cells are normally resistant to even 30 minutes of transient global ischaemia (Pulsinelli and Brierley, 1979). In ischaemia-susceptible regions the extent and type of cells destroyed depends on the duration of the ischaemic insult (Pulsinelli and Brierley, 1979). The subpopulations of neurones which are known as ischaemia-susceptible and which are destroyed by ischaemic periods of 10 minutes or less (Pulsinelli et al., 1982; Smith et al., 1984) lie within the CA1 region of the hippocampus, and also include some neurones in the dorsal hippocampus. Cortical neurones show little or no damage following 10 minutes of ischaemia, but 30 minutes can destroy some neurones in the cortex layers designated as layers 3, 5 and 6. The dorsolateral striatum region of the brain can show neuronal degeneration of after approximately 20 minutes of ischaemia (Pulsinelli et al., 1982). The general pattern is that specific populations of neurones exhibit degeneration and necrosis whereas glial cells remain viable at this stage.

<u>1.4</u> Brain Development

There is a marked species variation in the morphological, histological and biochemical changes occurring in the brain during the pre- and postnatal periods (see Flexner, 1955, for review). The concept has been established which states that certain animals are born in a relatively mature state of neurological competence. These species have been termed precocial, and include the horse, sheep and other herd animals, and the guinea pig. In contrast, other species are born in a relatively poor state of neurological development and are termed the non-precocial species. Such species include the mouse, rat, rabbit, dog and cat (Booth *et al.*, 1980). It is into the latter, non-precocial category that humans fit, although the human newborn is relatively mature at birth compared to other mammalian species within this category (Nyakas *et al.*, 1996).

The development of the mammalian nervous system involves a series of highly regulated, sequential changes, which include cell division (neurogenesis and gliogenesis), cell migration to target areas in the brain, cellular differentiation, deposition of myelin into sheaths surrounding axons, formation of synapses and the synthesis and release of neurotransmitters. The fundamental result of these maturational events is an overall increase in higher brain activity (Morgane *et al.*, 1992).

Referring to non-precocial species, a major part of brain development occurs postnatally during a period called *the brain growth spurt* (Dobbing, 1968). Thus during much of its development the brain does not have the protection afforded the foetus by the homeostatic mechanism of the dam. The postnatal brain growth spurt is known as one of the *vulnerable periods* in development because of these circumstances (Davison and Dobbing, 1966). In describing the increased ischaemic resistance of the neonatal brain, at a stage which is referred to by some as a vulnerable period, contradictions seem to arise. However, in contrast to the generalised increased resistance hypothesis, there is some evidence, albeit limited at this stage, for a transiently enhanced susceptibility to seizures during hypoxia in immature rats of age 9-13 days (Jensen *et al.*, 1991). This coincides with a period of rapid differentiation of axons and dendrites in the cortical brain areas (Nyakas *et al.*, 1996). During this period a severe but transient reduction in brain phosphocreatine and ATP contents after hypoxia was found, which was larger than during the preceding and following postnatal periods (Jensen *et al.*, 1993). Thus it appears that the correlation between brain development and susceptibility to glucose and oxygen lack may be more complex than a generalised statement of 'developing brain is more resistant to ischaemia than adult brain'.

Extensive work over many decades has described the development of the brain. An overview of the work relating to the development of adult brain structure, brain cellular development and of the development of many biochemical and molecular brain systems is outside the scope of this thesis. Therefore, the reader is referred to a number of excellent books and reviews which encompass many of these areas of brain development (for example, Dobbing and Sands, 1979; Reinis and Goldman, 1980; Wiggins *et al.*, 1985; Clark, 1990; Girard *et al.*, 1992; Morgane *et al.*, 1992; Rust, 1994; Nyakas, 1996).

Areas of brain development directly relevant to this thesis include the development of calcium homeostasis, glutamate homeostasis, enzyme systems connected with energy metabolism, and the brain's oxidative processes and defense mechanisms. These areas will be discussed in chapters 3, 4, and 5 in terms of how these maturing neurochemical processes may cause the neonatal nerve terminal to respond differently to adult nerve terminals during ischaemia and reperfusion.

<u>1.5</u> Aims of the Project

This thesis is primarily based on the well documented observation that young, immature mammals show a heightened resistance to episodes of ischaemia in comparison with fully matured adults. The original question underlying the thesis was whether this heightened resistance could in any way be accounted for, or reflected by, differences in response of neonatal and adult brain nerve terminals, during ischaemia/reperfusion.

The aims of the studies presented in this thesis were therefore:-

- to characterise a valid model of neonatal and adult nerve terminals which could be subjected to conditions relevant to ischaemia and reperfusion;

- to examine a select number of nerve terminal activities, which have been reported to change in adult neurones during ischaemia, in both neonatal and adult nerve terminals;

- to directly compare, and where applicable contrast, any changes in these activities between the adult and neonatal nerve terminals;

- to examine possible interplay between some of these activities in adult and neonatal nerve terminals, and determine where differences may occur between the two types of nerve terminal.



"A problem is an opportunity in work clothes"

Henry J Kaiser, Jr.

THE MODEL

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INTRODUCTION
CHAPTER 2

THE MODEL

2.1 Introduction

2.1.1 The need for a scientific model.

Clinical studies of post-mortem tissue derived from patients that have died following ischaemic incidents, such as stroke, can provide us with some useful biochemical information. However, such biochemical information is often limited because general cellular death may mask much of the detail which researchers are trying to uncover. Samples that are obtained from live patients recovering from ischaemic episodes can also be of use to medical researchers, but these samples are always obtained some time after the ischaemic event itself. The careful study and comparison of mechanisms occurring within cells and organelles prior to, during and after an episode of ischaemia is needed to fully elucidate the processes which cause injury. Coupled with this is the need to be able to manipulate the cellular environment, with chemicals and drugs, in order to gain knowledge for potential therapies. In light of ethical and moral considerations, it becomes obvious that scientific models are a necessity because such biochemical research cannot and should not be carried out using human subjects alone.

2.1.1.a Animal models

Many animal models have been successfully used to study cerebral ischaemia in a variety of species. Animal models have provided us with considerable insight into the pathophysiology of cerebral ischaemia, and will continue to do so. However, for the study of cerebral ischaemia there is no such thing as an "ideal" animal model.

As far as possible the animal model should:

- closely mimic the development of the clinical insult in humans,
- cause a reproducible injury, i.e. experiments should elicit similar responses in each individual animal tested,
- be closely physiologically monitored, i.e. core temperature, respiration rate, blood glucose and blood pressure,
- show similar pathology as a result of the experimental insult to that pathology seen in humans.

Nevertheless, even if the above principles for animal models can be closely adhered to, there are a number of limitations which must be borne in mind. Firstly, reproducible results are often very difficult to achieve because of anatomical and physiological variability both within and across species. It is also often arduous to tightly regulate physiological parameters, but a lack of strict control can lead to spurious, misleading results. This has now been realised to have been the case with some of the *in vivo* testing of the NMDA receptor antagonist MK-801. In some of the studies a lowered animal body temperature, caused by MK-801, accounted for a number of the cerebroprotective effects which were incorrectly attributed to the direct action of MK-801 on the NMDA receptor (Choi, 1992). Another important point to consider with animal models is that the animals are usually anaesthetised and have undergone surgery, which may profoundly alter the tissue response to an ischaemic episode.

2.1.1.b "Reduced preparations"

Intact animal models are not always ideal for basic cellular research. A large number of researchers study brain function by working on what may be termed "reduced preparations". These include cultured neurons and glia, brain slices and synaptosomes. In some cases such preparations are used simply because they are easier to manipulate than the intact physiological system. However, there are also a number of scientifically valid reasons for using these reduced systems.

The use of reduced systems can be justified when the investigation necessitates altering the extracellular *milieu* of the tissue. This ability to manipulate extracellular composition is lacking in *in vivo* studies of the adult brain. *In vivo* the intact, semi-permeable blood-brain barrier limits the ability to alter ion and metabolite concentrations surrounding brain cells, and makes quantifying any changes difficult. Another inherent problem with *in vivo* work is that the study of the cellular response and the separate quantification of metabolic products generated by both normal and injured cells is relatively difficult. Thus the reduced preparation becomes preferable when a study involves altering and measuring the composition of the extracellular environment.

When using intact systems it becomes hard to dissect out the mechanisms that are occurring in individual cell types or organelles. The use of culture systems and synaptosomes can be very important in enabling the investigator to assign functions to either a specific cell type, or to localise a function to synaptic endings. Another approach which is commonly used by investigators to enable such dissection and assigning of functions to specific cell types or organelles, is the isolation of cells and organelles from animal models that have undergone an ischaemic insult. However, this approach more often than not uses well established methods that have been optimised for healthy, non-pathological animals. It is important to realise that in using such an approach, researchers could be isolating the damaged cells and organelles incorrectly. This is particularly important when swelling of cells or organelles is a possibility following an ischaemic insult. Any changes in the density of the pathological cells and organelles (such as mitochondria) will have implications for the effectiveness of isolation methods using density gradients, which achieve separation by exploiting the different densities of cells and organelles. A further limitation of using the approach of post-ischaemic isolation is that only the post-ischaemic changes can effectively be studied, and the isolation procedure itself may allow a short time window for some post-ischaemic recovery. The manipulation of in vitro reduced systems is advantageous in this case, because they enable the investigator to study responses as they occur, rather than being limited to some time-period after isolation.

Any decision to use a reduced preparation is made at a cost because the ideal situation is always to study the intact physiological system. However, *in vitro* models are useful tools for studying cellular and molecular mechanisms of ischaemic damage. Scientific models, whether involving *in vivo* animal work or *in vitro* cell work, have their own unique advantages and limitations. It is imperative that such limitations are borne in mind and that extrapolation back to the human situation proceeds with extreme caution. However, if the researcher is aware of the applications and limitations of a particular model, much useful information can be gleaned from carefully designed experiments using *in vitro* scientific models.

<u>2.1.2</u> The synaptosome.

2.1.2.a What is a synaptosome?

A synaptosome is an isolated, detached presynaptic nerve terminal which arises from the homogenisation of neural tissue. Neurones do not survive the employed homogenisation techniques intact, and it is possible by careful, controlled homogenisation of brain tissue to shear the nerve axon just prior to the nerve terminal. The neck of the axon at the point at which it enters the nerve terminal appears to be fragile, perhaps due to changes in the cytoskeletal organisation (Nicholls, 1993), and it readily pinches off to leave an isolated nerve terminal. The plasma membranes of these neuronal fragments are "sticky" and may reseal to form osmotically active, intact nerve terminals which contain all of the organelles of the functional *in-situ* nerve terminal (McMahon and Nicholls, 1991). Figure 2.1 details both the main properties of the presynaptic terminal and also the planes of cleavage during the synaptosomal preparation.

2.1.2.b The history of synaptosome isolation.

The preparation of synaptosomes was first described thirty-four years ago (DeRobertis *et al.*, 1962; Gray and Whittaker, 1962). Since then much information about these preparations has become available, and a considerable amount of effort has been put into investigating the components and biochemical activities of synaptosomes.



Figure 2.1 The Nerve Terminal

The synaptosome has been widely used as an *in vitro* tool for studying biochemical events at the nerve terminal, with over eight thousand five hundred and eighty studies on the current Medline database which either use or refer to synaptosomes.

The synaptosomal preparation has gained a dubious reputation among some neuroscientists, primarily because many early experiments were conducted under conditions that were difficult to relate to the in vivo physiological events of neurotransmission, and because the synaptosomal preparation was considered to be in some way 'damaged' (McMahon and Nicholls, 1991). The original techniques of synaptosome preparation involved long periods of high-speed centrifugation on discontinuous high density sucrose gradients. This led to preparations that were often almost 50% contaminated with structural elements other than synaptosomes. The purity was slightly improved by the use of continuous gradients, but in either case, the hyperosmolarity of the solutions used led to shrinkage and the final state of the synaptosomes being somewhat unsatisfactory for functional studies (Booth and Clark, 1978). Density gradient materials, such as Ficoll and Percoll, that can achieve high density without the concomitant high osmolarity represents a milestone in the comparatively short history of subcellular fractionation. There were reports of isolation of free (non-synaptic) brain mitochondria and synaptosomes prior to 1970 (Abood, 1969). However, these earlier attempts at using Ficoll gradients suffered from several disadvantages, including long isolation procedures and the need to use high Ficoll concentrations. Later isolations using various Ficoll/sucrose procedures produced synaptosome fractions which were more metabolically competent, however purity still remained a problem. Contamination by non-synaptic mitochondria was often 25-40% of the synaptosome fraction.

The introduction, in 1978 by Booth and Clark, of a method based on flotation in a Ficoll/sucrose gradient enabled the rapid, routine isolation of synaptosomes which are predominantly intact, minimally contaminated with non-synaptic mitochondria and metabolically active.

The yields produced by this method, and other methods following it which are also based on Ficoll/sucrose and Percoll/sucrose gradients, are large enough for metabolic studies (Booth and Clark, 1978) which have enabled great insight into the workings of the nerve terminal under normal and pathological conditions.

2.1.2.c Disadvantages and advantages of synaptosomes

Disadvantages and limitations

A 100% pure synaptosomal preparation is impossible to achieve in practice. The maximum purity of synaptosomes prepared using the state-of-the-art techniques rarely exceeds 80%. Contamination can be by:- membrane fragments; microsomes; vesicular bodies from pinched off dendrites; "gliosomes" derived from glia which may contain non-synaptic mitochondria; non-synaptic free mitochondria that cosediment with synaptosomes in the density gradients. All of these contaminants can complicate the interpretation of results which must be borne in mind when assigning particular mechanisms and functions to the nerve terminal, following synaptosome studies. Careful characterisation of synaptosome preparations can help keep this disadvantage to a minimum. Although electron micrographs of synaptosomal preparations can look heterogeneous, the non-synaptosomal membrane fragments are generally non-active from a functional point of view. Therefore for studies which require an intact membrane, mitochondria, glycolytic pathway and maintenance of ion gradients, the membrane fragment contaminants do not interfere with the synaptosome responses seen.

Some researchers have reported that purified synaptosomes consist of at least two distinct populations. One population is synaptosomes which contain fully functional mitochondria. These have a submaximal glycolytic rate and a minimal lactate production. The other population is synaptosomes which are devoid of mitochondria, and which have been designated as "cytosolic particles" (Kyriazi and Basford, 1986). These cytosolic particles lack the full capacity for oxidative phosphorylation and do not have sufficient glycolytic enzymes to increase their glycolytic rate to that required to maintain ATP turnover. It has thus been suggested

that such cytosolic particles engage to a large degree in anaerobic respiration and are responsible for the high level of lactate accumulation found in synaptosome fractions (Kyriazi and Basford, 1986). Several reports have demonstrated that >30% of the glucose utilised by synaptosomes accumulates as lactic acid (Bauer and Braind, 1982). It has been further suggested that a limited supply of oxygen cannot be the explanation for this high degree of anaerobic respiration when compared with the whole brain in vivo or with brain slices in vitro because synaptosomes are easily maintained in a well oxygenated state (Ksiezak and Gibson, 1981). It is important to be aware that synaptosome fractions may contain a number of cytosolic particles which are devoid of mitochondria. However, this does not preclude the use of synaptosomes as a valid and good model of the nerve terminal. Indeed, it has also been observed that both neuronal and non-neuronal cells in culture have excess glycolysis in comparison to respiration, leading to accumulation and release of lactate in the presence of oxygen (Kauppinen et al., 1989). Therefore, unlike the situation in vivo where nearly all of the glucose utilised by the brain is fully oxidised to water and carbon dioxide, in vitro models of neurones inherently have a higher degree of lactate accumulation. The careful use of controls prevents this property of in vitro neuronal preparations from having any relevance to synaptosomal responses under metabolic stress. In the studies reported in this thesis, each single synaptosome preparation is split into a control and test sample. By using this experimental design, any variation in contaminants between preparations is minimised.

Synaptosomes from the mammalian central nervous system are heterogeneous in terms of their neurotransmitter content because they result from the homogenisation of nervous tissue which is composed of various types of nerve terminals. This disadvantage is least severe for studies involving glutamate, primarily because this is the dominant neurotransmitter in most synaptosome preparations (Nicholls, 1993).Careful experimental design can minimise this disadvantage by overcoming the natural variation between preparations. However, it should be borne in mind that not all of a response, for example an evoked change in calcium signal, can be coupled to the release of one specific neurotransmitter. Finally synaptosomes have a finite life-span. They can be maintained on ice for at least six hours with no deterioration in their metabolic integrity (McMahon and Nicholls, 1991; Nicholls, 1993). However once incubation at room temperature or greater has commenced, synaptosomes are unable to sustain themselves for more than three or four hours. Prolonged periods of incubation lead to a decrease in the integrity of the synaptosomal plasma membrane, and the concomitant release of cytosolic material.

Advantages and applications

Brain function is centred at the synapse and, consequently, substantial scientific effort is directed towards exploring synaptic function under normal and pathological conditions. The synaptosome is the simplest system in which various neuronal events, from plasma membrane depolarisation to neurotransmitter release, can be studied. It is also the most complex neuronal preparation which is sufficiently homogeneous to allow detailed biochemical analysis of events occurring at the nerve terminal alone. This is because the synaptosomal preparation is largely depleted of functional glia and neuronal cell bodies, which are inherent in brain slice preparations and neuronal cell cultures respectively.

The methodology for isolation of synaptosomes is relatively straightforward, widely used and accepted. The synaptosomal preparation has been extensively exploited for biochemical studies and hence a large amount of data is available for reference to, and comparison with, current work.

Manipulation of synaptosomes is relatively simple. Nerve terminals are bioenergetically autonomous and synaptosomes continue to function effectively for sufficient periods after preparation when supplied with glucose. The synaptosomal preparation can yield a wealth of information on the mechanisms and regulation of neurotransmitter uptake, storage and release. The control of metabolic pathways at the synapse and the effects of metabolic stress can also be easily studied. This is highly relevant because *in vivo*, an energy depletion will probably not effect all parts of the

neurone equally, and because the presynaptic terminals have a very high metabolic rate due to neurotransmitter regulation and maintenance of ionic gradients they are likely to be most susceptible. A further important advantage of using synaptosomes is that precise developmental and ageing studies are easily achieved.

2.1.2.d Characteristics and properties of the synaptosome

Synaptosomes have a characteristic appearance, with a diameter within the range of $0.5 - 1 \mu m$. They contain mitochondria, small synaptic vesicles (SSV's) of approximate 50 nm diameter, and occasional large dense cored vesicles (LDCV's). Synaptosomes contain little or no internal membrane corresponding to endoplasmic reticulum (Nicholls, 1993). Synaptosomal SSV's contain neurotransmitters classified as either type 1 (amino acids: GABA, glycine and glutamate); or type 2 (catecholamines: adrenaline, noradrenaline, dopamine, serotonin, acetylcholine). Neuropeptides are co-transmitters at many terminals, and are stored in the LDCV's.

Synaptosomes contain all of the machinery necessary for transducing an action potential into an influx of calcium ions followed by the release of neurotransmitters from synaptic vesicles. They possess a number of highly active ion channels, receptors and carriers in their plasma membrane and hence display the majority of the properties of the *in situ* nerve terminal. To fully appreciate the functioning of synaptosomes, an understanding of the characteristics of the nerve terminal is necessary.

Synaptosomes contain ATP (1.4 - 5.6 nmoles / mg protein) and ADP (0.4 - 2.45 nmoles / mg protein) giving an ATP/ADP ratio of 1.4 - 6. This range of values is cited in a recent review (Erecinska *et al.*, 1994) and concerns a variety of different preparative methodologies and animal species. The rate of synaptosomal oxygen consumption lies within the range of 2-4 nmoles/min/mg protein at 25° C, and 5.2 - 11 nmoles /min /mg protein at 37° C. The level of lactate production ranges between 0.3 - 0.5 and from 0.8 - 2.7 nmoles/min/mg protein respectively, at the same temperatures.

The synaptosomal concentrations of high-energy phosphate compounds and the glycolytic and mitochondrial oxidative activity of synaptosomes are 5 - 10 fold lower than the values for *in vivo* intact brain. Some researchers explain this by the heterogeneity of the preparation and the presence of sealed nerve terminals that do not contain functioning mitochondria. However, the activities *per se* of several mitochondrial enzymes have been found to be lower in synaptic mitochondria than in non-synaptic mitochondria (see Clark and Lai, 1989, and references cited therein for discussion). It may be that the intrinsic activity of the energy producing pathways in mitochondria functioning in nerve endings are lower than in those mitochondria which remain in the neuronal cell body or those present in glial cells.

Synaptosomes have limited endogenous substrates, which are incapable of supporting anaerobic ATP synthesis (Erecinska *et al.*, 1994). Even in the presence of exogenous substrates, respiratory inhibition of synaptosomes by cyanide causes an approximate 70% decrease in ATP levels, illustrating that oxidative phosphorylation is more effective than anaerobic glycolysis in maintaining energy levels in synaptosomes. Like the intact brain, synaptosomes are highly sensitive to "ischaemia-like" conditions. When oxidative phosphorylation is blocked there is a 10-fold increase in glycolysis (the 'Pasteur effect'). However, in synaptosomes the glycolytic pathway not only cannot maintain ATP/ADP ratios, but this Pasteur effect fails to function continuously when the mitochondrial respiratory chain is highly reduced, ie. during anoxia or inhibition of cytochrome c oxidase.

There is some controversy in the current literature as to the extent in which other substrates can replace glucose in synaptosomal energy production. Exogenous pyruvate can effectively substitute for glucose as an *in vitro* substrate, demonstrating again that synaptosomal respiration is essentially aerobic and intimately linked to mitochondrial metabolism. Whilst it is agreed that glucose is the key physiological substrate and that pyruvate can also be utilised, there is no clear agreement as to the role of certain amino acids.

Some state that neurones appear unable to utilise the high concentrations of glutamate and aspartate in their cytoplasm as fuel (Kauppinen and Nicholls, 1986; McMahon and Nicholls, 1991). However, recent work with radiolabels showed that transamination from glutamate to aspartate is very active in synaptosomes (Erecinska *et al.*, 1994). It is suggested by some (Bradford *et al.*, 1978; Erecinska *et al.*, 1994) that glutamine can be a major substrate for nerve endings. A series of reactions may operate, in which glutamate is produced from glutamine, and is then transaminated to aspartate with the concomitant production of α -ketoglutarate. α -ketoglutarate then cycles through the TCA cycle leading to ATP synthesis and the regeneration of oxaloacetate. It is known *in vivo* that hypoglycaemia causes a decrease in the levels of glutamine and glutamate and an increase in aspartate. Current research is therefore suggesting that this pathway has an important role to play in the synaptosome.

In contrast to this, the activity of glutamate dehydrogenase is very low. This enzyme is part of the major mechanism for breakdown of glutamate with the consequential production of ammonia. The low activity of this enzyme in synaptosomes suggests that nerve endings conserve glutamate.

Glutamate is present at a concentration of approximately 10 mM in the cytosol of synaptosomes, and a 10 - fold gradient exists across the vesicular membrane thus ensuring that the concentration of glutamate in SSV's is approximately 100 mM. Synaptosomes possess an acidic amino acid transporter which is sodium-coupled and extremely active. This transporter is responsible for removing glutamate from the synaptic cleft and hence maintaining an extracellular glutamate concentration close to 1 μ M (Nicholls, 1993).

ATP is utilised by synaptosomes for a number of processes. These include the maintenance of a proton electrochemical gradient across the synaptic vesicle membrane, and an undefined aspect of vesicular exocytosis and neurotransmitter release.

The majority of ATP is expended in maintaining the correct resting ion concentrations across the plasma membrane. Synaptosomes contain an internal concentration of 45 - 65 mM potassium and approximately 20 mM sodium (Erecinska et al., 1994). However, some estimates for the internal concentration of sodium have been much lower at approximately 10 mM (Deri and Adam-Vizi, 1993). Whether these differences are due to different methodology or whether the sodium concentration in nerve terminals is lower than in the neuronal cell body is still under investigation. Most of the ATP is used by the sodium/potassium ATPase in maintaining the resting concentrations of these ions. This is the key enzyme for the maintenance of ion gradients, and it extrudes 3 equivalents of sodium and accumulates 2 equivalents of potassium with the hydrolysis of one ATP molecule. The estimated activity of this enzyme in synaptosomes is 160 -200 nmoles/ min/mg protein at 37°C (Kimelberg et al., 1978). ATP is also utilised for calcium homeostasis. The concentration of calcium ions in the synaptosome cytosol has been widely measured to be between 0.1 and 0.35 μ M, depending on the preparation and the methodology used. Measurements using synaptosomes indicate that the concentration of protons is slightly higher in the nerve ending than in the external environment (Boakye et al., 1991; Erecinska et al., 1991; Richards et al., 1984). Resting synaptosomes in a physiological medium maintain a plasma membrane potential of approximately -70 mV, and a mitochondrial membrane potential of approximately -150 mV.

In summary, synaptosomes possess the morphological and biochemical characteristics and properties of the *in situ* nerve terminal.

2.1.2.e The decision to use synaptosomes for this study

In any scientific research there are underlying questions to be answered, along with an informed decision as to how to best go about investigating these questions. This thesis is primarily questioning whether the heightened resistance of immature animals to episodes of ischaemia in comparison to adults can in any way be accounted for by different biochemical responses of immature and mature neurones.

The decision to use a reduced preparation for this investigation was taken to enable manipulation and measurement of the external and internal cellular environments, and so that biochemical changes during and after metabolic stress could be determined. Brain slices were discounted because of their inherent heterogeneity and the desire to focus on neuronal events alone. Cell culture techniques have been invaluable in neurobiological research and pure neuronal cultures are widely used for the analysis of function at the single cell level. However, a neuronal cell culture model was not used for this study, because a direct comparison between the responses of fully matured adult nerves and immature developing nerves was the main aim of the investigation. Whilst continuous clonal cell lines may be regarded by many as representative of matured neurones, these cell lines often do not behave as normal tissue due to the loss or gain of certain characteristics over prolonged culture time. Neuronal primary cultures are cultured from foetal or neonatal brain, and although they undergo a maturing process over a number of weeks in culture, it is uncertain which culture times are representative of specific stages of neuronal development. It was therefore decided to isolate synaptosomes from precisely aged rat brain and focus on the response of immature and mature nerve terminals to metabolic stress in vitro.

The decision to concentrate on the nerve terminal alone was an informed one because many years ago it was postulated that the synapse might be the primary area of neurological failure during oxygen deprivation (Harkonnen et al., 1969). Following this postulation, it was expected that hypoxia and/or similar insults *in vivo* would alter the characteristics of derived synaptosomes; or that synaptosomes from control animals might be vulnerable to treatments *in vitro* that mimic hypoxia or similar insults. This has formed the basis for many investigations which use adult synaptosomes as a model. There is little information in the literature regarding the function of synaptosomes from immature brain which have undergone metabolic stress. In light of the well documented relative resistance of immature animals compared with adults to conditions of hypoxia/ischaemia, any information on the function of neonatal synaptosomes under metabolic stress compared with data from adult synaptosomes in identical conditions is of potentially great interest.

2.1.2.f Validation of the synaptosome models used in this study

To ensure that direct comparisons between adult and immature synaptosomes were entirely valid the removal of synaptosomes from control animals followed by characterisation of these preparations was the critical first step in the study. The purity of synaptosome preparations should ideally be assessed by the appropriate marker enzyme assays and by electron microscopy. In practice, the assessment of the purity of different synaptosomal preparations isolated by "standard" methods is rarely reported. Such "quality control" studies are advisable because a degree of variation may be found even when a standard method is employed. Standard methods are often modified to suit the equipment available in a particular researcher's laboratory and the lack of quality control data makes it difficult to compare results from various laboratories, even though the same "standard" method for synaptosome preparation has been employed. It is important to complement the structural studies of electron microscopy and purity studies of enzyme markers with biochemical assessment of metabolic integrity. It is possible that a preparation of synaptosomes may appear to be relatively pure and structurally intact, and yet be metabolically inactive and unable to maintain ion gradients. Therefore, for both adult and neonatal synaptosome preparations electron microscopy, enzyme marker studies and oxygen uptake studies were conducted.

2.1.3 In vitro metabolic stress

To enable the investigation of ischaemic damage in the brain a number of experimental approaches can be taken. In large animals global ischaemia has been modeled in a number of ways including the induction of cardiac arrest, using a neck tourniquet and raising the intracranial pressure to levels greater than the mean arterial blood pressure. In smaller animals, such as the rat, global ischaemia has often been modeled by decapitation although this precludes studies concerned with reperfusion. Transient experimental ischaemia is often induced in smaller animals by reversibly occluding the blood vessels that perfuse the brain.

The most obvious problem with *in vitro* models of ischaemic damage is that the model lacks vasculature. Hence although the term "ischaemia" is often applied to *in vitro* models, there is actually not a "reduced blood flow" because the model inherently lacks blood flow of any sort. Oxygen and glucose deprivations are considered to be the major perturbations of ischaemia, and these elements can be quite adequately studied *in vitro*. In the normal control situation, synaptosomes are incubated in a physiological medium (refer to section 2.2.7) which contains adequate oxygen and glucose to support respiration. The levels of either oxygen or glucose can be manipulated to provide a hypoxic or hypoglycaemic environment for the synaptosome. If both oxygen and glucose are reduced this is effectively an *in vitro* model for the physiological condition of ischaemia.

The term ischaemia is well defined as reduced blood flow. However, it can refer to a wide spectrum of oxygen and glucose levels depending on the extent of the residual blood flow that is seen by the brain tissue. Great care should be taken in using the terms hypoxia and hypoglycaemia to describe in vitro models of ischaemia. This is because precise definitions of the oxygen and glucose concentrations experience by the brain tissue are more informative than these terms alone and enable more accurate comparison of work between researchers. For example, it can be confusing when comparing a number of studies concerned with the effects of hypoxia on the release of glutamate from neurones in culture. Often at first sight it may appear valid to compare and contrast these different studies, however further examination of the methodology frequently highlights that some of the studies were in fact looking at anoxic (complete oxygen lack) conditions or hypoxia exacerbated by a lack of glucose as well. It is the responsibility of the researcher to fully define and explain the extent of glucose and oxygen lack that is involved in a particular in vitro model of ischaemia. Similarly it is also very important to make oneself aware of the precise conditions involved in ischaemic studies when scrutinising and reviewing the literature.

METHODS

<u>2.2</u> <u>Methods</u>

2.2.1 Materials

Ficoll 400 was obtained from Pharmacia, Ltd., Uppsala, Sweden.

Bio-Rad DC Protein Assay Reagent kit was purchased from Bio-Rad, Hemel Hempstead, Hertfordshire, U.K. and included:-

reagent A - an alkaline copper tartrate solution.

reagent B - a dilute Folin reagent.

All other chemicals and enzymes used were of analytical grade and were supplied by either BDH Limited, Dagenham, Essex, U.K., the Sigma Chemical Company, Poole, Dorset, U.K., or Boehringer Mannheim U.K. Limited, Lewes, East Sussex, U.K. All solutions were made up in deionised double distilled water.

Medical oxygen (100%), air, and pure nitrogen (100%) were purchased from British Oxygen Company, Great West Road, Brentford, Middlesex, U.K.

2.2.2 Adult synaptosome preparation.

2.2.2.a Animals

Adult male rats of the Wistar strain were used for all experiments, and were purchased from B and K Universal, Aldborough, England. These mature animals were provided with a standard laboratory diet and water *ad libitum*.

2.2.2.b Ficoll preparation

Ficoll 400 is a neutral, highly branched, synthetic, hydrophilic polymer of sucrose. It is made by the copolymerization of sucrose and epichlorohydrin. Ficoll provides high density solutions of low viscosity and osmotic pressure, and it has significant applications in the separation and isolation of organelles. Ficoll can be made iso-osmotic with intracellular contents by the addition of saline or tissue culture media. However, since subcellular particles tend to aggregate in the presence of salts and because sucrose is assumed to have a stabilising effect on synaptosomal membranes (Sperk and Baldessarini, 1977) Ficoll/sucrose based gradients are normally used for subcellular fractionation.

Ficoll 400 is provided as a spray-dried powder and it was prepared for experiments as follows:-

- 80 g of Ficoll powder was dissolved by stirring in 160 ml of water. The resulting solution was dialysed in dialysis tubing with pore size 24Å (inflated diameter 19 mm) against double distilled water for 5-7 hours at room temperature, and with constant stirring. The water was changed at least once during this period of dialysis.
- Following dialysis, the density of the Ficoll solution was determined using a specific-gravity bottle. A standard curve of density as a function of concentration (provided by Pharmacia) was used to determine the concentration of the Ficoll solution. The Ficoll solution was then diluted to a stock concentration of 20% (w/w) which was stored at -20°C. From this stock solution a 12% Ficoll/sucrose solution was prepared with isolation medium (0.32 M sucrose, 10 mM Tris/HCl, 1 mM potassium EDTA, pH 7.4 at 4°C), and the pH was finally adjusted to 7.4 at 4°C.

2.2.2.c Centrifugation

All of the centrifugation procedures were carried out at 4°C. Either a Beckman J2-21M/E Centrifuge with a JA20 fixed angle rotor, or a Beckman L8-70M Ultracentrifuge with a swinging bucket SW28 rotor was used for all procedures. Average g values are quoted throughout.

2.2.2.d Procedure for synaptosome preparation

The following procedure was carried out at 4°C, and it is also illustrated in figure 2.2.



Figure 2.2 Adult synaptosome preparation

Preparation of brain homogenate

For each separate synaptosomal preparation either 4 adult rat brains or 8-12 rat pup brains were used. Animals were killed by stunning and cervical dislocation. The whole brain minus the cerebellum was rapidly removed and placed in approximately 25 ml of ice-cold isolation medium (IsM), which consisted of 320 mM sucrose, 1 mM EDTA, 10 mM Tris/HCL, pH 7.4 at 4°C. The brains were finely scissor-chopped while being frequently washed with fresh IsM to ensure adequate removal of blood and debris. One volume of chopped forebrains was then homogenised manually with 9 volumes of IsM using a Dounce-type glass-glass homogeniser (total clearance 0.1 mm). 12 up-and-down strokes were used for this homogenisation.

Preparation of the crude synaptosomal/mitochondrial pellet

The homogenate was centrifuged at 1,300 g for 3 minutes, and the resultant supernatant was carefully decanted. The supernatant was then respun at 17,000 g for 10 minutes to obtain the crude synaptosomal/mitochondrial pellet (P2 pellet).

Principles of synaptosome purification

Purified synaptosomes were produced by centrifugation through a Ficollsucrose gradient. This preparative centrifugation technique utilised a discontinuous (or step) gradient which has been found to be the most suitable for the separation of subcellular organelles from animal tissue homogenates (Griffiths, 1986). The method is based on the flotation of organelles in a homogenous suspension so that they rise or sediment until reaching a region where the solution density is equal to (isopycnic position) or greater than their own buoyant density. Hence the organelles band to form discrete zones. This form of isopycnic centrifugation depends solely upon the buoyant density of the organelle, and not on its shape or size. Subcellular organelles can thus be effectively separated by such a technique due to their different buoyant densities.

Procedure for synaptosome purification

As illustrated in figure 2.2., the P2 pellet was resuspended in a total of 6ml IsM, then diluted to 36 ml with 12% Ficoll-sucrose medium, pH 7.4 at 4°C, and then gently homogenised by hand with a loose Potter-type homogeniser. 18 ml of this 10% Ficoll/crude synaptosomal suspension was introduced into two thin walled ultracentrifuge tubes. Onto this was layered 10 ml of 7.5% Ficoll-sucrose medium (7.5% Ficoll, IsM). Finally, 9 ml of IsM was layered on top of this. The tubes were centrifuged at 99,000 g for 30 minutes in a SW28 swing out rotor.

The myelin (Mye) fraction banded at the interphase between the IsM and the 7.5% Ficoll medium. The synaptosomal (Ss) fraction banded at the interphase between the 7.5% and the 10% Ficoll media. The free mitochondria pelleted below the 10% Ficoll medium. An ideal preparation was taken to be one where a small but noticeable amount of Ss (off-white) pelleted on top of the free mitochondria, ensuring that mitochondrial contamination of the synaptosomes was minimal.

The synaptosomal pellet

The myelin fraction was aspirated and discarded. The synaptosomal band was carefully sucked off from the interphase, diluted with IsM to a final volume of approximately 50 ml and pelleted by centrifugation at 17,000 g for 10 minutes. The resultant synaptosomal pellet was kept on ice until use.

2.2.3 Neonatal synaptosomes preparation.

2.2.3.a Animals

Rat pups of the Wistar strain were used for all experiments, and were either purchased from B and K Universal, Aldborough, England, or were bred in house from adult Wistar rats. The mature breeding animals were provided with food and water *ad libitum*. Pups were used at either post-partum day 5, 10, 15, or 20.

2.2.3.b Principles

The separation, and hence the purification, of synaptosomes relies on the buoyant density of organelles and their behaviour in a discontinuous Ficoll gradient. The density and composition of brain tissue alters markedly during development (Dobbing and Sands, 1979), and because of this a different gradient is required to enable sufficient purification of synaptosomes from the developing brain.

2.2.3.c Procedure

The procedures used were identical to those for the adult synaptosome preparation up until the point of purification on a Ficoll gradient. The modified Ficoll gradient for preparations from these age animals is detailed below and is also illustrated in figure 2.3. The P2 pellet was resuspended in a total of 4ml IsM, then diluted to 24 ml with 12% Ficoll-sucrose medium, pH 7.4 at 4°C, and then gently homogenised by hand with a loose Potter-type homogeniser. 12 ml of this 10% Ficoll/crude synaptosomal suspension was introduced into two thin walled ultracentrifuge tubes. Onto this was layered 10 ml of 6% Ficoll-sucrose medium (6% Ficoll, IsM). Onto this was then layered 9 ml of 4% Ficoll (4% Ficoll, IsM). Finally, IsM was layered on top of this until the level was approximately 5 mm from the top of the tube. The tubes were centrifuged at 99,000 g for 30 minutes in a swing out rotor.

Following centrifugation, a small discrete band of myelin (Mye) was found at the interphase between the IsM and the 4% Ficoll medium. There is considerably less myelin in this preparation than in the adult synaptosome preparation because laying down of myelin occurs during brain development. A band of membrane material and cytosolic particles (resealed nerve terminals not containing mitochondria) banded at the interphase between the 4% and the 6% Ficoll media. The synaptosomal (Ss) fraction banded at the interphase between the 6% and the 10% Ficoll media. The free mitochondria pelleted below the 10% Ficoll medium.





12 ml of 10% Ficoll/crude synaptosomal suspension

Figure 2.3 Modified Ficoll gradient for neonatal synaptosome preparation

The Model.

To isolate the synaptosomal pellet, the myelin and membrane bands were aspirated in the same way as for the adult synaptosome preparation. The synaptosomal band was sucked off from the interphase, diluted with IsM to a final volume of about 50 ml and pelleted by centrifugation at 17,000 g for 10 minutes. The resultant synaptosomal pellet was kept on ice until use.

2.2.4 Marker enzyme assays for the determination of synaptosome purity and integrity.

2.2.4.a Principle

Ideally, a synaptosome preparation should contain only synaptosomes. In practice they are contaminated to varying extents by free mitochondria, segments of axons, myelin and glial cell fragments. It is necessary, therefore, to assess the degree of contamination. This can be done by the use of an enzyme or other biochemical marker whose association with a particular structure has been previously determined. Hence, the degree of contamination and also the level of integrity of a synaptosome fraction can be determined by measuring the activities of a number of marker enzymes in that synaptosome fraction. In this study, to fully assess the status of synaptosome fractions from different age rat brains, a selection of marker enzymes for subcellular constituents including mitochondria, myelin and microsomes have been determined in both a pure reference fraction of each subcellular constituent and the synaptosome fraction. The amount of contamination of synaptosomes by various non-synaptosomal particles is then calculated by taking the ratio of the specific activity of marker enzymes measured in the synaptosomal fraction to that measured in the reference fraction. This value is then expressed as the percentage of contamination.

2.2.4.b Procedure

All of the marker enzymes were assayed in a Kontron Uvikon 941 spectrophotometer at 30°C. Synaptosome samples were taken directly from the gradients, washed, pelleted and resuspended in isolation medium (IsM, see section 2.2.2). Protein was assayed as detailed in section 2.2.5.

2.2.4.c Microsome contamination

The microsomal contamination of the synaptosomal fractions was assessed by measuring the NADPH:cytochrome c oxidoreductase (NCR) activity. This enzyme is specific to the endoplasmic reticulum (Gurd *et al.*, 1974). The assay measures the NADPH-dependent reduction of cytochrome c at 550 nm. The rate of increase in A_{550nm} is measured. Often the activity of this enzyme is measured in isolated mitochondria (Hatefi *et al.*, 1967). However if those same conditions were used here, the hypo-osmotic conditions would cause synaptosomal lysis. Hence the activity measured would be very high because the intrasynaptosomal enzyme would have access to the substrates. Therefore for assessing contamination, the assay was essentially by the same method as in the original characterisation of adult synaptosomes produced by the flotation method (Booth and Clark, 1978).

1 ml total reaction mixture contained:-

200 mM potassium phosphate buffer, pH 7.4 at 30°C.
1 mM KCN
0.1% (w/v) cytochrome c
250 μM NADPH

The molar extinction coefficient of cytochrome c used was 19.2 mM^{-1} cm⁻¹.

The activity of this enzyme under the same conditions was also measured in a purified microsomal fraction, prepared at the same time as the synaptosomes. The microsomal pellet was prepared by centrifuging the supernatant which was removed from the top of the crude synaptosomal/mitochondrial (P2) pellet. Centrifugation was at 100,000 g for 60 minutes. The resultant pellet was used as the purified microsomal fraction.

2.2.4.d Non-synaptic mitochondria contamination

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The non-synaptic or 'free' mitochondrial contamination of the synaptosomes was assessed by measuring the rotenone-insensitive NADH: cytochrome c oxidoreductase activity. This enzyme is a marker of the mitochondrial outer membrane (Duncan and Mackler, 1966). When using this method for estimating the

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contamination by free mitochondria, one has to consider that this enzyme is located in both the mitochondrial outer membrane and also in the membrane of microsomes. This can be overcome by measuring the activity of NCR as detailed above, which is specific to microsomes. By calculating the difference between the NCR activity and the rotenone-insensitive NADH: cytochrome c-oxidoreductase activity, an estimate of the amount of free mitochondria in the sample is given. The activity of the rotenoneinsensitive NADH: cytochrome c oxidoreductase was measured in a reference mitochondrial sample. The purified mitochondrial sample was prepared by the method of Lai and Clark (see Lai and Clark, 1989 for review of methods). This method utilises a discontinuous Ficoll gradient of the same percentages and steps as for the Booth and Clark method for adult synaptosome preparation (Booth and Clark, 1978). However, in the Lai and Clark method, the P2 pellet is layered onto the Ficoll gradient and the organelles spin down and sediment during centrifugation. The resultant mitochondrial pellet is less contaminated with synaptosomes than the pellet produced by the flotation method. Hence, this mitochondrial pellet is more suitable as a reference sample for assessing the activity of rotenone-insensitive NADH: cytochrome c oxidoreductase.

The free mitochondrial contamination is thus calculated as:-

enzyme activity

rotenone-insensitive NADH: cytochrome c oxidoreductase		NCR
Synaptosomes (A)	**1	**
Mitochondria (B)	**	**
Microsomes (C)	**	**
Contamination		
TOTAL	$A/B \ge 100 = \$\%$	$A/C \ge 100 = \sqrt{6}$

TOTAL	A/B x $100 = x\%$	$A/C \ge 100 = y^{0}$
Mitochondrial	\mathbf{x} % - \mathbf{y} % = % contamination	

^{**} denotes the particular enzyme activity assayed in the fraction A, B, or C.

The assay for determining the activity of rotenone-insensitive NADH: cytochrome c oxidoreductase was by the method as in the original characterisation of adult synaptosomes (Booth and Clark, 1978). The assay measures the NADH-dependent reduction of cytochrome c at 550 nm. The rate of increase in $A_{550 nm}$ is measured.

1 ml total reaction mixture contained:-

200 mM potassium phosphate buffer, pH 7.4 at 30°C.
1 mM KCN
0.1% (w/v) cytochrome c
250 μM NADH
5 μM rotenone

The molar extinction coefficient of cytochrome c used was 19.2 x 10^3 M⁻¹ cm⁻¹.

2.2.4.e Membrane fragment contamination

Measurement of the activity of the enzyme acetylcholinesterase in the synaptosomal sample may be taken as a marker of membrane contamination (Cotman & Matthews, 1971). This synaptic membrane enzyme hydrolyses acetylcholine to acetate and choline. In so doing, its role is to terminate the action of the neurotransmitter acetylcholine at the post-synaptic membrane. A large proportion of membrane fragment contaminants would increase the activity of this enzyme in the synaptosomal fraction. This assay was carried out as in the original adult synaptosome characterisation (Booth and Clark, 1978) and is based on the original method by Ellman (1961). The measured values (measured at 25°C for this particular assay) were compared to previous values found in synaptosomal preparations, to enable a direct comparison of the extent of contamination to be made.

1 ml total reaction mixture contained:-

100 mM potassium phosphate buffer, pH 8.
0.4 mM Ellman's reagent (DTNB - 5,5'-dithiobis-2-nitrobenzoic acid)
750 μM acetylthiocholine iodide

The assay is based upon the hydrolysis of acetylcholine in membranes by the enzyme acetylcholinesterase. The hydrolysis reaction products react with DTNB, and this reaction leads to the formation of a mercaptide ion which absorbs light at 412 nm. The increase in absorbance at 412 nm was measured. The rate of change of absorption at 412 nm is proportional to enzyme concentration. Changes in DTNB concentration do not affect the rate of reaction. The molar extinction coefficient used was 13.6 x 10 3 M⁻¹ cm⁻¹ (Ellman, 1961)

2.2.4.f Synaptosome integrity

The integrity of the synaptosomal fraction can be assessed by monitoring the amount of extrasynaptosomal lactate dehydrogenase (LDH). This is a characteristic cytosolic marker enzyme which is widely used to assess the membrane integrity of many tissue preparations. When wanting to check the integrity of a preparation the assay must be carried out as soon as possible on the day of synaptosome isolation. The samples need to be kept on ice and treated as delicately as possible.

The activity of LDH is determined essentially by the method of Clark and Nicklas (1970). The reaction that occurs during the assay is:-

LDH $pyruvate + NADH + H^{+} \rightleftharpoons lactate + NAD^{+}$

The reaction is catalysed by LDH and the assay is based on the absorbance changes that occur when NADH becomes oxidised in the reaction. The oxidation of NADH to NAD⁺ results in a decrease in absorbance at 340 nm. Both NADH and NAD⁺ exhibit a major absorption peak at 260 nm, characteristic of their nucleotide structure.

However, the spectrum of the reduced form also shows a smaller absorption band with a maximum at 340 nm whereas the oxidised form does not. The loss of NADH is in a stoichiometry of 1:1 with the reduction of pyruvate to lactate and hence the rate of change of absorption at 340 nm is proportional to enzyme concentration. The molar extinction coefficient for NADH used was $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

The assay of LDH activity is utilised to determine synaptosome integrity in the following way:

- The rate of reaction is monitored in the freshly prepared sample. For a good preparation there should be very little activity of the enzyme at this stage.

- A volume of 1% Triton (a detergent which causes membrane lysis) equal to the sample volume is added, and the reaction rate is again monitored for a few minutes.

The addition of Triton ruptures the intact synaptosomes and a measure of the total enzyme activity is thus found.

- The pre- and post- Triton addition LDH activities are calculated.

- The activity LDH in the synaptosome sample is expressed as a percentage of the total activity of the enzyme present (post-Triton addition).

This gives an indication of the integrity of the preparation. The lower the percentage 'leak' of this cytosolic enzyme, the more intact a preparation is. This assay can also be used to determine the effects of an incubation or a treatment on the integrity of a synaptosomal suspension. This is determined by taking a sample of the suspension prior to the incubation which can be mixed with Triton and hence be used to assay the total activity of the preparation. Samples are then taken during the incubation or treatment period, and the activity of LDH is determined in the supernatant after the synaptosomes have been pelleted. If this activity in the supernatant is given as a percentage of the total activity, an indication of the release of LDH is given. The recorded level of leak is important in determining whether membrane damage is occurring during an incubation or treatment period.

2.2.5 Protein determination

2.2.5.a Principle

The Bio-Rad protein assay is a colorimetric assay for protein concentration which is based on the well-documented Lowry assay (Lowry *et al.*, 1951). It does have the following improvements as compared with the original Lowry assay:

- the reaction reaches 95% of its maximum colour development within 15 minutes, which is more rapid than the original assay.
- there is no more than 5% colour change within one hour after the addition of reagents, and thus the assay has a high stability.

The assay is based on the reaction of protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper treated protein. Proteins effect a reduction of the Folin reagent by loss of 1, 2 or 3 oxygen atoms from the reagent, thus producing a number of several possible reduced species. These reduced species lead to colour development which can be measured spectrophotometrically because they have a characteristic blue colour with maximum absorbance at 750 nm and minimum absorbance at 405 nm. This colour development is primarily due to the presence of the amino acids tyrosine, tryptophan and to a lesser extent cystine, cysteine and histidine.

2.2.5.b Procedure

A standard curve of 10 - 200 μ g / ml bovine serum albumin was routinely prepared each time the assay was performed. Initial controls showed that preparation of standards in double distilled water or the same Krebs Buffer (see 2.2.6) as synaptosome samples did not lead to significantly different results. For each assay the following procedure was followed:-

 μ l of samples and standards were pipetted into clean, dry test tubes. μ l of reagent A was added into each test tube and all tubes were vortexed. μ l of reagent B was pipetted into all tubes which were then vortexed immediately. All of the tubes were then kept in the dark and at room temperature to ensure that maximal colour development occurred.

The absorbance of all standards and samples were read on a Kontron Uvikon 941 spectrophotometer at 750 nm. The protein concentration of the unknown samples were calculated by using the standard curve. Standard curves were only accepted if $r \ge 0.995$.

<u>2.2.6</u> The oxygen electrode.

The measurement of oxygen consumption rates was performed using a 250µl capacity incubation chamber with a water jacket and a Clark electrode (Yellow Springs Instrument Ltd., Yellow Springs, U.S.A.) fitted into the top of the chamber.

2.2.6.a Introduction

The utilisation or evolution of oxygen is fundamental to so many biological processes. Electrodes which are capable of measuring changes in oxygen concentration are very valuable tools for biochemical studies. The oxygen electrode is commonly used in studies of mitochondrial function, but it can be used in any reaction in which oxygen is either evolved or absorbed by a system.

2.2.6.b Mitochondrial respiration

A more detailed discussion of the mitochondrial electron transport chain and its function is given elsewhere in section 4.1.

Consumption of oxygen forms the basis for polarographic measurements of mitochondrial respiration with an oxygen electrode. Electron transfer, and therefore the activities of the entire respiratory chain enzymes, working in concert together, are assayed in terms of the amount of oxygen consumed by a sample. Thus mitochondrial respiration is conveniently measured by an oxygen electrode.

2.2.6.c Principles of the oxygen electrode

Oxygen electrodes operate on the principle that a measurable current is produced in the system which is proportional to the partial pressure of oxygen in a sample.

There are four basic types of commercial oxygen electrodes;

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- the open electrode
- the recessed microelectrode
- the Galvanic probe
- the Clark type electrode

of which the Clark type electrode is the most common and widely used. This normally consists of a silver/silver chloride reference anode surrounding a platinum cathode. Both electrodes are immersed in the same solution of concentrated potassium chloride, and are separated from the test solution by a thin Teflon membrane. The cathode and anode are polarised by the application of an external voltage of 0.5 - 0.8V across them, and electrons become generated. At the platinum electrode these electrons reduce oxygen molecules to water. The oxygen tension at the cathode then drops to zero and this acts as a sink, so that oxygen diffuses towards it to make up the deficit. Chloride anions migrate to the anode and release electrons. The overall result is that a transfer of electrons between the cathode and the anode occurs and a current is therefore generated by this electron movement. The reactions that occur are:-

 $4H^+ + 4e^- + O_2 \rightarrow 2H_2O$ and $4Ag + 4Cl^- \rightarrow 4AgCl + 4e^-$

The flow of electrons can be measured in an external circuit. The measured current is proportional to the amount of oxygen which permeates the thin membrane. The probe actually measures oxygen pressure. Since oxygen is rapidly consumed at the cathode, it can be assumed that the oxygen pressure inside the membrane is zero. Thus, the force causing oxygen to diffuse through the membrane is proportional to the absolute pressure of oxygen outside the membrane. If the oxygen pressure increases, more oxygen diffuses through the membrane and more current flows through the electrode. The relationship between external oxygen pressure and probe current is linear.

Figure 2.4 details the type of electrode used for respiration experiments in this study.



Figure 2.4 The Oxygen Electrode

The electrodes can be conveniently enclosed in a casing as a small probe, which is introduced into a chamber containing the test sample, and effectively acts not only as the oxygen probe but also as a close fitting seal keeping the chamber in isolation from atmospheric oxygen. To enable precise quantification of respiration the oxygen electrode must first be calibrated.

2.2.6.d Calibration procedure

The current produced in an oxygen electrode system is proportional to the partial pressure of oxygen in a sample, and because the response is linear only two calibration points are necessary. Calibration must be done routinely and under the precise temperature conditions that are to be used for the experimental investigation. Calibration was always carried out before experiments commenced, as follows:-

- $250 \,\mu$ l of distilled water (at 30°C) was pipetted into the electrode chamber. The chamber was maintained at 30°C by a water jacket around it, which was fed by a thermostated water bath.

- A few crystals of sodium dithionite $(Na_2S_2O_4)$ was added to the chamber and the oxygen concentration rapidly fell to zero, with a consequential response by an attached chart recorder. The position of the pen on the chart recorder was set to zero.

- The electrode chamber was carefully washed out with water a number of times, to fully remove any traces of $Na_2S_2O_4$.

- A further 250 μ l of distilled water, air equilibrated at 30 °C, was introduced into the electrode chamber. The chart recorder response was adjusted to 90% by suitable sensitivity controls. This provides two known oxygen concentrations of zero and 440 ng atoms oxygen/ ml, from which other concentrations can be determined.

2.2.6.e Respiratory Control Ratio (RCR)

Mitochondrial electron transport, monitored by measuring the rate of oxygen consumption by a suspension of mitochondria or cells, can occur at rapid rate only following the addition of an oxidisable substrate and ADP plus inorganic phosphate. The "active" state in the presence of substrate and ADP has been designated *state 3* (Chance and Williams, 1956), and is a situation where rapid electron transfer, rapid oxygen consumption and rapid ATP synthesis all occur. This rate is measured and compared to the resting state when all of the ADP has been consumed, and which is designated as *state 4* (Chance and Williams, 1956). The ratio of the state 3 to the state 4 rates of respiration is referred to as the *respiratory control ratio* (*RCR*) and is a measure of the "tightness" of the coupling between electron transfer and oxidative phosphorylation. The integrity of the mitochondrial membrane is required for tight coupling, and damaged mitochondrial preparations show low RCR values.

2.2.6.f Procedure for assessing synaptosome respiration

The oxygen consumption studies with synaptosomes were conducted at 30 °C in respiration medium (pH 7.4) of the following composition:

100 mM KCl
75 mM mannitol
25 mM sucrose
10 mM potassium phosphate
10 mM Tris-HCl
50 μM K⁺-EDTA

Approximately 1-2 mg of synaptosomal protein and 0.5 mg bovine serum albumin (BSA) were incubated in the electrode chamber in a total volume of 250 μ l. The medium in the chamber was constantly stirred with a magnetic stirrer. The synaptosomes were added in a minimal volume of isolation medium (see section 2.2.2). Substrates were added into the chamber in small volumes (5 - 20 μ l) to give final concentrations of :-

10 mM glutamate + 2.5 mM malate 10 mM pyruvate + 2.5 mM malate

or
After equilibration, 62.5 nmoles (250 μ M final concentration) ADP was added to enable state 3 respiration to be assessed. When all of the ADP was consumed, a resting state was measured for a few minutes, then a further addition of ADP was made to induce state 3 respiration again. This cycle was repeated three or four times for each synaptosome sample.

2.2.6.g Expression of results

From a calibrated trace the amount of oxygen consumed per minute by the synaptosome sample added to the electrode chamber could be calculated. By measuring the protein content of the synaptosome sample a protein baseline was used so that all results were expressed as *ng atoms of oxygen consumed per minute per mg of synaptosomal protein*. These values for state 3 and state 4 respiration can be used to calculate the RCR for the synaptosome samples.

2.2.7 Gassing protocol for the induction of metabolic stress.

2.2.7.a Physiological Incubation Medium

For all experiments, synaptosomes were suspended and incubated in a modified Krebs Phosphate Buffer (KPB) pH 7.4, of the following composition :-

sodium chloride	NaCl	141	mΜ
potassium chloride	KCl	5	mΜ
magnesium sulphate	MgSO ₄	1.3	mМ
sodium hydrogen phosphate	Na ₂ HPO ₄	10	mМ
calcium chloride	CaCl ₂	1.2	mМ
glucose ²		10	mΜ

2.2.7.b Procedure

Following preparation, the synaptosomes were kept on ice as a pellet. For all experiments this synaptosomal pellet was gently resuspended in approximately 2 - 3 ml of KPB which did not contain any CaCl₂.

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for aglycaemic and ischaemic experiments, glucose was omitted from the incubation medium.

The synaptosomes were then incubated at 37° C for 5 - 10 minutes. This was to allow the plasma membrane to polarise and voltage-dependent calcium-channels to close (Nicholls, 1993). After this pre-incubation, for some experiments the synaptosomes were pelleted in a bench-top centrifuge (4,000 g for 2 minutes).

Control incubations

For all control incubations synaptosomes were incubated in KPB which contained 10 mM glucose and also contained oxygen, so that aerobic respiration could be fully supported. In early pilot experiments the KPB had been pre-gassed and hence equilibrated with 100% oxygen at 37°C for at least one hour prior to the synaptosomal incubation. In later experiments the KPB was equilibrated with air at 37°C for at least one hour, and hence the oxygen tension of the buffer was lower than in the original experiments.

In all control experiments synaptosomes were incubated in a shaking water bath at 37°C in either Eppendorf tubes or in 25 ml glass vials with plastic stoppers. Pure oxygen was supplied via a glass gassing manifold that was connected to a gas supply with controlled flow rate for the period of the incubation. Gas was delivered through hypodermic needles attached to the gassing lines and which pierced the tops of the tubes or vial lids. Hence the gas was in equilibrium with the air space above the synaptosomal incubation rather than bubbling through the suspension itself. This avoided excessive synaptosomal lysis and excessive evaporation of the KPB. Using this protocol <2 % evaporation of the buffer occurred during the duration of the experiments.

Incubations to induce metabolic stress

Synaptosomes were made aglycaemic by using essentially the same protocol as for control incubations but ensuring that the buffer contained no glucose.

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Synaptosomes were rendered hypoxic by incubating in KPB which did contain 10 mM glucose but which had a very limited oxygen tension. This limited oxygen tension was achieved in one of 2 ways:-

2.2.7.b(1). In early experiments, the KPB was pre-gassed and equilibrated with 100% pure nitrogen for over one hour. However, there was a residual oxygen tension in this buffer. To keep this to a minimum the synaptosomes were pelleted following their pre-incubation and the resultant oxygen containing supernatant was removed. The pellet was then resuspended in the nitrogen gassed KPB and hence oxygen availability was limited. However, each pelleting and resuspension cycle which the synaptosomes were subjected to caused increasing damage of the synaptosomal membranes because of the shearing forces created during the resuspension. Hence this protocol was modified (see below) to ensure that damage was limited.

2.2.7.b(2). In the modified protocol, a volume (200 μ l) of the highly concentrated synaptosomal pre-incubation was diluted approximately 1:5 with the appropriate KPB. Hence in the hypoxic situation 200 μ l of control synaptosomes were diluted with the nitrogen gassed KPB. Unfortunately, although this protocol limited membrane damage, it also led to an unsatisfactory amount of oxygen in the incubation at time zero.

To rectify this situation the hypoxic KPB was fully depleted of oxygen before it was added to the synaptosomal incubation. This was achieved by the addition of the reducing agent sodium dithionite (0.05 mM final concentration) and continuous bubbling with pure nitrogen. This is a widely accepted protocol for rendering physiological buffers anoxic (Bright and Ellis, 1992). The oxygen tension of this buffer was zero. Control synaptosomes were then diluted with this anoxic buffer and were incubated for the duration of the experiment in a shaking water bath at 37°C in the same manner as for the control incubation. However, in this case 100% pure nitrogen was supplied for the incubation period rather than oxygen.

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In all of these oxygen limited experiments, synaptosomes were initially hypoxic because of the oxygen present in the 200 μ l of synaptosomes that was added to the anoxic buffer. The incubation became anoxic by 15 minutes due to the active respiration of the synaptosomes and hence the consumption of the remaining oxygen. These incubation conditions are therefore best described as hypoxia/anoxia.

Synaptosomes were rendered hypoxic/aglycaemic by adhering to the same gassing protocol as for the hypoxic synaptosomes, but by also ensuring that no glucose was present in the incubation buffer. Hypoxic/aglycaemic incubations were used as a model for *in vitro* ischaemia.

Hence, synaptosomes were classified as either:-

control aglycaemic hypoxic/anoxic ischaemic

The conditions underlying these classifications are defined as follows:-

control:

incubation in glucose and oxygen containing KPB, with 100% oxygen gas supplied throughout.

aglycaemic:

incubation in KPB containing oxygen but no glucose, with 100% oxygen gas supplied throughout.

hypoxic/anoxic:

incubation in KPB containing glucose but little or no oxygen, with 100% nitrogen gas supplied throughout.

ischaemic:

incubation in KPB without glucose and with little or no oxygen, with 100% nitrogen gas supplied throughout.

These conditions were adhered to for all synaptosome preparations from different age rats. Lengths of exposure to these conditions varies and are detailed for individual experiments. The most routine exposure time was 30 minutes.

2.2.8 Gassing Protocol for reperfusion and recovery

2.2.8.a Principle

After the synaptosomes had been through a period of metabolic stress (as outlined), in some instances the effect of a recovery period was assessed. This was akin to ischaemia/reperfusion or hypoxia/reperfusion. It involved the reintroduction of oxygen and glucose into the synaptosomal incubation, and also the removal of the extrasynaptosomal medium. Hence, any metabolic products released from the synaptosomes during metabolic stress were removed as would occur *in vivo*.

2.2.8.b Procedure

After the initial incubation period under conditions of metabolic stress, synaptosomes were pelleted in a bench top centrifuge (4,000 g, 1 minute). 700 µl of the supernatant was removed and replaced with control KPB, which contained oxygen and glucose and had been kept at 37°C. The synaptosomes were gently resuspended into this medium with a Gilson pipette by gently sucking up the KPB into the pipette and washing it over the synaptosome pellet. Using this procedure the synaptosomes were subjected to the same procedure. All synaptosome suspensions were then incubated at 37°C in a shaking water bath, with 100% oxygen supplied via the gassing manifold as for the original control incubations. The period of incubation was always 30 minutes.

2.2.9 Statistical analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) (Sokal and Rohlf, 1981). Differences between groups were tested using the Least Square Difference (LSD) test (Sokal and Rohlf, 1981).

RESULTS

2.3.1 Assessing the purity of the synaptosome fraction.

2.3.1.a Contamination by microsomes and free mitochondria

(Tables 2.1, 2.2 and 2.3)

The microsomal and free mitochondrial contamination of synaptosomes fractions isolated using the original Ficoll gradient are shown in table 2.1. This original Ficoll gradient was optimised for the isolation of synaptosomes from mature adult rat brain (Booth and Clark, 1978). The data show that using this gradient for the isolation of synaptosomes from immature rat brain is unacceptable. Both the microsomal and free mitochondrial contamination is significantly higher in the synaptosome preparations from PND 5-15 rats when compared with the contamination present in the adult preparation.

The original Ficoll gradient was therefore modified to decrease both the mitochondrial and microsomal contamination. A wide range of combinations of Ficoll steps were assessed. The most satisfactory combination was a gradient with 10%/6%/4% Ficoll steps. The microsomal and free mitochondrial contamination of synaptosomes fractions isolated using this modified Ficoll gradient are shown in Table 2.2. Using this modified gradient there was no statistical difference between the degree of contamination in any of the preparations.

It was important to ensure that the comparable degree of contamination between all of the preparations was not an artefact due to developmental profiles of the enzymes leading to an underestimation of contamination in the younger preparations. The activities of these enzymes in reference fractions of the pure organelle in question were therefore measured. Reference fractions of microsomes and free mitochondria were made from rat brains of PND 5 - adult. There was no significant difference between the enzyme activities in any of the ages. Hence there was no change in these enzyme activities with age, which could have created further confusion when assessing the degree of contamination across these preparations.

Microsome and free mitochondria contamination of synaptosome fractions prepared from different postnatal day (PND) rat brain using the original 10%/7.5% Ficoll gradient.

	PND 5	PND 10	PND 15	PND 20	Adult
Total					
microsome &	35.8 %	33.4 %	29.2 %	14.2%	12.0%
mitochondria					
contamination	а	а	а		
Microsome					
contamination	21.1%	17.0%	16.7%	8.7%	7.8%
	а	а	а		
Free					
mitochondria	14.7%	16.4%	12.5%	5.5%	4.2%
contamination					
	а	а	а		A Carrier

Contamination is given as a percentage. This is calculated from a ratio of the enzyme activity measured in the synaptosome sample to the activity in a pure reference sample. These reference values are given in Table 2.3.

Values given are calculated from the ratios of means of enzyme activities. The standard errors of these means were less than 10% of the mean in all cases. Means were taken from 8 separate synaptosome preparations, with each assay value taken in duplicate.

a - denotes a statistical significance ($p \le 0.01$) when compared to the adult synaptosome contamination.

Statistical analysis was by unpaired student t-test.

Microsome and free mitochondria contamination of synaptosome fractions prepared from different postnatal day (PND) rat brain using the modified 10%/6%/4% Ficoll gradient.

	PND 5	PND 10	PND 15	Adult
Total microsome & mitochondria contamination	15.9 %	14.7 %	14.5 %	12.0%
Microsome contamination	8.8%	10.4%	10.2%	7.8%
Free mitochondria contamination	7.1%	4.3%	4.3%	4.2%

Contamination is given as a percentage. This is calculated from a ratio of the enzyme activity measured in the synaptosome sample to the activity in a pure reference sample. These reference values are given in Table 3. The shaded column gives the contamination values of adult synaptosomes prepared by the original Ficoll gradient.

Values given are calculated from the ratios of means of enzyme activities. The standard errors of these means were less than 10% of the mean in all cases. Means were taken from 8 separate synaptosome preparations, with each assay value taken in duplicate.

Values were tested for statistical significance against the adult synaptosome contamination. Statistical analysis was by unpaired student t-test.

Reference values of enzyme activities in purified organeties	Reference	values of	enzyme	activities	in	purified	organelles.
--	-----------	-----------	--------	------------	----	----------	-------------

Reference organelle	Enzyme	activity nmoles/min/ m protein
Free mitochondria	rotenone-insensitive NADH: cytochrome c oxidoreducatase	46.2 ± 3.9
Microsomes	NADPH:cytochrome c oxidoreducatse	17.4 ± 1.5

Values given for the mitochondria and microsomes are the means \pm standard errors of pooled data. Means were from 15 separate organelle preparations. 3 preparations from each age group (PND 5, 10, 15, 20 and adult) were made. When no significant differences were found between the ages the data was all pooled to give one reference value.

Statistical analysis was by one way analysis of variance (ANOVA). Significane was assessed, at the level of P<0.05, by using Fishers Least Square Difference (LSD) test after analysis.

The reference values of the enzyme activities in the purified organelles are detailed in Table 2.3. Data from all preparations were pooled to give these values, which were then used for calculation of the contamination percentages shown in tables 2.1 and 2.2.

2.3.1.b Other contaminants

The results shown in tables 2.1 and 2.2 determined that the modified Ficoll gradient was acceptable for the preparation from rats age PND 5 - 15, and the original Ficoll gradient was acceptable for rats above this age. The activity of acetylcholinesterase was measured in the preparations from immature rat brain and compared with the previously reported activities in adult synaptosomes (Booth and Clark, 1978). In all measured preparations the activity of this enzyme was less than 60 nmol/min/mg protein. At least three preparations per age group were assessed. There was no significant difference in the enzyme activity in any of the preparations when compared with the adult preparation. Hence, the membrane contamination was shown to be comparable between all preparations.

2.3.2 Assessing the integrity of the synaptosome fraction

The amount of the cytosolic enzyme LDH present in the extrasynaptosomal medium of isolated synaptosomes is detailed in table 2.4. The amount is expressed as a percentage of the total enzyme activity in that particular preparation. Results are given for preparations of synaptosomes from PND 5 to adult rats. The preparations were isolated on the appropriate Ficoll gradients which give acceptable contamination as detailed in section 2.3.1. The data indicate that synaptosome preparations from all different age rats show an acceptable integrity.

Synaptosomal integrity, as assessed by the percentage leak of the cytosolic enzyme LDH. Synaptosome fractions were prepared using the appropriate Ficoll gradient which gave an acceptable degree of contamination for the different postnatal day (PND) rat preparations.

	PND 5	PND 10	PND 15	PND 20	Adult
percentage LDH activity in synaptosomal preparation	3.8 %	3.8 %	3.2 %	4.1%	3.0%
Enzyme latency	26	26	30	24	34

The leakiness of the synaptosome preparation is given as a percentage. This is calculated from a ratio of the enzyme activity measured in the synaptosome sample to the total activity present in the preparation following membrane disruption by Triton.

Percentage values given are calculated from the ratios of means of enzyme activities. The standard errors of these means were less than 10% of the mean in all cases. Means were taken from 6 or 8 separate synaptosome preparations.

Statistical analysis was by one way analysis of variance (ANOVA). Significance was assessed, at the level of P<0.05, by using Fishers Least Square Difference (LSD) test after analysis.

The percentage of this cytosolic enzyme which has leaked out from synaptosomes, possibly as a result of membrane damage, is less than 10% in all cases. Hence all preparations are over 90% intact and would be suitable for using in further experimentation. The original adult synaptosome preparation was over 90% intact (Booth and Clark, 1978) although in the original paper the data was expressed as enzyme latency. The latency is calculated by merely dividing the total enzyme activity by the activity in the intact preparation. The higher the enzyme latency the more intact a preparation is. For ease of comparison the latency values for the synaptosome preparations under test in this study are also given in table 2.4.

2.3.3 Morphological assessment.

Electron microscopy was kindly performed on the various synaptosomes fractions by Mr Brian Young of the Institute of Neurology, Queen Square, London. Before fixation of synaptosome pellets, the synaptosomes were incubated in physiological control KPB at 37°C for 10 minutes. The synaptosomes were pelleted in buffered glutaraldehyde (pH 7.3).

Random sections of the entire pellet were examined in all cases. For all preparations the population appeared relatively homogenous throughout. Morphologically, all pellets consisted primarily of synaptosomes. Randomly cut sections of all different preparations indicated that the primary contaminant consisted of nonsynaptic membrane limited particles and membrane fragments. Small amounts of free mitochondria were evident. Myelin contamination was negligible in all cases. Synaptosomes appeared intact and because of the presence of EDTA in all isolation media, adhering postsynaptic material was rarely seen (Van Leeuwen et al., 1976). Most of the synaptosomes were very densely packed with synaptic vesicles and contained well-preserved intrasynaptosomal mitochondria. There appeared to be no obvious differences in the extent of contamination, type of contamination, or appearance of the synaptosomes between different age preparations.

2.3.4 Functional assessment - synaptosome respiration.

Table 2.5 indicates the rates of respiration of the different synaptosome preparations in a high potassium medium. The substrates used were pyruvate or glutamate. The high potassium medium enabled exogenous ADP to gain access through the opened synaptosomal plasma membrane and hence be available to the mitochondrial respiratory chain. The medium chosen is one that is used for respiration studies of isolated mitochondria. It is important to enable exogenous ADP to gain access to the intrasynaptosomal mitochondria so that the extent of coupling of respiration and oxidative phosphorylation can be established. The intrasynaptosomal mitochondria of all of the synaptosome preparations showed oxygen uptake when supplied with exogenous substrate. Furthermore they showed an increase in respiration when supplied with ADP, illustrating that mitochondria were coupled and functional. The respiratory control ratios were similar to those reported for adult synaptosomes in the original characterisation (Booth and Clark, 1978). Although these are lower than for isolated free and synaptic mitochondria this is to be expected because it is generally more difficult to conduct respiration studies with synaptosomes. Differences in the amount of protein and access of exogenous substrates to the mitochondria means that RCR's are often lower. However, the isolation of synaptic mitochondria from the synaptosome preparations would have been problematic. If the mitochondria from immature brain proved less functional than those from adult brain it would have been more difficult to dissect out whether this was due to the preparations themselves or to their reactions to the further isolation methods.

In summary synaptosomes from all age preparations proved to be functional to a similar extent, due to their ability to respire when supplied with exogenous substrate.

Assessing synaptosomal function: measured rates of oxygen uptake by synaptosomes.

	PNI	D 5	PNI	D10	PND15		PND20	
	st3	st 4	st 3	st 4	st 3	st 4	st 3	st 4
10 mM glutamate	4.9 ± 0.5	1.0 ± 0.2	6.4 ± 0.7	1.7 ± 0.2	7.6 ± 1.0	2.3 ± 0.4	9.3 ± 0.6	2.5 ± 0.3
10 mM pyruvate	7.1 ± 0.5	2.2 ± 0.5	7.2 ± 0.9	3.0 ± 0.3	9.2 ± 0.7	2.5 ± 0.3	11.2 ± 1.2	3.1 ± 0.3

Synaptosome respiration rates are shown. Oxygen uptake was measured at 30° C either in the presence of 250 μ M ADP (st 3) or in the absence of added ADP (st4). The results are expressed in units of ng atoms of O uptake/ min per mg of protein.

Results are expressed as the means \pm standard deviations for 4 separate preparations on different days.

2.3.5 Characterisation of the incubation conditions during periods of metabolic stress and under normal conditions

The oxygen levels in the control buffers were calculated from known equations and the oxygen levels in the buffers used for the incubation of synaptosomes under conditions of metabolic stress were assessed using a Clark type oxygen electrode.

Control incubations

In early, pilot experiments the Kreb's Phosphate Buffer (KPB) was equilibrated with 100% oxygen at 37°C for 1 hour. The pressure, in mmHg, of this 100% O_2 saturated gas can be calculated from the following information (Bachelard, 1976):-

vapour pressure of water at $37^{\circ}C = 47 \text{ mmHg}$

Therefore conversion of 100% O_2 at 37°C to mmHg in a water saturated solution occurs as follows:-

Standard atmospheric pressure = 760 mmHg water vapour pressure at $37^{\circ}C = 47$ mmHg Residual O₂ pressure = 760 - 47 = 713 mmHg

The oxygen concentration of the KPB buffer equilibrated with the 100% oxygen is equivalent to 760 torr (Ksiezak and Gibson, 1981). The oxygen content of this buffer can also be expressed by using calculations involving gas solubility relationships. For example, it is known that the volume of oxygen dissolved in aqueous KPB at 1 atmosphere at 37°C is 23.9 μ l/ml⁴. Using the following gas solubility relationships the oxygen concentration at standard atmospheric pressure can be calculated:-

1 μ l oxygen/ml = 1.430 μ g atoms oxygen/ml at 0°C (273° absolute)

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data taken from the Yellow Springs incorporated Biological Oxygen Monitor data manual

A correction for the temperature is necessary, therefore at 37°C:-

<u>23.9</u> = 21.0 μ l oxygen/ml [(273 + 37)/273]

So,

 $21.0 \text{ x } 1.43 = 30 \ \mu\text{g}$ atoms oxygen/ml.

1 μ g atom oxygen/ml = 0.031 μ mol/ml.

Therefore,

 $30 \ge 0.031 = 0.933 \ \mu \text{mol/ml}$, at 1 atmosphere (760 mmHg)

At 713 mmHg,

713/760 x 0.933 = 0.875 μ mol/ml = 875 μ M oxygen.

In later experiments the control KPB was equilibrated against air $(21\% O_2)$. The conditions labelled as mild metabolic stress were characterised as containing 11% O_2 oxygen, measured by the oxygen electrode. The conditions labelled as severe metabolic stress contained 4% O_2 . The oxygen levels of these can be calculated as above, to allow comparison with other literature.

<u>Oxygen levels in buffers when equilibrated</u>						
	percentage	torr	mmHg	٨L		
control	21%	160	150	184		
'mild' stress	11%	83	-	96		
'severe' stress	4%	30	-	35		

These calculated values are similar to others quoted in the literature, for example air saturated Kreb's Ringer solution at 37°C was taken to be 200 μ M by Park *et al.*, (1987), and 100% oxygen saturated KPB was estimated to be 900 μ M by Boakye (1992) at 37°C.

In all of the metabolic stress incubations where oxygen became limiting for the synaptosomes, anoxia was reached by 15 minutes of incubation, due to active synaptosomal respiration. This was assessed by use of the oxygen electrode in synaptosome preparations from all age rats. All of the different protein concentrations used in the variety of experiments presented throughout this thesis were also tested, to ensure that by the first time-point of measurement conditions were anoxic in all experiments.

As detailed in section 2.2.7.b some of the incubations were oxygen depleted by addition of 0.05 mM sodium dithionite. Concentrations below 1 mM of this compound have been assessed by other authors to deplete oxygen but not lead to other effects which are unrelated to anoxia (Bright and Ellis, 1992; Pang and Eyzaguirre, 1993). However, to ensure this was the case for the experiments presented in this thesis the following procedure was carried out for all experiment groups (eg. calcium measurements, or nitrite/nitrate measurements):-

control KPB to be used for one of the control incubations was firstly oxygen depleted by addition of 0.05mM sodium dithionite. This buffer aliquot was then gassed with 100% oxygen to replete the oxygen content of the buffer (as assessed by the oxygen electrode). This control buffer was equilibrated against air at 37°C alongside the normal control KPB, and was used for one of the control incubations on that day.

This was repeated at least 3 times for each of the different studies undertaken in this thesis. In all of the studies, there was no significant difference between these results and those from the normal control incubations, indicating that the presence of 0.05 mM sodium dithionite *per se* had no influence on the anoxic incubations.

DISCUSSION

2.4 Discussion

2.4.1 Comparison between adult and immature synaptosomes

The primary consideration in the research undertaken in this thesis is whether a valid comparison between adult and neonatal nerve terminals can be conducted. It is important to ascertain that responses of adult and neonatal synaptosomes to ischaemia and related conditions are not due to preparative differences which would lead to variation in the status of control synaptosomes. Therefore the studies presented in this chapter were undertaken to fully characterise the status of neonatal synaptosomes and compare them with adult synaptosomes prepared as in the original method (Booth and Clark, 1978).

Using the modified Ficoll gradient for immature rats and the original gradient for adult rats, the purity of synaptosomes prepared from all age rats was within an accepted level (McMahon and Nicholls, 1991), and was not significantly different between any of the ages. Contamination by non-synaptic mitochondria was less than 10% in all preparations, therefore ensuring that interpretation would not be significantly complicated by the presence of a large amount of non-synaptic mitochondria. All synaptosome preparations were more than 90% intact and showed minimal myelin contamination. Free membrane contamination was comparable with the original adult preparation (Booth and Clark, 1978).

Synaptosomes prepared by these methods were assessed to be functional due to their oxygen consumption and hence respiration under appropriate conditions. There was a distinct developmental profile with the rates of respiration, as would be expected because of the development of enzymes associated with energy producing metabolic pathways during postnatal development (Clark *et al.*, 1994; Almeida *et al.*, 1995). However, synaptosomes of all ages exhibited active respiration and also showed similar stimulations to the original adult preparation upon addition of ADP.

Brain mitochondria exhibit much higher specific activities to oxidise the substrates used in these studies (glutamate and pyruvate) than synaptosomes, and therefore even a few percent contamination of the synaptosomes by free mitochondria will contribute a disproportionate increase in oxygen uptake on the addition of ADP. However, on comparison with literature the rates of respiration here are much lower than some reported, for example Verity (1972) showed 49 ng atoms of O/min per mg of protein at 30°C for glutamate and ADP. The respiration rates reported here are comparable with those of the original method for adults (Booth and Clark, 1978) suggesting that the amounts of free mitochondrial contamination are similar between these preparations.

When the original Ficoll gradient (Booth and Clark, 1978) was used for the preparation of immature synaptosomes, the purity of the neonatal preparations was significantly different from that of the adult synaptosomes. This illustrates the importance of optimising a preparative method for application to different tissues or as in the case here to the same tissue at a different developmental stage. The modified Ficoll gradient used here for the preparation of neonatal synaptosomes has not been reported elsewhere in literature. Following characterisation of the preparations it became clear that neonatal and adult synaptosomes prepared by the modified and original Ficoll gradients, respectively are comparable and that further work comparing and contrasting the responses of these isolated nerve terminals to conditions of metabolic stress would be valid.

2.4.2 Incubations conditions used to induce in vitro metabolic stress

This study involved insults that resulted in complete glucose lack and a low residual oxygen availability to isolated nerve terminals. During all periods of incubation the residual oxygen would be consumed by actively respiring nerve terminals attempting to conserve energy. This is an *in vitro* study and these conditions may differ slightly from those occurring in an *in vivo* ischaemic or hypoxic episode. However, there can be a variety of conditions arising during *in vivo* ischemic models.

For example in some models there can be a residual blood flow throughout the whole ischaemic episode, whereas other models of decapitation for example can lead to complete cessation of blood flow. Therefore the limitations of the model used in this study do not preclude valid conclusions and comparisons with other studies from being drawn.

Some authors have investigated the effects of inhibition of aerobic and anaerobic metabolism in the brain by utilising the chemicals iodoacetate, rotenone and/or sodium cyanide (Bickler et al., 1993; Uto et al, 1995). Whilst it has been stated that these chemical forms of anoxia or ischaemia cause neurones to exhibit characteristics similar to those of energy independent ischaemic cell death in vivo (Uto et al., 1995), some caution with these methods is required. The use of cyanide and other toxins such as rotenone and iodoacetate which interfere with the mitochondrial electron transport chain or glycolysis are unlikely to be truly analogous to conditions of hypoxia or ischaemia (Bachelard et al., 1993). This is primarily because these metabolic toxins may interfere with enzymes of diverse metabolic pathways. For example, a potentially pathological effect of iodoacetate is the inactivation of thiolcontaining enzymes which play an important role in the scavenging of free radicals (Uto et al., 1995). Similarly, the metabolic poison cyanide can lead to increased free radical production from the mitochondrial respiratory chain. Studies using cyanide to mimic anoxia (Ashley et al., 1984; Gibson et al., 1989) have been found to report different results to studies using alternative methodology (Dagani et al., 1989; Boakye et al., 1990). These differences may well arise from the effects of cyanide on enzymes other than those of the mitochondrial respiratory chain and also from the fact that in the presence of cyanide the ability of cells to utilise oxygen becomes irreversibly inhibited even though adequate oxygen tension is available. The studies presented in this thesis do not suffer from these problems because the oxygen tensions are truly decreased and more closely represent a valid hypoxic or ischaemic episode.

The oxygen tensions in the ischaemic and anoxic incubations at the start of the incubation period were either 11% in the original experiments or 4% in the later (see page 53) experiments (Hence at the start of the incubation period the synaptosomes were hypoxic. Although the oxygen tensions at which effects on cerebral intermediary metabolism can first be detected are lower than those used here (below 25 torr)

(Luft, 1965; Park *et al*., 1987) it is well documented that other nerve terminal function is impaired by levels of hypoxia milder than those at which changes in the energy state can be observed (Gibson and Blass, 1976; Booth *et al.*, 1983).

Care must be taken when comparing the oxygen tensions of hypoxic conditions which induce a metabolic response. Synaptosomes and brain slices differ in their responses to reduced oxygen tensions (Ksiezak and Gibson, 1981; Park et al., 1987). While the first metabolic changes in brain slices have been observed at 152-53 torr, in synaptosomes these same metabolic variables remained unchanged within a range of 760 - 19 torr. The differences in metabolic response may be explained in part by a difference in the availability of oxygen (Ksiezak and Gibson, 1981). However, the metabolic rate and hence oxygen requirement of synaptosomes and brain slices are also different (Cremer, 1964; Booth and Clark, 1978). Thus, the differences in response between synaptosomes and brain slices have been suggested to originate from basic metabolic differences between these preparations (Ksiezak and Gibson, 1981). Indeed, under 100% oxygen ATP levels, ATP/ADP ratios and CO₂ production are different between these preparations (Ksiezak and Gibson, 1981). It is accepted protocol to expose brain slices to five times the oxygen concentration in vivo to offset diffusion difficulties with brain slices (Park et al., 1987). There is not the same problem with diffusion in the synaptosome preparation, however many researchers incubate synaptosomes under 100% oxygen (Boakye et al., 1992). In the studies here there was no significant differences in the metabolic characteristics of synaptosomes, including ATP levels and mitochondrial enzyme activities, if the synaptosome preparations were incubated under 100% oxygen or air.

The Model

In the metabolic stress experiments in this thesis involving oxygen depletion, synaptosomes became anoxic by 15 minutes of incubation in all preparations. The main emphasis of this thesis is the comparison of the response of adult and neonatal nerve terminals to ischaemia and reperfusion, with additional comparisons of the response to the anoxic component of ischaemia. The incubation conditions used have been fully defined in terms of oxygen and glucose concentrations and are identical for adult and neonatal synaptosomes. Therefore, although this is an *in vitro* study which may differ from *in vivo* ischaemia in a number of respects, the direct comparison between adult and neonatal nerve terminals under the conditions here is valid and may elucidate important and useful biochemical information.



"A single idea of a single brain is mightier than Niagara, for we can chain it and use it."

J. Brierly

<u>Chapter 3</u>

ISCHAEMIA, CALCIUM AND GLUTAMATE

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INTRODUCTION

<u>CHAPTER 3</u>

ISCHAEMIA, CALCIUM AND GLUTAMATE

3.1 Introduction

3.1.1 Calcium - an introduction

Calcium is the most abundant cation in vertebrates (20-30 g/kg body weight, in humans) (Pozzan *et al.*, 1994). Most of the Ca²⁺ of higher organisms is immobilised in the bones and teeth as hydroxyapatite $[Ca_{10} (PO_4)_6 (OH)_2]$. A much smaller amount of Ca²⁺ is contained in the extracellular and intracellular fluids of the body (Carafoli, 1987). The concentration of Ca²⁺ in the extracellular compartment is controlled by the movement of Ca²⁺ in and out of the bone deposits, and is fixed at about 3 mM, of which approximately half is ionised Ca²⁺ (Pozzan *et al.*, 1994). It is the intracellular Ca²⁺ which performs the fundamental task of carrying signals to a large number of biochemical activities in the various subcellular compartments. Hence Ca²⁺ acts as a ubiquitous intracellular signal. The precise relationship between extracellular and intracellular Ca²⁺ is not completely understood, but the maintenance of the extracellular reservoir of Ca²⁺ within a narrow concentration range ensures that a constant source of Ca²⁺ will always be available to cells (Borle, 1981; Meyer, 1989).

There is an abundant and comprehensive literature available on the intricate details of calcium homeostatic mechanisms. A detailed treatment is beyond the scope of this introduction and readers are directed to standard biochemistry textbooks and a number of excellent reviews (Carafoli, 1987; Meyer, 1989; Miller, 1991; Pozzan *et al*, 1994). A superficial coverage of some areas of calcium homeostasis will be undertaken to aid the reader. However, this introduction will primarily focus on those areas of neuronal calcium homeostasis which pertain to calcium involvement in ischaemic neuronal cell death.

3.1.1.a Characteristics of cytosolic calcium

Cytosolic calcium concentration $([Ca^{2+}]_c)$ is much lower in all types of animal cells in which it has been measured than the free Ca²⁺ concentration in serum or interstitial space $([Ca^{2+}]_e)$. $[Ca^{2+}]_e$ levels are found in the low millimolar range (approximately 1 - 3 mM) in humans (Carafoli, 1987; Meyer, 1989; Pozzan *et al.*, 1994). Typical resting $[Ca^{2+}]_c$ levels are in the nanomolar range, of approximately 100 nM (Fiskum, 1985), although reported values do differ between 50 nM (Gunter *et al.*, 1990) and 400 nM (Fontana and Blaustein, 1995), depending on the cell type and the method of measurement employed. A Ca²⁺ concentration gradient of over four orders of magnitude thus exists across the plasma membrane.

The low level of cytosolic Ca^{2+} is altered under two conditions;

Firstly, under pathological conditions, such as ischaemia, when the plasma membrane becomes permeable to Ca^{2+} . When inward leakage of Ca^{2+} increases, a new steady state may be reached between influx and efflux through specific Ca^{2+} pumps and exchangers. When cytosolic Ca^{2+} exceeds 200-300 nM, mitochondria begin to accumulate Ca^{2+} (Borle, 1981; Meyer, 1989; McCormack *et al.*, 1990; Gunter *et al.*, 1994;), and it is suggested that they can accumulate enough Ca^{2+} to "uncouple" oxidative phosphorylation (Farber, 1981).

Secondly, under physiological conditions, pulses of Ca^{2+} are often observed following depolarisation of the plasma membrane, or exposure to a primary messenger (Carafoli, 1987; Berridge, 1994). The characteristics of these pulses vary considerably between cell types in terms of frequency of pulse, pulse duration, and the intensity (i.e. Ca^{2+} concentration reached) (Berridge, 1994). These physiological Ca^{2+} signals have been associated with a myriad of functions.

3.1.1.b Calcium as a second messenger

The high concentration of Ca^{2+} in the extracellular milieu as compared with the intracellular Ca^{2+} level, and the resultant large electrochemical force on Ca^{2+} are convenient to its role as an intracellular second messenger (Harris *et al*, 1981). This is because even minor changes in the permeability of the plasma membrane, in response to a primary physiological stimulus, will produce very significant fluctuations in cytosolic concentrations. In order to function effectively as an intracellular messenger Ca^{2+} must undergo large fluctuations in concentration around the targets of the messenger function (Carafoli, 1987). Significant concentration swings can only be achieved rapidly if Ca^{2+} is poised to exert the signalling function in a very low concentration range.

Hence, it is imperative for cells to have a number of ways to keep their ionised internal concentration of Ca^{2+} very low (Harris *et al.*, 1981; Meyer, 1989).

Turning specifically to the neurone, fluctuations in the neuronal $[Ca^{2+}]_c$ act as signals which are enormously important for a variety of reasons (Miller, 1990). Ca^{2+} is vital for a number of neuronal processes including:

- triggering neurotransmitter release (Smith and Augustine, 1988)
- control of neuronal excitability (Marty, 1989)
- integration of electrical signals (Llinas, 1988; Mart, 1989)
- synaptic plasticity of various types (Malenta *et al.*, 1989)
- gene expression (Szekely *et al.*, 1990)
- metabolism (McCormack *et al.*, 1990).

The complexity of the neurone make the study of neuronal Ca^{2+} signals particularly challenging. There is no universal pattern of neuromodulation shared by all neurones, and even within a particular neurone the segregation of various functions into different sections of that neurone requires it to segregate Ca^{2+} signals in a similar manner (Tank *et al.*, 1988; Ross *et al.*, 1990).

Hence, neuronal Ca^{2+} second messenger function and homeostasis is involved and complex. A Ca^{2+} signal within a neurone initially requires the activation of processes that increase $[Ca^{2+}]_c$ and then that subsequently decrease it, thus returning $[Ca^{2+}]_c$ to resting levels. We firstly turn to the processes which increase $[Ca^{2+}]_c$.

3.1.2. Calcium influx through neuronal Ca²⁺ channels

In many instances increases in Ca^{2+} are produced by transiently increasing the Ca^{2+} permeability of the plasma membrane. This is achieved by opening various types of Ca^{2+} permeable ion channels that allow Ca^{2+} to enter the cell down its electrochemical gradient (Mayer and Miller, 1990; Miller, 1990; Miller and Fox, 1990; Llinas *et al.*, 1992). These Ca^{2+} permeable channels may be gated by changes in membrane potential (Miller and Fox, 1990) and are thus termed **voltage-gated**. Alternatively they may be **ligand-gated**, when their integral ion channels are opened by the binding of neurotransmitters (Mayer and Miller, 1990; Miller, 1990) or other physiological primary inputs (Tsien and Tsien, 1990; Tsien *et al.*, 1991). These two categories are not mutually exclusive since some channels may require both ligand binding and depolarisation to open (Nicholls, 1994), such as the NMDA receptor (Mayer *et al.*, 1984).

In some instances, the segregation of channels into various regions of the neurone may serve to produce highly localised Ca^{2+} influx and consequently highly localised Ca^{2+} signals (Miller, 1987; McMahon and Nicholls, 1990; Westenbroek *et al.*, 1990).

A great deal is now known about the properties of neuronal Ca^{2+} channels, although key questions about them still remain (Miller, 1991). These properties have been extensively reviewed (Miller, 1987; Tsien and Tsien, 1990; Tsien *et al.*, 1988).

3.1.2.a Voltage-gated calcium channels

In 1987, Fox *et al.* proposed that voltage-activated Ca²⁺ channels (VACCs) could be broadly classified into three groups, depending on their electrophysiological characteristics. This original classification was derived from studies on neuronal cell bodies, and it does not necessarily follow that the channels in the presynaptic nerve terminal conform to this classification. However, a definitive classification of VACCs will only be possible when the individual peptides have each been purified, sequenced and expressed *in vitro*. Such work is still ongoing and the current classification of VACCs relies on a combination of pharmacology combined with electrophysiological criteria. The situation is unclear in terms of the properties of these channels in presynaptic nerve terminals because the electrophysiological criteria are defined for the neuronal cell body and do not take account of channels on the nerve terminal.

The original classification of VACCs was into L-type, T-type or N-type channels (Fox, *et al.*, 1987). Since these provisional classifications P-type and Q-type channels have also been defined (Nicholls, 1994) according to the sensitivity of these channels to specific neurotoxins. The L-channel is the only one for which complete molecular information is available and it has become apparent that the classification of the other channels is incomplete (Siesjö, 1992). This is especially apparent in the case of channels associated with nerve terminals, which except in atypical cases are not accessible to electrophysiological classification.

Studies of VACCs at mammalian synapses have become a major area of investigation (McCleskey, 1994). As synaptosome preparations come from many different kinds of synapses, they cannot divulge the pharmacology of particular synapses. However, these preparations have yielded some useful information on presynaptic Ca^{2+} channels. Turner *et al.* (1992) demonstrated that P-channels play a major role in glutamate release from mammalian synaptosomes. They also found that no combination of blockers could stop all of the glutamate release from synaptosomes, thus indicating that other Ca^{2+} channels, for which there are no known inhibitors, are important in neurotransmitter release in the mammalian central nervous system.

The roles of the many VACCs are becoming evident. P and N channels appear most important in mammalian synaptic transmission, although their importance varies depending on the particular synapse (McCleskey, 1994). L-channels are relatively unimportant for neurotransmitter release although it is suggested that they may be located nearby, but not immediately at, presynaptic active zones and thus they could serve as a 'backdoor' for Ca²⁺ entry to the active zone when they are sufficiently stimulated. Other Ca²⁺ channels, not yet pharmacologically defined are also important in synaptic neurotransmission (McClesky, 1994).

3.1.2.b Ionotropic calcium channels

Ligand-gated ion channels are also termed ionotropic receptors (Nicholls, 1994). Ionotropic receptors have integral ion channels and act in a submillisecond timescale. Cytoplasmic Ca^{2+} can be modulated by such ionotropic receptors in two ways (Meyer, 1989). Firstly, for those ionotropic receptors with predominantly monovalent cation permeability, Na⁺ will enter the cell following ligand binding to the receptor, and membrane depolarisation will ensue as a result. If this depolarisation is sufficient to activate voltage-activated Ca²⁺ channels in the vicinity of the ionotropic receptor then Ca²⁺ will enter the cell. Alongside this somewhat indirect effect some ionotropic receptors can conduct Ca^{2+} directly through their integral ion channels, thus elevating Ca²⁺ independently of voltage-gated channel activity (Cotman and Iversen, 1987). Such ionotropic receptors include the glutamatergic NMDA receptor and some isoforms of the AMPA/KA (non-NMDA) receptors (Hollman et al., 1991), although most kainate and AMPA receptors usually allow movement of Na⁺, K⁺ and H⁺ but not Ca²⁺ (Mayer and Westbrook, 1987; Siesjö, 1992). Calcium influx through such ionotropic Ca²⁺ channels as the NMDA receptor has been a widely implicated mechanism for neuronal ischaemia/reperfusion damage (reviewed in Pulsinelli, 1992; Siesjö, 1992).
3.1.2.c Metabotropic receptors

Metabotropic or G-protein coupled receptors can also affect Ca^{2+} flux across the plasma membrane, and can also lead to the release of Ca^{2+} from internal stores. In contrast to ionotropic receptors, metabotropic receptors are slower acting but they can initiate a wide range of modulatory responses in the neurone. In the last years there has been an enormous proliferation in the number of G-protein coupled receptors which have been identified (Nicholls, 1994). Metabotropic receptors activate a range of heterogenous trimeric G-proteins and allow the activated α -subunit of the G-protein to dissociate from the $\beta\gamma$ -subunits and interact with an effector system. The G-protein activation is very rapid and many molecules of G-protein can be activated by a single activated metabotropic receptor. The effector systems may be enzymes such as adenyl cyclase or ion channels such as voltage-activated Ca^{2+} channels.

This receptor type can affect Ca^{2+} fluxes and levels in a number of ways. Gproteins activated by metabotropic receptors can activate VACCs (G_s) or inhibit such channels (G_o, G_{i1}, G_{i2}, G_{i3}). The presence of presynaptic metabotropic receptors which can modulate Ca^{2+} entry allow a fine control of depolarisation at individual terminals (Nicholls, 1994). The regulation of Ca^{2+} channels can be by a mechanism described as 'membrane delimited', which is a direct interaction between G-proteins and ion channels. There are a number of ways in which G-proteins activated by presynaptic metabotropic receptors may directly control ion channels. The G-protein may regulate the presynaptic Ca^{2+} channel itself, by affecting the probability of opening, the voltage-dependency or some other kinetic parameter. Alternatively, the G-protein may modulate the permeability of presynaptic Na^+ or K^+ channels and hence control the duration or intensity of the action potential. This would then indirectly regulate Ca^{2+} entry through the voltage-activated Ca^{2+} channels.

G-proteins activated by presynaptic metabotropic receptors can also control ion channels via generated second messengers and kinases. The activated α -subunits of G-proteins can diffuse and collide with enzymes which synthesise or degrade second messengers. Although somewhat simplified, in terms of its influence on Ca²⁺

homeostasis, the enzyme phosphatidylinositol specific phospholipase C (PI-PLC) can be effected in this manner, and can then hydrolyse the phospholipid phosphatidylinositol 4,5 bisphosphate (PIP₂) to generate the second messengers diacylglycerol (DAG) and a soluble inositol 1, 4, 5 trisphosphate (1,4,5 IP₃). The latter second messenger can release Ca²⁺ from intracellular stores, whereas DAG plays a central role in the activation of |**protein** kinase C (PKC) isoenzymes. Calcium, Na⁺ and K⁺ channels often contain consensus sequences which may be phosphorylated by PKC and thus modulated to some degree. There is no consistent effect of PKC activation on neuronal Ca²⁺ channels. Inhibition, no effect and activation have all been reported (Coffey *et al.*, 1993; Nicholls, 1994) and further research is currently focusing on this area.

3.1.3 Calcium efflux through the plasma membrane

Following a period of activity, Ca^{2+} that has entered a neurone must ultimately be expelled across the plasma membrane. This involves Ca^{2+} moving out of the cytosol against a large electrochemical gradient and therefore it must be linked in some way to the expenditure of energy. There are two processes which have been identified as being capable of producing net Ca^{2+} transport out of the neurone (Baker and DiPolo,1984; Carafoli, 1987; Blaustein, 1988). The first of these is the Ca^{2+} -ATPase and the second system involves coupled Na⁺/Ca²⁺ exchange.

3.1.3.a The Ca²⁺-ATPase

This Ca^{2+} pump was first discovered in 1966 in erythrocytes (Schatzmann and Bürgin, 1978), and was subsequently found in a number of other eukaryotic plasma membranes (reviewed in Carafoli, 1990). Following its discovery in 1966, for a number of years the plasma membrane used in most studies of the Ca^{2+} -ATPase was the erythrocyte, and most of the properties of this enzyme have been established on this preparation (Carafoli, 1987). Only relatively recently the studies were extended to other cell types and it was found that the pump is ubiquitously distributed in eukaryotic cells. The pump has been found in cultured neurones (DiPolo, 1979). It was first purified by Niggli *et al.* in 1979 using a calmodulin affinity column. Subsequent

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biochemical and molecular biological studies have revealed the sequence of the pump from several tissues including the brain (Shull and Greeb, 1988; Verma *et al.*, 1988; Greeb and Shull, 1989).

The Ca²⁺-ATPase of plasma membranes is clearly related to other ion translocating membrane ATPases including the Na⁺/K⁺ATPase, and the Ca²⁺ pump from the sarcoplasmic reticulum in muscle, all of which are known as P-type ion pumps (Carafoli, 1988; Schatzmann, 1989). All of these P-type pumps share similar mechanisms of action. The Ca²⁺ pump is a high-affinity enzyme which interacts with Ca²⁺ with a K_m well below 1 μ M (estimated to be approximately 0.1-0.2 μ M (Miller, 1991), but which transports Ca²⁺ with a low total capacity. It seems most likely that the resting cytosolic Ca²⁺ concentration is set by this Ca²⁺ pump. The Ca²⁺ pump presumably removes low levels of Ca²⁺ from the cell (Miller, 1991). Synaptosomal studies (Sanchez-Armass and Blaustein, 1987) have concluded that the Na⁺/Ca²⁺ exchange system is generally utilised for the clearance of large Ca²⁺ loads following a period of stimulation, whereas the Ca²⁺ pump is capable of working at the lower end of the physiological range and thus "fine-tuning" the [Ca²⁺]_c around the normal resting level.

Of great importance is the fact that the Ca^{2+} pump is activated by calmodulin (Carafoli, 1988, 1989). Calmodulin stimulates both the rate of phosphorylation and of dephosphorylation of the enzyme (Jeffery *et al.*, 1981). The effects of calmodulin are critical in allowing the pump to function in the physiological range of $[Ca^{2+}]_c$ (Carafoli, 1991). When no calmodulin is bound the enzyme has a K_m of 10-20 μ M. When $[Ca^{2+}]_c$ rises, calmodulin is activated and binds to the enzyme, thus increasing its maximum K_m for Ca^{2+} some 20 to 30 times. The pump can also be activated by other factors in addition to calmodulin, including acidic phospholipids and polyunsaturated fatty acids (Carafoli, 1991).

3.1.3.b Sodium/calcium exchange

The other plasma membrane system, aside from the Ca^{2+} -ATPase, which is capable of producing net Ca^{2+} transport out of the neurone is the Na⁺/Ca²⁺ exchange system (Carafoli, 1987; Blaustein, 1988).

The Na⁺/Ca²⁺ exchange is particularly active in excitable plasma membranes, like those of neurones. The system is a carrier-mediated transport process in which the movement of Ca²⁺ ions across the plasma membrane is directly coupled to the movement of Na⁺ ions in the opposite direction. The exchange system is electrogenic with a stoichiometry of $3Na^+$ per Ca²⁺ (Reeves, 1990).

The realisation that the movement of Ca²⁺ across the plasma membrane could be directly linked to that of Na⁺ was first suggested in the late 1950's (Luttgau and Niedergerke, 1958) and was subsequently developed in studies during the late 1960's. These experiments were conducted on cardiac muscle (Reuter and Seitz, 1968) and on the squid giant axon preparation (Baker and Blaustein, 1968). From the original studies other research soon showed that the exchanger, although undoubtedly most active in heart and brain cells, is also present in nonexcitable tissues (Carafoli, 1987). The properties of the Na⁺/Ca²⁺ exchange system have been extensively characterised in the squid giant axon preparation (DiPolo and Beauge, 1990). However, results with squid axons have also been shown to generally hold true using synaptosomal preparations (Nachshen, 1985; Sanchez-Armass and Blaustein, 1987).

Logically, the role of the Na⁺/Ca²⁺ exchanger should be the control of the intracellular free Ca²⁺ concentration. The commonly held view is that Na⁺/Ca²⁺ exchange provides a relatively low affinity, but high capacity, system for the expulsion of Ca²⁺ from the neurone and that this system is generally utilised for the clearance of large Ca²⁺ loads following a period of stimulation (Miller, 1991).

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The exchange system can mediate Ca^{2+} fluxes in either direction across the plasma membrane, depending upon the prevailing conditions. The major physiological role of the exchange system is to pump Ca^{2+} out of the cell, using the inwardly-directed electrochemical gradient for Na⁺as an energy source (Reeves, 1991). Na⁺/Ca²⁺ exchange activity is determined by three factors:

- the transmembrane Na⁺ gradient;
- the transmembrane Ca²⁺ gradient;
- the membrane potential.

The reason for the first two is obvious. The reason for the third is that the exchanger carries more charge on its Na⁺ transporting arm than on its Ca²⁺ transporting arm, i.e. 3 Na⁺ and $1Ca^{2+}$, therefore 3 positive charges for 2 positive charges and thus a net movement of one positive charge. Thus the exchange will produce a current when it operates, and will be partially influenced by the prevailing membrane potential. The predicted effect of membrane potential on the activity of the exchanger is that Ca²⁺ extrusion is favoured at negative membrane potentials and inhibited at depolarised potentials. This effect has been observed in synaptosomes (Turner and Goldin, 1985; Taglialatela, 1990).

The activity of the Na⁺/Ca²⁺ exchange system has been found in neuronal tissue as made clear by experiments with squid axons and synaptosomes. However, several studies have examined the activity of the system in buffering Ca²⁺ loads in the cell bodies of cultured neurones (Thayer and Miller, 1990) and acutely isolated rat neurones (Duchen *et al.*, 1990) and have failed to find evidence for the operation of this system. This is possibly because the exchanger is preferentially localised in nerve terminals and has low activity in the cell soma (Miller, 1991).

Several studies have indicated that $[Ca^{2+}]_c$ and $[ATP]_c$ have separate regulatory roles to play in the activity of the Na⁺/Ca²⁺ exchanger (Baker and DiPolo, 1984). At higher ATP cytosolic concentrations the exchanger exhibits high affinity for Ca²⁺. Indeed, ATP increases the Ca²⁺ affinity of the exchanger about tenfold, and the sodium

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affinity about twofold (Carafoli, 1987), possibly due to the presence of a calmodulin directed phosphorylation step. Although activators have been identified, no specific inhibitor for the exchanger has been found to date, although non-specific compounds such as the anthracycline Doxorubicin and its derivatives do possess inhibitory activity (Carafoli, 1987).

Although *in vivo* experimental evidence for the operation of the exchanger in the reverse mode to produce Ca^{2+} influx is sparse, a model for the exchanger activity in nerve terminals has been proposed (Blaustein, 1985) which includes Ca^{2+} influx as well as extrusion. Calcium influx can be clearly observed in squid axons and synaptosomes (Turner and Goldin, 1985). In this proposed model, as depolarisation occurs the membrane potential favours Ca^{2+} influx however the $[Ca^{2+}]_c$ is too low to occupy the regulatory site in the exchanger. If Ca^{2+} enters the neurone through VACCs or is released from internals stores then the Ca^{2+} regulatory site becomes occupied and extracellular Ca^{2+} will also enter the cytosol via the exchanger. When the membrane potential reverts to negative, Ca^{2+} extrusion is favoured and will occur until the $[Ca^{2+}]_c$ falls below the level at which Na⁺/Ca²⁺ exchange operates effectively.

It should be noted that the precise role of this system is potentially very flexible, and conditions such as ischaemia may have a profound influence on the function of this exchanger at any one given moment.

3.1.4 Neuronal calcium buffers

There are a number of processes which serve to terminate or "buffer" cytosolic Ca^{2+} signals. Calcium extrusion across the plasma membrane has already been considered, but there are other mechanisms which are of importance in the nerve terminal.

These are:-

- 1] The mitochondria
- 2] The endoplasmic reticulum and associated organelles
- 3] Calcium binding proteins

All of these mechanisms exist in nerve terminals and are capable of handling a Ca^{2+} load. Processes 1 and 2 can also act as sources of Ca^{2+} to potentially increase $[Ca^{2+}]_c$ as well as acting as buffers to terminate Ca^{2+} signals. It is clear that the buffering mechanisms **can** function, however it is not totally clear **when** they function in nerve terminals.

3.1.4.a The mitochondria

The notion that isolated mitochondria accumulate large amounts of Ca^{2+} goes back to the 1950s and 1960s (Slater and Cleland, 1953; Vasington and Murphy, 1962). It is now well known that mitochondria can take up Ca^{2+} into the mitochondrial matrix (Crompton, 1985; Denton and McCormack, 1985; Nicholls, 1985, 1986; Carafoli, 1987; Brand, 1988; McCormack and Denton, 1990; McCormack *et al.*, 1990). Indeed, under appropriate experimental conditions mitochondria can be shown to accumulate huge quantities of Ca^{2+} (Miller, 1991). However, there has been a constant debate about the physiological significance of this Ca^{2+} uptake system. In order to understand these issues we will first briefly review the components concerned in the movement of Ca^{2+} in and out of the mitochondria (Brand, 1988; Carafoli, 1987; Crompton, 1985, 1990; Hansford, 1985; Nicholls, 1985, 1986; Denton and McCormack, 1985, 1990; McCormack and Denton, 1990; McCormack *et al.*, 1990).

Mitochondrial calcium transport systems

The calcium transport systems of the mitochondrial inner membrane of mammalian tissues is illustrated in figure 3.1. Mitochondrial calcium transport is mediated by a complex system comprising at least three separate mechanisms. Calcium influx is primarily via a very fast Ca^{2+} uniporter and efflux is via both Na⁺-independent and Na⁺-dependent efflux mechanisms (Gunter and Pfieffer, 1990).



Figure 3.1 The Ca²⁺ transport systems of mitochondria

Calcium influx mechanisms

An electrophoretic uniporter is responsible for the uptake of Ca^{2+} into mitochondria. This is driven by the membrane potential component of the protonmotive gradient set up by proton extrusion by the respiratory chain (Lehninger et al., 1967). The uniporter can be inhibited physiologically by magnesium (McCormack *et al.*, 1990). The first evidence that the Ca^{2+} influx mechanism is a uniporter came from studies showing that Ca²⁺ influx utilises an internally negative membrane potential (Scarpa and Azzone, 1970) and is not coupled to the transport of any other ion (Selwyn *et al.*, 1970). Although no other ions are coupled to Ca^{2+} influx, in practice it is accompanied by the movement of phosphate anions. Indeed, above certain levels of Ca^{2+} uptake, Ca^{2+} and phosphate are believed to form some sort of complex within the mitochondrial matrix (Miller, 1991), the nature of which remains uncertain at his time. Nevertheless this complex is fully reversible which allows Ca²⁺ to leave the matrix once more under appropriate conditions (Zoccarato and Nicholls, 1982). Kapus *et al.*, (1991) have reported that the uniporter cannot sequester Ca^{2+} in the absence of a membrane potential, even in the presence of an eightfold Ca^{2+} concentration gradient. They concluded that the membrane potential maintains the uniporter in a transport-competent conformation. However, many workers have reported rapid uniporter-mediated release of Ca²⁺in the absence of a membrane potential. The uniporter is activated by ADP and Ca^{2+} (Gunter *et al.*, 1994). For a review of the known activators and inhibitors of the uniporter see Gunter and Pfieffer (1990). The mitochondrial uptake of Ca^{2+} in response to an increase in cytoplasmic $[Ca^{2+}]$ is driven by the protonmotive force and can reach very high levels. Indeed, in the face of a large cytoplasmic Ca^{2+} load, the entire activity of the respiratory chain can be diverted from the synthesis of ATP to the accumulation of Ca^{2+} by the mitochondria (Miller, 1991).

The number of uniporters in the mitochondrial membrane has been estimated by inhibitor titration to be as low as 0.001 nmol/mg protein (Reed and Bygrave, 1974). The uniporter has been suggested to be one of the fasted gated pores known (Gunter *et al.*, 1994), primarily because Ca^{2+} sequestration is extremely rapid

(Wolkowicz and McMillin-Wood, 1981). Numerous studies on the properties of the Ca^{2+} influx mechanism have been conducted (Gunter and Pfieffer, 1990). These studies include investigations into the calcium concentration dependence of uptake, the membrane potential dependence of uptake and the temperature dependence of uptake. In summary, such investigations have shown that the uniporter behaves like a very fast gated Ca^{2+} pore (Gunter and Pfieffer, 1990).

Calcium efflux mechanisms

The inner mitochondrial membrane also possesses systems for the egress of Ca^{2+} from the matrix in exchange for Na⁺ or H⁺ (Nicholls, 1986; Crompton, 1990). These sytems are known as the Na⁺-dependent and Na⁺-independent mechanisms respectively (Nicholls, 1986; Crompton, 1990). This outward transport of Ca²⁺ from the matrix requires energy either from the electrochemical gradient of a cotransported or exchanged ion, from chemical energy (eg. ATP hydrolysis) or from conformational energy transferred from some step in the electron transport chain for example. The necessary energy may actually be supplied by components from both the electrochemical gradient of an ion and from active sources.

Na⁺*-independent mechanism*

The Na⁺-independent egress mechanism is much slower than the Ca²⁺ influx mechanism (Wingrove and Gunter, 1986). It is inhibited by cyanide, low levels of uncouplers and very high levels of ruthenium red (Gunter and Pfieffer, 1990). Na⁺- independent Ca²⁺ efflux involves direct proton exchange and is considered as a selective carrier or gated pore occurring in the energised mitochondrion (McCormack and Denton, 1993). This egress pathway currently remains poorly characterised although it is known to be of much lower activity than the Na⁺-dependent pathway in brain mitochondria with physiological Ca²⁺loads (McCormack *et al.*, 1990). At present there remains very little substantial molecular detail about the Na⁺-independent Ca²⁺ transport proteins (McCormack *et al.*, 1992). Research has identified this system of Ca²⁺ egress as being either passive or active, depending on the prevailing conditions. Studies and calculations showed that there is sufficient energy under physiological

conditions for the Na⁺-independent mechanism to function as a passive $Ca^{2+}/2H^+$ exchanger. However, conditions were found in which this Na⁺-independent efflux of mitochondria pumped Ca^{2+} outwards against a concentration gradient, and required more energy than that available from the exchange of H⁺ (Gunter *et al.*, 1991). Evidence has accrued which shows that the most likely source of this extra energy is the electron transport chain (Gunter *et al.*, 1994). Thus, it seems that the Na⁺-independent mechanism can function as an active mechanism, even though under physiological conditions sufficient energy exists in the H⁺ electrochemical gradient to support passive exchange of Ca^{2+} for 2 H⁺.

Na⁺-dependent mechanism

In the nervous system Na⁺-dependent Ca²⁺ efflux is by far the dominant of the two egress systems (Miller, 1991). The Na⁺-dependent Ca²⁺ efflux mechanism has been identified as a Ca²⁺/2Na⁺ exchanger (Crompton *et al.*, 1977; Crompton *et al.*, 1978; Li *et al.*, 1992). Although this process is therefore electroneutral exchange, it is also driven by the respiratory protonmotive gradient, via subsequent re-export of Na⁺ by the much more active Na⁺/H⁺ exchanger (McCormack and Denton, 1993). As with the Na⁺-independent mechanism, under normal physiological conditions there should be enough energy available for exchange of Ca²⁺ with 2Na⁺ in a passive exchanger, but the system does not appear to function in this way (Gunter *et al.*, 1994). Studies have shown that this transporter demonstrates the ability to transport Ca²⁺ against an electrochemical gradient greater than that which could be achieved using only the energy from the electrochemical gradient of 2Na⁺ (Gunter *et al.*, 1994). It is thus suggested that this mechanism is an active rather than passive transporter *in vivo* (Gunter *et al.*, 1994).

Mitochondria and calcium accumulation

Two basic positions concerning the significance of the mitochondrial Ca^{2+} transport mechanisms have been discussed. The first is that accumulation of Ca^{2+} by the mitochondria helps to buffer large increases in cytoplasmic [Ca^{2+}], thus acting as a sink before subsequently releasing it again, allowing Ca^{2+} to be removed slowly

from the cell (Nicholls, 1985; 1986). In this way the mitochondria are viewed as being able to protect neurones from high sustained Ca^{2+} concentrations which can be pathological (Miller, 1991). On the other hand it has been argued that the mitochondrial Ca^{2+} uptake system could relay fluctuations in the cytoplasmic [Ca^{2+}] within the physiological range into the mitochondrial matrix where they would control the activity of several key matrix enzymes that play an important role in oxidative metabolism, (Denton and McCormack, 1985; 1990; McCormack and Denton, 1990). In fact it seems most likely that both of these processes occur and may be important under different circumstances.

Calcium has been reported to activate dehydrogenases associated with the TCA cycle, electron transport and the F_1 ATPase. The evidence is particularly strong that dehydrogenase Ca^{2+} activates pyruvate dehydrogenase and α -ketoglutarate *L*, leading to an increased rate of NADH and FADH₂ production (McCormack and Denton, 1994). Furthermore, there is also evidence that increases in NADH can lead to stimulation of oxidative phosphorylation (Gunter *et al.*, 1994). Hence there is growing evidence that the regulation of key matrix enzymes by micromolar concentrations of Ca^{2+} is an important physiological means of matching energy production with demand in stimulated cells (McCormack and Denton, 1993).

The precise relationship between the cytoplasmic $[Ca^{2+}]$ and the mitochondrial matrix $[Ca^{2+}]$ will depend on the relative activity of the influx and efflux pathways. For small increases in cytoplasmic Ca^{2+} (<1 μ M) the matrix $[Ca^{2+}]$ will be similarly increased. Increased Ca^{2+} uptake by the matrix will be followed by an increased rate of efflux. If the cytoplasmic $[Ca^{2+}]$ begins to rise further into the micromolar range the rate of Ca^{2+} efflux from the matrix becomes constant. Under these circumstances the mitochondria will begin to accumulate Ca^{2+} , as uptake will continue at very high levels alongside a constant rate of efflux. Calcium accumulation will continue until the cytoplasmic Ca^{2+} has fallen to a level at which the rate of mitochondrial Ca^{2+} influx and efflux match each other. This is known as the **set-point**. As the cytoplasmic $[Ca^{2+}]$ falls below this level due to other Ca^{2+} buffers within the cell, the Ca^{2+}

accumulated by the mitochondria will now exit, serving to slow the decline in the cytoplasmic $[Ca^{2+}]$. Finally, as the mitochondria are depleted of Ca^{2+} , the cytoplasmic $[Ca^{2+}]$ will fall to resting values once more. It should be emphasised that changes in the cytosolic $[Ca^{2+}]$ within the range 0.1 - 1 μ M produce similar changes in the matrix $[Ca^{2+}]$ and these changes are within the range that has been shown to activate mitochondrial enzymes involved in respiration. The linkage of neuronal activity and metabolism coupled through Ca^{2+} homeostasis is now well accepted (Miller, 1991). However, there is greater controversy concerning the operation of neuronal mitochondrial Ca^{2+} metabolism above the set point.

The accumulation of Ca^{2+} by mitochondria may occur to some degree when a neurone is operating under physiological conditions. In the case of mitochondria in the nerve terminal this latter property may contribute to a relatively long term enhancement of neurotransmitter release (Zucker, 1989). It is supposed that when action potentials are fired at frequent enough intervals buffering of the intracellular Ca^{2+} resulting from the first action potential will not be complete before the arrival of the second spike and subsequent Ca^{2+} influx. Thus, the second Ca^{2+} load will be added to any Ca^{2+} remaining from the previous influx. If Ca^{2+} rises above the set-point for a significant period of time, allowing mitochondrial Ca^{2+} accumulation to occur, then the period of potentiation could be greatly increased; ie. the length of the intracellular Ca^{2+} signal is greatly enhanced by the plateau operating at the mitochondrial set-point. In effect, the mitochondria are acting as a kind of synaptic memory storage system.

Pathological conditions

There is little doubt that neuronal mitochondria accumulate large amounts of Ca^{2+} in association with several pathological states (Choi, 1990; Meldrum and Garthwaite, 1990). In conditions of cellular Ca^{2+} overload mitochondria can accumulate Ca^{2+} , effectively allowing the cell some time to "bale itself out" (McCormack *et al.*, 1990). However, this property is not limitless and can lead to the damage of the mitochondria themselves (McCormack and Denton, 1993). This appears to occur by the opening , dependent in part on matrix $[Ca^{2+}]_{,}$ of a non-

specific pore in the inner membrane. This then allows the passage of all molecules up to and around 2,000 Dalton, and obviously leads to de-energisation of the mitochondria (Crompton et al., 1988). It has been proposed that opening of this pore, known as the permeability transition (PT) pore, can be source of irreversible injury (Gunter et al., 1994) and is enhanced by P_i and oxidative stress (Crompton et al., 1988). There has been a lot of interest in this mechanism, and recently it has been shown that cyclosporin A may prevent pore-opening and further that it may have cytoprotective effects in cardiac tissue or cells exposed to hypoxia/reoxygenation or ischaemia/reperfusion (Nazareth et al., 1991; Halestrap et al., 1993). Permanent opening of the PT destroys the mitochondrial membrane potential and therefore ADP phosphorylation. The resulting energy deprivation may result in cell injury and death (Gunter et al., 1994). There is considerable evidence, some direct, much of it currently indirect that an important source of both ischaemic and reperfusion injury is a population of mitochondria that have irreversibly undergone the PT (Gunter et al., 1994). However, there is currently little research specifically directed towards studying pore-opening in mitochondria from brain tissue.

3.1.4.b The endoplasmic reticulum

In addition to Ca^{2+} accumulation in mitochondria, Ca^{2+} accumulation in the neurone is also normally detected in organelles associated with the endoplasmic reticulum (ER). These organelles are found within various regions of the neurone including the soma (Hwang *et al.*, 1990), dendrites (Andrews *et al.*, 1987), axons (Henkart, 1980) and the presynaptic nerve terminal (Andrews *et al.*, 1987).

Early studies observed that microsomal preparations from neurones could take up Ca^{2+} in an ATP dependent manner (Trotta and DeMeis, 1975). This was gradually developed to the site of storage being associated with the ER (Henkart, 1980) and having properties which were similar to those of muscle sarcoplasmic reticulum (SR). The ER associated organelles are described as "cistern-like", and often appear as stacks which are connected by bridging structures and appear to be continuations of the ER (Miller, 1991). Several descriptions have noted that these organelles are often

found in close proximity to mitochondria thus possibly enabling the two organelles to cooperate in the control of Ca^{2+} homeostasis (McGraw *et al.*, 1980).

Several studies on neurones have histologically demonstrated the association of a Ca²⁺-ATPase with these organelles (Hartter *et al.*, 1987), and the presence of Ca²⁺ binding proteins within them (Hashimoto *et al.*, 1988). These intracellular Ca²⁺ storage sites are caffeine and ryanodine sensitive and appear to have a similar Ca²⁺ release channel to the muscle SR which is known as the "ryanodine receptor" (Imagawa *et al.*, 1987). Thus neurones appear to contain intracellular Ca²⁺ storage sites that may act either as neuronal Ca²⁺ buffers when empty, or as neuronal Ca²⁺ amplifiers when they are full.

The caffeine sensitive ER stores which are activated by changes in the $[Ca^{2+}]_c$ are not the only stores in neurones. It is well established that Ca^{2+} can also be regulated by the second messenger IP₃ (Berridge, 1987). IP₃ can be shown to release Ca^{2+} from intracellular stores in neurones (Nahorski, 1988), and it has been proposed that this IP₃ mediated Ca^{2+} release occurs from a unique organelle named a **calciosome** (Hashimoto *et al.*, 1988). The calciosome appears to be associated with the ER, to contain Ca^{2+} binding proteins and to possess an associated Ca^{2+} -ATPase.

Neurones clearly contain Ca^{2+} buffering organelles associated with the ER which may play important roles in processing and buffering Ca^{2+} signals and which could become targets for ischaemia/reperfusion perturbation.

3.1.4.c Calcium binding proteins

It is generally thought that Ca^{2+} entering the nerve terminal would initially encounter buffering systems, other than mitochondria and the ER, which would be more rapid than these organelles. A variety of soluble Ca^{2+} binding proteins are the best candidates for such a system. The Ca^{2+} binding proteins that are involved in the regulation and processing of a Ca^{2+} signal belong to two groups; the soluble proteins and the membrane intrinsic proteins. Both types contribute to the buffering of cell

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 Ca^{2+} , however it has been stated that the role of Ca^{2+} modulated proteins such as calmodulin in the buffering of cell Ca^{2+} is of minor importance as compared with their function in the processing of a Ca^{2+} signal (Carafoli, 1987).

In the nerve terminal attention has focused on four proteins - calmodulin, parvalbumin, calbindin and calretin (Heizmann and Hunziker, 1990) which are thought to occur in high enough concentrations that they might serve to buffer internal Ca²⁺ loads. Calmodulin has numerous functions (England, 1986) but it exists in high enough concentrations (>1 μ M) so that it could also act as a buffering protein. No clear cut functions apart from Ca²⁺ binding have been uncovered for the other three proteins (Rogers, 1989)

It has been suggested that these proteins may temporarily bind Ca^{2+} entering neurones, thus rapidly limiting its sphere of influence prior to the Ca^{2+} being further buffered by an intracellular organelle or expelled across the plasma membrane (Miller, 1991). Much effort has been expended on characterising the localisation of these proteins, and there do not seem to be any obvious rules as to which neurones contain which binding proteins (Miller, 1991). Authors have attempted to produce correlations between Ca^{2+} binding proteins content and cell viability under pathophysiological conditions (Sloviter, 1989; Sloviter *et al.*, 1989 Johansen *et al.*, 1990;). However, most of the data concerning Ca^{2+} binding proteins in the nervous system is descriptive. There is currently little data on when these proteins play a role in buffering Ca^{2+} *in vivo*.

3.1.5 <u>Calcium and the pathophysiology of ischaemia/reperfusion</u>3.1.5.a The calcium hypothesis

There is good evidence from the scientific research literature for a pivotal role of increased calcium content in adult ischaemic neuronal cell death (Siesjö, 1981). Indeed, the wealth of experimental data showing elevations of intracellular calcium levels after ischaemia has led to the "Ca²⁺-hypothesis". This hypothesis predicts that cellular Ca²⁺ overload occurs during ischaemia and ultimately leads to cell death

(reviewed by Siesjö *et al.*, 1989). Events known to be involved in the biochemistry of ischaemic cell death include depolarisation, disturbance of the energy state and release of neurotransmitters such as glutamate (Pulsinelli and Duffy, 1983; Siesjö and Wieloch, 1985; Siesjö, 1989; Choi and Rothman, 1990; Haddad and Jiang, 1993). Many of these processes are thought to be mediated by an increase in the intracellular Ca^{2+} concentration, thus triggering a cascade of damaging biochemical reactions (Siesjö, 1989).

It is also believed that Ca^{2+} plays a primary role in the delayed neuronal death characteristic of many ischaemic episodes (Nyakas *et al.*, 1996). The increased cytosolic Ca^{2+} influences the state and integrity of cytoskeleton and plasma membrane structures (Nyakas *et al.*, 1996). Under pathological conditions the elevated Ca^{2+} levels appear to kill cells by activation of proteases, lipases, protein kinase C, and by generation of free radicals (Choi, 1987; Orrenius *et al.*, 1989). These factors are thought to act synergistically over minutes to hours to produce cellular necrosis.

3.1.5.b Calcium changes during ischaemia/reperfusion

Studies have shown that during the first 1 to 2 minutes following the onset of ischaemia there is a small rise in intracellular free Ca^{2+} (Silver and Erecinska, 1990). Extracellular concentrations of calcium have been shown to remain relatively unchanged (Siesjö, 1992), and it is thought that this initial increase in intracellular Ca^{2+} is due to the release of Ca^{2+} from intracellular stores

(Silver and Erecinska, 1990; Siesjö, 1992) secondary to the rise in intracellular pH which is triggered by PCr hydrolysis. These changes in calcium in the first minute of ischaemia occur in the absence of complete energy failure. They also appear to be associated with an increased potassium conductance through the plasma membrane (Siesjö, 1992), possibly as a consequence of Ca²⁺ activated increased K⁺ conductance, although activation of ATP-dependent K⁺ conductances cannot be excluded as the possible mechanism (Katsura *et al.*, 1994).

After the first 1 to 2 minutes of ischaemia the extracellular concentration of

calcium decreases significantly (Hansen and Zeuthen, 1981; Harris et al., 1981). Calcium influx into cells occurs when extracellular K⁺ has risen to approximately 15 μ mol.ml⁻¹, probably because the concomitant depolarisation then opens VACCs and thus allows Ca^{2+} influx. This second phase of calcium concentration change is preceded by extensive energy failure, and hence impairment of energy dependent ion translocation, such as the plasma membrane Ca²⁺-ATPase. Intracellular measurements using ion-selective electrodes have shown rapid increases in cytoplasmic Ca²⁺ in neurones from both ischaemia-susceptible and ischaemia-resistant populations, beginning some 2 to 4 minutes after the onset of ischaemia (Silver and Erecinska, 1990). These increases in cytosolic calcium may stabilise at concentrations as high as 50 μ M in some neurones (Silver and Erecinska, 1990). Interestingly, higher concentrations of intracellular Ca²⁺ were often observed in these studies in neurones of the susceptible CA1 region of the hippocampus. Although many neurones of the resistant CA3 region also showed marked Ca²⁺ redistribution the overall increases were much lower, and reached a maximal value of approximately 3 μ M (Silver and Erecinska, 1990, 1992).

Increased cytosolic calcium during ischaemia is a consequence of :

- Ca²⁺ release from intracellular stores;

- increased entry of Ca^{2+} into the cytoplasm at multiple sites;
- failure to remove the accumulated intracellular Ca^{2+} because of energy depletion and the consequential failure of the energy dependent transporters of Ca^{2+} at the plasma membrane and intracellular buffering organelles.

As previously stated, increased Ca^{2+} entry into the cytoplasm is likely to occur during ischaemia via VACCs as a consequence of membrane depolarisation. Calcium may also enter through ionotropic receptors, in particular the NMDA receptor. It has also been suggested that AMPA and kainate receptors containing certain combinations of subunits may also be permeable to Ca^{2+} (Hollmann *et al.*, 1991).

The accumulated intracellular Ca^{2+} is not adequately sequestered into buffering organelles because energy depletion leads to failure of the Ca^{2+} -ATPase of the endoplasmic reticulum (Sims and Ziadan, 1995) and a reduced mitochondrial membrane potential decreases the mitochondria's ability to remove Ca^{2+} from the cytosol during ischaemic conditions (Peterson *et al.*, 1985). In contrast to Ca^{2+} sequestration by these organelles, Ca^{2+} release may occur from mitochondria (Duchen *et al.*, 1990) or endoplasmic reticula (Bickler *et al.*, 1993) during ischaemic conditions.

The accumulated Ca^{2+} is also not removed via plasma membrane extrusion mechanisms because of failure of the Ca^{2+} -ATPase due to ischaemic energy depletion (Sims and Zaidan, 1995). Reduced intracellular ATP concentrations reduce the affinity of the Na⁺/Ca²⁺ exchanger for intracellular Ca²⁺ (Carafoli, 1987) and the depolarised membrane potential inhibits Ca²⁺ extrusion (Turner and Golding, 1985). In combination with these effects on the Na⁺/Ca²⁺ exchanger, depolarisation favours Ca²⁺ influx and the high intracellular Ca²⁺ occurring during ischaemia ensures the Ca²⁺ regulatory site on the exchanger is occupied. Thus these conditions, coupled with an influx of Na⁺ during ischaemic conditions may reverse the Na⁺/Ca²⁺ exchanger which then moves Ca²⁺ inwards (Blaustein, 1985; Huang *et al.*, 1991).

3.1.5.c Consequences of ischaemic Ca²⁺ changes

The ischaemic depletion of extracellular interstitial Ca²⁺ may have a variety of dramatic and potentially pathological consequences, including:

- Calcium stabilised structures of intracellular contacts and of the extracellular matrix as well as of intraneuronal cellular and subcellular membranes, neurotubuli and neurofilaments may be disrupted or "frozen" in functionally insufficient conformations (Kluge, 1991).

- Calcium mediated enzymatic reactions of ligand-receptor interactions may run out of control by an inappropriate activation (Kluge, 1991).

In addition, increased cytoplasmic Ca^{2+} during ischaemia has the potential to trigger a number of events which could be deleterious to the survival of the cell (Fleckenstein *et al.*, 1974; Chien *et al.*, 1979; Farber *et al.*, 1981; Choi, 1988; Meyer, 1989; Siesjö and Bengtsson, 1989; Siesjö, 1992). Calcium plays an important role in the regulation of many enzymatic pathways. Of these, four have gained prominence in attempting to explain calcium-mediated cytotoxic effects. They are:-

- 1] Calcium activated phospholipases;
- 2] Calcium activated proteases;
- 3] Calcium activated protein kinases;
- 4] Calcium phosphatases.

For example, Ca^{2+} overactivation of phospholipase A_2 leads to the release of free fatty acids, including arachidonic acid, from cell membranes (Siesjö, 1981), which can be precursors of prostaglandins, thromboxanes and leukotrienes. These latter compounds can be a trigger of ischaemic damage (Selman *et al.*, 1982; Wolfe, 1982; Bazan *et al.*, 1991) at least partly due to free radical production (Gelmers, 1985). Increased cytoplasmic Ca^{2+} can lead to increased production of the free radical nitric oxide and can also cause the production of free radicals from the xanthine oxidase dependent conversion of hypoxanthine to xanthine.

Aside from influencing free radical production, increased cytoplasmic Ca^{2+} can have a number of other deleterious effects on cell metabolism. By activation of some of the enzyme systems mentioned previously, Ca^{2+} can cause breakdown of cytoskeletal structures via activation of the protease calpain (Kluge, 1991) and changes in properties of membrane channels can occur via protein kinase C activation and changes in phosphorylation states due to the actions of phosphatases (Kluge, 1991). Receptor functions have been reported to be altered by increased cytoplasmic Ca^{2+} (Cheung *et al.*, 1986; Meyer, 1989).

Calcium is also known to uncouple mitochondrial oxidative phosphorylation, and an overload of mitochondria with Ca^{2+} can lead to their own damage and hence the derangement of cellular energy production (McCormack and Crompton, 1991).

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Increased cytoplasmic Ca^{2+} levels are evident in ischaemic conditions (Siesjö, 1981), numerous studies have shown that ischaemic mitochondria exhibit deranged function (Sims and Pulsinelli, 1987) and deleterious effects of Ca^{2+} on mitochondrial function have been reported (Sciamanna *et al.*, 1992).

Reperfusion Ca²⁺ changes

Although recirculation following ischaemia normally leads to prompt normalisation of some ion gradients, such as a quick return to expected extracellular K^+ levels, the recovery of extracellular Ca^{2+} values occurs much more slowly (Katsura *et al.*, 1994).

Normalisation of extracellular Ca²⁺ typically occurs in two stages:

- firstly with a fast recovery to approximately 70% of the normal value, which probably reflects extrusion of free and bound Ca^{2+} ;

- followed by a slower recovery stage which probably reflects the gradual release of Ca^{2+} from sequestration sites such as mitochondria (Katsura *et al.*, 1994).

Hence Ca^{2+} gradients are seen to eventually return to normal, often by 20 minutes of restoration of blood flow (Hansen, 1985; Silver and Erecinska, 1992). However, brief periods of transient ischaemia often lead to delayed neuronal cell death, occurring some hours or days after the primary insult (Kassura *et al.*, 1994). Very probably, this delayed neuronal death reflects metabolic cascades set in motion by the primary insult. Although at present these cascades remain a matter of some speculation, it is plausible that a major factor in this process is a lingering perturbation of cell Ca^{2+} metabolism (Siesjö, 1992). It is of interest to note that during extended periods of recirculation following transient ischaemia, modest increases in cytoplasmic Ca^{2+} have been noted in ischaemia susceptible regions which are lacking in resistant regions (Silver and Erecinska, 1992). Evidence that intracellular accumulation of Ca^{2+} develops in many neurones, and particularly in mitochondria, during recirculation has also been found with both Ca^{2+} staining (Dux *et al.*, 1987) and post-ischaemic intramitochondrial Ca^{2+} measurements (Zaidan and Sims, 1994).

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Further abberations in Ca^{2+} homeostasis during ischaemia, reperfusion and delayed neuronal death remain to be fully explored.

3.1.5.d Evidence for the involvement of Ca²⁺ in ischaemic damage

Calcium, and particularly the accumulation of intracellular Ca^{2+} which occurs during ischaemia, is widely proposed to contribute to neuronal death following short term ischaemia (Siesjö and Bengtsson, 1989; Siesjö, 1989, 1992). The likely role of Ca^{2+} as a common factor in degeneration of many cells and more specifically the Ca^{2+} dependence of at least some types of neuronal death produced by excitotoxic substances (Choi, 1992) provides support for the key role of Ca^{2+} as a causative factor in neuronal death. However, definitive evidence for the contribution of Ca^{2+} to ischaemic neuronal death is not yet available (Sims and Zaidan, 1995). There are several lines of evidence though, which do suggest that disruption of Ca^{2+} homeostasis may be involved in ischaemia-induced neuronal degeneration.

Evidence has been accrued from both *in vivo* and *in vitro* ischaemic studies. Many studies of *in vivo* ischaemia have addressed changes in cellular Ca²⁺ content by the use of histochemical stains for Ca²⁺ performed on the post ischaemic or reperfused brain (Simon *et al.*, 1984; Hossman *et al.*, 1985; De Leo *et al.*, 1987; Dux *et al.*, 1987; Hashimoto *et al.*, 1992; Bonnekoh *et al.*, 1992). Intracellular Ca²⁺ accumulation has been detected in such studies. Radiochemical techniques have also been used to examine alterations in tissue accumulation of ${}^{45}Ca^{2+}$ (Dienel, 1984; Sakamoto *et al.*, 1986). Dienel (1984) showed that accumulation of ${}^{45}Ca^{2+}$ was detectable in rat brain following 30 minutes of ischaemia and during the first few hours of recirculation. Interestingly, in this study ischaemia resistant regions showed less accumulation during recirculation than ischaemia susceptible regions (Dienel, 1984). This technique provides a sensitive and quantifiable indicator of altered Ca²⁺ homeostasis, but it is not possible to determine whether accumulation is due to increased uptake or reduced turnover of Ca²⁺.

Direct *in vivo* measurements of intracellular Ca²⁺, using Ca²⁺-sensitive microelectrodes have been obtained in only a few studies during ischaemia and recirculation. This is primarily because of the difficulties in placing microelectrodes in central nervous system neurones and maintaining these for extended periods (Uematsu *et al.*, 1988; Silver and Erecinska, 1990, 1992). In a well conducted study intracellular Ca²⁺ measurements were achieved (Silver and Erecinska, 1992), and were shown to increase in ischemia susceptible CA1 hippocampal neurones from < 100 nm to 30μ M after an 8 minute ischaemic episode. Restoration of blood flow was shown to normalise these Ca²⁺ levels, but subsequent small increases in cytoplasmic Ca²⁺ levels (to approximately 400 nm) were detected between 2 and 4 hours of recirculation (Silver and Erecinska, 1992). These later small elevations of Ca²⁺ during recirculation may precede changes in mitochondrial Ca²⁺ which have been reported in ischaemia sensitive brain regions within a few hours of recirculation (Zaidan and Sims, 1994)

The isolation of post-ischaemic mitochondria has shown Ca^{2+} accumulation in these organelles, by the use of histochemical staining (Simon *et al.*, 1984; Hossman *et al.*, 1985; Dux *et al.*, 1987;) and Ca^{2+} sensitive fluorescent dyes (Zaidan and Sims, 1994). In an *in vitro* study (Sciamanna *et al.*, 1992) of mitochondria isolated from post-ischaemic rats, a decrease in NAD⁺-linked mitochondrial respiration was seen after 12-30 minutes of ischaemia, which could be restored to control values if a Ca^{2+} chelator such as EGTA was included in media, thus suggesting a role for Ca^{2+} in the decreased respiration. The authors (Sciamanna *et al.*, 1992) also found a significant decrease in the Ca^{2+} uptake of ischaemic mitochondria, and a significant increase in Ca^{2+} located externally to the mitochondrial membrane. They thus suggested that increased cytosolic Ca^{2+} as a consequence of ischaemia results in an excessive accumulation of Ca^{2+} on the mitochondrial membrane and a consequential inhibition of the mitochondrial respiratory chain (Sciamanna *et al.*, 1992).

Alterations in both extracellular and intracellular Ca^{2+} accompany *in vivo* ischaemia (Uematsu *et al.*, 1989). The changes in Ca^{2+} homeostasis following ischaemia differ between presynaptic and postsynaptic processes (Huang *et al.*, 1991),

and these compartments cannot easily be distinguished *in vivo. In vitro* studies have thus been helpful in providing evidence of ischaemic Ca^{2+} changes in these compartments. Studies with brain slices have shown increased cellular Ca^{2+} accumulation during glucose and oxygen lack (an *in vitro* model akin to ischaemia), (Bachelard *et al.*, 1993; Badar-Goffer *et al.*, 1993; Bickler *et al.*, 1993; Brooks and Kauppinen, 1993;). Neuronal cell culture studies have reported increased cellular Ca^{2+} following ischaemia (Choi, 1989; Gibson *et al.*, 1989; Carroll *et al.*, 1992;). Ischaemic effects on the presynaptic compartment of neural tissue has been investigated using synaptosome preparations, and such studies have reported increased Ca^{2+} in the nerve terminal following glucose and oxygen lack (Boakye *et al.*, 1991) and chemical ischaemia (Dagani *et al.*, 1989; Gibson *et al.*, 1991). The removal of extracellular Ca^{2+} from bathing media during chemical ischaemia has also been shown to prevent ischaemia-induced neuronal degeneration in cell culture models (Ginsberg, 1989; Friedman and Haddad,1992).

Direct evidence for the involvement of Ca^{2+} in post-ischaemic neuronal death has been provided by numerous studies with Ca^{2+} channel antagonists and other pharmacological strategies. A detailed consideration of all the pharmacological manipulations that have been investigated is beyond the scope of this introduction. However, some of the interesting generalisations emerging from studies with Ca^{2+} modifying neuroprotective agents will be presented.

Perhaps not surprisingly, there are a number of studies in which Ca^{2+} modifying treatments have been reported not to achieve neuroprotection during ischaemia and/or reperfusion (Newberg *et al.*, 1984; Sakabe *et al.*, 1986; Vibulsreth *et al*, 1987). Negative results are more difficult to evaluate however because the lack of protection may arise from other factors such as incorrect dosage or incomplete access to the relevant site in the brain (Sims and Ziadan, 1995).

Studies of Ca²⁺ channel blockers and other modulators of Ca²⁺ content have shown a degree of ischaemic neuroprotection, or have been shown to attenuate some of the changes associated with ischaemia which may be contributing to neurodegeneration (Steen et al., 1983; Deshpande and Wieloch, 1986; Beck et al., 1988; Hadani et al., 1988; Nuglisch et al., 1990; Welsch et al., 1990; Alps 1992, Takakura et al., 1992; Brooks and Kauppinen, 1993). The most impressive reductions in neuronal damage have been reported in some studies in which there was postischaemic administration of the drug as well as prior to or during the ischaemic insult (Alps and Hass, 1987; Poignet et al., 1989). Similar protection has been achieved with post-ischaemic administration alone (Zhang et al., 1993; Deshpande and Wieloch, 1986; Izumiyama and Kogure, 1988). These findings of effective early post-ischaemic administration suggests that changes in Ca²⁺ during early recirculation are important in the neurodegenerative process. In one study, the pre-ischaemic administration of nifedipine (An L-type channel blocker) into rats had no effect on intracellular Ca²⁺ at the end of ischaemia, but accelerated the recovery of cytoplasmic Ca^{2+} in the early post-ischaemic period (Silver and Erecinska, 1992). An N-type channel blocker -SNX-111- has also been reported to produce marked protection in CA1 hippocampal neurones when administered post-ischaemically (Valentino et al., 1993) Antagonists for the NMDA receptor have also been shown to reduce the initial rate of cytoplasmic Ca²⁺ inflow and also to reduce the overall Ca²⁺ accumulation (Silver and Erecinska, 1990, 1992) and damage in ischaemic neurones. Mitani et al., (1993) showed amelioration of ischaemic neuronal damage in brain slices following treatment with the pharmacological agent Dantrolene, which blocks the mobilisation of Ca^{2+} from intracellular stores.

Although the Ca²⁺ hypothesis has substantial experimental support, a number of reports have actually cast some doubt on its potential generality (Hansen and Zeuthen, 1981; Harris *et al.*, 1981; Dubinsky and Rothman, 1991; Friedman and Haddad, 1992; Haddad and Jiang, 1992). Kristian *et al.* (1992) found that intracellular Ca²⁺ accumulation during ischaemia is little affected by hypothermia, although this treatment dramatically reduces neuronal damage. Some researchers state that ischaemic

neuronal injury does not have to be preceded by an increase in Ca^{2+} and therefore that other mechanisms leading to injury have to be implicated (Haddad and Jiang, 1992). However, the Ca^{2+} hypothesis has strong support and is widely accepted and implicated in ischaemic neuronal death (Siesjö, 1989), but definitive evidence is still lacking. Increased Ca^{2+} may prove to be one of a number of causative factors acting synergistically with others to lead to neuronal degeneration, or alternatively increased Ca^{2+} may be a consequence of generalised cellular damage and the causative factor(s) remain to be fully elucidated.

<u>3.1.6</u> <u>Calcium and the developing brain</u>

Whilst there is substantial knowledge of the processes concerned with calcium homeostasis in the adult brain, less is known about these processes in the immature brain. Additionally, many of the changes in Ca^{2+} homeostasis thought to be involved in the pathophysiology of ischaemic neuronal death are identified in the adult brain. Again, less is known about the involvement of Ca^{2+} in neonatal ischaemic brain damage.

The roles of the cations Na⁺, K⁺ and Ca²⁺ in early brain development are undoubtedly important but are not so well defined as they are for the functioning of mature brain. The electrolyte composition of rat brain was found to change over the postnatal development period (Vernadkis and Woodbury, 1962), although Ca²⁺ was not measured specifically. The homeostatic buffering of Ca²⁺ appears critical for adequate neuronal growth (Nyakas *et al.*, 1996). Several lines of evidence point to the specific role of intracellular free Ca²⁺ in the growth process of the developing nerve cell (Mattson and Kater, 1987; Mattson *et al.*, 1990; Kater and Mills, 1991).

Neurotransmitters and electrical activity affect neurite outgrowth and synaptogenesis, in large part by altering the local level of intracellular Ca^{2+} concentration (Mattson, 1988; Nyakas *et al.*, 1996). Voltage-dependent Ca^{2+} influx is a signal for both growth cone expansion and neurite elongation in primary dissociated neurones in culture (Suarez-Isla *et al.*, 1984). The optimal development of nerve cell

growth cones takes place in a narrow intracellular Ca^{2+} concentration range (Angus Silver *et al.*, 1989; Kater and Mills, 1991). Many neuronal cultures require an elevated intracellular Ca^{2+} concentration for optimal growth and neurite expression. Such elevations are often routinely created by activation of Ca^{2+} transporting NMDA receptors, or by partial depolarisation with KCl, and such partial depolarisation has been shown to remove partially a requirement for nerve growth factor (Solem *et al.*, 1995). If intracellular Ca^{2+} deviates from a narrow range in cultured neurones, then growth and motility cease, and the neurones may begin a process of programmed cell death (apoptosis) (Nicholls, 1994).

During brain development there are considerable increases in activities of some of the ion transport mechanisms, including an increase in activity of the Na⁺, K⁺-ATPase during the first 4 weeks of postnatal development (Abdel-Latif *et al.*, 1967) There are few studies detailing postnatal changes in calcium homeostatic mechanisms and Ca²⁺ transporter activities (Nyakas *et al.*, 1996). The activity of the Na⁺/Ca²⁺ exchanger increases between day 1 and 6 in cultures of primary neurones (Michaelis *et al.*, 1994), suggests a change in activity with neuronal maturation. Mailleux *et al.*, (1993) suggested a change in calcium homeostasis during brain development, following studies showing that synaptogenesis corresponded with an increase in mRNA content for the inositol 1,4,5-triphosphate receptor (which is concerned with mobilisation of intracellular calcium stores). Carpenter *et al.* (1990) have reported that during the process of early development, neuronal voltage-gated calcium channels and neurotransmitter channels allowing calcium entry increase greatly in abundance and diversity.

In terms of the involvement of Ca^{2+} in neonatal ischaemic brain damage, only a few studies have focused on direct measurements of Ca^{2+} in neonatal brain tissue (Stein and Vannucci, 1988; Kass and Lipton, 1989; Bickler *et al.*, 1993; Friedman and Haddad, 1993; Nyakas *et al.*, 1996). The importance of elevations in Ca^{2+} in severe hypoxic-ischaemic brain damage has been established in immature rats (Stein and Vannucci, 1988), however there do seem to be major differences in the Ca^{2+} response of neonatal as compared with adult brain (Nyakas et al., 1996).

Interestingly, Kass and Lipton (1989) noted less calcium accumulation in neonatal brain than in adult brain following hypoxia. In brain slice preparations, Bickler *et al.* (1993) showed differences between the response of adult and neonatal brain to cyanide poisoning, anoxia and glutamate addition. The neonatal brain slices exhibited less increases in intracellular Ca^{2+} than the adult brain. Bickler *et al.* (1993) concluded that the regulation of Ca^{2+} in response to these metabolic insults changes significantly during early development. Friedman and Haddad (1993) have presented evidence, using dissociated CA1 neurones, that neonatal rat neurones differ from adults in their sensitivity to anoxia in terms of their handling of cytosolic free Ca^{2+} . This was in line with earlier observations showing that depolarisation and changes in extracellular Ca^{2+} , Na⁺ and Cl⁻ take much longer to develop during anoxia in the neonate than in the adult (Haddad and Donelly, 1990; Jiang and Haddad, 1991; Jiang *et al.*, 1992).

Only a small number of studies have investigated the actions of Ca^{2+} antagonists in the immature brain. In 7 day old rat pups Silverstein *et al.*, (1986)
showed that the antagonist *flunarizine* was neuroprotective, as judged by histology.
Sundvall *et al.*, (1994) have reported that there is an increase in extracellular Ca^{2+} during the first few minutes of anoxia in the neonatal brain which is larger than that
reported in adult brain (Hansen, 1985). These authors also reported that the NMDA
receptor antagonist MK-801 did not prevent Ca^{2+} influx into anoxic cells in the
neonatal brain. They have hence questioned whether the neuroprotective effects of
MK-801 in neonatal ischaemic brain damage reported in many studies (Wong *et al.*,
1986; McDonald *et al.*, 1987; Ford *et al.*, 1989; Hattori *et al.*, 1989; Olney *et al.*,
1989) involves a blockage of Ca^{2+} uptake or whether other mechanisms lie behind the
neuroprotective effect of this compound.

Ischaemia, calcium and glutamate

In summary, Ca^{2+} homeostasis in the immature brain is of importance in the regulation of growth and development. Studies suggest that mechanisms involved in Ca^{2+} homeostasis may change as the brain develops into maturity, and this may be implicated in the different responses of the immature and adult brain to hypoxic and ischaemic neuronal damage. More investigation of Ca^{2+} homeostasis in the developing brain and its implications for pathological conditions, such as ischaemia, is needed.

3.1.7 An introduction to glutamate

3.1.7.a What is glutamate?

Glutamic acid, or glutamate, is an amino acid with multiple biological roles (Erecinska and Silver, 1990). It is an important component in the synthesis of both small (for example, glutathione) and large (for example, peptides and proteins) molecules. In the mammalian central nervous system glutamate is a major neurotransmitter in its own right, and it is also a precursor of the neurotransmitter - gamma-aminobutyric acid (GABA).

It is estimated that up to 90% of the numerous synapses in the human brain utilise glutamate as their neurotransmitter (Nicholls, 1993) and hence it is stated that glutamate plays the dominant role in fast information transfer (Nicholls, 1993). Glutamate receptors are found throughout the mammalian brain and glutamate is the major excitatory transmitter in the brain (for review see Fonnum, 1984; Foster and Fagg, 1984). Glutamate is classed as an excitatory amino acid because it acts predominantly on depolarising post-synaptic receptors, whereas inhibitory neurotransmitters such as glycine and GABA act on hyper-polarising receptors. The glutamate system is involved in the first steps of learning and memory acquisition by way of synaptic facilitation (for review see Collingridge and Bliss, 1987). Facilitation implies that presynaptic neurotransmitter release, postsynaptic receptor sensitivity, or both increase as a result of synaptic activity.

<u>3.1.7.b</u> <u>Glutamate receptors</u>

Glutamate mediates cellular responses by interacting with metabotropic glutamate receptors and ionotropic glutamate receptors. A family of metabotropic glutamate receptors coupled to G-proteins has been identified (Sladeczek *et al.*, 1985; Sugiyama *et al.*, 1987; Schoepp *et al.*, 1990) and cloned (Masu *et al.*, 1991; Tanabe *et al.*, 1992). These receptors may be either post- or presynaptic and play a predominantly modulatory role in neurotransmission (Nicholls, 1993).

Currently, this family of receptors has seven members. The receptors may either be coupled positively to inositol phosphate turnover (mGluR1, mGluR5) or negatively to adenylyl cyclase activity and/or Ca²⁺ channel activity (mGluR2, mGluR3, mGluR4, mGluR6 and mGluR7) (Anwyl, 1991). The distribution of these receptor subtypes within the brain is complex and, furthermore, the role which these receptors play in neurotransmission varies with brain area, even for the same receptor subtype. It is therefore impossible to generalise about the actions of these receptors. It is known that metabotropic glutamate receptors are present and functional on the synaptosomal membrane (Nicholls, 1993)

Glutamate also interacts with three classes of postsynaptic ionotropic glutamate receptors, namely NMDA, kainate (KA), α -amino-3-hydroxy-5-methyl-4-isoxazole-4-propionate (AMPA). The AMPA /KA receptors are responsible for fast information transfer while the NMDA receptor is only operative when the postsynaptic membrane is independently depolarised by another receptor. The presence of functional ionotropic glutamate receptors on the synaptosomal membrane is currently controversial and a matter of some debate. There is presently a lack of definitive evidence for the presence of the NMDA, KA and AMPA receptors in the synaptosome preparation,however the statement that these receptors are purely postsynaptic (Nicholls, 1993) is yet to be proved.

The NMDA receptor

NMDA receptors are widely distributed in mammalian brain and spinal cord, with particularly high densities in cerebral cortex and hippocampus (Iversen, 1994). The receptor is a ligand-gated ion channel composed of at least two different protein sub-units known as NMR_1 and NMR_2 , each containing almost 1,000 amino acid residues.

The NMDA receptor has multiple ligand recognition sites: L-glutamate and related agonists bind and promote the opening of a high conductance channel which permits entry of sodium and calcium into target cells. L-glutamate is virtually ineffective, however, unless a glycine recognising modulatory site is also occupied. In a target cell with normal resting membrane potential, L-glutamate is unable to activate the NMDA receptor because the ion channel is blocked by magnesium (Mg²⁺), and this block is only removed when the target cell is partially depolarised through activation of other synaptic inputs. This "conditional" nature of the NMDA receptor is highly unusual. Indeed, it is thought that this particular property may play a role in the involvement of NMDA receptors in the synaptic plasticity associated with learning and memory (Collingridge and Bliss, 1987).

In addition to the inhibitory action wielded by Mg^{2+} at a site deep in the ion channel, there is evidence that zinc (Zn^{2+}), acting at a site near the mouth of the channel, is also an inhibitory modulator of channel function (Westbrook and Mayer, 1987).

A large amount of research interest has been focused on the NMDA receptor, in particular, over recent years. This is primarily because of its involvement in glutamate mediated toxicity.

3.1.7.c Glutamate toxicity

Prolonged or excessive stimulation of the ionotropic glutamate receptors can damage and eventually kill the cell or nerve terminal in a phenomenon known as excitotoxicity. The precise mechanisms involved in excitotoxicity remain unclear. although avid research interest is continually adding to current knowledge. The general consensus does appear to agree that excitotoxicity involves a large and ultimately irreversible increase in intracellular Ca²⁺ caused by NMDA receptor activation. The process of excitotoxicity has been widely studied and is the subject of a number of excellent reviews (see for example Olney, 1978, 1988; Rothman and Olney, 1986; Choi, 1988, 1992; Siesjö and Bengtsson, 1989; Meldrum and Garthwaite, 1990; Coyle and Puttfarcken, 1993).

Since glutamate is found in high concentration in the CNS and serves a number of important metabolic functions, early reports that systemic administration of glutamate to infant mice destroys neurones in the retina (Lucas and Newhouse, 1957; Olney, 1969a), or in certain regions of the brain (Olney, 1969b) were met with disbelief. However, the toxic effects of glutamate following either oral or subcutaneous administration was readily reproduced in a number of animal species, including primates (Olney *et al.*, 1972).

In addition, it was found that amino acids related to glutamate, which also elicit excitatory responses, could reproduce the neurotoxic effects of glutamate (Olney, 1978). Furthermore, specific analogues known to share the neuroexcitatory properties of glutamate were found to mimic its neurotoxic effects also, and that those analogues lacking excitatory activity also lacked neurotoxicity (Olney, 1991). For example, Nadler *et al.*, (1981) found that the injection of kainic acid, a non-metabolisable glutamate analogue, into the hippocampus produced lesions remarkably similar to those produced by glutamate. Thus, a firm link was established between excitatory glutamate and neurotoxicity.

Ischaemia, calcium and glutamate

Early studies stressed the importance of glutamate and neuronal excitation in cell killing (Olney 1969, 1978) but did not explore more cellular mechanisms of injury. The link between calcium and glutamate neurotoxicity was first firmly established by Choi (1987). Choi (1987) experimented with neuronal cell cultures by exposing them to glutamate for five minutes, then returning them to normal extracellular fluid, and thus finding that most neurones died within a day. He found that if the glutamate treatment was carried out in buffer lacking calcium, cell mortality was greatly reduced. Of interest, the cells would still swell when bathed in glutamate without added calcium, but the swelling proved to be reversible. Rothman *et al.*, (1987) reported similar results showing a distinct role for calcium in glutamate neurotoxicity. From these initial experiments much research has focused on the cellular mechanisms of glutamate toxicity, and in particular the role of the NMDA receptor and calcium influx through it (reviewed in Choi, 1992).

A speculative description of glutamate neurotoxicity was proposed in 1990 by Choi, and there has been a large amount of work published pre- and post- this proposal which would lend support to his description.

Choi (1990) proposed that glutamate neurotoxicity may have three stages:-

Induction:	overstimulation of glutamate receptors, leading to a set of
	immediate cellular derangements;
Amplification:	events that intensify these derangements and promote the glutamate involvement of additional neurones;
Expression:	the destruction cascade directly responsible for neuronal cell degeneration.

The glutamate receptor system and glutamate excitotoxicity has been implicated in a number of degenerative diseases and also in the neuronal cell death that takes place after insults to the brain such as head trauma and epileptic seizures. The observations over many decades that glutamate is toxic to neurones in culture and *in vivo* led to the suggestion that many neurological accidents involving strokes and the consequential loss of oxygen and glucose result in neuronal loss involving glutamate toxicity (for a review see Olney, 1989). The following section will briefly look at the area of glutamate toxicity in ischaemia/reperfusion.

3.1.8 Glutamate and the pathophysiology of ischaemia/reperfusion

3.1.8.a Release of neurotransmitters during ischaemia/reperfusion

As a result of the collapse of ionic gradients during ischaemia the reversal of a number of ion-dependent reuptake processes occurs, as does the release of neurotransmitters from neurones (Waniewski and Martin, 1984). The extracellular concentrations of most neurotransmitters have been reported to increase many fold during ischaemia, although in most cases the concentrations have also been shown to return to normal or near normal within the first thirty minutes of recirculation (see individual references detailed below). These neurotransmitters include aspartate (Beveniste *et al.*, 1984; Hagberg *et al.*, 1985), dopamine (Globus *et al.*, 1987), gamma-aminobutyric acid (GABA) (Hagberg *et al.*, 1985; Globus *et al.*, 1988), glycine (Globus *et al.*, 1991), noradrenaline (Globus *et al.*, 1989), adenosine (Hagberg *et al.*, 1987) acetylcholine (Bertrand *et al.*, 1993) and serotonin (Phebus and Clemens, 1989; Sarna *et al.*, 1990).

The extracellular concentrations of glutamate have also been reported to change during ischaemia and reperfusion (Bosley *et al.*, 1983; Drejer *et al.*, 1985; Hagberg *et al.*, 1987). The reported increase in extracellular glutamate during ischaemia/reperfusion along with evidence showing that the death of neurones under ischaemic and hypoxic conditions could be prevented by pretreatment with glutamate antagonists (Goldberg *et al.*, 1987; Rothman *et al.*, 1987) opened the way for a large amount of ischaemic research to focus on glutamate antagonists as possible neuroprotectors.

3.1.8.b Evidence for a role of glutamate in ischaemic damage

There is now considerable evidence indicating that an excitotoxic response arising from increased extracellular glutamate is likely to be important in determining the extent of damage in tissue affected by prolonged focal ischaemia (reviewed in Pulsinelli, 1992; Siesjö, 1992).

In short-term global ischaemia, the marked accumulation of extracellular glutamate during ischaemia also potentially provides the stimulus for an excitotoxic response. Most of the evidence suggesting a role for glutamate in ischaemic damage comes from studies aimed at providing neuroprotection via selective glutamate receptor antagonists. A number of NMDA antagonists have been shown to prevent or reduce neuronal damage in many studies of focal ischaemia (Kochhar et al., 1988; Ozyurt et al., 1988; Park et al., 1988; Buchan et al., 1992) and in primary cultures of neurones deprived of oxygen and glucose (Rothman et al., 1987; Choi et al., 1988). In global ischaemia the situation is less clear. A number of studies have demonstrated a marked cerebroprotective effect of the NMDA receptor antagonist MK-801 (Church et al., 1988; Gill et al., 1988; Izimiyama and Kogure, 1988; Rod and Auer, 1989; Gill and Woodruff, 1990; Swan and Meldrum, 1990). However, many other studies have failed to find neuroprotection with this compound (Block and Pulsinelli, 1987; Wieloch et al., 1988; Fleischer et al., 1989; Michenfeld et al., 1989; Buchan et al., 1991; Nellgard and Wieloch, 1992). The reasons for these differences have proved to be somewhat controversial. In some studies demonstrating protection with MK-801 the body temperature of the animals was not monitored (Gill et al., 1987; Rod and Auer, 1989), and it has been found that MK-801 treatment in combination with ischaemia reduces the body temperature of gerbils by up to 3°C

(Buchan and Pulsinelli, 1990). Furthermore, the maintenance of body temperature during MK-801 treatment was found by some researchers to eliminate the protective effects of MK-801 (Buchan and Pulsinelli, 1990), indicating that the original protection involved the well documented neuroprotective effect of hypothermia (see for example Mitani and Kataoke, 1991). Nonetheless, protection with MK-801 has been reported in instances where body temperature was adequately maintained (Gill and Woodruff, 1990).

Marked reductions in neuronal damage have been consistently reported following treatments with the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7sulfamoylbenzo [F]-quinoxaline (NBQX) in both focal and global ischaemia models (Sheardown et al., 1990; Buchan et al., 1991; Dimer et al., 1992; Gill et al., 1992; Nellgard and Wieloch, 1992; Le Peillet et al., 1992). Interestingly, NBOX treatment initiated some 24 hours into recirculation following an ischaemic episode has been found to have neuroprotective effects (Sheardown et al., 1990; Pulsinelli and Cho, 1992; Sheardown et al., 1993). This ability to reduce neuronal damage at such a late stage of reperfusion is difficult to reconcile with the traditional picture of glutamate excitotoxicity. It is of course possible that the normal cellular response to glutamate may be modified by an ischaemic insult and the protective action of NBXQ is concerned with preventing this later response to glutamate. It should be emphasised however, that the mechanisms of neuronal loss caused by glutamate under some conditions may be delayed for many hours after the initial insult (Sims and Zaidan, 1995), and that ischaemia involves many different mechanisms, all of which may interact and modify cellular responses to biological agents.

There are several observations that do not fit easily with a conventional excitotoxic type of damage following ischaemia. No obvious correlation has yet been identified between susceptibility of cells in different brain regions and either the extent of glutamate accumulation or the distribution of the major glutamate receptor subtypes. A role for glutamate in ischaemic damage is not eliminated by such observations.
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However, other research including a study comparing the effects of metabolic deprivation with the effects of excitotoxic amino acids on energy failure and calcium homeostasis (Bachelard *et al.*, 1994) has suggested that some ischaemic changes cannot simply be attributed to the release of excitatory amino acids. There is undoubtedly a role for the glutamate system in the pathophysiology of ischaemia/reperfusion, but questions remain as to whether it is a causative factor, a secondary phenomenon or one of a concert of factors involved in ischaemic neuronal death.

<u>3.1.9</u> Glutamate and the developing brain

There is currently more knowledge of the processes concerned with glutamate homeostasis and its role in excitotoxic damage during ischemia in the adult brain than in the developing neonatal brain. However some studies have enabled the following about these processes in the developing brain to be elucidated.

Glutamate concentrations progressively increase during maturation, reaching the adult level by the 25th postnatal day (Vernadkis and Woodbury, 1962). During the process of early development, neuronal Ca²⁺ channels increase greatly in abundance and diversity (Carpenter *et al.*, 1990). These data were gathered from studies on messenger RNA (MRNA) coding for rat brain glutamate receptors. The authors found that there was a dramatic increase in mRNA for these receptors from birth to day 30. Data from the laboratory of Friedman and Haddad (1993) show that, in the neonate, levels of the ion-carrying NMDA and AMPA channels are low, increasing with age.

Studies which allow separate investigation of pre- and postsynaptic receptors have observed that presynaptic markers of the EAA systems are immature in the brain of newborn rat (Campochiaro and Coyle, 1978; Kvamme *et al.*, 1985). On the contrary, postsynaptic EAA system activities, particularly those of the NMDA and quisqualate receptors are transiently enhanced early in life (Hattori and Wasterlain, 1990; Insel *et al.*, 1990).

This transient enhancement has been observed in a number of studies. Stein and Vannucci, (1988) reported that the density of NMDA receptors is transiently high during certain stages in the immature brain. The number of glutamate receptors and their functional reactivity to agonists overshoots adult values during early postnatal development around the period of 6-15 postnatal days in several brain areas (Baudry et al., 1981; Tremblay et al, 1988; Hattori and Wasterlain, 1990, Insel et al., 1990). This has been connected with increased neurotoxicity in neonates. It has been confirmed repeatedly that infant animals are much more vulnerable than adults to the toxic action of glutamate (Olney, 1991). NMDA has been found to be more neurotoxic in 7-day old rats than adults (McDonald et al., 1988). Intracerebral injections of NMDA cause larger damage in the immature brain than in the adult (McDonald et al., 1988). It has also been observed that the NMDA receptor is more easily activated in immature brain due to less magnesium block (Bowe and Nadler, 1990). In summary, these results, and others, indicate that right after birth the sensitivity of glutamate receptors is very low, while it sharply increases and becomes hypersensitive around 1-2 weeks of age (Hattori and Wasterlain, 1990).

There do appear to be a number of changes in the properties of these receptors as the brain matures. A number of studies found that during postnatal development of rat cerebellum, changes take place in the relative depolarising potency and efficacy of selective glutamate receptor agonists, and that in particular there is an apparent loss of chemosensitivity to NMDA in neurones (Dupont *et al.*, 1987; Garthwaite *et al.*, 1987). Friedman and Haddad (1993) have reported that the electrophysiological response of neurones to glutamate in neonates is much smaller in magnitude than that of adults. Southam *et al.*, 1991 have found that the coupling of glutamate receptors to the formation of nitric oxide (NO) from arginine changes markedly with postnatal brain development. They found that the exogenous agonist became less effective in producing an NO response in brain slices as the brain developed, although whether this is a true indication of the *in vivo* situation is as yet still unclear (Southam *et al.*, 1991).

Important developmental changes have also been observed in elevations in intracelllular Ca²⁺ caused by exogenous glutamate addition. Bickler *et al.*, 1993 reported that for a given concentration of glutamate, neonatal brain slices accumulated significantly less Ca²⁺ than adult brain slices. Although it is thus suggested that less Ca²⁺ flux occurs through glutamate receptors in the neonatal brain, data still shows that the immature brain is hypersensitive to stimulus of NMDA receptors (McDonald and Johnston, 1990; Sundvall *et al.*, 1994), and therefore the role of Ca²⁺ influx in this sensitivity is questionable.

The postsynaptic enhancement of some glutamate receptor functions during certain periods development renders the immature brain vulnerable to pathological enhancement of glutamate release as observed during neonatal hypoxia-ischaemia (Nyakas et al., 1996). In terms of knowledge about the involvement of glutamate in neonatal ischaemic neuronal damage, NMDA receptor antagonists are proved to be highly neuroprotective in neonatal hypoxic-ischaemia (Wong et al., 1986; McDonald et al., 1987; Ford et al., 1989; Hattori et al., 1989; Olney et al., 1989). MK-801 given before hypoxic-ischaemic insults in postnatal day 7 and 10 rats has been seen to completely prevent brain damage, and even given up to one hour after the insults it still showed a neuroprotective effect. These studies were conducted on rats at the postnatal ages reported to have transient peaks in glutamate receptors (Greenamyre et al., 1987). Hypoxic-ischaemic brain injury at postnatal day 7 has been reported to result in a considerable release of EAAs including glutamate both in vitro (Silverstein et al., 1991), and in vivo (Andiné et al., 1990; Gordon et al., 1991). Interestingly though, (Cherici et al., 1991) found no increase in glutamate release in neonates under ischaemia. Some studies appear to be contradictory in this point and hence further investigation remains necessary. In a model of hypoxic-ischaemia in 7 day old rats, EAAs and glycine were released to the extracellular space (Andiné et al., 1991), calcium was accumulated in brain regions that will eventually undergo infarction (Stein and Vannucci, 1988), and neurones that expressed EAA receptors were reported to be particularly vulnerable (Silverstein et al., 1987). In the case of hypoxicischaemic brain damage at postnatal day 7 the striatal and neocortical structures are

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notably damaged, whereas the hippocampus remains relatively spared (Hattori *et al.*, 1989). This difference in structural sensitivity cannot however be explained with the altered densities of glutamatergic receptors, as hypothesised by some (reviewed in Nyakas *et al.*, 1996). It therefore seems that at this stage the role of glutamate and its receptors during ischaemia in the developing brain is unclear and requires further investigation.

There are apparent differences in glutamatergic processes during anoxia as opposed to ischaemia in the developing brain. Whilst they did not directly measure released amounts of glutamate under anoxia, Friedman and Haddad (1993) suggested from their data that glutamate blockers do not attenuate the increase in Ca^{2+} seen in dissociated CA1 neurones during anoxia, and thus indirectly suggest that glutamate is not involved in anoxic disturbance of Ca^{2+} homeostasis. Other studies have reported that there is a limited release of glutamate upon hypoxia in hippocampal slices *in vitro* at the age of 4-9 days compared to the adult level (Cherici *et al.*, 1991).

In summary, studies suggest that mechanisms involved in glutamate and excitotoxicity change as the brain develops into maturity. The role of these different responses of the immature and adult brain to neuronal damage remains as yet unclear. Further investigation of these processes in the developing brain and their implications for pathological conditions, such as ischaemia and hypoxia, is needed.

METHODS

3.2 Methods

3.2.1 Materials

Fura-2/AM, Triton X-100, NADH and NADP⁺ in prepacked, sealed vials, N,N,N'N'tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), and L-glutamate dehydrogenase (GDH) (EC 1.4.1.3) were purchased from Sigma Chemical Company Ltd. (Poole, Dorset, U.K.). GDH was dialysed against a 50:50 (V/V) glycerol: 100 mM sodium phosphate buffer, pH 7.3 to remove contaminating glutamate.

All other routine chemicals and enzymes used were of analytical grade and were supplied by either the Sigma Chemical Company Ltd., or from BDH Ltd. (Dagenham, Essex, U.K.)

All solutions were made up in deionised double distilled water. Composition of routine buffers were as detailed in section 2.2.7.

3.2.2 Measurement of intrasynaptosomal calcium concentration

3.2.2.a Principle

The investigation of the numerous roles of calcium requires the accurate, quantitative measurement of calcium concentrations. The presence of high extracellular concentrations of calcium, and the considerable amount of bound calcium within cells has made the assaying of intracellular free calcium concentrations difficult. This problem has been overcome to a large extent over the last few years. This has been because of the use of fluorescent techniques that employ dyes with known calcium affinity, and specific measuring instruments. The fluorescent dyes that have been introduced can be easily accumulated by isolated cells and retained by them. These dyes form a complex with intracellular free calcium and give an easily detectable, characteristic fluorescent signal.

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Using the technique of fluorometry to determine calcium concentrations is advantageous in comparison with other methods in use. Unlike techniques which employ radioactive isotopes for calcium measurements, there are no specific handling problems incurred with fluorometry. Furthermore, unlike techniques utilising microelectrodes, there is no cellular damage caused, and measurement is not restricted to large cells or to local responses. Fluorometry permits very fast tracking of cation concentration changes, and it can be easily coupled with sophisticated imaging techniques. Imaging techniques can pinpoint the location of site-specific calcium events within a cell and distinguish heterogenous behaviour in an apparently uniform sample, including calcium effects in specific organelles such as mitochondria. Without imaging capability, fluorometric analysis of micro-samples can still give fast, accurate changes in average intracellular calcium changes over time.

Ca²⁺- indicators

The standard method for determining intracellular free Ca^{2+} concentration involves a family of tetracarboxylic acids which can be easily loaded into cells as the ester form. The first in this family was Quin2, and a number of additional Ca^{2+} sensitive dyes have since been introduced, each with a number of improved properties (Grynkiewicz *et al.*, 1985). These dyes have been modelled on the widely used calcium selective chelator EGTA. Figure 3.2 shows the structures of EGTA, Quin2 and the more recently introduced and widely used Fura-2, for comparison. While the tetracarboxylic acid Ca^{2+} -binding site is largely unchanged in the fluorescent indicators, additional groups have been added which act as fluorescent reporters. Binding of Ca^{2+} withdraws the lone pair electrons from the aromatic rings resulting in altered fluorescence properties. For most biological applications, any one or a combination of the following altered fluorescence properties are appropriate:

- a change in fluorescence yield.
- a shift in the excitation spectrum.
- a shift in the emission spectrum.







Figure 3.2 The structures of EGTA, Quin2 and Fura-2

Dye loading

In some experiments, dyes can be introduced into cells by microinjection. However, the indicators also lend themselves to loading as membrane permeant esters, which allow for a decreased disruption of the cell membrane (Grynkiewicz *et al.*, 1985). Each carboxyl group on the Ca²⁺-indicators can be esterified to form acetoxymethyl (AM) esters. The resultant esterified indicator is sufficiently hydrophobic to readily cross the plasma membrane. Once in the cytosol, endogenous, non-specific esterases cleave off the AM groups sequentially, regenerating the free acid from of the indicator. This free acid form cannot permeate the cell membrane and results in the accumulation of trapped indicator in the cytosol. For accurate results, care must be taken to ensure that any fluorescent probe that has leaked from the cell is diluted or washed away prior to the measurement. Once extracellular dye has been removed, fluorescence signals from the cytosolic dye can be recorded and used to calculate intracellular Ca²⁺ concentrations.

3.2.2.b Fura-2

Fura-2 is the dye that has been used to measure intrasynaptosomal free Ca^{2+} concentration in this study. It is currently the most widely used of the fluorescent Ca^{2+} indicator dyes. Ca^{2+} binding to this dye is a function of the free Ca^{2+} concentration, and the dissociation constant (Kd) for Fura-2. In the case of Fura-2, half-maximal binding occurs at about 200 nM free Ca^{2+} concentration. Figure 3.3 shows the spectral properties of Fura-2. The excitation spectrum of Fura-2 is shifted some 40nm to a lower wavelength when Ca^{2+} binds. This shift in excitation spectrum allows the proportion of free-fura and calcium-bound fura to be calculated from the ratio of emission intensity, following excitation, at two different wavelengths. Hence this allows Fura-2 to be utilised as a dual excitation indicator. For optimal separation of fluorescence from the two forms of Fura-2, Ca^{2+} free Fura-2 is monitored at 380 nm and Ca^{2+} bound Fura at 340 nm excitation. Fura-2 also has an isobaetic point at 360 nm which is useful for monitoring Ca^{2+} -independent fluorescence.



Figure 3.3 Effect of Ca⁺⁺ on the excitation spectrum of Fura-2

legend for figure 3.3

This figure, adapted from Grynkiewicz *et al*, (1985), shows the excitation spectrum of Fura-2 when exposed to varying concentrations of calcium, shown in nM units in italics. The fluorescence peak of unbound Fura is at 380 nm and that of calcium-bound Fura at 340 nm.

Typically, Fura-2 is used at intracellular concentrations in the micromolar range. It has a Kd of approximately 225 nm, and therefore Fura-2 is suitable for intrasynaptosomal free Ca²⁺ concentrations up to several micromolar. Fura-2 can be easily loaded into cells as its acetoxymethyl ester form Fura-2/AM. After typically a 30 minutes incubation, most of the original Fura-2/AM has accumulated in the cytoplasm as free Fura-2. Fura-2 does not affect the plasma membrane potential of synaptosomes (Komulainen and Bondy, 1987), which was an important consideration for studies involving metabolic stress.

3.2.2.c Advantages and disadvantages of fura-2

Advantages of fura-2

Fura-2 offers several advantages over its predecessor Quin2, which have made the measurement of calcium more reliable.

[1] Fura-2 offers a 30-fold more intense fluorescence than Quin2.

Fura-2 has a greater quantum efficiency, resulting in a given intensity of fluorescence requiring about 30 times less Fura-2 than Quin2 (Grynkiewicz *et al.*, 1985). Quin2 was typically used in the millimolar range, whereas Fura-2 is in the tens of micromolar range. This decreases the danger of cytotoxicity being incurred by the presence of the indicator (Tsien, 1981).

[2] Photobleaching is not a significant problem with Fura-2.

Fura-2 loaded synaptosomes can be illuminated continuously for longer than 15 minutes without significant photobleaching, whereas this is not possible with quin2 loaded synaptosomes (Komulainen and Bondy, 1987).

[3] Mitochondrial hydrolysis of Fura-2 is not thought to significantly contribute to synaptosomal calcium measurement.

It is suggested that because of the higher loading and subsequent hydrolytic capacity of synaptosomes relative to mitochondria, at the low loading concentrations used Fura-2/AM is more likely to be hydrolysed in the cytosol of synaptosomes than in extraor intrasynaptosomal mitochondria (Rink & Pozzan, 1985). Contamination of synaptosomal preparations by mitochondria has therefore been found not to affect measurements of intrasynaptosomal calcium concentration $[Ca^{2+}]_i$ (Komulainen and Bondy, 1987). Hence, Fura-2 measurements of $[Ca^{2+}]_i$ are routinely carried out using a crude synaptosome/mitochondria (P2) pellet.

[4] Low concentrations of Fura-2 can be used.

The low concentrations of Fura-2 loaded into cells means that this dye has much less of a calcium-buffering effect than quin2, and calcium transients are easier to detect than with Quin2.

[5] The affinity for cations is different between Fura-2 and Quin2.

Fura-2 has a slightly lower affinity for Ca^{2+} than Quin2. This improves the resolution of changes in Ca^{2+} levels above 1µM. The generally reduced heavy metal affinity of Fura-2 compared to Quin2 also means that toxicity due to chelation of essential heavy metals is less likely to occur (Tsien, 1981). Fura-2 also has a better discrimination than Quin2 for Ca^{2+} over competing divalent ions such as magnesium, manganese and zinc (Grynkiewicz *et al.*, 1985). Artifactual readings due to high levels of exchangeable heavy metals are therefore less likely to occur with Fura-2.

[6] Fura-2 measurements can be made as a ratio.

The utilisation of the ratio of two separate wavelengths for Fura-2 measurements rather than a single wavelength leads to a number of distinct advantages over Quin2. The ratiometric approach ensures that a number of variables that perturb measurements are eliminated.

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In particular, ion-independent factors that affect the signal intensity are cancelled out in the ratio measurements, since these parameters have a similar effect on intensities at both wavelengths. These include variations in cell thickness, dye loading, probe leakage, dye bleaching and instrument efficiency. The problem of sedimentation of synaptosomes is also overcome, since the ratio remains constant in the face of diminishing total fluorescence. The ratio method is also less sensitive to potential errors due to correction for extrasynaptosomal dye (Komulainen and Bondy, 1987).

Disadvantages

Fura-2 has many advantages over its predecessor Quin2 and most of the problems with Fura-2 can be resolved using suitable controls and calibrations. However, it is important to be aware of the following disadvantages of Fura-2 and to have an understanding of where they could become problematic.

[1] Fura-2 has a tendency to become compartmentalised in subcellular organelles during prolonged loading periods. This can be easily assessed and the loading protocol modified to ensure that most of the dye remains cytosolic.

[2] The main problem that has been encountered with Fura-2 relates to the incomplete hydrolysis of the ester form. The by-products of incomplete ester hydrolysis are the most likely cause of cell damage or abnormal cellular function. It is relatively straightforward to assess whether incomplete hydrolysis is a problem. The fluorescence can be monitored after calcium saturation of the dye, for example following addition of a calcium ionophore. A continuously rising fluorescence indicates ester forms that are hydrolysed progressively during measurement. Loading conditions can easily be manipulated to ensure that maximum hydrolysis of ester forms is occurring before measurements are taken.

The long list of advantages and the ease of overcoming the few disadvantages have made Fura-2 the preferred fluorescent indicator for many intracellular applications, including measuring calcium changes in the isolated nerve terminal.

3.2.2.d Sample preparation

Crude synaptosomes (P2), or purified synaptosomes were prepared as outlined in section 2.2.2 for adults, and in section 2.2.3 for pups aged 15 days and below.

Original experiments - 60 minutes, mild metabolic stress

In the experiments when calcium levels were measured following a 60 minute period of metabolic stress, synaptosomes were incubated in the appropriate Kreb's Phosphate Buffer (KPB) at 37°C, as outlined in section 2.2.7.b.(1). This set of experiments has been labelled as 60 minutes of 'mild metabolic stress', because oxygen tensions in these incubations were initially higher than for later experiments.

Incubation conditions

This original set of experiments was conducted on brains of all age rats, under all conditions. Synaptosome pellets were resuspended into the appropriate pre-gassed, KPB at 37°. This was after a short pre-incubation in control KPB without calcium (see section 2.2.7.b). The pre-incubation suspension was split into eight equal volumes of 500 μ l, and pelleted. These pellets were then resuspended into eight different vials, containing the appropriate buffer for each incubation. For some sets of experiments there was not enough material available for eight vials, and therefore different combinations of control and insults were used, but controls were always present. For four insults to be conducted in one experiment, four vials were necessary so that incubations to correct for autofluorescence could be conducted. Protein content in the incubations was approximately 5 mg/ml (see section 2.2.5. for protein determination). The oxygen content of the hypoxic/anoxic and ischaemic incubations at time zero was approximately 11% of control incubations in these experiments. During all incubations oxygen or nitrogen was supplied as detailed in section 2.2.7.b.

Fura-2/AM addition

After 30 minutes of the incubation Fura-2/AM was added to four of the vials labelled control, anoxia, algycaemia and ischaemia which were then wrapped in aluminium foil to exclude light and incubated for a further 30 minutes in the same

conditions as the previous 30 minutes. Fura-2/AM final concentration was 5 μ M dissolved in anhydrous dimethyl sulphoxide (DMSO). The remaining four vials had the same volume of DMSO (typically 10 μ l into 1 ml total) added to them to enable autofluorescence measurements on the various conditions to be performed. These vials were also labelled control etc., but were also clearly labelled as DMSO samples. They were wrapped in aluminium foil and incubated as for the Fura-2 containing incubations. At the end of the incubation period, Fura-2 loading was terminated by a rapid ten-fold dilution with the appropriately gassed, warmed buffer for each incubation. i.e. dilution with aglycaemic buffer for the aglycaemic incubation. The diluted incubations were centrifuged at 10,000g for two minutes in 1.5 ml aliquots. The supernatant was discarded and all pellets were washed with warm pregassed KPB to eliminate any extrasynaptosomal fluorescent probe. Pellets were kept on ice in the dark until fluorescent measurements were performed, which were always within fifteen minutes of pelleting. To enable this the start times of incubations were often staggered.

Later experiments - severe metabolic stress

In later experiments, only adult or PND 10 synaptosomes were used and only control, anoxic and ischaemic conditions were used. These experiments have been labelled as 'severe metabolic stress', because the oxygen tensions were initially lower than for the original experiments of mild metabolic stress. Synaptosomes were incubated in KPB at 37°C as outlined in section 2.2.7.c.(2), i.e. sodium dithionite was present in the anoxic and ischaemic buffers. The initial oxygen tensions of these incubations were approximately 4%, always becoming totally anoxic within 15 minutes of incubation. Calcium measurements were made under these conditions at 15, 30 and 60 minutes.

15 minutes

For the 15 minute incubations, Fura-2/AM or DMSO was added at time zero whilst all incubations were under control conditions. After 15 minutes, synaptosomes were pelleted at 10,000 g in a bench-top centrifuge, and the supernatant discarded.

Fresh KPB was then added to the pellets, and incubation proceeded as detailed in section 2.2.7.b. The KPB used was the appropriate buffer for the appropriate sample (i.e. ischaemic buffer was added to the incubation tube which labelled as ischaemic). To ensure that the oxygen tensions were similar to the 30 and 60 minute incubations, 200 μ l of control buffer and 800 μ l of the appropriate buffer were used to resuspend the pellets, because the protocol here was slightly different. A second addition of Fura-2/AM or DMSO, at the same concentration as originally, was made to these incubations because these compounds would have been discarded with the supernatant which was removed at 15 minutes. At 30 minutes the incubations were terminated. They were then treated exactly the same as for the initial set of 60 minute mild metabolic stress experiments. The incubation conditions used here allowed a 30 minutes loading time for fura-2/AM, coupled with a 15 minute period of metabolic stress.

30 minutes

For the 30 minute incubations, Fura-2/AM or DMSO was added at time zero when all incubations were put under conditions of metabolic stress, as outlined in section 2.2.7.b.(2). After 30 minutes the incubations were terminated, and then treated exactly the same as for the initial set of 60 minute mild metabolic stress experiments and the 15 minute severe metabolic stress experiments.

60 minutes

For the 60 minute incubations, synaptosomes were put under conditions of metabolic stress, as outlined in section 2.2.7.b.(2). After 30 minutes of the incubation Fura-2/AM or DMSO was added, exactly as for the 60 minute mild metabolic stress experiments, and the incubations proceeded as before. After 60 minutes the incubations were terminated, and then treated exactly as for all previous calcium experiments.

3.2.2.e Fluorescence measurement

To determine the free Ca²⁺ concentration in the treated synaptosome samples, fluorescence was measured using a Perkin-Elmer LS50B Luminescence Spectrometer. Fluorescence was measured by the method of Ashley *et al.*, (1984) using the formula of Grynkiewicz *et al.*, (1985). Synaptosome pellets were removed from the dark. The supernatant was discarded and the pellet was washed with the appropriate KPB. Using the appropriate KPB, four pellets were resuspended into 1.5 ml total, leading to a final protein concentration of approximately 2mg/ml. The sample was transferred to a quartz cuvette which was capped and placed in the spectrometer. The temperature was kept constant at 37°C and constant stirring was applied to the sample. The stirring mode was low and a red stirrer was used to keep synaptosomal lysis and light scattering to a minimum. The instrument was operated in the ratiometric mode.

For all measurements the parameters were set as follows:-

slit widths - ex., em.	5, 10 nm
emission wavelength	510 nm
excitation wavelength (2)	380 nm
excitation wavelength (1)	340 nm

Measurements were taken over a 2 - 5 minute period, and all data points were used to arrive at a mean value for the calcium concentration. During analysis, a basal unstimulated recording was taken. This was followed by addition of 50 mM KCl (final concentration) directly into the cuvette. Addition was made through a microsyringe with a long needle to enable addition without having to open the chamber, and thus light entry was kept to a minimum. The addition of KCl caused a clamped depolarisation of the synaptosomes to occur, and this depolarised level was recorded for a further 2-5 minutes.

Following the fluorescence measurements of the sample, the autofluorescence of a similarly treated sample was measured (i.e., the DMSO containing sample). The autofluorescence measurement was subtracted from the sample prior to calibration.

The autofluorescence correction was particularly important for samples which had undergone metabolic stress. This was because any changes in the NADH/NAD⁺ status of the synaptosome preparation would lead to changes in the emission value following absorption at 340 nm and could interfere with the assay of calcium levels, for which a correction is necessary.

3.2.2.f Calibration

Each sample was individually calibrated to correct for any slight differences in protein concentration or dye loading between samples. A calibration run is collected separately from the data run. To calibrate the sample, a value for the maximum fluorescence ratio, when all Fura-2 present in the sample is saturated with calcium (R_{max}) must be determined. The minimum fluorescence ratio, when no calcium is bound to the Fura-2 (R_{min}) must also be determined. The values for R_{max} and R_{min} can easily be obtained for each sample by manipulating the calcium levels surrounding the fluorescent indicator. R_{max} was determined by addition of Triton X-100 (0.5% final concentration). Triton X-100 is a non-ionic detergent which causes lysis of the synaptosome plasma membrane. This allows all of the fura-2 in the synaptosome sample to come into contact with the 1.2 mM CaCl₂ in the medium. Hence all of the fura-2 rapidly becomes saturated, and a value for R_{max} is obtained. Following determination of R_{max} , R_{min} was also obtained for each sample. R_{min} is given by the ratio at steady state following addition of EGTA-Tris (30 mM final concentration). This excess EGTA results in all of the calcium ions becoming stripped off the fura-2 molecules. The alkaline medium, created by the presence of Tris causes the EGTA to have an increased affinity for calcium. All of the calcium ions become chelated by the EGTA, and hence the minimum fluorescence ratio when no calcium at all is in contact with fura-2 is determined. Following synaptosome lysis by Triton X-100, R_{max} and R_{min} may be obtained in any order. Note that for calibration of the samples, the fluorescence values do not need to be corrected for extracellular dye. This is because the ratio is independent of dye concentration.

3.2.2.g Treatment of results

During the fluorescence assay of a sample, raw data for the fluorescence intensities at each of the discrete wavelengths are saved and stored in ASCII format files using the Perkin-Elmer software. Ratios of the intensities at the 340 nm and 380 nm excitation wavelengths are then calculated, also taking into account the values for autofluorescence. This ratio data must then be calibrated in order to calculate the concentration of the metabolite - which in this case is calcium. Calibration occurs as detailed in section 3.2.1.f. The ratio values for R_{max} and R_{min} are thus collected and then stored. Calcium concentration is then calculated from the ratio data collected during sample measurement, and also the calibration ratio data.

Calcium concentration is calculated according to the following equation:-

$$Ca_{T} = Kd x \quad (R_{T} - R_{min}) x \quad Sf2$$

$$(R_{max} - R_{T}) \quad Sb2$$

Where:-

Ca_T	is the concentration of calcium at time T.	
Kd	is the dissociation constant of the Fura-2 at the temperature of	
measurement.	ent. It is 224 nM for the conditions employed in this study	
R_T	is an experimental ratio value of 340 /380 nm at time T.	
R _{min}	is the ratio value from the completely unbound Fura-2 probe, that is in	
	the absence of calcium.	
R _{max}	is the ratio value from the completely bound Fura-2 probe, that is in the	
	presence of saturating calcium.	
Sf2/Sb2	is the ratio of fluorescence of Fura-2 at zero calcium and saturating	
	calcium at the excitation wavelength of 380 nm. This measurement	
	corrects for any wavelength biasing due to instrumental artifacts.	

3.2.3 <u>Validation of the accuracy of calcium measurements with Fura-2.</u>3.2.3.a. Synaptosome integrity

The integrity of the synaptosome preparation was monitored during the periods of incubation that were employed for calcium measurements. It was imperative to assess whether the permeability of synaptosome membranes was increased during the period of calcium measurement in the cuvette in the fluorometer. If there was increased membrane permeability this could lead to increased leakage of the Fura-2 which had been previously loaded into the synaptosome cytosol. During a period of fluorescence measurement, if Fura-2 was passing through the synaptosome membrane it would come into contact with the extrasynaptosomal calcium concentration of 1.2 mM. This would give an artifactual increase in the levels of fluorescence during the measurement period, and hence an artificial increase in calcium levels would be recorded that had no connection with cytosolic changes in calcium.

The integrity was therefore monitored in two ways. Firstly, the activity of LDH released during the period of incubation and Fura-2 loading was measured. The principle and assay for LDH release as a marker of membrane integrity is detailed in section 2.2.4.g. Secondly, after a few minutes of fluorescence measurement under the same conditions as all measurements were conducted, (*i.e.* at 37°C and with stirring) a synaptosome incubation was pelleted and the supernatant assessed for fluorescence due to the presence of leaked Fura-2. This was assessed in at least 3 separate experiments for separate adult preparations and postnatal day (PND) 10 pups.

3.2.3.b Cytosolic localisation and ester hydrolysis.

When wanting to study changes in intrasynaptosomal calcium levels, it is important to establish that the majority of the active Fura-2 is indeed cytosolic, and also that complete hydrolysis of the ester has taken place.

A number of parameters need to be taken into account when establishing a protocol for the loading of Fura-2 as an ester form. The hydrolysis products of the deesterification process may be toxic and care must be taken in the amount of ester

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added to an incubation and the length of time over which these products can be in contact with the synaptosomes. The synaptosome density can affect the level of loading, which is often only 50% efficient. Thus with high protein concentrations which reflect a high synaptosome concentration a higher concentration of Fura-2/AM is required. Loading proceeds most rapidly at 37°C, but lower temperatures can reduce compartmentation. Therefore, a systematic approach involving many preliminary experiments is often needed to establish the optimal conditions for loading and hydrolysis in previously untested cases.

It has been noted previously that the hydrolytic capacity of synaptosomes for the Fura-2 ester is limited (Komulainen and Bondy, 1987), and it is therefore important to determine whether hydrolysis is occurring to a sufficient extent to allow calcium measurement. The time course and extent of de-esterification of Fura-2 can be determined by repeated wavelength scans on an incubation to which the ester form has been added. This can provide some information because the spactrum of the ester form is sufficiently different from the free-acid form of the dye. However, this approach does not distinguish between dye hydrolysed inside the synaptosome and that hydrolysed in the extracellular medium. In addition the spectra may not reveal the presence of partially hydrolysed species. The most direct approach to assess the hydrolysis of the ester groups is to wash away extracellular dye, release the dye from the synaptosomes after the incubation period, and to test its calcium sensitivity. Ideally the loaded Fura-2 is released from the synaptosomes by membrane lysis using TritonX-100 or a similar detergent.

A series of spectra are then taken in buffer with differing calcium concentrations and compared to similar spectra of the free acid form of Fura-2, which is commercially available. Alternatively, the fluorescence of synaptosomal suspensions can be followed after saturation with calcium by addition of a calcium ionophore such as ionomycin. If a continuously rising fluorescence becomes apparent over time, this indicates that part of the indicator content consisted of ester forms that are hydrolysed progressively during measurement. Hence this would highlight that the loading protocol used did not allow full conversion of the ester form to the free acid form of Fura-2.

Hydrolysis of the Fura-2/AM loaded into adult and immature synaptosomes was assessed during optimisation of the protocol used in these experiments. The results are detailed in section 3.3.1.

The localisation of the free acid form of fura-2 was also assessed in the preparations. This was assessed by differentially permeabilising the synaptosome plasma membrane with 0.1 % (v/v) digitonin so that only cytosolic entrapped dye would be released. In the presence of saturating calcium, the increase in fluorescence ratio caused by the addition of digitonin corresponds to the free acidic Fura-2 formed in and confined to the cytosol. Any remaining Fura-2 which is compartmentalised, in the mitochondria for example, can be released by the addition of 0.5% Triton X-100. By taking a ratio of the initial increase in fluorescence after digitonin treatment to the total maximum fluorescence, and expressing it as a percentage, the extent of cytosolic localisation can be determined. This was assessed and compared between adult and immature synaptosome preparations.

3.2.3.c Artifactual results caused by interfering divalent cations.

Fura-2, along with the other fluorescent indicators which have been largely modelled on the structure of EGTA, can bind a number of metal ions. An important property of the Ca^{2+} -indicators is their selectivity for Ca^{2+} over other divalent transition metal ions. However, a number of ions including manganese (Mn²⁺),

iron (Fe²⁺) and zinc (Zn^{2+}) can bind to the fluorescent indicators and hence interfere with the accuracy of the results obtained. In the case of Quin2, Zn^{2+} , Fe^{2+} and Mn^{2+} have all been reported to have at least an order of magnitude greater affinity for this dye, than has Ca²⁺ (Grynkiewicz et al., 1985; Komulainen and Bondy, 1987). Binding of such cations quench, the fluorescence of Quin2 and at low Quin2 concentrations such quenching can become significant and cause an underestimation of [Ca²⁺]. Fura-2 is different in a number of respects. Firstly, Fura-2 has considerably less affinity for divalent ions other than Ca^{2+} , than Quin2 has. Therefore, when Fe^{2+} and Mn²⁺ do bind to Fura-2 there is also quenching of fluorescence as with Quin2, but this is much less of a problem in the case of Fura-2. However, Zn^{2+} in particular can tightly bind to Fura-2 and form a complex with it. Unlike any complexes formed between Zn^{2+} and Quin2 which quench fluorescence, the complex formed between Zn²⁺ and Fura-2 causes fluorescence changes similar to those resulting from calciumbinding. Hence, an increase in fluorescence signal can occur in the presence of Zn^{2+} . Although most cells contain very little free Zn^{2+} , its presence may therefore induce experimental artifacts which become crucial to evaluate. The use of TPEN, a permeant trace metal chelator (Arslan et al., 1985), with high affinity for the interfering divalent cations being discussed here, can establish whether $[Ca^{2+}]$ is being incorrectly estimated due to the presence of these cations.

In adult synaptosomes under normal conditions the overestimation of $[Ca^{2+}]$ by Fura-2 has been established to be approximately 10% (Komulainen and Bondy, 1987) and does not preclude the use of this dye. However, in light of the developmental aspect of this study it was important to establish whether the same low level of artifact occurs in immature synaptosomes. It also became vitally important to repeat some of the experiments studying the effects of metabolic stress on calcium levels and to assess possible artifacts due to Zn^{2+} . This was in light of published work detailing metabolic resonance spectroscopy studies of Ca^{2+} , Zn^{2+} and energy metabolism in superfused brain slices (Bachelard, 1994). A release of free intracellular Zn^{2+} was observed after exposure to excitotoxic amino acids, but not after metabolic deprivation in these studies.

These studies used brain slices and although synaptosomes were the model of choice for the studies in this thesis it remained important to assess for possible artifacts due to increased free Zn^{2+} in synaptosomes under metabolic stress. To enable this, control and ischaemic samples from adult and postnatal day 10 synaptosomes were assessed in the presence and absence of TPEN. The results are detailed in section 3.3.1.

3.2.4 Determination of the glutamate concentration in the extrasynaptosomal medium

3.2.4.a Principles of assaying glutamate in the supernatant

Hypoxia, hypoglycaemia and ischaemia can each lead to a pathological increase in extracellular glutamate. Glutamate exerts its effects on glutamate receptors in the pre- or post-synaptic membrane when it comes into contact with them. Although this may seem an obvious statement, it underlies the reason for choosing to assess the concentration of glutamate present in the extrasynaptosomal medium during conditions of metabolic stress. There is compelling evidence to show that extracellularly elevated glutamate worsens neuronal survival *in vivo* under conditions of energy failure caused by hypoxia or ischaemia (Kauppinen, 1994). It has been suggested that the origin of excitotoxic glutamate is likely to be the nerve terminal (Kauppinen, 1988) *In vivo* the amount of this excitotoxic glutamate that is available to glutamate receptors can vary when changes occur in either/both:-

[1] the amount of glutamate released from the nerve terminals,

[2] the rates of re-uptake of glutamate from the synaptic cleft.

Under a situation of imposed metabolic stress on isolated nerve terminals, glutamate release may decrease or increase. However, changes may also occur in the pathways of re-uptake of glutamate into the nerve terminals. It is a combination of these two distinct parameters which will ultimately determine whether the amount of glutamate in the synaptic cleft is increased or decreased under conditions of metabolic stress.

Ischaemia, calcium and glutamate

This study was concerned with determining the amount of glutamate that would be present in the extrasynaptosomal *milieu* during conditions of metabolic stress. By comparing the outcome in adult and PND 10 pup synaptosome preparations, any possible differences in the amount of glutamate available to glutamate receptors in the brain under the same conditions of metabolic stress, but at different stages of maturity, could be assessed.

3.2.4.b Principle and procedure for assaying glutamate in the supernatant

All synaptosome samples were incubated for 30 minutes under conditions of severe metabolic stress as detailed in section 3.2.3.d 'later experiments - severe *metabolic stress'*. Following incubation of the synaptosomes, all samples were pelleted at 10,000g for 5 minutes in a bench top centrifuge. The supernatants were immediately removed and snap frozen in liquid nitrogen. Samples were then stored at -80°C until the day of assay.

Glutamate was determined in the supernatants by a fluorometric assay using a Perkin-Elmer LS-B50 luminescence spectrometer. Exogenous NADP⁺ and the enzyme glutamate dehydrogenase (GDH) are added to the test sample. Hence, this assay is based on the following reaction:-

GDH

glutamate + NADP⁺ $\Rightarrow \alpha$ -ketoglutarate + ammonia + NADPH + H⁺

The change in fluorescence due to the reduction of NADP⁺ and the resultant appearance of NADPH is followed at 340 nm excitation and 460 nm emission. This method has been widely used for a number of years. Although NAD⁺ can also be used in the place of NADP, and indeed both nucleotides are equally active with GDH, NADPH does not undergo the slow reoxidation that has previously been observed to happen with NADH under some circumstances (Nicholls et al., 1987).

Glutamate was assayed in the supernatant alone, after removal of the synaptosomes for the following reasons:-

[1] The glutamate present at the end of the incubation can more accurately be assessed if the synaptosomes and therefore the opportunity for further changes in glutamate status are removed, i.e. accurate timings of glutamate status at 30 minutes can be achieved by supernatant removal and snap freezing.

[2] The rate of α -ketoglutarate production by GDH does not occur instantaneously because the equilibrium favours the back reaction. The possibility therefore exists that if synaptosomes were still present a proportion of the glutamate could be reaccumulated into the synaptosomes, leading to an underestimation of the total extrasynaptosomal glutamate present in the incubation.

For the quantitative studies required here, which do not involve precise kinetics of rates of glutamate release, the fluorometric assay is sufficiently accurate if a standard curve is also run on the day of assay. The response of the assay to known concentrations of added glutamate is used to convert the fluorescence changes displayed by the test samples to nanomoles of glutamate per milligram of protein.

Procedure

A stirred 3 ml fluorometry cuvette kept at 30°C throughout the assay, contained the following (final concentrations):-

1.25 mMNADP+50 UGDH50 - 200 μ ltest supernatantcontrol KPB is added to make a final volume of 2 ml.

A baseline fluorescence reading was taken for a few minutes. The reaction was then initiated by the addition of the test supernatant. After reaching a plateau the fluorescence was monitored for a few minutes.

Ischaemia, calcium and glutamate

A standard addition of 5 nmoles of glutamate was added at the end of each sample analysis to ensure that conditions of the assay had not altered during the period of incubation. The concentration of glutamate was calculated by use of a standard curve of 1-10 nmoles on the day of assay, before running the test samples. Assaying of samples only proceeded if the standard curve was satisfactory and linear under the conditions used ($r \le 0.996$).

The spreadsheet package Quattro-Pro was used to analyse the data. Means of the fluorescence changes were calculated and converted to nmoles of glutamate by use of the standard curve. The dilution factor was then taken into account (i.e. 200 μ l sample assayed but the synaptosome incubation volume was 1 ml, therefore the calculated glutamate concentration was multiplied by 5). The protein concentration of the synaptosomes incubation was then measured (as detailed in section 2.2.5) and expressed as mg per ml. With all of this data, the results could be expressed as nmoles glutamate per mg protein, and comparisons between groups made. Results are detailed in section 3.3.5. Results are expressed as mean \pm s.e.m. of 4 separate experiments. Statistical analysis was performed using analysis of variance (ANOVA).

3.2.5 <u>The role of metabotropic glutamate receptors in intrasynaptosomal</u> <u>calcium changes</u>

3.2.5.a Principle

The amino acid glutamate can be excitotoxic, primarily due to chronic activation of post-synaptic NMDA receptors. Hence, much of the neuronal injury caused by exposure to increased glutamate is focused around post-synaptic events involving elevations of $[Ca^{2+}]_i$ and the glutamate receptors.

Part of this study has focused on the amount of glutamate that would be in the extrasynaptosomal milieu during and following metabolic stress. This is mostly relevant to the post-synaptic receptors which would be in contact with any glutamate present in the synaptic cleft.

However, one must question and examine the effects that increased glutamate in the extrasynaptosomal environment may have on the state and function of the synaptosome itself. This is important because pre-synaptic glutamate receptors are present on the synaptosome membrane, and when studying synaptic function following metabolic stress one cannot rule out that changes in glutamate concentrations could occur and affect synaptic function via such glutamate receptors. Exactly which functional glutamate receptors are present on the synaptosome membrane is currently a matter of debate, as detailed in section 3.1.7. However, the possibility of changes in extrasynaptosomal glutamate concentrations influencing synaptic state and function should be explored, even if the exact receptor types and subtypes that may be involved are somewhat unclear at this stage.

The effect of metabolic stress on the levels of cytosolic calcium in synaptosomes have been researched in this study. The levels of glutamate present in the extrasynaptosomal environment under the same conditions have also been studied. Following such investigations, it became important to ascertain whether the presence of increased glutamate in the synaptosomal incubation could be influencing the calcium changes that became apparent under the conditions of metabolic stress. Experiments were therefore conducted in which any glutamate released under the conditions of metabolic stress was removed. Changes in the intrasynaptosomal calcium were then assessed in the presence and absence of glutamate to determine whether removal of the amino acid would influence these changes in any way.

3.2.5.b Removal of glutamate from synaptosome incubations

To assess the potential role of pre-synaptic glutamate receptors in calcium changes during conditions of metabolic stress, it was necessary to remove any released endogenous glutamate from the synaptosome incubations. For this purpose, 2 mM NADP⁺ and 75 units of GDH were added to relevant incubations. The reaction that occurs in the incubation as glutamate is released from the synaptosomes is the same reaction which forms the basis for the assay detailed in section 3.2.4.

Ischaemia, calcium and glutamate

As glutamate is released into the extrasynaptosomal medium it becomes oxidised to α -ketoglutarate by the action of GDH. Hence, glutamate is prevented from exerting its effects on the synaptosomes via glutamate receptors.

The amount of ammonia that is produced in this reaction is obviously dependent on the amount of glutamate that is released from the nerve terminals. High concentrations of ammonia can be detrimental to cellular function. Hyperammonaemia can shift the equilibrium of the reaction outlined previously to such an extent that α ketoglutarate becomes limiting for the TCA cycle and ATP depletion may occur as a consequence. It was important to ascertain whether excessive amounts of ammonia would be produced in the incubations when glutamate was removed. If very high levels of glutamate were released and hence high levels of ammonia would be formed, synaptic function could become compromised as a consequence of ATP depletion. Thus, artifactual results could be gained which were not directly connected to the effects of glutamate via pre-synaptic glutamate receptors, and which would confuse the issues being investigated. Normal cellular and blood concentrations of ammonia are within the micromolar range. Previously conducted experiments established that the levels of glutamate released under the conditions outlined in section 3.2.3 were in the micromolar range. Therefore the same conditions were used for this investigation, and protein concentrations were kept to the 5 - 10 mg/ml range to ensure that the levels of glutamate and hence ammonia produced remained safely in the micromolar range.

Adult synaptosome incubations were assessed to see if glutamate removal attenuated the changes in cytosolic calcium that were apparent under conditions of metabolic stress. These synaptosomes were incubated for 30 minutes under conditions of severe metabolic stress as detailed in section 3.2.3.d '*later experiments - severe metabolic stress'*.

The following incubations were set up in an experiment.

- i) control incubation
- ii) control, autofluorescence incubation
- iii) ischaemic incubation
- iv) ischaemic, autofluorescence incubation
- v) ischaemic incubation with released glutamate removed
- vi) autofluorescence for ischaemic incubation with released glutamate removed

Five separate experiments were conducted. Results can be found in section 3.3.4. Results are expressed as mean \pm s.e.m.

3.2.6 Statistical analysis

The effects of incubation under different conditions of metabolic deprivation on the parameters measured were tested using one-way analysis of variance (ANOVA) (Sokal and Rohlf, 1981). Differences between groups were tested using the Least Square Difference (LSD) test (Sokal and Rohlf, 1981). In studies requiring comparisons between only two groups , Student's t test was used (Sokal and Rohlf, 1981).

RESULTS

<u>3.3</u> <u>RESULTS</u>

3.3.1 Validation of calcium assay

3.3.1.a Synaptosome integrity

To ensure that the results seen throughout the studies of calcium levels were not influenced by leakage of Fura-2 from the synaptosomes (which would hence gain access to extracellular calcium concentrations) lactate dehydrogenase (LDH) activity was measured as a marker of synaptosomal integrity. The LDH release following a 1 hour normoxic incubation including 30 minutes of Fura-2/AM loading was less than 10% of total LDH activity at all ages, with no significant difference between adult and neonatal preparations, thus indicating that synaptosomal integrity was similar for all ages.

The release of Fura-2 into the extrasynaptosomal medium during an incubation period was also assessed in adult and PND 10 pup synaptosomes. Fluorescence due to leaked Fura-2 into the supernatant following pelleting of the synaptosomes was below the detection of the assay used in both age preparations tested.

3.3.1.b Autofluorescence, cytosolic localisation and ester hydrolysis

Potential differences in autofluorescence of immature and adult samples were accounted for because autofluorescence was routinely checked and subtracted from final fluorescence in all samples. Fura-2/AM loading was also assessed to ensure there were no differences across the ages. This was done by taking R_{max} as an indicator of Fura-2 loaded and converted to the free-acid form from the ester. There were no significant differences in the amount of Fura-2 loaded into the synaptosomes throughout development under the conditions used.

Cytosolic localisation of Fura-2

Fura-2 loading was assessed to be approximately $83\% \pm 4\%$ (n=7; 3 adult and 4 PND 10 pup preparations) confined to the cytosol. This was assessed by a method based on that used by Roe *et al.* (1990). The synaptosome plasma membrane was differentially permeabilised with 0.1% (v/v) digitonin and then the remaining non-cytosolic Fura-2 released with 0.5% Triton-X100. A dose response curve of the required concentration of digitonin to give maximal release of the cytosolic enzyme lactate dehydrogenase but minimal release of the mitochondrial enzyme citrate synthase was performed to find the concentration of 0.1% digitonin. For this, five milligrams per ml of synaptosomal protein was incubated with different concentrations of digitonin for 5 minutes on ice. After mixing, the pellet was spun down and the supernatant assayed for released enzymes. The Citrate Synthase measurements were kindly performed by Dr. Angeles Almeida Parra. The percentages of cytosolic Fura-2 were similar in the adult and PND 10 preparations, and this data was pooled to give an average percentage of cytosolic Fura-2.

3.3.1.c Effects of divalent ions

The addition of N,N,N'N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a permeant heavy metal chelator (Ashley et al., 1984) did not significantly modify the fluorescence signals in samples prepared from either immature (10-day) or adult animals (60-day) indicating there was no overestimation of values due to the presence of high concentrations of trace metals (Ashley et al., 1984).

3.3.2 Effect of 60 minutes of metabolic stress on intrasynaptosomal calcium concentrations

3.3.2.a Basal calcium levels

(Figure 3.4)

The effects of anoxia, aglycaemia and ischaemia on basal (resting) $[Ca^{2+}]_i$ are presented in figure 3.4. Control (normoxic) values are shown as 100% and results from the metabolic stresses have been expressed as a percentage of the control values.



Legend for Fig. 3.4

Resting intrasynaptosomal calcium levels were measured as described in 3.2.2 in different age rat synaptosome preparations. Incubation conditions were control, anoxic, aglycaemic or ischaemic as detailed in 2.2.7.

Results are expressed as mean % of control values. Control means were: 453, 436, 458, 415, 395 and 260 nM for animals 5, 10, 15, 20, 25 and 60 days old respectively. All SEMs of the mean values were less than 10% of the mean.

n > 4 separate preparations.

* represents significant difference (P<0.05) from control in same age.

** represents significant difference (P<0.01) from control in same age.

Ischaemia, calcium and glutamate

The calcium data clearly presents a difference in response with increasing age in two of the metabolic deprivations (aglycaemia and ischaemia), but not in the third (hypoxia/anoxia).

3.3.2.b Depolarised calcium levels

(Figure 3.5)

In all ages and all conditions, the addition of KCl led to a significant (p<0.05) increase in $[Ca^{2+}]_i$ compared to the resting levels. The effects of anoxia, aglycaemia and ischaemia on the depolarised $[Ca^{2+}]_i$ are presented in figure 3.5. Control (normoxic) values are again shown as 100% and results from the metabolic deprivations have been expressed as a percentage of the control. The results mirror those under resting conditions; there is a significant difference in response with increasing age in two of the metabolic deprivations (aglycaemia and ischaemia), but not in the third (hypoxia/anoxia).

Anoxia

There was no significant change in resting or depolarised $[Ca^{2+}]_i$ following 60 minutes of oxygen deprivation, in all of the different age animals studied.

Aglycaemia

A lack of glucose in the incubation medium led to significant (p<0.01) increases in both basal and depolarised $[Ca^{2+}]_i$ in 25 and 60 day old animals, when compared to controls.

Ischaemia

In preparations from 5 and 10 day old pups a change in $[Ca^{2+}]_i$ was notably absent under these conditions. The combination of a lack of oxygen and glucose caused markedly larger increase in $[Ca^{2+}]_i$ than a lack of glucose alone in preparations from animals that were 15 - 60 days old.



Legend for Fig. 3.5

Depolarised intrasynaptosomal calcium levels were measured as described in 3.2.2 in different age rat synaptosome preparations. Incubation conditions were control, anoxic, aglycaemic or ischaemic as detailed in 2.2.7.

Results are expressed as mean % of control values. Control means were: 606, 554, 586, 533, 509 and 380 nM for animals 5, 10, 15, 20, 25 and 60 days old respectively. All SEMs of the mean values were less than 10% of the mean.

n > 4 separate preparations.

* represents significant difference (P<0.05) from control in same age.

** represents significant difference (P<0.01) from control in same age.
In synaptosomes from animals age 15 days and upwards the combined insult led to a significant (p<0.05) increase in $[Ca^{2+}]$ compared to control, however the level of significance was lower than with the ages above 15 days. There is an obvious change in the severity of the rise in calcium as age increases from 15- to 25-days, both in resting and depolarised conditions. The rise in resting calcium following ischaemia continues to increase from 25-days (279%) to 60-days (306%), but not for depolarised conditions, where the levels are very similar for both 25 and 60 day old rats (265% and 250% respectively).

3.3.2.c The developmental profile of synaptosomal calcium levels

Figure 3.6 shows the developmental changes of resting and depolarised intrasynaptosomal calcium levels under control conditions of normoxia and normoglycaemia. Resting and depolarised calcium levels decrease with age of the rat. Statistical analysis of the data by ANOVA followed by Fisher's Least Square Difference Test revealed that results obtained from animals age 5 to 25 days were significantly different to those obtained from mature 60 day old brain (P<0.01).

<u>3.3.3</u> LDH release over the 60 minute incubation

(Figures 3.7, 3.8 and 3.9)

Adult and immature (PND 10 pup) synaptosomes were assessed under all conditions to see if LDH release became significantly increased during incubation. This would indicate that synaptosomal membrane integrity had become compromised and that damage had occurred to the membrane via some process.

Under all conditions in both age groups the release of LDH during the 60 minute incubation remained below 10%. There was no significant effect on LDH release by the conditions of metabolic stress endured by both groups of synaptosome preparation, indicating that these conditions did not lead to cellular membrane damage. Figure 3.9 illustrates how low the level of LDH release was during all incubations, when compared with the total of LDH which can be released by membrane disruption induced by Triton X-100 treatment.





Resting and depolarised intrasynaptosomal calcium levels were measured, as described in 3.2.2., in synaptosomes from different age rats under control conditions.

Results are expressed as mean ± SEM.

n > 4 separate preparations.

****** represents significant difference (P<0.01) from adult (day 60) value.



Time of incubation (minutes)

Legend for Fig. 3.7

The amount of LDH released from synaptosomes incubated under control, anoxic or ischaemic conditions was measured as described in 2.2.4.

Results are expressed as mean percentage of the total LDH activity in the synaptosome preparation (ie. following lysis with Triton). All SEMs of the mean values were less than 10% of the mean.

n = 6 separate preparations, measurements performed in duplicate.



The amount of LDH released from synaptosomes incubated under control, anoxic or ischaemic conditions was measured as described in 2.2.4.

Results are expressed as mean percentage of the total LDH activity in the synaptosome preparation (ie. following lysis with Triton). All SEMs of the mean values were less than 10% of the mean.

n = 6 separate preparations, measurements performed in duplicate.



The amount of LDH released from synaptosomes was measured as detailed in figures 3.8 & 3.9.

Results are expressed as mean percentage of the total LDH activity in the synaptosome preparation, and are shown on the same axis as the 100% value to illustrate the low level of LDH activity in the synaptosome incubations.

****** represents significant difference (P<0.01) from the total LDH activity.

3.3.4 Effect of severe metabolic stress on intrasynaptosomal calcium concentrations

(Figures 3.10, 3.11 and 3.12)

3.3.4.a Adult synaptosomes

An increasing period of incubation had no effect on the control intrasynaptosomal calcium levels, which remained similar throughout all time periods. In the adult synaptosomes, by 15 minutes of ischaemia the calcium levels were significantly increased as compared to control levels. Anoxic conditions for this time period did not lead to a significant increase in synaptosomal calcium concentration however. By 30 minutes, both ischaemia and anoxia had caused a significant increase in calcium levels. The calcium levels at 30 minutes of ischaemia were also significantly (P<0.01) higher than those levels at 15 minutes of ischaemia. By 60 minutes of the conditions of metabolic stress, anoxic synaptosome calcium levels had decreased from those measured at 30 minutes and were no longer significantly different to control levels. Ischaemic calcium levels in these synaptosomes remained significantly high when compared to control levels. The levels at 60 minutes were not significantly increased compared with those at 30 minutes, thus suggesting that no significant further increase in synaptosomal calcium levels had occurred in the latter 30 minutes of the 60 minute ischaemic incubation period.

3.3.4.b PND 10 pup synaptosomes

In the synaptosomes prepared from PND 10 rat pups there was no effect of the incubation period on control synaptosomal calcium levels. When compared to these control calcium levels, both anoxic and ischaemic incubations had no effect on the calcium levels in these synaptosomes. At 60 minutes of ischaemia there appeared to be a trend towards an increase in calcium levels, but this did not reach significance. Therefore it seems that the synaptosomal calcium response to ischaemia is very different in the adult and immature nerve terminals.



Intrasynaptosomal calcium levels were measured as described in 3.2.2 in adult synaptosomes during conditions of severe metabolic stress as described in 2.2.7.

Results are expressed as mean \pm SEM. n > 4 separate preparations.

** represents significant difference (P<0.01) from control.



Intrasynaptosomal calcium levels were measured as described in 3.2.2 in pup synaptosomes during conditions of severe metabolic stress as described in 2.2.7.

Results are expressed as mean \pm SEM. n > 4 separate preparations.



Intrasynaptosomal calcium levels were measured as described in 3.2.2 in adult and pup synaptomes during conditions of severe metabolic stress as described in 2.2.7.

Results are expressed as mean % of control values. Control means are as detailed on figures 3.11 & 3.12 for adult and pup synaptosomes respectively.

n > 4 separate preparations.

** represents significant difference (P<0.01) from control.

Ischaemia, calcium and glutamate

At first sight, the response to a 60 minute anoxic incubation seems similar between the adult and immature synaptosome preparations because there is not a significant increase in calcium in either age group. However, the response to anoxia is different between the two groups, in terms of the calcium levels, because an increase does occur in the adults but control levels become re-established, whereas in the pup synaptosomes there is not a calcium increase at any of the incubation stages assessed here.

The differences in calcium response to ischaemia and anoxia between the two different age synaptosome preparations can be clearly seen in figure 3.11. The calcium levels are expressed here as percentage of control so that the effects of the conditions of metabolic stress can be easily compared.

3.3.5 Concentration of glutamate present in the extrasynaptosomal medium during severe metabolic stress

(Figure 3.13 and 3.14.)

The results presented in figure 3.13 indicate that in the adult synaptosomes under ischaemic conditions there is a significantly (P<0.01) increased amount of glutamate in the extrasynaptosomal medium. This was at both 15 and 30 minutes of an ischaemic incubation. In these adult synaptosomes, at 15 minutes of anoxic incubation there is a decrease in glutamate in the medium, which is significant (P<0.05) from the control levels. However, by 30 minutes of the anoxic incubation the glutamate in the extrasynaptosomal medium had risen to the extent that it was now significantly (P<0.01) increased when compared with control levels.

Figure 3.14 show that In the PND 10 pup synaptosomes neither anoxia or ischaemia at either incubation time caused a significant difference in the amount of glutamate present in the extrasynaptosomal medium, when compared with control levels.







Glutamate concentration in the extrasynaptosomal medium of adult synaptosomes was measured as described in 3.2.4., under conditions of severe metabolic stress as detailed in 2.2.7.

Results are expressed as mean + SEM

n = 4 separate preparations, measurements performed in triplicate.

** represents significant difference (P<0.01) from control .



Glutamate concentration in the extrasynaptosomal medium of pup synaptosomes was measured as described in 3.2.4., under conditions of severe metabolic stress as detailed in 2.2.7. Results are expressed as mean \pm SEM

n = 4 separate preparations, measurements performed in triplicate.

3.3.6 Effect of glutamate removal on changes in calcium concentrations (Figure 3.15)

Experiments were performed in order to assess whether presynaptic metabotropic glutamate receptors play a part in the increases in $[Ca^{2+}]_i$ seen in the older aged rat synaptosomes. Any endogenous glutamate released from adult synaptosomes during a 30 minute ischaemic incubation was removed enzymatically. These incubations were compared to control and ischaemic incubations performed on the same preparations but without the enzyme and cofactor involved in glutamate removal present. The removal of glutamate, and hence any possible involvement of metabotropic receptors, attenuated the $[Ca^{2+}]_i$ rise seen after ischaemia. The insult led to intrasynaptosomal calcium levels of 814 nM, which was decreased to 631 nM when glutamate was removed. The latter value was still highly significant (P<0.01) when compared to the control level of 231 nM. Thus stimulation of glutamate receptors does play a part in the calcium rise seen in the adult synaptosomes under 30 minutes of ischaemic conditions. However it does not fully account for the calcium rise induced by ischaemic conditions, which remains even if all stimulation of synaptic glutamate receptors is prevented by the removal of extrasynaptosomal glutamate.



conditions (30 minutes incubation)

Legend for Fig. 3.15

Intrasynaptosomal calcium levels were measured in adult synaptosomes as described in 3.2.2. Incubation conditions were control or severe ischaemia as detailed in 2.2.7, or ischaemia with enzymatic removal of glutamate as described in 3.2.5. Results are expressed as mean <u>+</u> SEM

n = 5 separate preparations.

** represents significant difference (P<0.01) from control .

DISCUSSION

3.4 Discussion

CALCIUM

The results show that control levels of $[Ca^{2+}]_i$ in nerve terminals change with brain development *per se*. Furthermore, that regulation of $[Ca^{2+}]_i$ in nerve terminals during ischaemia changes dramatically during early postnatal development. These results may in part explain why the neonatal brain tolerates periods of ischemia that produce serious neurological injury in adult animals.

3.4.1 Critique of methods

With the present model, recovery from the metabolic insults was not studied, and so important further difference between adults and neonates might exist. Recovery was not studied primarily because further incubation under reperfusion conditions led to an increased accumulation of Fura-2 into intrasynaptosomal organelles. This resulted in less of the signal being derived from cytosolic Ca^{2+} changes. Hence it became questionable as to whether comparisons between the insult stage and the recovery stage would be valid in this model, using Fura-2 as the calcium indicator. Preliminary experiments showed that extensive reoxygenation of the hypoxic/anoxic and ischaemic samples did not occur during the experimental manipulations and Ca^{2+} measurements. Therefore the measurements truly reflected the status of $[Ca^{2+}]_i$ at the end of the metabolic insult and no recovery period was allowed.

In the original set of comparisons between adult and neonatal animals, a 60 minutes insult period was focused upon. This period was chosen because 60 minutes of these conditions has been reported to result in the most significant differences in adult synaptosome properties (such as Ca^{2+} and lactate levels) between ischaemic and anoxic conditions as compared with control conditions (Boakye *et al.*, 1990; 1991). In later comparisons the oxygen tension in the incubations was reduced, and the insults were felt to be more severe under these conditions than in the original 60 minute incubations.

Ischaemia, calcium and glutamate

Hence a time course of these conditions was performed of 15, 30 and 60 minutes. The trend of maturational changes in $[Ca^{2+}]_i$ under metabolic stress conditions was reflected in all of these incubation times.

Following the original developmental profile of rat pups age 5 days through to adult rats of 60 days, only one age of neonatal pup (day 10) was selected to compare with adult animals in all experiments. This reflected an age of neonatal animal in which there was a significant difference from the adult animal, but in reducing the amount of ages studied it allowed many more biochemical processes to be focused on during the 3-year study period.

<u>3.4.2</u> Maturational changes in $[Ca^{2+}]_i$

This work demonstrates an age-dependent decrease in calcium levels measured in rat brain synaptosomes made from animals aged 5 to 60 days in both resting and depolarised conditions. The resting $[Ca^{2+}]_i$ reported here for adults (60-day old rats) are comparable with values measured previously (100-300 nM) in synaptosomes made from adult rat brain (Boakye *et al.*, 1991; Nicholls, 1993; Erecinska *et al.*, 1994). It has been shown that damaged nerve terminals give rise to spuriously high resting $[Ca^{2+}]_i$ (Fontana and Blaustein, 1995). However the low and consistent values of LDH release from preparations from rats of all ages used suggest that this is not the reason for the age-dependent decrease in $[Ca^{2+}]_i$. In addition synaptosomes at all ages under normoxic conditions exhibit a rapid increase in calcium following depolarisation by KCl, which also indicates that they are "healthy" preparations able to maintain normal ion gradients (Boakye *et al.*, 1990; Fontana and Blaustein, 1995).

Resting calcium concentration within synaptosomes is tightly regulated. *In vitro* studies of adult synaptosomes have shown that they possess all of the mechanisms concerned with Ca^{2+} homeostasis. Namely, Ca^{2+} channels (Naschen and Blaustein, 1979, 1980, 1982), a $3Na^{+}/Ca^{2+}$ exchanger (Gill *et al.*, 1981) and a plasma membrane Ca^{2+} -ATPase (Javros *et al.*, 1981).

This is the first demonstration of a developmental change in resting Ca^{2+} levels in the nerve terminal. Bickler et al., (1993) did not find any effect of age on intracellular Ca²⁺ in brain slices from neonatal through to adult rats. However, brain slices are heterogenous in their cellular content and the study presented here focuses upon nerve terminals alone. In light of observations that intracellular free Ca^{2+} has a specific role in the growth process of the developing nerve cell (Mattson and Kater, 1987; Mattson et al., 1990; Kater and Mills, 1991), it seems likely that the higher levels of Ca²⁺ measured in the immature nerve terminals are connected with nerve terminal growth and maturation. Indeed, alterations in the local level of intracellular Ca^{2+} are known to affect neurite outgrowth and synaptogenesis (Mattson, 1988; Nyakas *et al.*, 1996). Voltage-dependent Ca^{2+} influx has been observed to be a signal for both growth cone expansion and neurite elongation in primary dissociated neurones in culture (Suarez-Isla et al., 1984), and the optimal development of nerve cell growth cones is known to take place in a narrow intracellular Ca²⁺ concentration range (Angus Silver et al., 1989; Kater and Mills, 1991), which in neuronal cultures (from immature brain) is an elevated intracellular Ca²⁺ concentration (Nicholls, 1994).

It is possible that in the developing nerve terminals calcium homeostasis changes with maturation so that higher resting levels of Ca^{2+} are available for optimal growth in the immature nerve terminals. During their studies on neuronal sensitivity to anoxia, Friedman and Haddad (1993) suggested that initial resting levels of Ca^{2+} may differ between adult and neonatal neurones, although they did not measure this during their experiments. A change in calcium homeostasis during development has also been suggested in other work (Mailleux *et al.*, 1993). Developmental changes in some of the processes concerned with calcium homeostasis would further support this notion, although there are few studies pertaining to developmental changes in Ca^{2+} homeostasis (Nyakas *et al.*, 1996). The activity of the Na⁺/Ca²⁺ exchanger increases between day 1 and 6 in cultures of primary neurons, which are cultures from neonatal brain (Michaelis *et al.*, 1994).

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Carpenter *et al.* (1990) also reported that during early development, neuronal voltagegated calcium channels and neurotransmitter channels allowing calcium entry increase greatly in abundance and diversity.

All of these observations suggest that the developmental decrease in $[Ca^{2+}]$ reported in the present study is a reflection of a more complex regulation of calcium fluxes during development, and that higher levels of $[Ca^{2+}]_i$ may be necessary in young nerve terminals for normal growth and development, than in mature nerve terminals where the levels of $[Ca^{2+}]_i$ measured in the immature synaptosomes could possibly be detrimental.

3.4.3 Calcium homeostasis during metabolic stress

3.4.3.a Hypoxia/anoxia

These results clearly demonstrate that in neonatal nerve terminals under conditions of hypoxia/anoxia no increase in intrasynaptosomal Ca^{2+} is apparent at any time point measured. In adult nerve terminals under hypoxia/anoxia, no increase in intrasynaptosomal Ca^{2+} levels are seen at 15 minute;, however a significant increase, compared to control values, is apparent at 30 minutes of this metabolic insult. However, by 60 minutes incubation under these anoxic conditions the intrasynaptosomal Ca^{2+} levels are decreased once again, and are no longer significantly increased when compared to control values.

Firstly, this study therefore presents a maturational change in the response of the nerve terminal to hypoxic/anoxic conditions. Although this is the only study reported in literature (Keelan *et al.*, 1996) which has focused specifically on Ca^{2+} changes in developing nerve terminals, the findings are in general agreement with a number of other studies. For example Friedman and Haddad (1993) showed that neonatal rat neurones differ from adults in their sensitivity to anoxia in terms of their handling of cytosolic free Ca^{2+} .

Kass and Lipton (1989) noted less Ca^{2+} accumulation in neonatal brain than in adult brain following hypoxia, and a number of studies have observed that extracellular Ca^{2+} takes much longer to change during anoxia in the neonate than in the adult (Haddad and Donelly, 1990; Jiang and Haddad, 1991; Jiang *et al.*, 1992). Bickler *et al.*, (1993) have also observed that there are maturational changes in the Ca^{2+} response of brain slices to anoxia. They found that brain slices from rats below 30 days of age showed significantly less intracellular Ca^{2+} accumulation than adult rats.

Friedman and Haddad (1993) found that neonatal neurones did accumulate Ca^{2+} in the same manner as adult neurones, however the latency of the Ca^{2+} rise was about five times longer than in the adult. Hence the major maturational difference of neurones to anoxia was that the onset of rise in cytosolic Ca^{2+} shows a delay in neonatal neurones. In the study presented here no increase in Ca^{2+} in neonatal synaptosomes was apparent at the times measured, however it is possible that an increase did occur at a time point in between those assessed here. Indeed, if the latency of Ca^{2+} increase is longer in neonatal neurones than adult neurones, a significant increase in Ca^{2+} may occur after 30 minutes, at which time point an increase was apparent in the adult nerve terminals.

The significant increase in intrasynaptosomal Ca^{2+} at 30 minutes of anoxia in adult neurones becomes diminished by 60 minutes, so that a normal Ca^{2+} gradient effectively becomes re-established over this 30 minute period. In relation to this, Carrol *et al.*, (1992) found with PC12 cells that a chemical hypoxia reduced ATP concentrations by 92%, and after 10 minutes $[Ca^{2+}]$ increased 2.5 fold above control, but that after 30 minutes the calcium gradient was re-established, and that at this time period and also at 60 minutes $[Ca^{2+}]$ was equivalent in hypoxic and control cells. Similarly, in the study by Friedman and Haddad (1993) an increase in fluorescence due to calcium was seen in adult neurons, followed by a decrease after 10 minutes even though the neurones were still anoxic.

Such observations involving adult neurones undergoing an increase in $[Ca^{2+}]$ although oxygen remains depleted, raise the question: "do pre-hypoxic levels of calcium become re-established under these conditions ?". Sequestration into intracellular stores or extrusion across the plasma membrane which require energy seem to be unlikely possibilities because ATP levels are depleted at the time points in question (see chapter 4 for full details).

In a number of other studies no increase in Ca^{2+} in adult neurones has been reported during hypoxia (Dagani *et al.*, 1989; Boakye *et al.*, 1990). The possibility remains however that under hypoxic conditions adult neurones exhibit a brief increase in Ca^{2+} in the cytosol, followed by a decrease to normal values and that the results achieved in a study may to some extent depend on the time point of measurement of the neuronal Ca^{2+} level.

The situation in isolated neurones appears to be different to overall intracellular Ca^{2+} changes in brain slices, where neurones are not the only cell type present. Bachelard *et al.*, 1993 reported an initial significant decrease of the measured free intracellular Ca^{2+} to 59% of control values, which then under continued hypoxic conditions returned to values similar to control, and remained so for over 3 hours of the insult. No increase above control values was reported under hypoxia in these adult brain slices. It is therefore possible that there are quite distinct differences in the Ca^{2+} handling of neurones and glial cells during hypoxic conditions.

3.4.2.b Combined oxygen and glucose lack

In the present study, a combination of both oxygen and glucose deprivation led to obvious maturational differences in the nerve terminal response to these conditions. Only in more mature preparations (above 10 days) did the combined insult cause a significant increase in cytosolic calcium levels. This is in agreement with a study of chemical ischaemia (using brain slices in the presence of metabolic poisons), where a similar developmental profile of calcium levels was observed (Bickler *et al.*, 1993). These authors showed that only brain slices from animals above two weeks old

showed rapid increases in intracellular calcium during ischemia. A potentially important explanation for these differences is that the ability of the neonatal nerve terminal to preserve ATP levels or produce ATP from alternative pathways could enable energy-requiring processes for Ca^{2+} homeostasis to be sustained for much longer periods. To address this issue, ATP concentrations in the synaptosome preparations were measured under these same conditions. These results of changes in ATP concentrations are detailed more fully in chapter 4 of this thesis, however in order to aid this discussion it is important to state that in both adult and neonatal preparations ATP levels declined to less than 20% of the control values by 15 minutes of all metabolic insults, with no significant differences in Ca^{2+} homeostasis during ischaemia in neonate as compared with adult nerve terminals is not merely a reflection of a difference in ATP status, as some authors have suggested (Friedman and Haddad, 1993).

The difference in pattern of Ca^{2+} changes between the neonate as compared with adult is most likely a reflection of different regulatory mechanisms of cytosolic free Ca^{2+} between the age groups. Possible differences include better buffering capacity (Blaustein, 1988; Mattson *et al.*, 1991), lower rate of inward Ca^{2+} leakage, fewer active calcium-conducting channels, increase extrusion of intracellular Ca^{2+} , or increased ability of sequestration into intracellular stores in the neonate. However, evidence for such differences is currently lacking.

The different patterns of change in $[Ca^{2+}]_i$ measured during anoxia and combined oxygen and glucose lack in the adult nerve terminals suggest that calcium changes in adult brain ischaemia are not due merely to a lack of oxygen.

The maturational change in intrasynaptosomal $[Ca^{2+}]$ during aglycaemia reflects the pattern seen during combined oxygen and glucose lack, however the absolute levels of $[Ca^{2+}]$ are higher in the combined insult. This is in agreement with previous work on adult synaptosomes (Boakye *et al.*, 1990). Therefore it appears that in the adult brain the hypoxic component of ischaemia does not primarily determine the Ca^{2+} handling of the cells, whereas the hypoglycaemic component has more of an influence. A combined lack of both oxygen and substrate leads to a much more highly perturbed Ca^{2+} homeostasis than glucose lack alone, therefore indicating that decreasing oxygen tension renders adult neurones more susceptible to the metabolic perturbation of substrate lack. This finding of combined glucose and oxygen lack altering intracellular Ca^{2+} homeostasis in adult brain but the individual components having different effects has been previously observe in brain slices (Bachelard *et al.*, 1993) and synaptosomes (Boakye *et al.*, 1990).

If the changes in intrasynaptosomal $[Ca^{2+}]$ seen in adult synaptosomes here are factors in nerve terminal damage, these data suggest that anoxia involves different mechanisms in relation to calcium metabolism than those initiated by combined oxygen and glucose lack. The patterns of Ca^{2+} changes in the two separate insults are quite markedly different in the adult nerve terminals. It remains to be seen whether these differences in calcium homeostasis in the two types of insult lead on to further differences in nerve terminal response and damage. Alternatively intrasynaptosomal Ca^{2+} changes may play no significant causative role in adult nerve terminal damage in one of or both types of these metabolic insults, and the differences observed here between the insults may not be of any specific significance.

When comparing neonatal synaptosomes with adult synaptosomes the data here show clearly that there are marked differences in the calcium status and handling of the different age nerve terminals. Whether such maturational differences in calcium metabolism under metabolic stress have a role in the superior survival of the neonatal brain under oxygen and substrate limiting circumstances remains to be elucidated. Studies in the following chapters of this thesis will further explore this possibility by

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investigating other changes in nerve terminal biochemistry under conditions of metabolic stress.

<u>3.4</u> <u>Discussion</u>

GLUTAMATE

The results show that the effect of anoxia and ischaemia *in vitro* on the amount of glutamate present in the extrasynaptosomal medium changes dramatically during brain maturation. Furthermore that the presence of increased glutamate in contact with adult nerve terminals increases the $[Ca^{2+}]$ in these nerve terminals slightly but does not account for all of the significant increase in intrasynaptosomal Ca^{2+} levels seen under ischaemic conditions, as discussed in section 3.4.2.b.

3.4.4 Developmental changes in glutamate in the extrasynaptosomal medium

In the immature nerve terminals of postnatal day 10 rat pups there is no significant change in the amount of glutamate in the extrasynaptosomal medium under any of the conditions of metabolic stress tested. However, in the adult nerve terminals there is a significant increase in glutamate levels under 15 minutes of ischaemia *in vitro* which remains at this significantly high level at 30 minutes of the insult. This developmental difference in the nerve terminal glutamate homeostasis has been observed in other studies. For example, Cherici *et al.* (1991) observed no increase in glutamate release in ischaemic neonates whereas a number of studies have recorded increased glutamate release in adult ischaemic brain tissue (Benveniste *et al.*, 1984; Hagberg *et al.*, 1985; Kaupppinen *et al.*, 1988; Globus *et al.*, 1991; Ginsberg *et al.*, 1992). Other studies (Silverstein *et al.*, 1991; Gordon *et al.*, 1991) have reported increased extracellular glutamate levels in neonatal brain, however one plausible reason for this discrepancy is that no distinction between glutamate released from nerve terminals and metabolic glutamate extruded from glial cells was made in these studies.

The data presented here only focuses on glutamate released from pre-synaptic nerve terminals.

A number of studies have linked a developmental difference in Ca^{2+} homeostasis in ischaemic or anoxic neurones with a developmental difference in the Ca^{2+} response of these neurones to exogenous glutamate (Friedman and Haddad, 1993; Bickler *et al.*, 1993; Sundvall *et al.*, 1994). However, few studies have allowed direct comparison between glutamate homeostasis in neonatal and adult neurones under conditions of metabolic stress (Nyakas *et al.*, 1996). The calcium dependency of glutamate release has been observed to change with development. Jennings *et al.*, (1995) reported that glutamate release from synaptosomes only became sensitive to calcium influx after 10 day postnatally, and that prior to this age increased Ca^{2+} influx following depolarisation did not stimulate glutamate release. Therefore the data reported in this thesis do suggest, in line with observations like those of Jennings *et al.* (1995), that the mechanisms underlying glutamate release from nerve terminals possibly change with brain development, hence leading to different amounts of external glutamate than those observed with adult nerve terminals under conditions of metabolic stress.

3.4.5 Effects of anoxia and ischaemia on extrasynaptosomal glutamate levels in adult nerve terminals

Aside from developmental differences in glutamate homeostasis, the data presented here also clearly show differences in the glutamate homeostasis of adult neurones under conditions of anoxia as opposed to ischaemia. Under ischaemic conditions the amount of extrasynaptosomal glutamate significantly increased whereas, in contrast anoxic conditions initially induced a significant decrease which then later increase significantly above ischaemic conditions. Such different patterns of glutamate homeostasis have previously been reported (Kauppinen, 1994). This researcher found that hypoxia in the presence of glucose did not lead to increased extracellular glutamate, whereas a lack of oxygen and glucose together caused an extensive leakage of glutamate. The extent of energy failure becomes an important consideration when

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comparing glutamate homeostasis under these conditions. The Ca²⁺-dependent release of glutamate is inhibited when energy failure occurs (Kauppinen *et al.*, 1988), however Ca²⁺-independent glutamate release, due to reversal of the Na⁺-dependent uptake pathway, increases under these conditions (Levi *et al.*, 1993; Kauppinen, 1994).

In the conditions used in this thesis it seems likely that the decrease in extrasynaptosomal glutamate is a consequence of anaerobic synaptosomal metabolism being able to partially drive nerve terminal glutamate homeostasis as suggested by Kauppinen (1994). Sufficient ATP will allow glutamate to be compartmentalised in glutamate vesicles in the nerve terminal, but Ca^{2+} -dependent glutamate release from such vesicles may not occur under these conditions because an increase in cytosolic Ca^{2+} in these nerve terminals does not occur and also because the probability of such release becomes decreased due to the ATP levels beginning to fall (Kauppinen, 1994) (see chapter 4 results for detail of ATP decreases under these conditions). From 15 minutes onwards, as ATP levels have diminished to the same extent as in ischaemic conditions glutamate homeostasis can no longer be controlled in the anoxic nerve terminals.

It is speculated that in the absence of sufficient ATP the proton gradient across the transmitter vesicles collapses, redistribution of glutamate into the cytosolic compartment occurs from where it becomes available to amino acid membrane carriers and leakage from the nerve terminal can occur (Kauppinen, 1994). Hence it is suggested that the significant increase in glutamate seen between 15 and 30 minutes of anoxia is primarily influenced by the energy status of these nerve terminals, which falls to below 50% of control for the first time after 10 minutes of anoxia (section 4.3.1.a). In contrast the ischaemic conditions cause a more immediate increase in glutamate which is significant by 15 minutes of this insult.

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Increased Ca^{2+} in the nerve terminal under these conditions would increase Ca^{2+} dependent release if ATP levels remain sufficient for the first few minutes of ischaemia. After this mechanism becomes inhibited by energy failure, Ca^{2+} independent leakage of glutamate will occur due to energy failure and lack of ionic homeostasis.

In summary then, the different patterns in extrasynaptosomal glutamate levels in anoxia and ischaemic adult synaptosomes are likely to be at least in part due to one of, or a combination of the following:

- differences in Ca^{2+} homeostasis under these conditions

- differences in the time taken for energy failure under these conditions.

3.4.6 The effect of glutamate removal on the ischaemia induced Ca²⁺ increase in adult synaptosomes

As the major increases in both extrasynaptosomal glutamate and intrasynaptosomal $[Ca^{2+}]$ were observed following ischaemia in adult synaptosomes, these conditions were used to evaluate the role of pre-synaptic glutamate receptors in the ischaemic $[Ca^{2+}]$ rise. The intrasynaptosomal $[Ca^{2+}]$ rise seen during ischemia in these adult synaptosomes was attenuated by glutamate removal . However, the Ca^{2+} levels remained significantly high compared with control values, thus indicating that other mechanisms not involving glutamate were involved in the ischaemic $[Ca^{2+}]$ increase. Therefore it is valid to say that stimulation of pre-synaptic glutamate receptors plays only a limited role in the calcium increases observed in ischaemic adult nerve terminals.



"Knowledge is the food of the soul".

Plato

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<u>Chapter 4</u>

ISCHAEMIA AND ENERGY METABOLISM

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INTRODUCTION

CHAPTER 4

ISCHAEMIA AND ENERGY METABOLISM

<u>4.1</u> Introduction

The biochemical pathways of energy metabolism in the brain are not particularly different to those of other tissues. In brain research, as in all tissues, great care must be taken in extrapolating from *in vitro* studies to reach conclusions about *in vivo* metabolic functions. *In vitro* studies are important in serving to identify pathways of intermediary metabolism and mechanisms. *In vivo* studies are also necessary to evaluate the actual performance of metabolic pathways. The normal substrates and products of cerebral energy metabolism as well as reliable estimations of their rates of utilisation and production can only be determined from *in vivo* studies. Hence our current knowledge of brain energy metabolism has been realised through years of scientific research involving both *in vivo* and *in vitro* work. The following sections will outline this knowledge of brain energy metabolism; how the pathways involved change during brain development; and finally the involvement of energy metabolism in the pathophysiology of ischaemia/reperfusion.

4.1.1 Energy requirements of the brain

Metabolically, the brain is one of the most active organs in the body. Adult brain weight is only approximately 2% of the total body weight and yet it receives about 15% of the basal cardiac output (McHenry, 1978) and consumes approximately one-fifth of the body's total oxygen utilisation (Ginsberg, 1992). Although the adult brain does not participate in conspicuous processes that require large amounts of energy, such as the mechanical work of skeletal muscle, or the extensive osmotic work of the kidney, it has almost as high a rate of oxidative metabolism as some tissues that are involved in these types of work. For example the rate of oxygen consumption of the brain is comparable with that of cardiac and skeletal muscle and also with the kidney.

This high oxygen consumption provides the energy required for the intense physicochemical activity of the brain. Most of this oxygen consumed by the brain is used for the oxidation of carbohydrate and the production of compounds, such as adenosine triphosphate (ATP), which contain high-energy phosphate bonds.

Normal brain has an enormous energy demand. Indeed, the cerebral oxygen consumption and hence energy production necessary to meet the brain's demands continues unabated both day and night. Surprisingly, during sleep there is only a very small decrease in cerebral metabolic rate, and at times, for example during the rapid eye movement (REM) period of sleep this rate may even increase over day-time conscious levels.

Not only does the brain have an enormous demand for energy, but it is also absolutely dependent on continuously uninterrupted oxidative metabolism for maintenance of functional and structural integrity. This raises the question as to what specific work and functioning of the brain causes it to require such a large, continuous supply of energy. The energy-utilising reactions of the brain can be broadly divided into two categories:- biosynthesis and transport.

Biosynthesis

The average protein turnover and the rate of protein synthesis in the mature brain are slower than most other tissues in the body. Although some energy is expended in biosynthetic pathways, this does not account for the majority of the brain's energy consumption. The biosynthesis that occurs in the brain appears to happen mostly in cell bodies (proteins, polypeptides, lipid *etc.*) or in nerve terminals (neurotransmitters).

Transport

Transport processes in the brain are especially active in axons and dendrites. Processes include the cytoplasmic transport of molecules and organelles (known as axoplasmic transport), the acquisition of essential nutrients and the reuptake of neurotransmitters. The transport of ions across membranes is probably the most active transport process in nervous tissue. The functions of nervous tissue are mainly excitation and conduction by virtue of the way nerve cells function. This is reflected by the unceasing electrical activity of the brain. The electrical energy of nervous tissue is ultimately derived from chemical processes.

The transport of ions across membranes enables the maintenance of ion gradients which are responsible for the maintenance of transmembrane electrical gradients and ultimately nervous electrical activity. It is likely that the majority of the brain's energy consumption is used for the active transport of ions to restore and conserve transmembrane gradients. Studies of barbiturate anaesthesia by Crane *et al.*, (1978) and Hawkins *et al.*, (1979) depressed cerebral metabolism by 50-60% and affected electrical events within the brain. Such work suggested that more than half of the normal cerebral metabolic activity and energy expenditure is associated with electrical events such as the maintenance of Na⁺ and K⁺ gradients (Hawkins *et al.*, 1979).

It is established that the brain has a high energy demand and that a continuous oxygen supply is necessary for normal brain function. To ensure that the energy demand of the brain is met, the brain requires specific substrates for energy production. It is to the substrates utilised by the brain that we now turn.

4.1.2 Substrate utilisation in the brain

Over many years, numerous studies have been undertaken to investigate the substrates used by the brain for energy production and anabolic purposes. The important parameters for consideration in determining the substrates utilised by the brain are:

- the available circulating substrates,
- the extent to which these can be accumulated by the brain,
- the intrinsic capacity of the brain to utilise these substrates.

Investigations into substrate supply to the brain have in the main been carried out on the whole animal and have involved the measurement of arterial-venous differences in substrate levels from blood vessels supplying the brain (Cremer, 1982). Analysis of cerebral arterial and venous blood first provided important information on the type of substrate used by the brain. In 1929, Himwich and Nahum studied the respiratory quotient (RQ) in cats. The RQ is the ratio between the arteriovenous differences for CO_2 and O_2 . Carbohydrates have an RQ value of unity, whereas other substrates such as proteins and triglycerides have lower values (0.8 and 0.7 respectively). The chemistry of the total oxidation of carbohydrates is:-

$$(CH_2O)_n + {}_nO_2 \rightarrow {}_nCO_2 + {}_nH_2O$$

so that the ratio of CO_2 produced to O_2 utilised will be unity.

The investigation in cats (Himwich and Nahum, 1929) found that the RQ of sampled blood from vessels across the brain was close to unity, thus suggesting that the primary substrate utilised by the brain was carbohydrate. In 1931 Lennox confirmed this observation by showing that the RQ for the human brain was 0.95, somewhat higher than the arm (0.86) or the leg (0.76) (Lennox and Leonhardt, 1931). Hence it began to emerge that the brain had a greater dependence on carbohydrate metabolism than the rest of the body. These semi-quantitative measurements of RQ values confirmed previous observations that convulsion resulting from insulin induced
hypoglycaemia in rabbits could be stopped by administration of glucose and mannose (Noble and Macleod, 1923).

In vitro experiments also aided in identifying the primary substrate for brain. In 1925 Loebel found that glucose, pyruvate and lactate were effective in maintaining respiration in cerebral tissue. This was further narrowed by Quastel and Wheatley (1932) who showed that glucose was better than fructose, mannose and galactose in maintaining cerebral respiration, and also that pentose sugars were practically inert. Glucose oxidation was found to be higher in brain slices than slices of heart, kidney or liver (Olsen *et al.*, 1950).

Gibbs *et al.*, (1942) reported on arterio-venous differences in oxygen, glucose, carbon dioxide and lactate in men. This study concluded that glucose is the principal energy source in the brain *in vivo* and that the net uptake of glucose is sufficient to supply the energy requirements of the brain. Gibbs *et al*, 1942 also concluded that a small amount of the glucose utilised (approximately 15%) is present as lactate and pyruvate, but by far the majority of glucose is fully oxidised through to CO_2 .

The development by Kety and Schmit (1948) of techniques for measuring cerebral blood flow by using inert gas, subsequently led to numerous studies of oxygen and glucose consumption by the brain. Such studies confirmed the findings that glucose is the main substrate for cerebral metabolism. This has subsequently been accepted as a central dogma, which in its most rigorous form states that glucose is the sole substrate for brain metabolism.

A number of studies thus concluded that glucose was the primary substrate for the brain. Glucose availability to the brain did not contradict these conclusions. Glucose is available to the brain and is kept within a normal range of 70-110 mg/dl in blood. The efficient transport of glucose across the blood-brain barrier is also essential to its utilisation by the brain. Glucose transport in the brain, as for most

metabolites, is dependent on a specific carrier process. This transport of glucose into the brain is a facilitated process (Reinis and Goldman, 1980)

The established view on overall cerebral metabolism considers that not only is glucose the main substrate but also that the glucose extracted from the blood supply is either oxidised to CO₂ and water (the major fraction) or metabolised to pyruvate or lactate (the minor fraction). However, this established view has been challenged by a number of studies. Researchers who have administered radioactively labelled glucose found that radioactivity appeared in a number of tissue compounds including glutamate, glutamine and aspartate (see Balázs and Cremer, 1973). Thus they speculated that a substantial fraction of glucose was used for synthesis of amino acids and other non-carbohydrate compounds. In order to remain compatible with the findings that the RQ of brain is unity, an amount of non-carbohydrate compounds corresponding to those produced form glucose would need to be simultaneously oxidised to CO₂ and water. Subsequent research has focused more intensely on the relationship between the metabolism of carbohydrates and amino acids (Siesjö, 1978). The present view seems to be that some of the glucose metabolised by the brain can be used for the synthesis of amino acids, and that the latter constitute labile pool of substrates that can be utilised under special circumstances, for example hypoglycaemia.

It is now accepted that under normal physiological conditions, in adults, the main substrate for brain metabolism in the postabsorptive period is glucose. However, Owen *et al.*, (1967) suggested that the adult human brain could utilise non-glucose metabolic fuels. They measured arteriovenous differences in ketones across the brain in obese people after prolonged fasts, and concluded that ketone bodies can be used by the brain for both oxidative and anabolic purposes when conditions such as hyperketonaemia prevail. Maran *et al.*, (1994) also demonstrated that the cerebral cortex can use non-glucose fuels. They found that cerebral function during hypoglycaemia could be preserved by administration of lactate. Thus studies in

humans, and rats, (Drahota *et al.*, 1965) show that alternative substrates to glucose can be utilised by the brain under exceptional circumstances. The developing brain is somewhat more complex than the adult brain in terms of its substrate utilisation and this is detailed in section 4.1.5. We now turn to the pathways of carbon utilisation which glucose feeds into to enable its oxidation and the concomitant production of ATP.

4.1.3 Metabolism of substrates in the brain

In this section an overall view of the pathways of substrate metabolism in the brain will be given. A number of biochemistry textbooks and reviews are available for a more detailed account of intermediary metabolism.

4.1.3.a Glycolysis

In aerobic cells, such as brain cells, which normally oxidise glucose to CO_2 and water, the glycolytic pathway functions to:

- provide the first obligatory and preparative step for the further oxidation of the glucose molecule,
- provide glucose-6-phosphate for the pentose phosphate pathway,
- contribute to energy production in situations of oxygen lack.

The glycolytic reactions in the brain essentially constitute a unidirectional pathway for carbohydrate breakdown. Figure 4.1 outlines the flow of glycolytic substrates in the brain. A key regulatory point in the metabolism of carbohydrate in the brain is the phosphorylation reaction of glucose to glucose-6-phosphate which is catalysed by hexokinase. The reaction is irreversible. Hexokinase may exist in the cytosol or be firmly attached to mitochondria. Binding to mitochondria changes the kinetic properties of hexokinase and its inhibition by glucose-6-phosphate so that the bound enzyme on mitochondria is more active. The extent of binding is inversely related to the ATP/ADP ratio, so that conditions in which energy utilisation exceeds demand will lead to a switch from the cytosolic to mitochondrially bound hexokinase



Figure 4.1 The Embden-Meyerhof glycolytic pathway

form. Thus the potential capacity for initiating glycolysis to meet the energy demand is increased.

Another key regulatory enzyme in the control of glycolysis is phosphofructokinase (PFK), whose substrates are fructose-6-phosphate and MgATP. PFK is modulated by a large number of metabolites and cofactors, including ATP, Mg^{2+} and citrate (inhibitory) and ADP, fructose 1,6-bisphosphate, K⁺, PO₄³⁻, 5'-AMP (stimulatory). The activity of PFK is greatly influenced by the prevailing conditions of the cell (Vannucci and Plum, 1975).

Following the reaction involving PFK, fructose 1,6 bisphosphate is split into 2 phosphorylated 3-carbon intermediates. The reactions up to this point constitute the *first stage of glycolysis* in which 3-carbon intermediates are formed from glucose at the expense of two molecules of ATP (priming reactions). After the reaction with glyceraldehyde 3-phosphate dehydrogenase so that glyceraldehyde 3-phosphate is metabolised further along the main route of glycolysis, glycolysis in the brain proceeds in the usual steps. *The second stage of glycolysis* is that involving oxidative reactions and that which leads to conservation of energy. Brain enolase (which catalyses the dehydration of 2-phosphoglycerate to phosphoenolpyruvate) is present as two related dimers, one which is specifically associated with neurons (γ isoform) and the other with glia (α isoform). The reaction involving brain pyruvate kinase controls an essentially irreversible reaction that requires Mg²⁺ and also K⁺ or Na⁺ (Volpe, 1987). This step may also be regulatory in the brain.

At the termination of the main glycolytic pathway, pyruvate is formed. If oxygen is available pyruvate is further oxidised by mitochondrial enzymes. Under anaerobic conditions however, pyruvate remains in the cytosol where it is converted to lactate. Brain tissue, even when at rest and well oxygenated does produce a small amount of lactate which is removed in the venous blood. The formation of lactate from pyruvate is catalysed by lactate dehydrogenase (LDH) of which there are five isomers present in adult brain (Clark and Sokoloff, 1993). LDH functions in the cytoplasm as a means of oxidising NADH, which accumulates as a result of the activity of glyceraldehyde 3-phosphate dehydrogenase in glycolysis. As a consequence glycolysis can continue without being hampered by loss of NAD⁺, under anaerobic conditions. LDH can also function under aerobic conditions because NADH cannot easily penetrate the mitochondrial membrane. Thus cytosolic oxidation of NADH in the cytoplasm can occur by this reaction.

4.1.3.b The pyruvate dehydrogenase complex (PDHC)

Pyruvate dehydrogenase controls the entry of pyruvate into the Kreb's TCA cycle as acetyl Coenzyme A (acetylCoA) (Malloch et al., 1986). It is actually a mitochondrial multienzyme complex which includes the enzymes pyruvate decarboxylase, lipoate acetyltransferase and lipoamide dehydrogenase; the coenzymes thiamine pyrophosphate, lipoic acid, CoA, and flavin and nicotinamide adenine dinucleotides (Randle, 1981). PDHC is inactivated by phosphorylation of the decarboxylase moiety by a tightly bound Mg²⁺ATP dependent kinase (Randle, 1981; Reed, 1981). This kinase is inhibited by pyruvate, which thus protects PDHC from inactivation. Increases in pyruvate and ADP, and decreases in acetylCoA and ATP make PDHC more active. NADH inhibits PDHC, for example decreasing acetylCoA formation during oxygen lack so that pyruvate can be converted to lactate and NAD⁺ formed to sustain glycolysis. PDHC is also activated, though more indirectly, by increase in free Ca^{2+} . Ca^{2+} stimulates a Mg²⁺ and Ca²⁺-dependent phosphatase, which is loosely bound to the PDHC (Randle, 1981; Reed, 1981). Thus increased phosphatase action will lead to an increased amount of dephosphorylated and active PDHC.

PDHC serves the purpose of delivering 2-carbon units from the pyruvate pool to the pool of Kreb's cycle intermediates, with the third carbon atom disappearing as CO_2 .



Figure 4.2 The Citric Acid Cycle

4.1.3.c Oxidation of pyruvate - The Kreb's TCA cycle

The Kreb's TCA cycle is a series of cyclical reactions which achieve the complete oxidation of acetylCoA to CO_2 and water. These reactions are outlined in figure 4.2. The primary function of the TCA cycle is the oxidation of acetyl groups under aerobic conditions to generate NADH and FADH₂ from NAD⁺ and FAD. The actual flux through the TCA cycle depends on glycolysis and acetylCoA production which can "push" the cycle, on the control at several enzymatic steps of the cycle, and on the local ADP level, which is known to be a prime activator of the mitochondrial respiration to which the TCA cycle is intrinsically linked (Williamson and Cooper, 1980). For one revolution of the TCA cycle, acetylCoA condenses with oxaloacetate (OAA) to form citrate. Since OAA appears in the last reaction of the cycle (*ie.* malate \rightarrow OAA), the cycle is truly regenerative and allows, at least in theory, the oxidation of an unlimited amount of acetyl groups from pyruvate. Not only pyruvate, but also fatty acids, ketone bodies and amino acids are oxidised by being introduced as acetyl units, and therefore the TCA cycle represents the final common pathway for oxidation of various substrates.

In the brain, the enzymes NADH-isocitrate dehydrogenase, α -ketoglutarate dehydrogenase and succinate dehydrogenase may have regulatory roles. The former two enzymes are activated by increase mitochondrial Ca²⁺ by a marked increase in their Km values for isocitrate and α -ketoglutarate respectively (McCormack and Cobbold, 1991)

The TCA cycle functions not only as an oxidative process for energy production, but also as a source of vital carbon containing intermediates, such as OAA and α -ketoglutarate, for the synthesis of amino acids such as glutamate, glutamine, γ aminobutyrate, aspartate, and asparagine. To export net amounts of α -ketoglutarate and OAA, the supply of these must be replenished by anapleurotic reactions. These can set the upper limit at which biosynthetic reactions can occur.

The TCA cycle is inherently connected with electron transport and oxidative phosphorylation to fully enable energy production.

4.1.3.d Electron transport and Oxidative phosphorylation

Up until this point we have considered how the carbon atoms of glucose in the brain are effectively disposed of by glycolysis and the TCA cycle, and ultimately excreted as CO_2 . The reducing equivalents are effectively removed in a series of oxidation reactions forming NADH and FADH₂ from NAD⁺ and FAD. The key reactions which enable efficient energy production lie in the subsequent handling of the NADH and FADH₂ by the electron transport chain in mitochondria. In order to transduce the reducing power of NADH and FADH₂ into utilisable energy, mitochondria have a series of electron carriers in or associated with the inner mitochondrial membrane, which convert reducing equivalents in the presence of oxygen into utilisable energy by synthesising ATP. This process is a combination of electron transport coupled to oxidative phosphorylation. NADH and FADH₂ oxidation in this process results in the production of 3 and 2 mol of ATP per mole of reducing equivalent transferred to oxygen, respectively.

Electron transport

The mitochondrial electron transport system, also known as the respiratory chain, is a sequence of linked oxidation -reduction reactions. The major enzymes or proteins functioning as electron-transfer components involved in the mitochondrial electron transport system are:-

NAD-linked dehydrogenases, flavin-linked dehydrogenases, iron-sulphur proteins, cytochromes.

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Figure 4.3 Mitochondrial Electron Transport Chain

The mitochondrial electron transport chain is outlined in figure 4.3. the enzymes of the chain are commonly referred to as "complexes", so that the accepted nomenclature is as follows:

- complex I NADH CoenzymeQ reductase
- complex II succinate CoenzymeQ reductase
- complex III ubiquinol cytochrome c reductase
- complex IV cytochrome c oxidase
- complex V ATP synthase.

Reducing equivalents from NAD⁺ linked reactions are fed into the electron transport chain at complex I. This is a flavin-linked dehydrogenase enzyme containing the group flavin mono-nucleotide (FMN), bound non-covalently. The FMNH₂ of reduced complex I becomes reoxidised as electrons are passed from it to the iron-sulphur centre of complex I. CoenzymeQ (CoQ), also called ubiquinone, is a lipophilic electron carrier, which serves as a "mobile" electron transport component. CoQ becomes reduced by the transfer of two electrons and protons. Single electrons are then transferred between CoQ and complex III.

The reducing equivalents from the flavoprotein containing succinate dehydrogenase (complex II) pass from the flavin adenine nucleotide (FAD) group of this enzyme to CoQ. Electrons are first transferred through the iron-sulphur centre of complex II.

Whether electrons have derived from NADH or $FADH_2$ the remaining steps in electron transport are the same. Single electrons are passed from CoQ to the cytochrome b present in complex III, and from this site to cytochrome c_1 of complex III. Single electrons are than transferred from complex III to the small protein cytochrome c.

Electrons are then transferred from cytochrome c to complex IV. Cytochrome a of complex IV acts as the electron acceptor from cytochrome c. Electrons are then transferred from cytochrome a to cytochrome a_3 of this complex.

The terminal electron acceptor in the electron transport chain is molecular oxygen which accepts two single electrons from cytochrome a_3 of complex IV. Complex IV is a dimer which spans the membrane between the matrix and the intermenbrane space or cytosol. Cytochrome c binds to complex IV from the cytosolic side of the membrane, whereas oxygen binds from the matrix side of the membrane during the electron transferring event. The cytochromes only carry reducing equivalents in the form of electrons, but at the end of the chain, the H⁺ ions, released at an earlier step recombine with electrons and react with molecular oxygen to form water.

The structures of the complexes involved in electron transport are intricate, and detail is still being elucidated. Complex I is composed of 41 currently known subunits, seven of which are encoded for by mitochondrial DNA. Complex II consists of five subunits that are encoded by nuclear DNA. Complex III has 11 subunits, with one subunit (cytochrome b) encoded by mitochondrial DNA. Complex IV is composed of 13 subunits, with three encoded for by mitochondrial DNA. Complex V is composed of 12 subunits, with two subunits encoded in the mitochondrial genome. Each of these mitochondrial complexes can be inhibited by specific compounds, such as, rotenone, antimycin, cyanide and oligomycin. Such inhibition of electron transport is often used as an experimental aid for studying mitochondrial respiration.

Oxidative phosphorylation

It is normally assumed that electron transport and oxidative phosphorylation are coupled in an obligatory fashion, *ie*. one does not occur without the other. However, under a number of circumstances electron transfer can become uncoupled from oxidative phosphorylation. With increasing degree of uncoupling a proportionally larger fraction of the oxidative energy can appear as heat, for example as an increase in body temperature during salicylate intoxication. Electron transport can also lead to active transport of Ca²⁺ across the mitochondrial membrane, instead of being coupled to oxidative phosphorylation (McCormack and Cobbold, 1991).

When the processes are coupled, the energy released during electron transport is harnessed to pump protons from the matrix side of the inner mitochondrial membrane to the cytosolic side of the membrane. Hence a proton gradient becomes established. There are two components of this proton gradient which cause energy to be generated. Firstly, energy is generated as a consequence of the separation of charges, just as energy may be stored in batteries because of the separation of positive and negative charges in the different components of a battery. The pH gradient caused by proton separation also generates energy. Once there is a substantial electrochemical gradient established because of the proton gradient, the subsequent dissipation of the gradient is coupled to the synthesis of ATP by complex V. The electron transport carriers and the F_1F_0 -ATPase are localised in such a fashion in the inner mitochondrial membrane that protons are pumped out of the matrix compartment during the electron transport phase of the process, and protons are driven back through the membrane during the ATP synthesis aspect of the process.

The ATP thus generated is then transferred from the mitochondria to the rest of the cell where it is used to drive a variety of metabolic processes.

4.1.3.e The Pentose Phosphate Pathway (PPP)

The PPP is an alternative pathway for glucose metabolism that occurs in most tissues, including the brain. Under basal conditions at least 5% to 8% of brain glucose is likely to be metabolised via the PPP in the adult monkey, and 2.5% in the adult rat. The flux is considered to be similar in the adult human brain (Hostetler *et al.*, 1970). The PPP has a higher activity in the newborn and developing brain, reaching a peak at myelination. Although *in vitro* measurements of PPP capacity show the pathway remaining constant throughout the life span of the rat, PPP activity could not be detected with physiological acceptors in middle-aged and older animals. It is possible that the PPP serves as a reserve pathway for the times when its reactions which are necessary for biosynthesis become needed. The PPP enzymes and metabolic flux are present in synaptosomes.

The main contributions of the PPP include the provision of a source of 5carbon sugars (riboses) which are needed for the synthesis of nucleotides; and the production of NADPH which is required for the reductive reactions necessary for lipid synthesis, with the latter being a more major contribution of the PPP. It is also clear that an important function of the PPP is to provide NADPH for maintaining the tripeptide glutathione in its reduced form (see section 5.1). This reaction is important for cellular defence.

4.1.3.f Glycogen metabolism

The compound glucose-6-phosphate is not only able to feed into glycolysis or the PPP, but it can also be converted to glycogen. In liver and muscle glycogen metabolism is very important. In brain tissue, this does not apply to the same extent. Glycogen stores in the brain are relatively small (Maker *et al.*, 1976). The accepted role of glycogen is that of a carbohydrate reserve utilised when glucose falls below the required level for energy production. However, there is a rapid, continual breakdown and synthesis of glycogen which accounts for approximately 2% of the flux of glycolysis in adult brain, and which is subject to elaborate, local control mechanisms. This suggests that even under normal conditions glycogen reserves are important for brain function. However, if this reserve became the sole supply of glucose, the normal brain glycolytic flux could only be maintained for a short period of less than 5 minutes (Lust *et al.*, 1985). Nevertheless, this cerebral glycogen metabolism is of considerable importance under some circumstances. The glycogen reserves, although low, may be energetically important in situations of increased glucose utilisation or decreased glucose supply in the brain. It has been suggested that the brain glycogen levels may serve to afford some degree of protection under conditions such as ataxia (McCandless and Schwartzenberg, 1981). Under certain circumstances, for example anoxia, glycogen is broken down at rates that approach those of normal glucose utilisation.

4.1.4 <u>Cerebral energy metabolism and the pathophysiology of ischaemia/</u> reperfusion

There is now an extensive and growing body of literature on the biochemical consequences of ischaemia/reperfusion. It is not an easy task to summarise what is currently known of the changes in cerebral energy metabolism during an ischaemic episode (whether with or without reperfusion). Many researchers have measured concentrations of ATP and other energy compounds, rates of flux of metabolic pathways, and activities of enzymes of energy metabolism during conditions of ischaemia. However, the species, model and techniques used, the types of ischaemia, and the duration of the insult period are numerous and preclude a tidy summary of current knowledge. Instead, this section seeks to review, outline and assess some of the studies that have been conducted and conclusions that have been reached over the years, and to briefly report on some of the better characterised changes in energy metabolism that can occur during ischaemia. When considering different models of ischaemia, it is important to note the impact of the density and the duration of the ischaemia on metabolism so valid comparisons are made. This survey of the literature will be confined to ischaemia of sufficient severity to cause a severe disruption of cerebral energy metabolism, that is to say complete and near-complete ischaemia.

4.1.4.a Brain function

With the onset of complete global brain ischaemia the first noticeable effects are changes in brain function. These occur prior to any measurable effects on brain tissue levels of high energy phosphate compounds. It is important to know how cerebral function responds to an ischaemic episode, In 1943 Rossen et al. conducted a thorough study of the functional effects of complete ischaemia in conscious human subjects. Blood flow to the brain was arrested by the use of a cervical inflatable cuff. Loss of consciousness occurred within approximately 7 seconds of ischaemic onset. At this point a change in the electroencephalogram (EEG) occurred (ie. a change in electrical activity of the brain) so that a slower rhythm frequency was seen. The pupillary reflex was lost in approximately 10 seconds. Interruption of blood flow for up to 100 seconds was followed by complete restoration of brain function in subjects. Comparable results have been obtained in experimental animals. EEG has been shown to be suppressed after 20 seconds of ischaemia in the cat (Hossman and Olsson, 1970), after 10 seconds in the rat (Swaab and Boer, 1972) and after 30 seconds in the gerbil (Cohn, 1979). From data quoted by Siesjö (1978), it appears that in rabbits, cats and dogs, EEG disappears in 15-25 seconds, the pupillary reflex in 35-90 seconds, and spontaneous respiration in 30-120 seconds. It seems then that functions depending upon integration of cortical events (e.g. consciousness) are more susceptible to ischaemia than those controlled by lower brain stem centres.

4.1.4.b Energy metabolism and metabolites

We have seen that ischaemia affects brain function, but what of energy metabolism? Ischaemia rapidly (within seconds) leads to oxygen depletion and cessation of oxidative metabolism. Articles published by Lowry and associates (Lowry *et al.*, 1964; Goldberg *et al.*, 1966) provided the first detailed accounts of changes in cerebral energy metabolism under these conditions, in mice. It was pointed out by Lowry *et al.*, (1964) that in the absence of a fresh source of oxygen, the brain can only obtain energy by using its meagre energy-rich phosphate reserves, and by metabolising its stores of glycogen and converting glucose to lactate.

During ischaemia these stores are utilised within less than 5 minutes. The energy reserves are only capable of sustaining ATP for approximately 1 minute in the absence of blood flow (Lust *et al.*, 1985) and a decrease in concentration becomes apparent. Tissue concentration of phosphocreatine (PCr), the storage form of ATP, is reduced to nil within about 1 minute (Siesjö, 1984) in an attempt to stabilise ATP concentration. In their study, Lowry *et al.*, (1964) found that the energy stores were tapped off in the order PCr, glucose, ATP and glycogen, and that essentially no useful energy sources remained after 2 minutes of ischaemia. In a recent review it was stated that within the first two minutes of ischaemia, the concentrations of glucose, ATP and PCr are all reduced by more than 80% (Sims and Zaidan, 1995). It is a generally accepted statement that the concentration of adenine nucleotides reaches a plateau approximately 10 minutes after ischaemia, and more specifically that tissue ATP content decreases to zero within 5 to 7 minutes (Siesjö, 1984).

4.1.4.c Anaerobic metabolism

Following oxygen depletion and cessation of oxidative metabolism, anaerobic ATP production is enhanced. As ATP and PCr decline, anaerobic glycolysis is stimulated. From analysis of the glycolytic intermediates it has been concluded that enhanced glycolysis is due to the activation of hexokinase the and phosphofructokinase (Lowry and Passonneau, 1964). In spite of the limited substrate reserves, glycolysis is increased by as much as seven-fold during ischaemia (Lowry et al., 1964). Lactate accumulates as a result of the anaerobic glucose metabolism, reaching values in normoglycaemic animals of 5-10 times the pre-ischaemic concentrations (Pulsinelli and Duffy, 1983; Nowak et al., 1985; Crumrine and LaManna, 1991). The lactate concentration reaches a maximum value within 2 to 3 minutes (Siesjö, 1984). This is the expected result in complete ischaemia during which lactate production is limited by the pre-ischaemic stores of glucose and glycogen. With this accumulation of lactate, pH has been estimated to fall to approximately 6.5 (Lunggren et al., 1974). In animals that are hyperglycaemic at the time of ischaemia there are much larger reductions in pH than in normoglycaemic animals, and more severe tissue damage is also seen (Kalimo *et al.*, 1981; Siesjö, 1988). Elevated lactate may be a factor in exaggerating the impact of ischaemia, however the precise role of elevated lactate in the development of brain damage remains unclear. The increase in lactate is quite rapid and peaks within minutes, however the severity of brain injury still increases past this time-point, and escalates with longer periods of ischaemia. It therefore seems unlikely that elevated lactate could account for the pattern of injury, and this is particularly relevant where a heterogeneous pattern of injury is noted even though lactate elevation is uniform.

4.1.4.d The Krebs TCA cycle

During ischaemia, the major alterations in the TCA cycle intermediates are a massive increase in succinate and marked decreases of α -ketoglutarate and OAA (Goldberg *et al.*, 1966). The loss of OAA and α -ketoglutarate are stimulated by the increase in NADH/NAD⁺ ratio. The explanation put forward, by Goldberg *et al.*, (1966), for the loss of these intermediates, along with slight reductions in citrate and malate was that TCA intermediates are diverted towards amino acid formation during ischaemia.

4.1.4.e Energy failure

The extent of energy failure depends on the depth and duration of the ischaemic insult, being less pronounced in incomplete and short-term ischaemic than in complete and long-term ischaemia (Nordström *et al.*, 1978; Jafar and Crowell, 1987).

It must be noted that energy failure itself is not the determinant of cell degeneration. Cellular energy depletion appears to be a triggering event for many of the damaging biochemical processes occurring during ischaemia, for example loss of ionic homeostasis. Figure 4.5 outlines ATP turnover in the nerve terminal.



Figure 4.4 ATP turnover in the nerve terminal

legend for figure 4.4

A schematic illustration of some of the processes in the nerve terminal which are involved in ATP homeostasis.

A & B: ATP is produced during the oxidation of metabolic substrates.

C, D & E: ATP is utilised in maintaining the resting ionic gradients of the nerve terminal.

F: ATP is utilised in the process of exocytosis.

G: ATP is converted to GTP which enables correct functioning of G-proteins.

H: ATP is utilised in the reactions of a number of protein kinase enzymes.

One can see, from the previous illustration, the processes which become affected following energy failure and loss of ATP during ischaemia. The active efflux of ions across the plasma membrane ceases, as does calcium sequestration into intracellular stores. The active process of exocytosis is also affected by a decrease in ATP concentration, and the biochemistry of G-proteins and kinase enzymes is also altered under such circumstances.

4.1.4.f Recovery of energy metabolism during reperfusion

It is unlikely that the brain has within it's homeostatic mechanisms a plan to deal with extreme energy deprivation. What apparently happens is that the brain reaches a lower state of order that is readily reversible once the metabolic processes are restored. If the ischaemic episode is extended, then reversibility is lost. The restoration of energy metabolism is a prerequisite for the recovery of brain function following ischaemia. The initial depletion of energy metabolites observed in ischaemia may be followed by recovery on reperfusion, the extent of which is related to the ischaemic insult. However, it must be stated that the relatively rapid recovery of energy metabolites during recirculation does not indicate a return of the relevant metabolic processes to their pre-ischaemic state.

The restitution of cerebral blood flow results in the rapid reversal of many of the metabolite changes which occur during ischaemia. PCr recovers to pre-ischaemic values, and is commonly seen to overshoot slightly, within the first 10-20 minutes of reperfusion (Lust *et al.*, 1985; Nowak *et al.*, 1985; Allen *et al.*, 1988; Crumrine and LaManna, 1991) The concentration of ATP and the other nucleotides is initially restored only partially (to between 60 and 90% of control values), and commonly requires several hours for full recovery (Pulsinelli and Duffy, 1983; Lust *et al.*, 1985; Crumrine and LaManna, 1991). This delay results from depletion of the adenine nucleotide pool as a result of breakdown to inosine and hypoxanthine during ischaemia. The extent of adenine nucleotide pool depletion and hence the period required for ATP recovery is dependent on the length of the ischaemic insult

(Pulsinelli and Duffy, 1983; Nowak et al., 1985; Crumrine and LaManna, 1991).

A second decrease in the concentration of ATP and PCr is seen to occur in what have been described as the susceptible regions of the brain, for example the dorsolateral striatum and the CA1 region of the hippocampus (Lust *et al.*, 1985). The initial pattern of recovery of energy metabolites on the initiation of recirculation is the same in susceptible and resistant brain regions. The secondary changes in these metabolites differentiating the ischaemia susceptible and resistant regions only develop with prolonged recirculation.

A number of studies suggest that there is an initial burst of oxidative glucose metabolism in the first few minutes of recirculation (Steen *et al.*, 1978; Michenfelder and Milde, 1990). By 1 hour of recirculation the available evidence strongly suggests that a long-term depression of glucose metabolism occurs (Pulsinelli *et al.*, 1982; Jørgensen *et al.*, 1990; Sims and Zaidan, 1995). Oxygen utilisation may also be reduced during the first few hours of recirculation (Pulsinelli *et al.*, 1982; Michenfelder and Milde, 1990), and oxidative glucose metabolism may be depressed (Sims and Zaidan, 1995). The ability of cells to rapidly restore the adenylate energy charge and eventually to recover their ATP content while glucose oxidation is apparently reduced suggests that ATP utilisation must also be depressed during this post-ischaemic period.

4.1.4.g Mitochondrial function

The precise mechanism causing ischaemic brain damage remains unclear, although it is known to involve many interconnecting factors (Siesjo, 1984). Mitochondria have been considered to be subcellular targets of ischaemic injury, leading to loss of respiratory control (Mergner *et al.*, 1977; Schweiger *et al.*, 1988), decline of ATP synthesis (Mergner *et al.*, 1977) and swelling and enhanced lipolysis in mitochondrial membranes (Nakahara *et al.*, 1991).

A number of studies have shown that mitochondria isolated from brains made ischaemic for more than a few minutes show reductions in the rates of oxygen uptake, which worsen as the ischaemic period is extended (Rehncrona *et al.*, 1979; Hillered *et al.*, 1984; Sims and Pulsinelli, 1987). The changes have primarily been found to affect state 3 respiration (*ie.* ADP and substrate present), and are detectable using either NAD-linked or FAD-linked respiratory substrates. A more recent study (Allen *et al.*, 1995a), which established the relationship between cerebral blood flow and mitochondrial function in a gerbil model of graded cerebral ischaemia, measured reductions in the specific activities of mitochondrial complex I and complex II-III (as a linked assay).

Reperfusion of the brain following ischaemia in normoglycaemic animals has been seen to result in reversal of the post-ischaemic decreased mitochondrial respiration in some studies (Rehncrona *et al.*, 1979; Hillered *et al.*, 1984; Sims and Pulsinelli, 1987). However, it has also been found that ischaemia-mediated mitochondrial damage is not completely reversible, and that although initial, short reperfusion periods did indicate some recovery, there was further mitochondrial deterioration following extended reperfusion periods (Almeida *et al.*, 1995). Rehncrona *et al.*, (1979) showed mitochondrial recovery during reperfusion, following complete cerebral ischaemia, but no comparable recovery was found after a similar period of reperfusion following incomplete cerebral ischaemia.

A reduction in mitochondrial function in the ischaemia-susceptible dorsolateral striatum has been noted during the first few hours of recirculation (but not during ischaemia itself), which seems to be at least in part due to a selective reduction in the pyruvate dehydrogenase complex (PDHC) activity (Zaidan and Sims, 1993). This selective reduction in PDHC activity is slow in appearing over an extended recirculation period, and does not involve a change in the phosphorylation state of the enzyme, but rather a decrease in the available enzyme activity (Zaidan and Sims, 1993).

It is suggested that following ischaemia/reperfusion there is a delayed reduction of mitochondrial function (Sims and Zaidan, 1995). This partial impairment of mitochondrial function within cells may be compatible with maintenance of energy state while energy utilisation is depressed, but would be likely to contribute to altered cellular response and deleterious changes as the energy requirements of the affected cells recover during prolonged recirculation.

4.1.5 Energy metabolism and the developing brain

In all of the processes concerned with energy metabolism that have been considered thus far, the majority are relevant to the mature adult brain. The mammalian brain goes through a complex developmental process to become fully mature. It therefore becomes necessary to discuss certain aspects of energy metabolism which may differ between the mature adult brain and the immature developing brain.

The postnatal growth of the brain varies markedly according to the species being studied, as further detailed in section 1.4. In rats, mice and humans most of the brain development occurs postnatally, and at birth the brain is largely immature. The maturity level of the postnatal day 10 rat approximates to the developmental stage of a full-term human infant's brain (Nyakas *et al.*, 1996).

4.1.5.a Energy requirements

In terms of the energy requirements of the brain, in newborn infants brain oxygen consumption accounts for 65% of basal metabolism compared with 20% for adults. This reflects changes in the brain to body ratio, which in humans is 12% at birth compared with 2% in adults. It has been assumed that there is a lower energy demand of the immature brain because the neurons are at a lower level of differentiation (Duffy *et al.*, 1975; Bickler *et al.*, 1993), and that this in part accounts for the immature brains resistance to hypoxia and ischaemia. Indeed, synaptic communication is moderate in the rat postnatal period when compared with the adult rat brain.

Although it has already been stated that synaptic communication in the neonate is moderate, the developing neurones do probably require more energy for structural differentiation and synapse formation compared to that which is necessary for synaptic transmission. It has been confirmed that the rapid rate of differentiation and synaptogenesis may account for the increased susceptibility of certain neurones to hypoxic-ischaemic injury (Slotkin *et al.*, 1986). Data does also point however, to a lower metabolic need of immature neurones for ATP-dependent pumping mechanisms (Friedman and Haddad, 1993). It appears that some processes in the immature brain require less energy and some require more when compared with the adult brain.

4.1.5.b Substrate utilisation

In humans and rats the brain glucose utilisation is lower in neonates than in adults. For example Girard *et al.*, (1992) found that the brain glucose utilisation rate is 30% lower in immature suckling rats than in adult rats. Vannucci *et al.*, (1989) reported the cerebral glucose utilisation and transmembrane transport is approximately 10-fold lower in 7 day old rats than in adult rats (Vannucci *et al.*, 1989). In both of these species both glucose and ketone bodies can be utilised (Dahlquist and Persson, 1976) to meet the developing brain's energy requirements. In human and rat neonates the circulating concentrations of ketone bodies are elevated. In human neonates the plasma concentrations of ketones at the end of a normal feeding interval are equivalent to the concentrations of ketone bodies and lactate are both accumulated more and utilised more by the immature rat brain than the adult brain (Dahlquist and Persson, 1976).

The neonatal brain immediately post-partum uses lactate for energy production, followed by a mixture of glucose and ketone bodies (Clark *et al.*, 1994). Lactate and ketone bodies are readily accessible to the immature brain because of the not yet complete blood-brain barrier, and both are readily metabolised by developing neurones (Nyakas *et al.*, 1996). The extraction coefficient for ketone bodies by the neonatal

brain is some four times greater than the adult brain (Cremer, 1982). In the brain, ketone bodies could decrease glucose oxidation through the TCA cycle and increase lactate production by inhibiting PDHC through the provision of acetyl CoA and NADH. In the brains of 20 day old rats, an increase in blood ketone body concentration decreases the brain glucose utilisation and increases the concentration of glycolytic intermediates. Although it is clear that ketone bodies thus have a major role in the newborn brain economy, they cannot totally replace glucose as a fuel. Studies by Cremer and Heath (1974) have suggested that in the 18 day old rat the glucose and ketone bodies may make approximately equal contributions to carbon influx to the TCA cycle.

4.1.5.c Glucose metabolism and oxidation

Certain aspects of brain energy metabolism have been studied in some depth, with many investigators having concentrated on measuring the enzyme activities of those enzyme systems associated with the oxidative metabolism of substrates used by the brain during the postnatal period. The activity of the enzymes involved in glucose (Booth et al., 1980; Leong and Clark, 1984), ketone bodies (Leong and Clark, 1984; Page et al., 1971), and fatty acid utilisation (Warshaw and Terry, 1976; Reichmann et al., 1988) have all been reported on in some detail. For the most part, the activity of the enzymes involved in the complete metabolism of glucose *ie*. the glycolytic and TCA cycle enzymes, in rat brain rise rapidly during the neonatal period and reach adult values at or shortly after weaning (Land et al., 1977; Leong and Clark, 1984; Clark, 1990). The relationship between the glycolytic and oxidative capacity in newborn brain has been an area of debate and controversy. It is thought by some that the developing brain is considerably enriched in the enzymatic apparatus for glycolysis; peak lifetime activity of the regulatory enzyme phosphofructokinase is achieved during suckling and declines thereafter (Kasten et al., 1993). It has been suggested that the newborn brain may favour the glycolytic pathway over the oxidative TCA cycle for energy production (Rust, 1994). However, an alternative and more viable option is that glycolytic mechanisms subserve developmental synthetic tasks during periods of undisturbed brain oxygenation, and become available during periods of oxygen deprivation (Rust, 1994).

Anaerobic glycolysis is remarkably operative at the postnatal age of 10 days, but not at the later stages of 14 or 21 days (Bomont *et al.*, 1992). Indeed, this anaerobic glycolysis appears to be sufficient to maintain energy requirements for ionic homeostasis in newborn rats (Ballanyi *et al.*, 1993). After postnatal day 10, when anaerobic glycolysis plays a prominent role in the reaction to hypoxic conditions, oxidative glucose metabolism begins to increase more rapidly (Nyakas *et al.*, 1996). The oxidative respiratory rate of rat cerebral slices has been seen to be close to its adult values at 14 days (Kibler and Brody, 1942; Samson *et al.*, 1960). This rise in cerebral respiration occurs at a time when the basal metabolic rate of the whole animal is still low in comparison with the fully matured adult rat. It is interesting to note that this rise coincides with the period when electrical activity is first found in the cerebral cortex. (McIlwain and Bachelard, 1971)

The newborn has a relatively rich anaerobic energy supply due to the more readily available cerebral glycogen sources (Nehlig and Pereira de Vasconselos, 1993). However, aerobic oxidative glucose metabolism becomes increasingly important after birth until its contribution to the overall supply of energy greatly intensifies and becomes the most important energy yielding process. Mitochondrial numbers have been seen to increase during the first few days post-partum (Kibler and Brody, 1942; Samson *et al.*, 1960) and the majority of the complexes of the mitochondrial respiratory chain have been shown to increase during the first 21 days of life (Bates *et al.*, 1995; Almeida *et al.*, 1995).

The developmental profile of the enzyme PDHC is an important consideration because of its key role in linking the glycolytic pathway to oxidative glucose metabolism. PDHC appears to develop somewhat later than the main bulk of the glycolytic, TCA cycle and respiratory chain enzymes. This is thought to be critical to the development of full aerobic glycolysis and neurological competence (Clark et al., 1994).

In contrast to the enzyme developmental profiles so far discussed, glucose-6phosphate dehydrogenase, a key enzyme involved in the PPP is high prior to postnatal day 10 and drops to a stable activity after this point. Also the key enzymes involved in ketone body metabolism are higher during the suckling period than in adulthood, suggesting a higher rate of the PPP and ketone body metabolic pathways during development than adulthood.

In summary then, it can be seen that many of the metabolic pathways and energy requirements of the brain alter during development. This is particularly important to consider when examining and contrasting the effects of metabolic perturbations, such as ischaemia, on the developing and adult nerve terminal.

METHODS

<u>4.2</u> <u>Methods</u>

4.2.1 Materials

ATP luciferin-luciferase monitoring reagent and standard were BioOrbit (Turku, Finland) products, purchased from LABTECH, U.K. (Uckfield, East Sussex, England).

Ubiquinone 1 (CoQ_1) was a kind gift of the Eisai Chemical Company, Tokyo, Japan.

All other chemicals used were of analytical grade, and were supplied by either BDH Limited, Dagenham, Essex, U.K., or the Sigma Chemical Company, Poole, Dorset, U.K., or Boehringer Mannheim U.K., Limited, Lewes, East Sussex, U.K.

All solutions were made up in deionised double distilled water.

4.2.2 The assay of mitochondrial enzymes

4.2.2.a Introduction and principles

The complete oxidation of pyruvate or of fatty acids to carbon dioxide and water, with the concomitant production of ATP is a very complex process. It is possible to measure the overall rates of oxygen consumption using polarographic techniques such as the oxygen electrode. Spectrophotometric methods, which use electron acceptors whose absorption in specific regions of the spectrum changes during oxidation-reduction reactions, can be used to study some mitochondrial oxidations. Spectrophotometric methods allow the activities of individual mitochondrial enzymes to be determined. This is important when assessing possible specific sites of damage to the overall process of mitochondrial respiration. An advantage of these spectrophotometric methods is that relatively small amounts of sample can be used effectively to determine specific enzyme activity. This enables these methods of determining the activities of individual enzymes to be used in concert with polarographic techniques or assays of ATP concentration on the same sample. Hence data on the extent of damage to particular enzymes and the effect of such damage on respiration and/or ATP production can be established.

4.2.2.b Sample storage

Following treatment of synaptosome samples, as detailed in sections 2.2.7 and 2.2.8, so that they have been subjected to metabolic stress alone or with a reperfusion period, synaptosomes were pelleted and the supernatant removed. These pellets were then snap frozen by dropping the Eppendorf tubes into liquid nitrogen. All samples were then stored at -80°C. It has been noted previously that extended storage at low temperatures does not have any affect on the activities of complexes I, IV, V, citrate synthase and pyruvate dehydrogenase (Bates *et al.*, 1995). However, the activity of Complex II-III does change with extended storage time (Almeida *et al.*, 1995). For this reason, control, ischaemic and anoxic samples were always assayed together in one assay run, and the complex activities were always compared to the control values. As far as possible, storage time was kept to a minimum time period of a less than two months, so that standard deviations of the sample population being assayed were as low as possible.

4.2.2.c Considerations for spectrophotometric enzyme assay techniques

The determination of the activity of an enzyme is based upon the rate of utilisation of substrate or formation of product under controlled conditions. Enzyme assays normally require the use of excess substrate and an appropriate control. The control is in all respects the same as the test assay but lacking either enzyme or substrate. All reaction mixtures should be incubated at a constant, controlled experimental temperature for at least two minutes before the reaction is started either by the addition of pre-equilibrated enzyme or substrate.

Synaptosome suspensions are quite turbid at concentrations useful for most spectrophotometric enzyme assays. This can cause several problems in measurements of enzyme activities. Addition of sufficient synaptosomal protein to achieve assayable amounts of the enzyme of interest will always add to the apparent absorbance due to light scattering by the synaptosomal sample. The best protocol for synaptosome measurements is therefore to use a control with all the assay components present including the synaptosome sample, but excluding one substrate which is used for

reaction initiation. Spectrophotometric assays are usually performed in disposable cuvettes with a 1 cm light path. Care should be taken that the outsides of the cuvettes are clean and that the optical faces are not touched. During assaying, the cuvettes should be kept in a thermostatted compartment at the required temperature. Mixing of the reaction mixture may be done either by inversion of the cuvette which is covered by "Parafilm" or by using a disposable plastic stirring rod after the last addition; stirring is a less desirable option since it may introduce air bubbles into the reaction mixture.

4.2.3 NADH ubiquinone oxidoreductase (complex I)

4.2.3.a Principles

Complex I can catalyse the reduction of many acceptors by NADH. Complex I specific activity is therefore measured by the oxidation of NADH, with the ultimate reduction of ubiquinone 1 (CoQ_1) to ubiquinol. The decrease in absorbance at 340 nm due to the conversion of NADH to NAD⁺ is followed in this assay.

The accurate measurement of complex I activity in a sample depends on the access of NADH to its binding site on the inner part of the inner mitochondrial membrane. To enable this, before assaying all samples were freeze-thawed three times and gently vortex-mixed between freeze-thawing cycles to ensure mitochondrial lysis was complete.

4.2.3.b Assay procedure

Complex I activity was measured using a modification of the method described by Ragan *et al.*, (1987).

A 1 ml total volume in a spectrophotometer cell contained the following (final concentrations):-

25 mM	potassium phosphate, pH 7.2, 30°C.
0.2 mM	NADH
10 mM	MgCl ₂
1 mM	KCN
2.5 mg	fat free bovine serum albumin
approx. 50-100 μg	synaptosomal protein

The reaction was initiated by the addition of 50 μ M (final concentration) CoQ₁ in a minimum volume (10 μ l). The spectrophotometer cell was read at 340 nm against a reagent blank which contained all of the assay components except CoQ₁. After monitoring the rate of NADH oxidation for 5 minutes, 10 μ l of 0.5 mM rotenone was added to the test cuvette and the inhibited, rotenone insensitive rate of reaction was monitored for a further 5 minutes.

4.2.3.c Expression of results

The spectrophotometer software enables the results of the assay to be displayed as a rate. i.e amount of NADH oxidised over a specific time period, usually a minute by convention. The inhibitor sensitive rate i.e. NADH - Ubiquinone Oxidoreductase specific activity is calculated by subtracting the rotenone insensitive rate (the rate after rotenone addition) from the overall initial rate.

The extinction coefficient for NADH in this assay is $6.81 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. Although the molar extinction coefficient for NADH at 340 nm is $6.22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, the contribution of ubiquinone reduction to the absorption of NADH at 340 nm means that $6.81 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ is the true extinction coefficient for NADH in this assay. The enzyme specific activity is calculated as:-

Activity = $\underline{A/\min}$ x <u>1000</u> 6.81x10³ volume of synaptosome sample (μ l)

where A is the change in absorbance and A/min is the rotenone sensitive rate.

Ischaemia and energy metabolism

The calculated enzyme activity can be related to the synaptosomal protein in the cuvette, so that a protein baseline enables comparison between samples. The calculated activity is divided by the protein concentration (mg/ml) of the synaptosome stock from which a sample was taken and pipetted into the cuvette. Hence the results are expressed as an activity per mg of protein. Similarly, rather than protein being used as a baseline another specific mitochondrial enzyme, such as citrate synthase, can be used as a baseline to express the complex activity against.

When using brain homogenates this latter approach is often taken so that the residual non-mitochondrial protein is effectively discounted from the sample. In the studies reported in this thesis, protein has been used as a baseline for the expression of enzyme activities primarily because developmental profiles of certain mitochondrial enzymes could lead to some confusion. However, initial studies found that expressing the complex activities against either protein or citrate synthase gave the same significant results regardless of which baseline was used.

Complex I activity is expressed as nmol/min/mg protein.

4.2.4 Succinate cytochrome c reductase (Complex II-III)

4.2.4.a Principles

Complex II-III activity was measured as the antimycin a-sensitive rate of cytochrome c reduction. This assay measures the activity of succinate-cytochrome c reductase. It measures the dehydrogenation of succinate and the transfer and flow of electrons through complex II to ubiquinone to form ubiquinol which is then reoxidised by complex III with the transfer of electrons to cytochrome c. The change in absorbance as cytochrome c becomes reduced is followed at 550 nm.

This succinate cytochrome-c reductase (SCR) assay is a linked or multicomponent assay. Studies (Taylor *et al.*, 1993) have found that damage to complex II is readily picked up by this linked assay.

4.2.4.b Assay procedure

Complex II-III activity was measured using a modification of the method described by King, (1967).

A 1 ml total volume in a spectrophotometer cell contained the following (final concentrations):-

100 mM	potassium phosphate, pH 7.4
0.3 mM	K ⁺ EDTA
1 mM	KCN
0.1 mM	cytochrome c
approx. 50-100 μ g	synaptosomal protein

The reaction was initiated by the addition of 20 mM (final concentration) succinate in a minimal volume. The spectrophotometer cell was read at 550 nm against a reagent blank which contained all of the assay components except succinate. After monitoring the rate of cytochrome c reduction for 5-10 minutes, 10 μ l of 1 mM antimycin a was added to the test cuvette and the inhibited, antimycin insensitive rate of reaction was monitored for a further 5 minutes.

4.2.4.c Expression of results

The overall rate of reduction of cytochrome c which reflects (SCR) activity is calculated by subtracting the antimycin a - insensitive rate from the antimycin a sensitive rate. The extinction coefficient for cytochrome c in this assay is 19.2×10^3 M⁻¹ cm⁻¹.

The enzyme specific activity is calculated as:-

Activity = $\underline{A/\min}$ x <u>1000</u> 19.2x10⁻³ volume of synaptosome sample (μ l)

where A/min is the antimycin a sensitive rate.

Ischaemia and energy metabolism

This calculated activity is then divided by the protein concentration of the synaptosome sample. Complex II-III activity is then expressed as **nmol/min/mg protein.**

4.2.5 Cytochrome c oxidase (Complex IV)

4.2.5.a Principles

Mitochondrial complex IV catalyses the transfer of electrons from reduced cytochrome c to molecular oxygen with the concomitant oxidation of cytochrome c. Complex IV consists of a dimer that spans the mitochondrial membrane between the matrix and the intermembrane space. Cytochrome c binds to the oxidase enzyme from the cytosolic side of the membrane, whereas oxygen binds from the matrix side of the membrane during the electron transferring event.

The assay of complex IV activity relies on measuring the absorbance change (\triangle A) at 550 nm as reduced cytochrome c becomes oxidised by the enzyme. The kinetics of cytochrome c oxidase activity are complex, but the rate of reaction can be determined either as an initial rate or as a first-order rate constant by using a spectrophotometric assay. Complex IV activity has been measured in the studies for this thesis by determining the first-order rate constant. This is because the reaction is vigorously first-order with respect to cytochrome c, and also the initial rate of the reaction means that the substrate is not saturating so that as the substrate concentration decreases (as it reacts with the enzyme) the reaction rate also decreases. *k* the first order rate constant relates *V* (reaction rate) to [S] (concentration of substrate), in the following relationship:-

$$\mathbf{V} = \mathbf{k} [\mathbf{S}]$$

where k is constant.

4.2.5.b Assay procedure Preparation of reduced cytochrome c

Cytochrome c (10mg/ml final concentration) was prepared in water. A few crystals of ascorbate were added to fully reduce the cytochrome c. A colour change in the cytochrome c solution from a dark red to a pale red colour indicated that reduction had occurred. It was necessary to fully remove the ascorbate from the reduced cytochrome c so that it did not interfere with the assay of complex IV activity. This was achieved by using a small gel filtration column of 3 ml volume. Removal is accomplished because the cytochrome c is eluted more rapidly from the column due to its **larger** molecular size, and can be collected separately from the ascorbate. The column was thoroughly washed with a 10 mM phosphate buffer, pH 7.0, both before and after use.

Determination of cytochrome c concentration

The following was placed into two cuvettes: 950 μ l water 50 μ l reduced cytochrome c

One cuvette was placed into the spectrophotometer as the reference cuvette, the other as the test cuvette. The reading was autozeroed, and then 10 μ l of 100 mM ferricyanide was added to the reference cuvette so that all of the cytochrome c in that cell became oxidised. The absorbance reading was used in the following equation derived from the Beer Lambert law of A = ϵ cl:-

c = <u>absorbance reading</u> x dilution factor of 20 (50 μ l into 1000 μ l) 19.2 x 10³

to determine the concentration of the reduced cytochrome c.
Complex IV assay procedure

Complex IV activity was measured using a method based on that described by Wharton and Tzagoloff (1967). The assay measured the oxidation of reduced cytochrome c, monitored at 550 nm.

A 1 ml total volume in a spectrophotometer cell contained the following (final concentrations):-

reference cuvette	
10 mM	potassium phosphate, pH 7.0
50 μM	reduced cytochrome c
1 mM	potassium ferricyanide
test cuvette	
10 mM	potassium phosphate, pH 7.0
$50 \ \mu M$	reduced cytochrome c

The spectrophotometer test cuvette was read at 550 nm against the reference cuvette. The cuvettes were autozeroed against each other before the addition of ferricyanide to the reference cuvette. A steady absorbance reading was monitored for a few seconds after the addition of the ferricyanide. The reaction was then initiated by the addition of approximately 50-100 μ g of synaptosomal protein to the test cuvette only. The reaction was measured for approximately 5 minutes.

After concluding the measurement, the data was displayed as a table on the spectrophotometer. The maximum absorbance reading after the addition of the synaptosomal sample was found and taken as t=0. The absorbance readings at three time points, normally 1, 2 and 3 minutes after t=0 were then noted.

The first-order rate constant k was calculated from these by the following equation:

$$k = \underline{\ln (A)t=0} - \underline{\ln (A t=0 - A t=x)}_{x}$$
 x dilution factor

where x is the time point of 1, 2 or 3 minutes, and A is absorbance value.

4.2.5.c Expression of results

Due to the fact that the change in absorbance (\triangle A) over time changes (in a linear rate assay it remains constant), the natural log of \triangle A was taken so that the result becomes linearised. The mean of the calculated k values were taken and the calculated activity was expressed in k/minute. This calculated activity was then divided by the protein concentration of the synaptosome sample. Complex IV activity was then expressed as k/min/mg protein.

4.2.6 ATP synthase (Complex V)

4.2.6.a Principles

Mitochondrial complex V catalyses the formation of ATP from ADP + Pi. Complex V activity is difficult to measure directly and was therefore measured in a linked enzyme system as the oligomycin-sensitive rate of NADH oxidation. The assay is used to evaluate the enzyme's ATP synthase activity by measuring its ATPase activity. The two different activities of the enzyme are proportional to each other because the action of the ATP synthase is fully reversible.

The assay of Complex V activity was measured by coupling the product of the ATPase reaction - ADP - to the oxidation of NADH by the action of lactate dehydrogenase and the following reactions:-

ATPase

pyruvate kinase

monitored at 340 nm

The hydrolysis of ATP by the ATPase liberates ADP which is converted back to ATP by pyruvate kinase. Thus a constant concentration of ATP is maintained and a low steady state concentration of ADP (which is an inhibitor of ATP hydrolysis). The pyruvate produced, via pyruvate kinase, is converted to lactate by lactate dehydrogenase via the oxidation of NADH which is monitored at 340 nm.

This assay is used to evaluate the ATPase activity of complex V. Cells contain many ATPases. Many membrane-bound ion pumps, such as the Ca²⁺-ATPase on endoplasmic reticulum, have ATPase activity. The different ATPases in a cell can differ with respect to their kinetics and sensitivity to inhibitors. Therefore it becomes important to be able to measure the complex V ATPase specifically. Oligomycin can be a specific inhibitor of complex V if the correct concentrations are used. Therefore in this method the oligomycin-sensitive rate of ATP hydrolysis is measured to account for the activities of other cellular ATPases which may be present in the synaptosomal sample.

The synaptosome sample will contain a certain amount of endogenous ADP and ATP. This causes the comparatively fast oxidation of NADH which is not limited by ATPase activity. Therefore, it is important to record changes in optical density and ensure that a stable baseline is evident before the addition of exogenous ATP and assay commencement. Under the assay conditions used, the inhibition by oligomycin can take place rather slowly. It is important to read the inhibited rate for over 5 minutes and to then discount the first two to three minutes of this rate until full inhibition has occurred and the rate becomes truly linear.

4.2.6.b Assay procedure

Complex V activity was measured using a modification of the method described by Soper and Pedersen, (1979).

A 1 ml total volume in a spectrophotometer cell contained the following (final concentrations):-

50 mM	Tris-HCL buffer, pH 8.0
5 mM	phophoenolpyruvate
0.3 mM	NADH
6 mM	MgCl ₂
2 mM	KCN
100 mM	KCl
$10 \ \mu M$	rotenone
25 Units	pyruvate kinase
25 Units	lactate dehydrogenase
approx. 50-100 μg	synaptosomal protein

The reaction was initiated by the addition of 6 mM (final concentration) ATP in a minimum volume (20 μ l of 300 mM stock solution). The spectrophotometer cell was read at 340 nm against a reagent blank which contained all of the assay components except added ATP. After monitoring the rate of NADH oxidation for 5 minutes, 20 μ l of oligomycin (5 μ g/ml) was added to the test cuvette and the inhibited, oligomycin insensitive rate of reaction was monitored for a further 5 minutes.

4.2.6.c Expression of results

The overall rate of oxidation of NADH which reflects complex V activity is calculated by subtracting the oligomycin - insensitive rate from the oligomycin sensitive rate. The extinction coefficient for NADH in this assay is 6.22×10^3 M⁻¹ cm⁻¹.

The enzyme specific activity is calculated as:-

Activity = $\underline{A/\min}$ x <u>1000</u> 6.22x10³ volume of synaptosome sample (μ l)

where A/min is the oligomycin sensitive rate.

This calculated activity is then divided by the protein concentration of the synaptosome sample. Complex V activity is then expressed as **nmol/min/mg protein**.

4.2.7 The determination of ATP concentration

4.2.7.a Principles of ATP measurement

The assay of ATP used for the studies in this thesis is based upon the quantitative measurement of a stable level of light produced as a result of an enzyme reaction catalysed by firefly luciferase. The enzyme reaction is:-

luciferase

ATP + luciferin + oxygen ------ oxyluciferin + AMP +PPi + CO_2 + light

Assay principle

Light emitted from a sample is measured in the measuring chamber of a luminometer. The light emitted is collected and reflected by the wall of the measuring chamber which is coated with chromium. The reflector is present to ensure efficient capture of the emitted light. The impact of the photons on the photocathode can be registered in two different ways, either by photon counting or by time-averaging of

the electron pulses which are converted to a continuous voltage. With the equipment used in these studies, the reflected light is converted by a photomultiplier into an electrical signal which is then amplified by a pre-amplifier. Results are expressed in mV.

The reagents contained in the BIO-ORBIT ATP assay kit are designed to provide a linear, time-independent light output over a concentration range of $10^{-11} - 10^{-6}$ M ATP.

The constituents of the reagents are as follows:-

ReagentsAssay buffer0.1 M Tris-acetate containing 2 mMEDTA.Titrated to pH 7.75 with acetic acid.ATP monitoring reagent (MR)firefly luciferaseD-luciferinbovine serum albumin 50 mgmagnesium acetate 0.5 mmolinorganic pyrophosphateATP standardATP 0.1 µmolmagnesium sulphate 2.0 umol

Reagent reconstitution

The ATP monitoring reagent (MR) was reconstituted by adding 10 ml of double distilled deionised water and mixing gently. The ATP standard was also reconstituted with 10 ml double distilled deionised water, to give a stock soln. of 1×10^{-5} M. This was stored at -20°C for less than 4 months. For use in the assay the standard was thawed and further diluted 1:10 with buffer to give an ATP stock concentration of 1 μ M.

4.2.7.b ATP extraction

ATP was acid extracted from synaptosomal suspensions using 3.5% (final concentration) ice-cold perchloric acid (PCA). Extracts were neutralized with 0.7M K_2 HPO₄ (final concentration), kept on ice for 30 minutes and then pelleted (10,000g for 2 minutes). Resultant supernatants were snap frozen in liquid nitrogen and kept frozen (-80^oC) until the time of assay.

Principle

When PCA is added to samples containing living cells, two effects are observed.

1] cell membranes are disrupted which results in the release of cell contents including the adenine nucleotides.

2] the enzymes that catalyse adenine nucleotide converting reactions are irreversibly inactivated.

The concentrations of PCA used should be high enough to produce the maximum extraction of ATP but low enough, after appropriate dilution, not to interfere with the bioluminescence of firefly luciferase reaction. Concentrations less than 2% do not lead to adequate nucleotide extraction. Concentrations less than 0.1% do not inactivate adenine nucleotide converting enzymes. Effects on the luciferin-luciferase reaction are low enough to be acceptable at 0.1% PCA after dilution. For this study, 500 μ l 10% PCA was added into 1 ml of synaptosome incubation, to give 3.5% final concentration. After neutralisation by addition of 800 μ l K₂HPO₄ (1 M), the samples were frozen.

Before assaying the ATP samples were diluted 1:100 with the assay buffer, so that the PCA concentration in the ATP assay reaction would be approximately 0.03%. This would give acceptable low values to prevent interference with the luciferin-luciferase reaction.

4.2.7.c Procedure

The ATP assay was performed at 30°C on a 1251 LKB Wallac Luminometer, according to the manufacturers' instructions. The ATP samples extracted from the synaptosome incubations were defrosted, diluted 1:100 with assay buffer to 100 μ l and placed in the measuring cuvettes. 650 μ l of assay buffer was added to all of the cuvettes, which were loaded into an auto-sampler which was computer controlled so that one sample was measured at a time. A baseline reading was taken for a few minutes, then 200 μ l of ATP MR was added to the cuvette and a reading monitored for a few minutes which was due to the concentration of ATP in the sample. At the end of the assay 50 μ l of ATP standard was added to the cuvette, and the increase in light monitored for a few minutes.

The concentration of ATP in the sample was calculated by the software package and given in micromolar units. This was possible because the concentration of the standard used (1 μ M stock) was programmed into the computer programme. The mV reading produced by the known concentration of the ATP standard was used to determine the concentration of ATP in the unknown sample which produced a particular mV reading.

The concentration of ATP was calculated by using the ATP standard, but a correction for this ratio of standard and sample was necessary because 100 μ l of sample was used and only 50 μ l of ATP standard was routinely used. Hence the result given by the luminometer was divided by two to correct for the volume differences. A correction for the dilution factor incurred by the neutralisation of the PCA sample was also necessary for each sample.

A protein measurement was always taken of the synaptosome incubation prior to the addition of PCA. The ATP results could therefore be expressed as **nmoles ATP produced per milligram of synaptosome protein**.

4.2.8 Statistical analysis

The effects of peroxynitrite treatment on the parameters measured were tested using one-way analysis of variance (ANOVA) (Sokal and Rohlf, 1981). Differences between groups were tested using the Least Square Difference (LSD) test (Sokal and Rohlf, 1981).

RESULTS

4.3 Results

4.3.1 Metabolic stress and intrasynaptosomal ATP levels.

4.3.1.a Effect of ischaemia

(Figure 4.5)

After five minutes of the incubation period, the ATP concentration was significantly (p<0.01) decreased in adult synaptosomes incubated under ischaemic conditions when compared to control synaptosomes. ATP levels in these adult ischaemic synaptosomes remained significantly decreased throughout the rest of the incubation period (30 minutes). By fifteen minutes of the incubation a plateau of approximately 15% of control levels was reached, and the ATP concentration remained at this low level for the following fifteen minutes.

In postnatal day (PND) 10 pup synaptosomes incubated under ischaemic conditions, the ATP concentration also dropped to some 15% of control levels by fifteen minutes and remained at this level until the end of the incubation. However, the time period required for the ATP levels to drop significantly was longer than in the case of the adults. In the pup synaptosomes under ischaemic conditions, ATP levels were maintained at relatively high levels until five minutes of incubation when levels began to decrease and reached significance by ten minutes of the incubation.

4.3.1.b Effect of reperfusion following ischaemia (Figure 4.5)

Following a 30 minute period of reperfusion, where glucose and oxygen were restored to the ischaemic synaptosomes, the PND 10 pup synaptosomes had shown a much greater degree of recovery of ATP levels than the adult synaptosomes. In the case of the adults, ATP levels only recovered slightly, to 33% of the control values. These low levels were still significantly decreased in comparison with the adult control preparations.



Legend for Fig. 4.5

ATP concentration was measured in adult and pup ischaemic synaptosomes as described in 4.2.7. R represents a 30 minute reoxygenation period. Results are expressed as mean % of control value at the same time point. SEMs of means were less than 10% of the mean. Control values were all between 1.5 and 2.2 nmoles/mg protein, and control levels were well maintained throughout the incubation period.

n = 5 separate preparations, measurements performed in duplicate. ** represents significant difference (P<0.01) from control.

The ATP levels recovered to 72% of control levels in the PND 10 pups. Although this was still significantly (p<0.05) decreased when compared to the control incubation, there was obviously a much greater degree of recovery in this case than in the case of the adult. Therefore, on restoration of oxygen and glucose supply it appears that the immature nerve terminals were able to generate ATP levels to a much greater extent than the fully matured adult nerve terminals, following an episode of ischaemia.

4.3.1.c Effect of anoxia

(Figure 4.6)

Under anoxic conditions, the ATP concentration was significantly (p<0.01) decreased in adult synaptosomes when compared to control synaptosomes by five minutes of the incubation period. Although this same level of significance was reached by the same time period of incubation in the adult ischaemic synaptosomes, the levels during anoxia were slightly higher at 55% as compared with 40% during ischaemia. The ATP levels in the adult synaptosomes during anoxia remained significantly decreased throughout the rest of the incubation period (30 minutes). However, between five and ten minutes of incubation there was only a small decrease from 55% to 49% in these synaptosomes. This suggests that the energy demand at this time could be met to an extent by the levels of ATP and hence a further decrease was not apparent. By fifteen minutes of the incubation a plateau of approximately 15% of control levels was effectively reached, and the ATP concentration remained at this low level for the following fifteen minutes. This was the same phenomenon as occurred in the case of the ischaemic adult synaptosomes.

In PND 10 pup synaptosomes incubated under anoxia conditions, the ATP concentration again dropped to approximately 15% of control levels by fifteen minutes of the incubation period and remained at this level until the end of the incubation. However, at five minutes of the insult the ATP levels were close to control levels and were not significantly decreased. Between five and fifteen minutes the ATP levels began to decrease but were still above 50% at ten minutes of the anoxia. This was higher than the adult synaptosomes under the same conditions, and also higher than

the pup synaptosomes under ischaemic conditions.

4.3.1.d Effect of reperfusion following anoxia

(Figure 4.6)

After the 30 minute period of reperfusion, the PND 10 pup synaptosomes had shown a large degree of recovery, to the extent that the ATP levels in this preparation were no longer significantly decreased when compared with control ATP levels, and were in fact almost at control levels. In the adult synaptosome preparation which had been reperfused following anoxia, ATP levels recovered to almost 50% of the control values. Although this was still significantly decreased in comparison with controls, and also was significantly less than the PND 10 pup synaptosomes under the same conditions, the recovery in these adult synaptosomes was to a greater extent than the ischaemic adult synaptosomes.

It seems then that, on restoration of oxygen and glucose supply following an anoxic insult the immature nerve terminals were again able to generate ATP levels to a much greater extent than the fully matured adult nerve terminals, as was the case following ischaemia/reperfusion. The extent of restoration of ATP levels was greater with the anoxia/reperfusion than with the ischaemia/reperfusion for both immature and adult nerve terminals.

<u>4.3.2</u> <u>Metabolic stress and the activity of the mitochondrial respiratory chain</u> <u>enzymes.</u>

4.3.2.a Complex I

(Figures 4.7 and 4.8)

For adult synaptosomes, complex I activity in the synaptosomal mitochondria was significantly (p<0.01) decreased following fifteen minutes of either ischaemia or anoxia, when compared with control adult synaptosomes. There was no significant difference in the effect that the two different insults had on the activity of this enzyme. The activity of complex I remained significantly depressed at 30 minutes of both insults. After a thirty minute period of reperfusion when glucose and oxygen



Legend for Fig. 4.6

ATP concentration was measured in adult and pup anoxic synaptosomes as described in 4.2.7. R represents a 30 minute reoxygenation period. Results are expressed as mean % of control value at the same time point. SEMs of means were less than 10% of the mean. Control values were all between 1.5 and 2.2 nmoles/mg protein, and control levels were well maintained throughout the incubation period.

n = 5 separate preparations, measurements performed in duplicate. ** represents significant difference (P<0.01) from control.



FIGURE 4.7 Effect of metabolic stress on adult synaptosome

Time of incubation (mins)

Legend for Fig. 4.7

Complex I activity was measured in adult synaptosomes as described in 4.2.4., following 30 minutes of severe metabolic stress with or without reperfusion, as detailed in 2.2.7.

Results are expressed as mean + SEM

n = 8 or more separate preparations, measurements performed in duplicate.

** represents significant difference (P<0.01) from control.



FIGURE 4.8

Time of incubation (minutes)

Legend for Fig. 4.8

Complex I activity was measured in pup synaptosomes as described in 4.2.4., following 30 minutes of severe metabolic stress with or without reperfusion, as detailed in 2.2.7.

Results are expressed as mean ± SEM

n = 8 or more separate preparations, measurements performed in duplicate.

** represents significant difference (P<0.01) from control.

once more became available to the synaptosomes, the activity of complex I was restored and was not statistically different from controls. This was the case following reperfusion after both ischaemia and anoxia. Therefore, the activity of complex I was decreased during a period of metabolic stress, but this decrease was reversible.

In the PND 10 pup synaptosomes, the activity of complex I was significantly decreased, compared to controls, after 15 minutes of ischaemia. This decrease became even larger by thirty minutes of ischaemia, and was to a greater degree than that occurring in the adult synaptosomes. During anoxia, the activity of complex I in PND 10 pup synaptosomes did not become significantly decreased, when compared to controls, until after fifteen minutes of this insult. By thirty minutes of anoxia complex I activity was significantly decreased in the PND 10 pup synaptosomes.

Following thirty minutes of reperfusion, complex I activity was restored and was not statistically different from controls in either post-ischaemic or post-anoxic PND 10 synaptosomes. Hence, similar to the adult synaptosomes, the decrease in complex I activity occuring during the period of metabolic stress was reversible following reperfusion.

4.3.2.b Complex II-III

(Figures 4.9 and 4.10)

In adult synaptosomes, both anoxia and ischaemia had no significant affect on the activity of complex II-III in the synaptosomal mitochondria at either fifteen or thirty minutes of the insult.

After a thirty minute period of reperfusion following these insults, the activity of complex II-III became significantly (p<0.01) decreased when compared to controls. Hence some process is initiated during reperfusion following both ischaemia and anoxia, which leads to a decrease in activity of this enzyme.



Time of incubation (minutes)

Legend for Fig. 4.9

Complex II-III activity was measured in adult synaptosomes as described in 4.2.4., following 30 minutes of severe metabolic stress with or without reperfusion, as detailed in 2.2.7.

Results are expressed as mean ± SEM

n = 8 or more separate preparations, measurements performed in duplicate.

** represents significant difference (P<0.01) from control.



FIGURE 4.10 Effect of metabolic stress on PND10 pup synaptosomes Complex II-III activity

Time of incubation (minutes)

Legend for Fig. 4.10

Complex II-III activity was measured in pup synaptosomes as described in 4.2.4., following 30 minutes of severe metabolic stress with or without reperfusion, as detailed in 2.2.7.

Results are expressed as mean ± SEM

n = 8 or more separate preparations, measurements performed in duplicate.

** represents significant difference (P<0.01) from control.

Unlike the situation in the adult synaptosomes, the activity of complex II-III was significantly decreased at both fifteen and thirty minutes of anoxia and ischaemia in the immature synaptosomes from PND pups. Although this enzyme activity was decreased during these insults, complex II-III activity was restored to control levels following a thirty minute period of reperfusion. This was similar after both ischaemia/reperfusion and anoxia/reperfusion.

4.3.2.c Complex IV

(Figures 4.11 and 4.12)

The thirty minute period of ischaemia and also the thirty minute period of anoxia had no effect on the activity of complex IV in the adult synaptosomes, and values of the enzyme under these conditions remained similar to control values. However, after a thirty minute period of reperfusion following both ischaemia and anoxia, complex IV activity became significantly decreased, compared to control levels. Thus, it seems that some process is occurring during reperfusion alone, but not during the insults themselves, which leads to a decrease in activity of this enzyme in the adult synaptic mitochondria.

In the synaptosomes from PND 10 pups there was no significant decrease in the activity of complex IV under either ischaemic or anoxic conditions. A period of reperfusion following these insults also had no effect on complex IV activity in these immature synaptosomes, unlike the adult synaptosomes.

4.3.2.d Complex V

(Figures 4.13 and 4.14)

Complex V activity remained unaffected by either ischaemia, anoxia or reperfusion following these insults in both the adult and immature synaptosomes.



Time of incubation (minutes)

Legend for Fig. 4.11

Complex IV activity was measured in adult synaptosomes as described in 4.2.5., following 30 minutes of severe metabolic stress with or without reperfusion, as detailed in 2.2.7.

Results are expressed as mean <u>+</u> SEM

n = 8 or more separate preparations, measurements performed in duplicate.

* represents significant difference (P<0.05) from control.



Legend for Fig. 4.12

Complex IV activity was measured in pup synaptosomes as described in 4.2.5., following 30 minutes of severe metabolic stress with or without reperfusion, as detailed in 2.2.7.

Results are expressed as mean + SEM

n = 8 or more separate preparations, measurements performed in duplicate.



Time of incubation (minutes)

Legend for Fig. 4.13

Complex V activity was measured in adult synaptosomes as described in 4.2.6., following 30 minutes of severe metabolic stress with or without reperfusion, as detailed in 2.2.7.

Results are expressed as mean ± SEM

n = 8 or more separate preparations, measurements performed in duplicate.



FIGURE 4.14

Time of incubation (minutes)

Legend for Fig. 4.14

Complex V activity was measured in pup synaptosomes as described in 4.2.6., following 30 minutes of severe metabolic stress with or without reperfusion, as detailed in 2.2.7. Results are expressed as mean \pm SEM n = 8 or more separate preparations, measurements performed in duplicate.

DISCUSSION

4.4 Discussion

The results primarily show that there are distinct differences between the effect of metabolic stress on specific mitochondrial enzymes in the nerve terminals of adult as compared with neonatal rat brain.

4.4.1 ATP levels during metabolic stress

4.4.1.a Ischaemia and reperfusion

Firstly it should be stated that the ATP concentrations of synaptosomes measured under control conditions in this study fall within the accepted published range of values (Erecińska *et al.*, 1994).

These results clearly show that conditions of oxygen and glucose lack cause a decrease in the free ATP concentration of both adult and neonatal nerve terminals. After 15 minutes under these conditions both the adult and neonatal synaptosomes exhibited very low ATP concentrations of only approximately 10% of control values. These low levels remained at a plateau during the following 15 minutes under these conditions in both preparations. There were however apparent differences in the time course of ATP depletion in these two different age preparations. In the adult synaptosome preparation the decrease was initially more rapid than in the neonatal preparation, with a drop of 60% during the first five minutes in the adult compared with only a drop of 13% in the neonatal nerve terminals.

Similar differences in the decrease of ATP in adult as compared with neonatal brain slices have been reported following inhibition of oxidative metabolism by cyanide (Bickler *et al.*, 1993). These authors found that in cyanide treated brain slices of rats above 14 days of age ATP levels dropped to 90% of control after 10 minutes whereas the ATP level in brain slices of younger animals had only dropped to 50% of control values. However these authors did not investigate further time points to see if ATP levels reached the same low values in both preparations at a later point.

To further probe whether age-related differences in ATP depletion during cyanide exposure were due to differences in ATP utilisation or production, Bickler *et al.*,(1993) treated adult and neonatal brain slices with both cyanide and iodoacetate to cause a combined blockade of aerobic and anaerobic metabolism. Again they found that the ATP decline was more gradual in the neonatal brain slices and thus concluded that ATP consumption was reduced in the neonatal brain slices.

In the study presented in this thesis the amount of oxygen available to both of these age preparations was initially the same and there was no exogenous glucose available at all in either preparation. The neonatal brain contains greater glycogen stores than the adult which could be reflected in the amount of endogenous glycogen in the synaptosomes in this study. Initially there was a residual oxygen tension in all incubations and thus it is possible that the slower ATP decline in the neonatal synaptosomes was due to initial aerobic glycogen utilisation. It is also possible that the inherent demand for ATP was higher in the adult synaptosomes under these conditions as compared with the neonatal synaptosomes, and this reduced ATP consumption in the neonatal nerve terminals allowed a longer time period for maintenance of ATP levels. Indeed, it is reported that there is a lower metabolic need of immature neurones for ATP-dependent pumping mechanisms (Friedman and Haddad, 1993). Perhaps a combination of lower ATP consumption and the potential for ATP production from glycogen stores in the neonatal synaptosomes led to these nerve terminals exhibiting conservation of ATP levels for a longer time period than adult nerve terminals.

After a thirty minute reperfusion period when oxygen and glucose was once again made available to synaptosomes, there was a degree of recovery of ATP levels in both adult and neonatal nerve terminals. The degree of recovery of neonatal synaptosomes was much greater than that of adult synaptosomes. Neonatal synaptosomes ATP values reached 70% of control compared with only 30% for the adult synaptosome preparation.

It is possible that the metabolic systems concerned with ATP production are less functional after a thirty minute ischaemic period followed by thirty minutes of reperfusion in the adult as compared with neonatal synaptosomes. Alternatively, or perhaps in combination with the above, it is possible that from the same low ATP level of 10% of control in both the adult and neonatal synaptosomes at the end of the ischaemic period, restoration of oxygen and glucose supply re-established ATP production but a higher energy utilisation in adult nerve terminals caused the overall ATP level to be lower in the adult synaptosomes. Direct comparison of the reperfusion results of this study with others in literature was limited because the studies which have directly compared the response of adult and neonatal neuronal preparations to metabolic stress (Kass and Lipton, 1989; Bickler et al., 1993; Friedman and Haddad, 1993) do not report details of ATP levels following reperfusion. Most of the results which detail ATP levels following ischaemia and reperfusion have been derived from nuclear magnetic resonance (NMR) studies. There is a wealth of literature on ATP levels in both adults and immature animals and it is not entirely appropriate to give a detailed comparison of this literature with the ATP results from the study presented here. However, suffice to say that an increased recovery of ATP levels in postischaemic neonatal brain as compared with post-ischaemic adult brain has been widely reported (Rice et al., 1981; Hope et al., 1987; Palmer et al., 1990; Brooks et al., 1995;).

4.4.1.b Hypoxia/anoxia and reoxygenation

Under conditions of adequate glucose supply coupled with a limited availability of oxygen, the ability of anaerobic glucose metabolism to support the energy requiring processes of synaptosomes could be assessed. Under these hypoxic/anoxic conditions neonatal synaptosomes were able to fully maintain their ATP levels for at least five minutes, however by ten minutes a decrease to 60% of control values had occurred which further decreased to less than 20% by fifteen minutes and then remained at this low level for the rest of the anoxic incubation.

The neonatal synaptosome pattern of decreases in ATP levels was somewhat different to that of the adult, as was the case under ischaemic conditions. Adult synaptosomes could not maintain ATP levels for even a short period of time after oxygen became limiting, and by five minutes of incubation the ATP levels had dropped to less than 60% of control. Thus, a transient deficit in oxygen supply may be less detrimental to the energy supply for the neonatal brain than is the case for the fully matured adult. Although the decrease in ATP levels in adult synaptosomes under oxygen lack was greater than neonatal synaptosomes under the same conditions, the decrease was notably more gradual than that seen in adult synaptosomes under ischaemic conditions. Thus, it appears that anaerobic glucose metabolism lessens the energy decline in these synaptosomes during the first ten minutes of incubation although this metabolic pathway alone is not sufficient to fully supply all of the energy demands of the adult synaptosomes and a decrease in ATP still occurs. In the neonatal synaptosomes this phenomenon is similar in that the decrease in ATP under ischaemic conditions is more rapid than under hypoxic/anoxic conditions. Indeed, it appears that the ability of anaerobic metabolism in the neonate is more capable of fully supporting the energy demands of neonatal synaptosomes for a short period of time, although after five minutes ATP levels do decrease to the same extent in both age nerve terminal preparations after fifteen minutes of oxygen lack.

Following a thirty minute period of reoxygenation, there was a full recovery of ATP levels to control values in the neonatal synaptosomes. The adult synaptosomes also showed a degree of recovery to over 40% of the control values, although this was obviously much less of a recovery than in the immature nerve terminals. As suggested in the ischaemia/reperfusion studies, it is possible that the metabolic systems concerned with ATP production are less functional after a thirty minute period of oxygen lack followed by thirty minutes of reoxygenation in the adult as compared with neonatal synaptosomes.

In both the neonatal and adult synaptosome preparations, the recovery of ATP levels in the hypoxic/anoxic synaptosomes was slightly larger than that of ischaemic synaptosomes. The overall drop in ATP levels after thirty minutes of metabolic stress was not significantly different in the ischaemic as compared with hypoxic/anoxic synaptosomes in the case of adults and neonates. The ATP producing pathways in the post-ischaemic synaptosomes may be slightly less functional than that of the post-anoxic synaptosomes at the time point of recovery measured here. Alternatively, or perhaps in combination with this, the energy demand of the post-ischaemic synaptosomes may be greater than that of the post-anoxic synaptosomes in both the adult and neonatal preparations.

4.4.1.c ATP and calcium

In combining the studies concerned with Ca²⁺ homeostasis detailed in chapter 3 with the ATP data detailed here, a number of discussion points become evident. Firstly the decline in ATP levels do not entirely explain the observed differences in Ca²⁺ homeostasis between adult and neonatal synaptosomes. At a thirty minute time point of metabolic stress ATP levels were not significantly different between adult and neonatal ischaemic synaptosomes, adult and neonatal anoxic synaptosomes or indeed between the same age preparation under ischaemia as compared to anoxia. However, there were distinct differences in the Ca²⁺ homeostasis of adult as compared with neonatal ischaemic and anoxic synaptosomes at this time point. Although ATP levels and Ca²⁺ homeostasis do not therefore correlate during the metabolic stress incubation itself, a further area to explore is whether there is a relationship between Ca^{2+} and ATP in the recovery of ATP levels in different preparations after thirty minutes of reperfusion/reoxygenation. In the adult synaptosomes a significantly increased cytosolic [Ca²⁺] was present under both ischaemia and anoxia at thirty minutes, however there was no significant increase in the neonatal synaptosomes under either of these conditions. It is therefore tempting to speculate that the lower degree of recovery of ATP levels in post-ischaemic and post-anoxic adult as compared with neonatal synaptosomes involves the lack of Ca²⁺ homeostasis in the adult nerve terminals. Indeed, once ATP production is restored during reperfusion/reoxygenation a significant amount of the ATP produced would be utilised in the restoration of ionic gradients and thus a return to resting levels of cytosolic Ca^{2+} . In the neonatal synaptosomes less ATP would be utilised in this process because Ca^{2+} homeostasis was not significantly perturbed.

In the case of adult preparations there was a greater degree of recovery of ATP levels in the post-anoxic as compared with post-ischaemic synaptosomes, which is also reflected by a lower cytosolic $[Ca^{2+}]$ in the anoxic synaptosomes than in the ischaemic synaptosomes. However, Ca^{2+} homeostasis cannot entirely explain the differences in ATP levels. In the neonatal synaptosomes there was a much greater degree of recovery of ATP levels in the post-anoxic synaptosomes as compared with the post-ischaemic synaptosomes although Ca^{2+} homeostasis was not compromised in either of these incubations and cytosolic $[Ca^{2+}]$ was not significantly different from each other. Hence, other factors must come into play to explain the increased ATP recovery in neonatal post-anoxic synaptosomes.

4.4.2 <u>Mitochondrial respiratory chain enzyme activities during</u> <u>metabolic stress</u>

4.4.2.a Adult synaptosomes during metabolic stress

This work demonstrates that under conditions of combined glucose and oxygen lack there is a decrease in the activity of complex I of the mitochondrial respiratory chain in adult synaptosomes. This decrease in complex I activity also occurs under conditions of oxygen lack alone in the adult synaptosomes. There was no significant decrease in activity of any of the other complexes of the mitochondrial respiratory chain measured in these preparations under either ischaemic or hypoxic/anoxic conditions. The decrease in complex I activity in adult synaptosomes was apparent by 15 minutes of metabolic insult and remained decreased during a further 15 minutes of incubation. In ischaemic studies by other researchers complex I has been found to be affected by oxygen and/or glucose lack. Allen *et al.*,(1995) found that ischaemia in adult gerbil brain caused a decrease in a number of mitochondrial enzymes, with complex I being the component of the respiratory chain most sensitive to decreases in cerebral blood flow.

A number of studies have also reported that mitochondria isolated from adult brains made ischaemic for more than a few minutes exhibit reductions in the rate of oxygen uptake with NAD-linked substrates (Rehncrona et al., 1979; Hillered et al., 1984; Sims and Pulsinelli, 1987; Allen et al., 1995). Complex I has also been found to be damaged during ischaemia in the heart (Veitch et al., 1991), reflected by a decrease in mitochondrial oxygen consumption with NAD-linked substrates and also a decrease in the activity of complex I itself. These authors reported that a lack of oxygen alone did not decrease complex I activity, therefore suggesting a tissue specific difference in the affect of anoxia on complex I activity. Veitch et al., (1992) also reported that neither increased lactate nor decreased pH have a detrimental effect on complex I activity. Although Hillered et al., (1985) showed a decrease in mitochondrial respiration with NAD-linked substrates this was not correlated with an increase in mitochondrial Ca²⁺ content in their study. This lack of correlation between mitochondrial Ca²⁺ content and mitochondrial function contrasts with *in vitro* studies (Roman et al., 1981; Nowicki et al., 1982; Hillered et al., 1983) where increased mitochondrial Ca²⁺ was seen to interfere with ATP synthesis. Hillered et al., 1985 did however find that increased tissue Ca²⁺ concentration was correlated with decreased mitochondrial respiration of NAD-linked substrates. This is in agreement with the ischaemic data presented in this thesis, where increased synaptosomal [Ca²⁺] is apparent in the adult synaptosomes alongside decreased complex I activity at both fifteen and thirty minutes of ischaemia. However, complex I activity is decreased in anoxic adult synaptosomes at fifteen and thirty minutes whereas increased synaptosomal $[Ca^{2+}]$ is only apparent in these synaptosomes at thirty minutes of anoxia.

Hence it seems that other factors besides calcium homeostasis are implicated in the decreased complex I activity.

The studies presented here did not lead to a decrease in the activities of any of the other complexes of the mitochondrial respiratory chain measured. This is in contrast with the studies of Allen *et al.*, (1995) who also found decreases in the activities of complex II-III and complex V in adult mitochondria and synaptosomes isolated from post-ischaemic gerbil brain. However, as well as the species difference, the experiments conducted by these authors involved a small amount of blood flow and hence a residual oxygen tension throughout the metabolic insult. This could have increased the likelihood of free radical production which may precipitate a different pattern of respiratory chain complex damage.

In a number of studies concerned with post-ischaemic mitochondrial function, oxygen consumption of mitochondria with FAD-linked substrates was decreased (Rehncrona *et al.*, 1979; Hillered *et al.*, 1984; Sims and Pulsinelli, 1987). In these studies the specific activities of respiratory chain complexes were not measured, and therefore although a decreased oxygen consumption (of both NAD-linked and FAD-linked substrates in these studies) showed that integrated respiratory chain function was compromised this could involve other enzymes such as mitochondrial dehydrogenase enzymes and may not necessarily be due to damage of complex II-III.

4.4.2.b Adult synaptosomes following reperfusion/reoxygenation

Following a thirty minute period of reperfusion after ischaemia or a thirty minute period of reoxygenation after hypoxia/anoxia there was a significant decrease in the activities of complex II-III and also of complex IV of the mitochondrial respiratory chain in adult synaptosomes. Neither of these complexes had been previously affected by ischaemia or anoxia in adult synaptosomes. Thus it seems that the mechanisms that mediate inhibition or possibly irreversible damage of these enzymes may be initiated during ischaemia/anoxia but do not lead to a decrease in

enzyme activity until a relatively long period of recovery has occurred, i.e. a form of delayed mitochondrial damage. Alternatively, and perhaps more plausibly, is that the mechanisms of inhibition or damage are only initiated during reperfusion/ reoxygenation. Indeed, upon reperfusion or reoxygenation free radicals are known to be produced (Phillis, 1994). Complexes II-III and IV have been shown to be particularly susceptible to damage by free radical production (Soussi et al., 1990; Bolaños et al., 1994), and therefore, in a rather indirect manner, evidence suggests that the decreases in activities of these enzymes following reperfusion/reoxygenation are concerned with free radical mechanisms initiated once oxygen is again made available to the system. These results of complex II-II and IV damage following reperfusion are in agreement with Allen et al., (1995), who reported a similar pattern of complex damage. Veitch et al., (1992) also reported a significant decrease in complex II, complex III and complex IV activity following a period of reperfusion after ischaemia. In the case of all of these complexes thirty minutes of ischaemia alone did not cause a decrease in enzyme activity. The authors discussed that a marked increase in free radicals in mitochondria during the initial stage of reperfusion could lead to this pattern of complex damage (Veitch et al., 1992). Similarly, Hillered et al., (1984) reported a decrease in respiratory activity with complex I and complex II linked substrates after thirty minutes of ischaemia and thirty minutes of reperfusion. When discussing this work, Almeida et al., (1995) suggested that damage to either complex III or complex IV was therefore implicated, and suggested a mechanism involving free radicals.

Elegant work by Davey and Clark (1996) has shown that the threshold effects for the complexes of the respiratory chain enzymes are such that up to 50% inhibition of the individual complexes I-IV will not adversely affect oxidative phosphorylation. However, these studies were mostly conducted on tissue homogenate or on isolated nonsynaptic mitochondrial fractions, and the authors suggested it highly probable that the threshold effects in synaptic mitochondria might be seen at lower levels of complex inhibition than in nonsynaptic mitochondria.

Hence the approximate 45% decrease in complex II-III activity and 30% decrease in complex IV activity seen here in adult synaptosomes may compromise synaptosome mitochondrial function after an ischaemic or anoxic episode. The additive contribution of inhibition of more than one complex, as is the case in the post-ischaemic/anoxic adult synaptosomes detailed here, would also lead to a greater probability of compromised oxidative phosphorylation and the consequential ATP production which could ultimately lead to decreased nerve terminal integrity and function.

In the post-ischaemic and post-anoxic adult synaptosomes it is notable that complex I activity is not significantly different from control values. The activity of this enzyme was significantly decreased during the metabolic insults themselves but shows a return to control values after a period of reperfusion/reoxygenation. In work with complex I from heart mitochondria Veitch *et al.*, (1992) suggested that ischaemic damage of complex I is dependent on the presence of oxygen during ischaemia because anoxic pre-perfusion affords protection to this enzyme. The initial ischaemic/hypoxic incubations used in the studies presented here in this thesis are known to contain a residual oxygen concentration for up to the first fifteen minutes, at which time point the decrease in complex I activity was measured. However, decreased complex I activity was also apparent at thirty minutes of incubation, at which time point no oxygen is present in the ischaemia/anoxic incubations. During reperfusion, when oxygen is obviously present, complex I activity was not decreased.

It is possible that the inhibition of complex I activity during ischaemia/anoxia initially requires the nerve terminal to be in a specific state such that a concert of factors are present, one of which being oxygen, in order to set a particular mechanism in progress. This mechanism, once initiated, will affect complex I activity whether in the presence or absence of oxygen. The recovery of activity following reperfusion suggests that the inhibitory mechanism is terminated. However, a recovery of activity at this time point does not necessarily preclude a damaging process to complex I having been set in motion during ischaemia/anoxia, which is ongoing during
reperfusion but which has not yet manifested as an irreversible inhibition of activity. A later decrease in complex I activity following extended reperfusion, but which was primarily caused by factors initiated during ischaemia/anoxia is not precluded by the data presented here.

It is also possible that an initial factor or combination of factors occurring during metabolic stress leads to complex I inhibition. The return of complex I activity to control values during reperfusion/reoxygenation may in fact indicate a true recovery of this enzyme. If this is the case, reperfusion/reoxygenation leads to a removal of the inhibitory mechanisms in place during metabolic stress, due to one or more of these factors no longer being present or active under these conditions.

In summary these results indicate that in adult nerve terminals, the mechanisms that mediate inhibition of mitochondrial function immediately after ischaemia/anoxia and after a period of reperfusion/reoxygenation are different, because the former affects complex I and the latter affects complex II-III and complex IV.

4.4.2.c Neonatal synaptosomes during metabolic stress

The pattern of complex inhibition in neonatal synaptosomes under conditions of ischaemia or hypoxia/anoxia is different to that seen in adult synaptosomes. Direct comparison of the results presented here with other reports of mitochondrial respiratory chain activities in ischaemic/anoxic neonatal nerve terminals is not possible due to a current lack of such reports in the scientific literature. Differences in the patterns of inhibition between adults and neonates reported here can be discussed.

There are similarities between adult and neonatal ischaemic/anoxic nerve terminals in that the activities of complexes IV and V are not significantly decreased in either of these preparations. In neonatal synaptosomes alone however, complex II-III activity is significantly decreased when compared to control values. This decrease is apparent under both ischaemic and hypoxic/anoxic conditions at fifteen and thirty minutes of incubation. This decrease is not present in the adult synaptosomes. It is possible that an inhibitory mechanism affecting complex II-III is present and active in the neonatal nerve terminals but not in the adult nerve terminals. Alternatively, the same factors and conditions may be present in both preparations, but the properties of complex II-III differ at the different stages of nerve terminal maturity so that the neonatal form of the enzyme is more susceptible to inhibition under these conditions. Although it is known that the maximum activities of complexes of the mitochondrial respiratory chain enzymes show distinct developmental profiles (Bates *et al.*, 1995; Almeida *et al.*, 1995) no currently published work has investigated developmental differences in the properties of these enzymes.

In both the adult and neonatal synaptosomes complex I activity is significantly decreased under ischaemic conditions at fifteen minutes of incubation and remains so for the following fifteen minutes of ischaemic incubation. Therefore in the neonatal nerve terminals, the activities of complex I and complex II-II are both decreased under ischaemic conditions. The mitochondrial respiratory chain is thus inhibited to a larger degree in neonatal ischaemic synaptosomes than in adult ischaemic synaptosomes. Although substrate, in the form of glucose, is not available to provide reducing equivalents to the respiratory chain during ischaemia this distinct difference between the status of the respiratory chain in neonatal and adult nerve terminals at the end of an ischaemic period may have important implications as to what biochemical mechanisms occur during the initial stage of reperfusion.

Under hypoxic/anoxic conditions neonatal nerve terminals exhibited a significant decrease in complex I activity only at the thirty minute time point, therefore showing that this enzyme remained functional for a longer time during an hypoxic episode than in the case of adult nerve terminals, where inhibition was apparent at fifteen minutes. Therefore during the first fifteen minutes of hypoxia in the neonatal synaptosomes, unlike in the adult ones, glucose present in the incubation could in theory be metabolised through glycolysis etc. and any NADH reducing

equivalents produced could feed into the still fully active complex I of the respiratory chain. Although the activity of the complex II-III linked assay is reduced in these neonatal preparations, this assay more readily reflects complex II activity (Taylor et al., 1994) and it is therefore likely that reducing equivalents will be passed through complex I to complex III and then through the rest of the still functional respiratory chain. Under these conditions ATP production can still occur in theory, as long as the terminal electron acceptor - oxygen -is still present, which in these incubations is the case initially for up to fifteen minutes of hypoxia. In the adult hypoxic synaptosomes NADH reducing equivalents will be fed into the respiratory chain to a lower degree because of the significantly decreased complex I activity at fifteen minutes. Unlike, neonatal synaptosomes, in hypoxic adult synaptosomes FAD-linked substrates could still be fed into the respiratory chain at this stage because complex II-III is fully active during the first fifteen minutes of incubation when residual oxygen would be present, glucose supplied during hypoxia terminals. Thus it appears that there is an important distinction between the two different age nerve terminal preparations under short term hypoxic conditions; assuming that other enzyme systems are intact so that glucose can be fully metabolised through glycolysis and the TCA cycle, neonatal nerve terminals can accept NADH reducing equivalents to a greater degree than FADH reducing equivalents, and the opposite holds true for adult nerve terminals.

4.4.2.d Neonatal synaptosomes following reperfusion/reoxygenation

Following a thirty minute period of reperfusion after ischaemia or a thirty minute period of reoxygenation after hypoxia/anoxia there was no significant difference when compared to control values in the activities of any of the complexes of the mitochondrial respiratory chain enzymes in neonatal nerve terminals. This is markedly different to the adult synaptosomes studies here. Therefore it seems that either complex II-III and complex IV in the neonatal nerve terminals are less susceptible to reperfusion damage than in adult nerve terminals, or alternatively that the mechanisms of damage which are occurring in the nerve terminal during reperfusion in the adult are not occurring in the neonate.



"No matter how big a question may seem, get rid of confusion by taking one little step toward solution. Do something. Then try again. At the worst, as long as you don't do it the same way twice, you will eventually use up all of the wrong ways of doing it and thus the next try will yield the correct answer."

G. F. Nordenholt

<u>Chapter 5</u>

ISCHAEMIA, OXIDATIVE STRESS AND THE DEVELOPING BRAIN

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INTRODUCTION

CHAPTER 5

ISCHAEMIA, OXIDATIVE STRESS AND THE DEVELOPING BRAIN

5.1 Introduction

5.1.1 Oxidative stress

A natural consequence of aerobic, oxygen utilising metabolism is the production of potentially damaging species known as *free radicals*. Such species are capable of interacting with biomolecules of all types (for example lipids, proteins, nucleic acids *etc.*) and causing widespread cellular and tissue damage. Such damage is known as *oxidative damage*. There are, however, intrinsic mechanisms in the body which oppose these potentially damaging free radical species in an effort to prevent oxidative damage from occurring. These mechanisms can either directly neutralise the damaging species, or otherwise limit their activity. The inherent systems which are involved in the protection against oxidative damage are collectively referred to as the *oxidative defense capacity* of the body. The equilibrium of the opposing mechanisms behind oxidative damage and oxidative defense is usually nearly balanced. The condition of *oxidative stress* occurs when there is an imbalance in the equilibrium, and the number of damaging species exceeds the capacity of the defense mechanisms to scavenge them (Siesjö, 1985). Cellular damage often results during conditions of oxidative stress.

5.1.2 Free Radicals

5.1.2.a What are free radicals?

In the electron orbitals of most chemical substances, electrons are paired and spin in opposite directions. What are referred to as *free radicals* are molecules which contain a single, unpaired electron in their outermost orbital. Because of the tendency for electrons to be paired, the unpaired electron in the outer orbital makes the molecule extremely reactive and unstable, and often very short-lived.

This is because, in an effort to become more stable, the free radical will quickly react with any available surrounding compounds or molecules to ensure that its single electron becomes paired and thus regains chemical stability.

5.1.2.b Reactive oxygen species

Free radicals which are formed from molecular oxygen play an important role in biology and medicine (Halliwell and Gutteridge, 1992). Oxygen is not toxic in itself, but oxygen toxicity can occur because during its chemical reduction to water a number of reactive intermediates can be produced which are potentially harmful to the body. These reactive intermediates arise in sequential fashion from molecular oxygen by a number of successive single-electron reduction reactions (Halliwell and Gutteridge, 1985). This univalent pathway requires four electrons to enable the complete reduction of oxygen to water. The intermediates so formed are collectively known as *reactive oxygen species (ROS)*. Several of these ROS are formed following the initial reduction of oxygen, by reactions such as those detailed in figure 5.1. Although the ROS hydrogen peroxide (H_2O_2) is not truly a free radical, its high reactivity is similar to those of free radicals, and therefore, it is often classed as such.

5.1.2.c Sources of ROS

Mitochondria

It has been demonstrated that between 2% and 5% of the electron flow in isolated mitochondria produces superoxide radicals (O_2^{-}) and H_2O_2 (Boveris and Chance, 1973). *In vivo*, oxidation of the diffusible electron transfer agent in the mitochondrial respiratory chain - the ubisemiquinone radical - yields O_2^{-} . During normal cellular metabolism the small amounts of ROS constantly produced by this pathway are effectively scavenged by the cell's defences. However, during conditions of oxidative stress, such as those created during ischaemia and reperfusion, the ROS produced by this pathway can lead to permanent cellular damage.



Figure 5.1 Reactions of oxygen derived species

Purine catabolism

In the normal situation the purine nucleotides go through a common catabolic pathway for their breakdown. The end product of purine degradation in humans is uric acid. ATP is metabolised by a nucleotidase to adenosine which is then converted to inosine by the adenosine deaminase enzyme. Nucleoside phosphorylase then converts inosine to hypoxanthine. In the normoxic brain hypoxanthine is metabolised by xanthine dehydrogenase (XDH) to xanthine and ultimately uric acid. In the ischaemic brain the metabolism of hypoxanthine proceeds via a different route. With the onset of ischaemia, a rise in cytosolic calcium causes a rapid proteolytic conversion of XDH to xanthine oxidase uses molecular oxygen as its electron acceptor and catalyses the production of H_2O_2 and O_2 . during reperfusion. A substantial conversion of XDH to XO has been seen during rat brain ischaemia (Kinuta *et al.*, 1989), and O_2 . formation by a XO-dependent mechanism during ischaemia-reperfusion has also been demonstrated (Terada *et al.*, 1991).

Arachidonic acid metabolism

ROS can also be formed from the metabolism of arachidonic acid. During ischaemia calcium entry into neurones activates cytosolic phospholipases including phospholipase A_2 . This enzyme can cleave a fatty acyl chain from membrane phospholipids, and in so doing produces arachidonic acid. The levels of free arachidonic acid do rise during brain ischaemia (Yasuda *et al.*, 1985). Arachidonic acid can be metabolised by either cyclooxygenase or lipoxygenase, thus forming a number of vasoactive substances which include prostacyclins, prostaglandins, thromboxanes and leukotrienes. Cyclooxygenase catalyses the addition of two molecules of oxygen to an unsaturated fatty acid to produce prostaglandins with the concomitant production of O_2^{--} (Kuehl and Ekgan, 1980). The lipoxygenase pathway which produces leukotrienes can also generate hydroxyl radicals ('OH).

Other sources

There are other potential sources of ROS in the body. Those that may have relevance to the brain, particularly under ischaemic conditions include:-

- [1] the auto-oxidation of catecholamines and the monoamine oxidase catalysed oxidation of dopamine which uses molecular oxygen as an electron acceptor thus generating quinones and H_2O_2 (Graham *et al.*, 1978; Evans, 1993).
- [2] the activation of macrophage type microglial cells, and neutrophils which generate O_2^{-} by NADPH oxidase during a respiratory burst.
- [3] ROS generation coupled to the action of released excitotoxic amino acids. Evidence for a ROS generating action of kainate (Dykens *et al.*, 1987), and an emphasis on the interconnection between excitotoxic amino acid release and free radical generation (Pellegrini-Giampietro *et al.*, 1990) implicate a cooperative relationship between excitotoxicity and ROS.
- [4] the generation of nitric oxide (NO) and peroxynitrite (ONOO⁻). This will be further explored in section **5.1.4**.

5.1.3 Oxidative defence mechanisms

A number of protective mechanisms within the body have evolved to guard against the potential damage incurred by the constant production of free radicals *in vivo*. These intrinsic antioxidant defences involve both molecules that remove free radicals by reacting directly with them in a non-catalytic manner, and also a number of antioxidant enzymatic systems.

5.1.3.a Non-enzymatic antioxidants

A number of endogenous antioxidants are present in the body which remove free radicals by reacting directly with them and neutralising them. Such free radical scavengers include α -tocopherol, ascorbate and reduced glutathione.

α -Tocopherol

This is one of a group of compounds collectively referred to as vitamin E. It is a fat soluble molecule found in membranes and lipoproteins in the body. α -Tocopherol blocks the chain reaction of lipid peroxidation by acting as a hydrogen donor and thus scavenging intermediate peroxyl radicals. The tocopherol radical is much less reactive in attacking adjacent fatty acid side-chains than the peroxyl radicals.

Ascorbate

Ascorbate (vitamin C) is a good scavenger of many free radicals and may help to detoxify inhaled oxidising air pollutants in the respiratory tract. It also assists α tocopherol in inhibiting lipid peroxidation by recycling the tocopherol radical back to the fully reduced form of tocopherol. Ascorbate does however have the potential for pro-oxidant effects, particularly if mixed with copper or iron ions as has been demonstrated *in vitro* (Halliwell, 1995).

Glutathione

Glutathione is a tripeptide consisting of glutamate, cysteine and glycine. It is a major constituent of cells and it has been adapted through evolution to perform many diverse independent functions. GSH functions in catalysis, metabolism and transport as well as the detoxification of oxidising species. The two characteristic features of glutathione - namely its sulphydryl group and its γ -Glu linkage - promote its intracellular stability and are intimately associated with its functions (Meister, 1988). Many of the functions of glutathione are based on the reducing powers of the sulphydryl group in this molecule. It is thought that cysteine, which is the sulphuryl containing amino acid, is stored as glutathione because cysteine is toxic in high concentrations and glutathione in a reduced form is much better tolerated in cells as the reservoir of this reducing power (Mehler, 1992). This reduced form of glutathione (GSH) is accepted as the conventional way for abbreviating this compound because it is the primary form of glutathione in the cell. The primary reductants are the pentose phosphate shunt reactions that produce NADPH which is used by the enzyme glutathione reductase to reduce the disulphide bond of oxidised glutathione (GSSG). The steady state within cells generally maintains a ratio of about 100:1 of GSH:GSSG.

GSH is synthesised and catabolized via enzymes of the γ -glutamyl cycle, all of which are present in the CNS (Makar *et al.*, 1994). Enzymes catalysing interconversion of GSH and GSSG, and formation of glutathione-S-conjugates are also present in the brain (Das *et al.*, 1981). GSH is synthesised from its free amino acids in two steps; firstly the γ -carboxyl group of glutamate is activated by ATP and forms an amide with the amino group of cysteine; secondly a similar condensation of the cysteinyl residue of the dipeptide permits condensation with glycine. The rate limiting enzyme in this biosynthesis is γ -glutamyl cysteine synthetase.

GSH is a substrate for a group of enzymes - glutathione S-transferases - that catalyse the transfer of the sulphydryl group from GSH to a variety of acceptor molecules, including a number of xenobiotic compounds. The process is a detoxification and results in the formation of mercapturic acids which are excreted in the urine (Mehler, 1992). The sulphydryl group of GSH also participates in a disulphide interchange reaction that rearranges disulphide bonds in proteins until the thermodynamically most stable structure is formed. GSH is also involved in a cofactor role with several enzymes including prostaglandin PGE₂ synthetase, and again it is the sulphydryl group which is involved in this function. As mentioned in section 5.1.3.b GSH is used as the reducing source for the glutathione peroxidase catalysed detoxification of H₂O₂.

The enzyme γ -glutamyl transpeptidase is involved in the degradation and translocation of GSH (Meister, 1983). Export of GSH functions in inter- and intraorgan transfer of cysteine moieties, in the protection of cell membranes, and as part of a pathway of transport for cyst(e)ine and probably other amino acids.

Mitochondrial GSH is an important factor in cellular homeostasis in the brain. Depletion of mitochondrial GSH has been shown to result in impaired mitochondrial function (Heales *et al.*, 1995). GSH directly inactivates a number of oxidising species including ONOO⁻ (Barker *et al.*, 1996), and *in vitro* studies have correlated loss of cellular GSH with susceptibility of neurones to ONOO⁻ (Bolaños *et al.*, 1995).

GSH can be released from rat brain slices in the presence of Ca²⁺ upon K⁺ depolarisation, suggesting that the origin is from nerve endings (Zängerle *et al.*, 1992). Indeed, GSH has been found to be enriched in synaptosomes (Reichelt and Fonnum, 1969). A further important point in the relationship between GSH and nerve terminals is that a role for GSH as an endogenous agonist at the NMDA recognition domain on the NMDA receptor ionophore complex has recently been suggested (Ogita *et al.*, 1995). However, an antagonistic action of this peptide on the NMDA receptor has also been hypothesised (Levy *et al.*, 1991). The former observation involved normal physiological (μ M) concentrations of GSH (Chen *et al.*, 1989), whereas the latter report used higher (mM) concentrations. It is hypothesised that accumulation of GSH in synaptic clefts to millimolar concentrations, following release from nerve terminals, could lead to prevention of opening processes of an ion channel associated with the NMDA receptor (Ogita *et al.*, 1995). This is of great relevance to ischaemia where GSH content has been found to decrease (Rehncrona *et al.*, 1980). Further exploration of the relationship between GSH and ischaemia in nerve terminals is warranted.

5.1.3.b Antioxidant enzymes.

The enzymes involved in the protection against oxidative stress are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx).

SOD

SOD catalyses the conversion of O_2^{-} to H_2O_2 and O_2 (Fridovich, 1989). Based on the metal ion requirements and the anatomical distribution there are three forms of SOD known to exist in brain cells (Chan, 1996). Copper-zinc-SOD is a cytosolic enzyme which requires both copper and zinc ions as cofactors. This is a dimeric protein which is coded for by a gene on chromosome 21 in human cells (Lieman-Hurwitz *et al.*, 1982). A tetrameric protein coded for by a gene on human chromosome 4 has also been fully characterised and cloned. This protein is Mn-SOD, which is a mitochondrial enzyme with a requirement for manganese. A copper containing SOD has also been identified in the extracellular space and its gene has been successfully cloned (Marklund, 1982). Although there are different forms of SOD the brain expresses only moderate activities of this enzyme (Ben-Yoseph *et al.*, 1996).

Catalase

Similarly to SOD, the brain does not express high activity of catalase. This enzyme converts H_2O_2 to water and molecular oxygen. Catalase activity is located in specific organelles called peroxisomes (Gaunt and DeDuve, 1976), although it is of limited importance in brain tissue.

GPx

It is stated by some that the most important antioxidant enzyme in brain is GPx (Jain *et al.*, 1991). GPx detoxifies H_2O_2 to molecular oxygen and water at the expense of GSH. This is an important reaction because once formed, H_2O_2 is capable of being converted into the highly reactive OH if ferrous iron is present. The conversion of H_2O_2 to OH *in vivo* is in fact O_2^{-} dependent because this anion generates ferrous iron from the ferric iron which is the bound form of iron found *in vivo*.

GPx also catalyses the reduction of fatty hydroperoxides to hydroxy fatty acids, again whilst converting GSH into GSSG.

5.1.4 Nitric Oxide and Peroxynitrite

5.1.4.a Nitric Oxide (NO)

Nitric oxide (NO) is a simple molecule which can be endogenously synthesised and which is increasingly recognised as being important in a vast array of physiological and pathophysiological functions.

The compound which is today commonly referred to as NO was referenced in the writings of early investigators such as J. B. van Helmont (1648), Robert Boyle (1701) and G. E. Stahl (1703) (Bonner and Stedman, 1996). The chemical properties of NO were first described by Joseph Priestly in 1772. From that time onwards the role of NO on the chemistry of the atmosphere, in pollution and its industrial role in nitric acid manufacture has been focused on. NO is an inorganic free radical gas which is thermodynamically unstable and hence highly reactive. Extensive reviews of the general chemistry of NO are available (Bonner and Stedman, 1996). The importance of NO as a free radical in biological systems will be further detailed here.

NO in biology and medicine

Recent discoveries, within the last decade, of the multiple roles played by NO in mammalian physiology have stimulated intense and vigorous research into this molecule. This is evidenced by the increasing number of research publications concerned with NO; in 1986 the number of published papers in NO research was eighteen; by 1995 this figure had reached three thousand five hundred and fifty published research articles concerned with NO¹. The original discoveries in 1987/1988 that vascular endothelial cellsware able to synthesis NO from L-arginine as a transcellular signal (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Palmer et al., 1988) was

¹data derived from MEDLINE

initially received with caution in the field of biology and medicine. However, the existence of the L-arginine: NO pathway has now been thoroughly documented and its relevance in biology is currently being more fully explored. Whilst some researchers are focusing on precise details of the chemical mechanisms and reactions concerned with NO, others are taking a more "holistic" approach looking at the consequences of NO production in biological systems.

It is becoming evident that the area of NO in biology and medicine is more complex than it may first appear. Reactions of NO that may be of primary importance *in vitro* may actually have only limited relevance *in vivo*. This is because target cells and tissues *in vivo* not only undergo the consequences of NO action, but they also respond to NO in specific and often different ways depending on the cell type. Hence the role of NO in the body can be a dynamic and complex one depending on the state and condition of the cells and tissues at the time of endogenous NO production. Whilst a lot of insight into the relevance of NO in health and disease remains to be found, there is increasing knowledge of some of NO's roles and also of the way it is produced in the body.

Nitric Oxide Synthase

The production of NO from L-arginine with the concurrent production of Lcitrulline is catalysed by an enzyme known as NO synthase (NOS). NOS is reported to exist in three major isozymic forms that have been purified, cloned and characterised. These isoforms are named after the tissue in which they were originally purified and cloned (namely neuronal, endothelial and macrophage), but they have since been identified elsewhere. The nomenclature of the isoforms has been somewhat confusing and a preferred designation of the three forms has now surfaced.

The isoforms are currently designated as follows:-

current	numerical	original	expression
ncNOS	Type I	nNOS	constitutive
ecNOS	Type II	eNOS	constitutive
iNOS	Type III	macNOS	inducible

The NOS isoenzymes are remarkable for the speed with which they have been first described in 1989 (Knowles *et al.*, 1989; Palmer and Moncada., 1989; Mayer *et al.*, 1989; Palacios *et al.*, 1989; Stuer *et al.*, 1989), first purified in 1990 (Bredt and Snyder, 1990) and first cloned in 1991 (Bredt *et al.*, 1991). The NOS isoforms known so far are homodimers of 125 - 155 kDa subunits (Knowles and Moncada, 1994). They all have comparable specific activities of approximately 1 μ mole/min/mg protein at 37°C (Feldman *et al.*, 1993).

NOS's are members of the cytochrome P450 enzyme family and possess both reductase and oxygenase activity (Vincent, 1993); however, they require a specific collection of cofactors. The enzyme requires flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) as cofactors which play an important role in shuttling electrons from the electron donor NADPH to a heme complex which is present at the active site of NOS. In addition to heme, flavins and NADPH, NOS requires the presence of tetrahydro-L-biopterin (BH₄) to function efficiently. Although the precise role of BH₄ in the function of the NOS isoforms is still currently under investigation, it is thought to act as both an allosteric effector and redox active cofactor participating in L-arginine oxidation (Mayer, 1993). Reduced thiol also seems to play an unspecified role in maintaining enzyme activity. Thiol may limit the 'wear and tear' of the enzyme generated by the oxidising environment, or perhaps serve to relieve potential inhibition at the active site occurring through nitrosylation of the heme centre (Stamler and Feelisch, 1996). NOS's also possess a calmodulin-binding consensus sequence, allowing for the regulation of enzyme activity by Ca^{2+} .

NOS types I and II are constitutively expressed in particular cell types and are essentially inactive at low free Ca²⁺ concentrations and become maximally active at about 1 μ M (Knowles *et al.*, 1989; Bredt and Snyder, 1990; Mayer *et al.*, 1990). Type III NOS is slightly different in that most cells express this common inducible gene product in response to inflammatory cytokines and its activity is Ca²⁺ independent (Vincent, 1993). The assembly of iNOS subunits has been studied in some detail (Ghosh and Stuehr, 1995) but less is currently known about the structural features and roles of cofactors in dimerisation of the other isoforms of NOS.

The assembly of active NOS

A model has recently been proposed (Stuer *et al.*, 1996)² that relates iNOS dimer assembly with prosthetic group binding and activation of their catalytic functions. The first step involves binding of calmodulin, FAD and FMN to iNOS protein and results in a functional reductase domain. That is to say that these monomers exhibit cytochrome c reductase activity but do not catalyse the heme-dependent reactions attributed to NOS. The second step in assembly of the active site in iNOS is said to involve insertion of heme and BH_4 into the iNOS oxygenase domain. This occurs along with subunit dimerisation and generates an iNOS fully capable of NO synthesis. For iNOS it appears that L-arginine is also required in most cases to drive subunit dimerisation.

Work focusing on the dimerisation of nNOS has shown that nNOS monomers containing FAD and FMN only exhibit reductase activity. Active nNOS dimers also contain heme and BH_4 , although heme proved to be the sole cofactor required for enzyme dimerisation (Klatt *et al.*, 1995; Mayer *et al.*, 1996²). BH₄ does however appear to be essential for the conversion of the heme-containing nNOS dimers to an active, tight binding conformation (Klatt *et al.*, 1995).

² preliminary publication in abstract form at the Second International Conference for the Biochemistry and Molecular Biology of Nitric Oxide, Los Angeles, July 1996.

It appears that at low BH_4 and L-arginine concentrations the activation of oxygen is uncoupled from NO synthesis so that oxygen-derived free radicals are formed (Mayer, 1993). Indeed it is suggested that in the absence of BH_4 binding, the heme-containing nNOS dimer which is **not** in the tight binding conformation effectively acts as a peroxynitrite synthase (Mayer *et al.*, 1996³). If NOS produces such oxygen derived species *in vivo* this may have important pathophysiological implications in conditions such as ischaemia/reperfusion.

Reactions of NO

The biochemistry of NO is complex and can be categorised into direct and indirect effects (Wink *et al.*, 1996). The former involves the direct reaction of NO with its biological targets, generally requiring small fluxes of NO. Indirect effects are reactions mediated by reactive nitric oxide species (RNOS's) which can lead to cellular damage via nitrosation or oxidation of biochemical targets. The direct reactions are relatively straightforward to outline, whereas in contrast the indirect effects are more complex. A complex balance between NO and ROS's exists which is critical to understanding these indirect effects.

NO reacts directly in biological systems with O_2 , O_2^{-} and transition metals (M). The products of these reactions are outlined in figure 5.2, and have various inclinations towards toxicity and/or physiological activity depending on the chemistry they undergo in a given biological environment (Stamler and Feelisch, 1996). Direct reactions between NO and transition metal centres are central to the biological activities of NO (Stamler and Feelisch, 1996). For example, NO reversibly inhibits cytochrome P450 (Wink *et al.*, 1996). Interactions of NO with metal targets may account for the cytotoxicity of activated macrophages and the growth arrest of tumour cells (Stamler and Feelisch, 1996).

³preliminary publication in abstract form at the Second International Conference for the Biochemistry and Molecular Biology of Nitric Oxide, Los Angeles, July 1996.



Figure 5.2 Reactions of nitric oxide in biological systems

NO can bind to non-heme iron in a number of mitochondrial and cytosolic ironsulphur-cluster-containing enzymes such as aconitase, NADH: ubiquinone oxidoreductase (complex I) and succinate: ubiquinone oxidoreductase (complex II) (Bredt and Snyder, 1994), all of which become inhibited directly by NO (Radi *et al.*, 1993).

The ability of NO to bind to iron enables this molecule to exert influence on iron metabolism within the body. Iron metabolism is regulated post-transcriptionally by specific mRNA-protein interactions between iron regulatory factor (IRF) and iron-responsive elements (IRE) which occur in the untranslated regions of the messenger RNA (mRNA) transcripts for iron binding proteins such as ferritin (an iron-storage protein) (Klausner and Rouault, 1993). Studies have found that NO stimulates the IRF, which causes a change in translation of IRE-containing mRNA. NO increases the RNA-binding activity of IRF by displacing its iron-sulphur cluster (O'Halloran, 1993; Bredt and Snyder, 1994). Interactions of the IRF at the 3' and 5' ends of the mRNA serves to promote or inhibit the rate of translation, respectively (O'Halloran, 1993). It seems that elevated NO levels are associated with cellular iron depletion (Bredt and Snyder, 1994). NO also binds to the non-heme iron of ribonucleotide reductase to inhibit DNA synthesis (Reif and Simmons, 1990).

There are several well-defined actions of NO that are dependent on the activation of soluble guanyl cyclase (GC) and the induction of the cGMP signal transduction pathway (Vincent, 1993). NO activates GC by binding to iron in the heme, which is at the active site of the enzyme, and in doing so alters the enzyme's conformation and thus increases catalysis (Ignarro, 1989). Numerous other metalloregulatory proteins involved in respiration, metabolism and the oxidative stress-response systems may also exhibit NO-responsive control (Stamler and Feelisch, 1996). Many cGMP-independent effects are likely to be regulated by such metallo-

proteins or thiol-containing proteins which have interacted with NO.

Chemically, the one-electron reduction of NO to nitroxyl (NO-) is thermodynamically favoured (Stamler and Feelisch, 1996). Under physiological conditions NO⁻ converts rapidly to N₂O through dimerisation and dehydration. Competing reactions for nitroxyl include reactions with thiol groups, reactions with transition metals and the rapid reaction with molecular oxygen leading to ONOO⁻ formation (Stamler and Feelisch, 1996). These competing reactions are more direct reactions of NO. Various products of NO reduction exert biological actions in their own rights, and these are within the category of indirect NO reactions. For example, NH₂OH has been shown to induce vasorelaxation (Demaster *et al.*, 1989). The biological activity of N₂O has also received some attention. The chemistry of N₂O with transition metal centres and also its incorporation into lipids and proteins enable diverse interactions of this compound with much biological importance. N₂O has also been reported to affect neurotransmission at physiologically relevant concentrations (Gillman and Liehtfed, 1994).

In biological systems NO is rapidly oxidised by way of reactions with O_2 , O_2^- and transition metals. The direct reactions of NO with O_2 and O_2^- lead to higher nitrogen oxides (NO_x) and ONOO⁻ respectively. Each of these oxidation pathways of NO in a biological environment can provide mechanisms for oxidative chemistry and also for nitrosative chemistry (*i.e.* the reaction or transfer of NO⁺ through electrophilic substitutions or addition to bases, including peroxide (Stamler, 1992).

In acidic conditions the major product of NO metabolism in oxygenated systems - NO_2^- - can act as an NO⁺ donor. Such nitrosation reactions, mediated by RNOS's derived from NO can modify proteins, resulting in enzyme inhibition. A number of reports have been published concerning NO stimulation of the S-nitrosylation of numerous proteins (Lipton *et al.*, 1992; Stamler, 1992). Nitrosylation

of metal centres by both NO and NO⁻ can lead to rapid changes in protein properties and also mediates interconversion between NO and NO⁻ themselves (Stamler *et al.*, 1992).

Although numerous biological targets are susceptible to nitrosative attack, thiols and amines are the most reactive (Wink *et al.*, 1994). Such thiol rich proteins include DNA repair enzymes with zinc finger motifs which are particularly susceptible to nitrosylation from reactive nitrogen species (RNOS) derived from NO (Wink *et al.*, 1996). S-nitrosylation of a thiol group in the enzyme glyceraldehyde-3-phosphate dehydrogenase has been implicated in its inhibition by NO (McDonald and Moss, 1993). DNA damage by NO is increasingly being found to involve nitrosylation (Tannenbaum, 1996)⁴. Nitrosylation can be a reversible modification of proteins that can influence function, though the extent to which this occurs *in vivo* is unclear to date. Examples include ras protein, cyclooxygenase and several receptor-coupled ion channels, and the inhibition of enzymes that contain active or allosteric thiols. Included among these is γ -glutamyl cysteine synthetase, which is the rate limiting enzyme for glutathione synthesis.

Although it has been stated that nitrosylation of thiols occurs preferentially, under conditions of oxidative stress however, where thiol depletion often occurs the likelihood of nitrosation of other nucleophilic centres, such as nucleic acids, becomes increased (Marletta, 1988). This illustrates a key point. The main factor in the extent of toxicity by nitrosative and oxidative NO chemistry within a biological environment is very much dictated by the prevailing condition at that time point. Generally speaking the reactions of NO with oxygen and related species have been associated with toxicity. (This will be further detailed when we look at the actions of peroxynitrite (ONOO⁻) in section 5.1.2.e). However, the situation is not as predictable as suggested by some of the literature.

⁴preliminary publication in abstract form at the Second International Conference for the Biochemistry and Molecular Biology of Nitric Oxide, Los Angeles, July 1996.



Figure 5.3 The influence of NO on oxidative and nitrosative stress

Suffice to say that NO can potentiate oxidative stress via formation of ONOO-, however it can also exert antioxidant and protective effects by virtue of its ability to chemically convert radical species to less reactive products, including some products involved in nitrosative chemistry. This is illustrated in the schematic presented in figure 5.3. For example, NO can offer protection by acting as a chain terminator in lipid peroxidation (Wink *et al.*, 1993). Wink *et al.*,(1993) have also shown that NO can protect mammalian cells against oxidative stress induced by H_2O_2 .

In summary then, NO generation will lead to a variety of oxidation and reduction products with distinct lifetimes and biological activities. The production and effects of such species will depend on the prevailing conditions within the biological milieu during NO generation.

5.1.4.b Peroxynitrite (ONOO-)

In a reaction of considerable biological significance for biological systems, NO has been shown to react with O_2^{-1} to form the anion ONOO⁻ (Beckman *et al.*, 1990). ONOO⁻ is not a free radical because the unpaired electrons on NO and O₂⁻ have combined during the reaction to form a new N-O bond in ONOO. ONOO is still, however, a highly reactive, potent oxidising species even though it is technically not a free radical. Indeed, ONOO is being increasingly recognised as a biologically generated reactive and toxic species (Radi et al., 1994). ONOO is thought to be responsible for many of the neurotoxic effects of NO (Lipton et al., 1993). Indeed, the reactivity of NO has now been suggested to have been exaggerated in vitro because of the secondary formation of much stronger oxidants like ONOO (Beckman, et al., 1996). In vivo, under normal physiological conditions the formation of ONOO is probably kept low by the rapid removal of NO through reactions with oxyhaemoglobin in red blood cells and by the scavenging of O_2^{-1} by SOD. Under some conditions ONOO- is produced in larger quantities by the diffusion limited reaction between NO and O_2^{-} . NO is the only molecule that is known to be produced in high enough concentrations to successfully compete with O_2^{-} for SOD. Once formed ONOO is

stable enough to diffuse over at least one cell diameter under physiological conditions (Beckman *et al.*, 1996). It is this unusual stability of ONOO⁻ as an anion which contributes to its toxicity by allowing it to diffuse far from its site of formation whilst being selectively reactive with numerous cellular targets.

Oxidising effects of ONOO-

During its decomposition at physiological pH, peroxynitrite can produce some of the strongest oxidants known in a biological system, initiating reactions characteristic of OH, NO⁺ and NO₂. The products can include nitrosylated (R-NO) and nitrated (R-NO₂) derivatives as well as more traditional oxidation products (such as hydroxylated aromatics). It is critical to recognize that many of the products formed following peroxynitrite attack will differ from those products that would result from simple hydroxyl attack.

Peroxynitrite has multiple pathways through which it can exert it's oxidising effects. Buffer composition, pH, temperature and a number of other parameters all dictate the end products of peroxynitrite decomposition. Peroxynitrite becomes highly reactive at physiological pH by at least 3 very distinct pathways:-

- hydrogen ion catalysed decomposition to form an intermediate with the reactivity of OH⁻ and NO₂,
- 2) direct reaction of the anion with sulphydryl groups,
- 3) reaction with metal ions to form a potent nitrating agent resembling nitronium ion.

In addition there is also a direct isomerization pathway whereby peroxynitrite rearranges to nitrate without oxidizing another molecule (this pathway predominates at pH values greater than 8, resulting in a decrease in the oxidative potential of peroxynitrite). Although the reactions characteristic of OH are commonly assumed to be the most toxic, other direct reactions are important in understanding the cytotoxicity of ONOO⁻. The rapid and specific reactions of ONOO⁻ with sulphydryls and metals

increase its likelihood of inactivating a key cellular target.

Why are there different oxidative pathways?

These different pathways are possible because the O-O bond of ONOO- can react as if it were cleaved both homolytically into HO· and \cdot NO₂ (pathway 1) or heterolytically into HO- and NO₂⁺ (pathway 3). Metal ions are necessary as a catalyst for pathway 3 because the initial heterolytic cleavage and consequential charge separation requires a high activation energy. In the absence of metal ions pathway 1 is followed, where homolytic cleavage of the O-O bond leads to a hydroxyl radical like reactive oxidant. Physical separation of the peroxynitrous acid into hydroxyl radical and nitrogen dioxide does not appear to occur. Rather, it reacts in a vibrationally activated state, whereby nitrogen dioxide is released if this vibrationally activated peroxynitrous acid bumps into a target molecule. Although physical separation and outright production of the hydroxyl radical does not occur, peroxynitrite is capable of initiating many of the reactions attributable to the hydroxyl radical if it decomposes via this oxidative pathway.

Other reactions of ONOO-

Carbon dioxide (CO₂) is known to react with ONOO- (Uppu *et al.*, 1996) and it is suggested that in biological systems ONOO- is trapped by carbon dioxide (CO₂) because the reaction of ONOO- with CO₂ is so fast. Indeed, it seems that this reaction is one of the fastest reactions of ONOO-, second only to reactions between ONOOand metals (Uppu *et al.*, 1996). It is possible that many of the reactions of ONOOmay be mediated via reaction with CO₂, thus forming distinct nitrocarbonates which are nitrating agents and powerful oxidants (Pryor, 1996)⁵

It has also been found that ONOO- can react with specific physiological compounds to form products which are able to regenerate NO, thus effectively acting as NO donors. ONOO- can react in this manner with sugars or other compounds which

⁵preliminary publication in abstract form at the Second International Conference for the Biochemistry and Molecular Biology of Nitric Oxide, Los Angeles, July 1996.

contain an alcohol functional group(s) to form such NO donor(s) with the characteristics of organic nitrate/nitrites (Moro *et al*, 1995). ONOO- can also react with GSH to form an S-nitrothiol which is able to regenerate NO (Moro *et al.*, 1994). Such reactions of ONOO- could act as physiological detoxification pathways for this oxidant.

Biological actions of ONOO-

While initial, and in fact most, interest in ONOO- has focused on its potential cytotoxic actions, more recent studies (Wolin *et al.*, 1994) have found evidence that low levels of ONOO- may also participate in cell signalling mechanisms. ONOO- is suggested to have numerous effects in biological systems, including the following:-

- [1] the oxidation or nitrosation of specific moieties of DNA, thus causing DNA damage (Wink *et al.*, 1996),
- [2] the oxidation of protein and non-protein thiols (such as glutathione),
- [3] the inactivation of specific sodium channels,
- [4] inhibition of some DNA repair mechanisms by reaction with thiol groups (Wink *et al.*, 1996),
- [5] the nitration by ONOO- of tyrosine residues in target proteins in the presence of iron or copper, for example ONOO- has been found to nitrate MnSOD leading to inhibition of its activity (MacMillan-Crow, 1996)⁶,
- [6] oxidation of membrane phospholipids and the initiation of lipid peroxidation (Radi *et al.*, 1991),
- [7] inhibition of the respiration of isolated heart mitochondria (Radi et al., 1994),
- [8] irreversible damage of mitochondrial enzymes in cultured astrocytes and neurones (Bolaños *et al.*, 1995),
- [9] inhibition of the reuptake of glutamate by glutamate transporters reconstituted in liposomes (Trotti *et al.*, 1996),

⁶preliminary publication in abstract form at the Second International Conference for the Biochemistry and Molecular Biology of Nitric Oxide, Los Angeles, July 1996.

[10] inactivation by ONOO- of catalase, by either protein oxidation or nitration - the exact mechanism is as yet unclear (Keng *et al.*, 1996)^{6,}

In summary then, ONOO- has many biological effects and no doubt has many which are yet to be elucidated. Considering the fact that O_2^{-} is continually produced in mitochondria and that NO can be produced in the CNS and is readily diffusible into cells and organelles, it seems likely that ONOO- is present in the CNS during particular prevailing conditions. The effect of ONOO- on cellular processes warrants further research, particularly in light of its current implication in a number of pathophysiological conditions, including ischaemia/reperfusion (Beckman and Crow, 1993).

- 5.1.5 The role of free radicals in the pathophysiology of ischaemia and reperfusion.
- 5.1.5.a The Flamm-Demopoulos free radical hypothesis of cerebral ischaemia/reperfusion injury

Free radicals and related oxygen species have been implicated in ischaemia/reperfusion injury in a number of tissues for many years (reviewed in Halliwell and Gutteridge, 1990; and Phillis, 1994). The free radical hypothesis of cerebral ischaemia/reperfusion injury was first formulated over a decade ago (Flamm *et al.*, 1978; Demopoulos *et al.*, 1980). This hypothesis of free radical involvement in cerebral tissue injury was rationalised to provide a conceptual basis for evaluating the sequence of events leading to delayed neuronal death in brains subjected to ischaemia/reperfusion. The hypothesis basically offers the framework that free radicals and reactive oxygen species are produced during ischaemia and particularly during the reperfusion phase, and that such species either directly incur cerebral oxidative damage or initiate processes such as lipid peroxidation which ultimately play a role in delayed ischaemic neuronal death.

5.1.5.b The vulnerability of the brain to oxidative damage

The brain, like all other tissues is susceptible to damage from ROS's. Indeed, there are several reasons why neural tissue may in fact exhibit a higher vulnerability to oxidative damage than other tissue (Harman *et al.*, 1976).

Molecular iron is an important factor in the generation of ROS's, particularly the highly reactive OH[•]. Certain brain areas are relatively enriched in their iron content, thereby increasing the risk of free radical damage (Reiter, 1993). The brain uses large amounts of oxygen relative to its size and therefore is capable of producing abundant ROS's. It also contains abundant quantities of unsaturated fatty acids which readily undergo peroxidation (Chan, 1996).

Neurons, unlike many other cells, are generally considered to be incapable of mitosis and thus damage to these cells by free radicals may cause permanent lesions. Furthermore, the brain is relatively low in its concentrations of antioxidant enzymes and compounds when compared with the highly active oxidative defence mechanisms characteristic of some other tissues (Phillis, 1994; Reiter, 1993).

5.1.5.c Evidence for oxidative damage in ischaemia/reperfusion

Establishment of the causative role of ROS's in ischaemic brain injury remains a difficult task for neuroscientists. This is primarily because of the methodological difficulties involved in directly demonstrating free radical involvement. The highly reactive nature of free radicals coupled with the low amounts produced means that their direct detection is often problematic. Therefore, in the field of ischaemic research, many studies have utilised free radical scavengers or inhibitors of free radical generating systems in assessing the causative role of ROS's in damage. Some of these studies will be summarised in section 5.1.5.d. Somewhat more direct evidence for ROS formation in ischaemia/reperfusion has been accrued using exogenous trapping reagents. With the application of spintrapping, electron spin resonance (ESR) and electron paramagnetic resonance (EPR) spectroscopy short live free radicals are "trapped" with one of a number of available nitrone or nitroso spin trapping reagents. The resultant adduct gives rise to a stable nitroxide free radical which can be efficiently measured by ESR or EPR. Using such techniques the production of OH in rat cerebral cortex has been measured following reperfusion (Phillis and Sen, 1993; Sen and Phillis, 1993). Free radicals have also been detected with these techniques in the cerebral spinal fluid from the brains of pigs subjected to global ischaemia/reperfusion (Lange *et al.*, 1990) and in striatal microdialysates from rats both during the ischaemic and early reperfusion period, but not under basal control conditions (Zini *et al.*, 1992). Tominaga *et al.*, (1993) and Sato *et al.*, (1994) measured NO spin adducts using specific spin trapping reagents and cryogenic EPR, and they showed that there was an increase in NO radicals in rat brain after a few minutes of cerebral ischaemia.

Because of technical difficulties, the production of ROS's during ischaemia/reperfusion has often been assessed by indirect methods assaying biochemical indices of oxidative damage. Indeed, the bulk of the more direct evidence supporting a role for free radicals in brain ischaemia/reperfusion damage has been obtained by detecting protein oxidation, lipid peroxidation and DNA strand breaks and base modification (Halliwell, 1992; Phillis, 1994; Halliwell, 1995; Sims and Zaidan, 1995; Chan, 1996). Another useful biochemical index of ischaemia/reperfusion induced free radical production is a reduction in the concentration of endogenous antioxidants in brain tissue. Data suggest that brain antioxidants are consumed in an attempt to quench ischaemia/reperfusion induced free radicals (Rehnerona *et al.*, 1980; Siesjö, 1984; Chan, 1996).

More recent advances in methodologies have allowed investigators to measure hydroxyl formation during ischaemia *in vivo* by the HPLC assay of 2,5-

dihydroxybenzoate resulting from the hydroxylation of infused salicylate (Coa *et al.*, 1988; Oliver *et al.*, 1990; Boisvert, 1992). Superoxide anion has also been detected indirectly during ischaemia/reperfusion by its reaction with nitro blue tetrazolium to yield an insoluble blue formazan precipitate which can be quantified spectrophotometrically (Nelson *et al.*, 1992; Dugan *et al.*, 1995). An increased production of NO in ischaemic brain tissue has been measured by Malinski *et al.* (1993) by means of a self-made porphyrinic microsensor. These results were the first direct evidence that NO concentration in the brain increases both during and after transient middle cerebral occlusion (MCA). The assay of the stable end products of NO metabolism has also shown an increase in the production of NO and related species (Kader *et al.*, 1993; Keelan *et al.*, 1996) following ischaemia/reperfusion.

5.1.5.d Pharmacological evidence for cerebroprotective effects of free radical scavengers and inhibitors of free radical production

Most of the experimental approaches mentioned so far in the realm of ROS and ischaemia/reperfusion injury do suffer from distinct disadvantages. Included in these disadvantages are that the techniques are invasive and also the detection of free radicals is extracellular. Furthermore, such techniques do not provide information regarding either the cellular capacity of the antioxidant enzymes or the metabolic response to an oxidative insult. Hence, these current methods alone provide a rather incomplete picture of the cellular response to oxidative stress following ischaemia/reperfusion. Studies which have examined the cerebroprotective effects of free radical scavengers and inhibitors of free radical production have added to our knowledge of ischaemia/reperfusion and oxidative damage. There have been a number of reports describing cerebroprotective actions of a variety of free radical scavengers, and of inhibitors of mechanisms of free radical production. At times when examining the literature, the results may appear contradictory, however the findings of these studies are generally supportive of an involvement of ROS's in cerebral ischaemia/reperfusion injury. The inconsistency and confusion resulting from some studies may be accounted for in part by the following:

- the different ability of compounds to reach the brain,
- the different types of ischaemia involved in the studies (*eg.* global or focal, complete or incomplete), along with varying degrees and periods of ischaemia
- different species used for the studies,
- to date, many of the pharmacological actions of the agents may not be fully understood.

A number of studies have investigated protection with free radical trapping agents such as α -phenyl-*t*-butylnitrone (PBN) and 5,5- dimethyl-L-pyroline-L-oxidase (DMPO). The rationale for using these compounds as protective agents was that they would react with free radicals and halt the cascade of lipid peroxidation. PBN administration attenuated CA1 hippocampal neuronal loss in ischaemic gerbils (Phillis and Clough-Helfman, 1990; Yue *et al.*, 1992). Interestingly this compound also protected cultured hippocampal neurones from glutamate toxicity, implicating free radicals in excitotoxicity (Yue *et al.*, 1992).The neuroprotective effects of PBN in rats undergoing MCA has also been evaluated and shown to have a positive role (Phillis and Cao, 1994). DMPO has proven to be somewhat less effective than PBN as a cerebroprotective agent. This may be due to the high lipophilicity of PBN allowing it adequate access to mitochondria to achieve quite high concentrations in this organelle which is the site of ROS formation (Cheng *et al.*, 1993).

A number of antioxidants, free radical scavengers and inhibitors of free radical production have been shown to be cerebroprotective in *in vivo* studies, mostly with gerbils and rats. These include; α -tocopherol (Kitagawa *et al.*, 1990; Uyama *et al.*, 1992), the xanthine oxidase inhibitors oxypurinol (Phillis, 1989) and allopurinol (Phillis, 1994), the antioxidant LY231617 (Clemens *et al.*, 1993), dimethylthiourea (Pallmark *et al.*, 1993) and 21-aminosteroids (lazaroids) specifically U74006F which is a potent inhibitor of lipid peroxidation (Hall *et al.*, 1990).
Other pharmacological agents to which numerous reports attribute cerebroprotective actions include; catalase. dimethylsulphoxide, ascorbic acid, mannitol and deferoxamine (a chelator of ferric iron) (see Ikeda and Long, 1990; Halliwell, 1992; Phillis, 1994).

Pharmacological studies examining the neuroprotective effects of inhibitors of NOS have caused some confusion as to the role of this free radical in cerebral ischaemia/reperfusion injury. Whilst some NOS inhibitors have ameliorated ischaemic brain damage it seems that others have attenuated the damage (reviewed in Nagafuji *et al.*, 1995). The inconsistency in the data, particularly in studies of global forebrain ischaemia, has been suggested to be due the usage of different inhibitors, doses and administration schedules and a call for further *in vivo* studies has been made (Nagafuji *et al.*, 1995). There is currently, however, a need to further dissect the effects of ischaemia/reperfusion on the different isoforms of NOS, their expression and activity in different cell types in the brain at varying times during an ischaemic/reperfused episode, and also on the response of cell types to released NO. At this stage of NO and ischaemia research it is undoubtedly too early to dogmatically state whether NOS inhibitors play a negative or positive role in preventing cerebral ischaemia/reperfusion damage.

Our present state of knowledge seems to suggest that the NO produced during ischaemia/reperfusion has both neurotoxic and neuroprotective effects on the brain (Verrecchia *et al.*, 1995). NO toxicity that occurs in the early stages is probably due to the activation of ncNOS. The later toxic effects of NO, some 24-48 hours into reperfusion, could involve the inducible form of NOS. The neuroprotective side of NO could be due to the early production of NO from the ecNOs form which would lead to enhanced cerebral blood flow and anti-platelet effects. Suffice to say that during ischaemia/reperfusion, released NO may be beneficial in terms of its effects on vasculature but it may also be deleterious to cellular metabolism either at the same or at a later stage in the insult. Further research in this area is necessary so that the

potential therapeutic benefits of NO related agents can be fully realised.

5.1.5.e Summary

In summary then, there is a growing body of evidence in support of the original Flamm-Demopoulos hypothesis of a role for free radicals in the pathophysiology of ischaemia/reperfusion. This condition is however very complex. Whilst it seems that oxidative damage is indeed a part of the pattern leading to neuronal death following ischaemia/reperfusion, much biochemical and molecular information remains to be uncovered. We need to fully understand where and how free radicals are produced during the stages of ischaemia/reperfusion, where the cellular targets of damage are and how such damage is incurred. Ultimately we need to realise whether oxidative damage is a key focus for aiming therapeutic agents during ischaemia/reperfusion or whether the *in vivo* situation is far too complex to enable this.

5.1.6 Oxidative stress, brain development and ageing

The overall maturation of the mammalian brain is complex. Many changes in brain function occur during development and ageing and many of the mechanisms underlying such changes have not been well elucidated. The role of free radicals in ageing is often researched (Viani *et al.*, 1991; Floyd *et al*, 1984). However, it is also important to assess fundamental changes in systems involved with oxidative stress during brain maturation *per se*. Such knowledge would ensure that developmental and age-related changes in response to pathological conditions, such as ischaemia, can be assessed in the light of normal age-related changes in oxidative processes.

Currently, there is evidence that iron accumulates in the brain during ageing (Hallgren and Sourander, 1958; Francois *et al.*, 1981) and this has consequences for iron-induced peroxidative processes in the brain. Membranes from aged brain show greater responsiveness to lipid peroxidation resulting in a higher degree of alteration and damage than in younger brain (Viani *et al.*, 1991). Further reports have been

published on the age-related changes in peroxidative potential in rat brain (Sawada and Carlson, 1987; Devasagayam, 1989), and evidence suggests that there is an increase in the formation of free radicals with advancing age (Leibovitz and Siegel, 1980; Harman, 1981). Ischaemic studies in gerbils of differing ages, have shown an enhanced susceptibility to oxidative damage with increasing age (Evans, 1993). Studies have also shown decreases in activities of SOD, catalase and GPx with ageing (Vitorica *et al.*, 1984; Scarpa *et al.*, 1987; Semsei *et al.*, 1991). The accumulating evidence therefore suggests that oxidative stress and damage may be more pronounced in aged brain than in younger animals. This has implications for the severity of pathological conditions involving ROS's in the aged brain.

In contrast to the numerous reports concerning oxidative stress and the aged brain, there is currently very little information regarding oxidative processes and the developing brain. ROS's are implicated in hypoxia/ischaemia and reoxygenation/reperfusion induced injury not only at adult age but also during brain development. At this stage however, direct evidence for changes in concentration of free radical species and for modulation of biochemical constants of scavenging enzymes and antioxidants is very limited.

In light of this area of research, Nanda and Collard (1995) examined the endogenous level of GSH in the developing and adult rat brain. Their study found that there is a gradual raise in total brain GSH levels from PND 10 to the adult, however there was a marked transient increase in levels at PND 7. Interestingly this correlates with the NMDA receptor ontogeny in rat brain. In contrast there is no distinct developmental profile for the antioxidant α -tocopherol; brain levels tend to remain constant throughout development and into adulthood (Goss-Samson, 1987). SOD activity has been shown to increase with brain maturation (Mavelli *et al.*, 1978) and it is generally thought that the immature brain may have inadequate antioxidant mechanisms (Sokoloff, 1977).

In terms of free radical production, it is known that the activity of brain NOS changes with development (Matsumoto *et al.*, 1993). In immature rat brain cerebrum particulate NOS activity increased during the first week of development but then decreased and became almost undetectable in adult rats. In contrast the cytosolic NOS in the cerebrum increased slightly during maturation. In the cerebellum cytosolic NOS showed low activity in newborns but increased during development, reaching an 8-fold higher level in adult rats. Cerebellum particulate NOS increased slightly during maturation (Matsumoto *et al.*, 1993). Further changes in processes connected to oxidative stress in the developing brain remain to be elucidated.

METHODS

5.2 <u>Methods</u>

5.2.1 Materials

N'. nitro-arginine (NNA) and ionomycin were purchased from the Sigma Chemical Company, Poole, Dorset, U.K.

All other chemicals used were of analytical grade, and were supplied by either BDH Limited, Dagenham, Essex, U.K., or the Sigma Chemical Company, Poole, Dorset, U.K., or Boehringer Mannheim U.K., Limited, Lewes, East Sussex, U.K.

All solutions were made up in deionised double distilled water.

5.2.2 Measurement of NO-related activities - general considerations

NOS activity can easily be measured in a number of ways by a range of methods which include the following:-

- 1] spectrophotometrically by exploiting the reaction of NO with oxyhaemoglobin to form methaemoglobin which absorbs at 401 nm.
- 2] quantification of [¹⁴C]-or[³H] citrulline from the respective radiolabeled Larginine.
- 3] measurement of nitrite (NO_2-) and nitrate (NO_3-) , the stable solution decomposition products of NO.

Several less routine methods are also available to assess NOS activity. NO can be detected directly by using porphyrinic electrodes (Malinski *et al.*, 1993), chemiluminescence (Palmer *et al.*, 1987) or spin trapping compounds with electron paramagnetic techniques (Mordvintec *et al.*, 1991). NO induced activation of guanylate cyclase can also be assayed effectively to reflect NOS activity (Schmidt *et al.*, 1991).

5.2.3 Determination of NO₂- and NO₃- by the Griess Reaction.

NO production from the synaptosomes was assessed by assaying NO_2^- and NO_3^- , the stable end products of NO⁻ degradation in aqueous solution. These oxygenation products of NO metabolism (Knowles and Moncada, 1994) are more straightforward to measure under the conditions employed here to assess the concentration of NO because the half-life of NO is so short (Snyder, 1992) and thus the molecule itself cannot be accurately assayed directly.

The formation of NO_2^- and NO_3^- can be measured by a variety of techniques. These include High Performance Liquid Chromotography (HPLC) with ultra violet detection of NO₂⁻ and NO₃⁻ concentrations. A potential advantage of the HPLC technique is that NO_2^- and NO_3^- can be measured simultaneously. The Griess reagent for NO₂- is most frequently used, and samples can also be assayed for NO₃⁻ if a simple reduction step is added into the procedure. These techniques have comparable sensitivity (0.1 - 1 μ M for both anions) and are amenable to automation. The most frequent reason for turning to alternative detection methods is a lack of sensitivity. Chemiluminescence methods can be used if the stable end-products are reconverted to NO. The sensitivity of such a technique is about 100-fold more sensitive than when using the Griess reaction. However, the application of chemiluminescence must proceed with caution because other compounds such as S-nitrothiols can be detected and also levels of NO_2^{-}/NO_3^{-} already present in buffers and tissue samples can limit the usefulness of this technique. The technique used for these experiments is the Griess assay. This is because the sensitivity offered is adequate and the technique also offers simplicity and low cost.

When assessing NOS activity and the concentration of NO formed, the concentration of arginine is important. If initial rate conditions (<10% product formation) are followed endogenous arginine concentration will be above the NOS K_m for arginine (2-16 μ M). However if these conditions are not obeyed and more product is allowed to form (as is the case for the 30 minute synaptosome incubations here), the concentration of arginine may need to be increased.

5.2.3.a Assay procedure

 $NO_2 + NO_3$ released from the intact synaptosomes was measured in the supernatant of the synaptosomal incubations by a colorimetric procedure using the Griess reagent as described by Green *et al.*, (1982) which has been previously used with synaptosomes (Gorbunov and Esposito, 1994).

Griess Reagent

This was prepared fresh on the day of assay by mixing equal parts of 0.1% naphthalethyenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid. These individual stock solutions were stored at 4°C in a light free environment.

Standard Curve

A standard curve across the range 0-20 μ M NO₂ was constructed using NaNO₂. The standard curve was prepared in control KPB in 1 ml disposable cuvettes. 400 μ l of standard was used in the reaction mixture for all standards. Similarly, 400 μ l of sample (supernatant) was used in this assay.

Reduction step

The method of NO_3^- reduction is based on that of Olken and Marletta (1993). Nitrate reductase and NADPH were added to the samples and standards so that the final concentrations were 0.12 U nitrate reductase and 25 μ m NADPH. The cuvettes were incubated at room temperature for 30 minutes to allow full conversion of NO_3^- to NO_2^- .

Griess Reaction

NADPH can interfere with the Griess reaction (Medina and Nicholas, 1957). Therefore to alleviate this problem any residual NADPH from the reduction step was consumed by addition of L-Glutamate dehydrogenase (400 mU), NH_4Cl (100mM), and freshly prepared α -ketoglutarate (4 mM). All concentrations quoted are final concentrations. The cuvettes were incubated at room temperature for 10 minutes to allow conversion of residual NADPH to NADP⁺.

Chapter 5

500 μ l of Griess reagent was added to all samples and standards. Following a brief (5-10 minutes) incubation period at room temperature the absorbance at 550 nm was recorded verses a blank which contained Griess reagent and buffer only. Results were obtained from a standard curve in μ m units. These were then expressed against a protein baseline from the synaptosome incubations so that the data was expressed as nmole/mg protein.

5.2.3.b Assay validation

To validate that the assay was adequately reflecting NO[•] production the calcium ionophore, ionomycin (10 μ M f.c.), was added to control synaptosomes. This was to establish whether NO₂⁻ + NO₃⁻ production would accurately reflect an increased calcium concentration and subsequent increased activity of NOS and thus NO production. Applications of the NOS inhibitor N'. nitro-arginine (NNA) were made to the ischaemic incubations (100 μ M f.c.) (Snyder, 1992) to assess whether increased NO₂⁻ + NO₃⁻ production was due to increased NOS activity.

5.2.4 The Citrulline assay

The formation of ³H or ¹⁴C-labelled citrulline from labelled L-arginine is the most widely used method for assessing NOS activity. It uses a simple exchange separation of substrate and product and the quantitative determination of labelled product. This assay is both sensitive, usually requiring the formation of less than 100 nM product, and also robust for it does not require optically clear enzyme extracts, and thus crude cell or tissue samples can be easily assayed. However, in order to obtain accurate quantitative results, such assays of crude cell or tissue extract NOS require the endogenous L-arginine in the sample to be removed prior to the assay.

5.2.4.a Assay procedure

NOS activity was determined in both brain cytosolic fractions and cytosolic fractions from isolated synaptosomes prepared from 10 and 60 day old rats. Synaptosomes were prepared as detailed in sections 2.2.2 and 2.2.3. Whole brain cytosolic fraction was prepared as follows:-

Whole rat brain was homogenised (25% w/v) in isolation buffer (320 mM sucrose, 10 mM Tris, 1 mM EDTA, 1mM dithiothreitol, pH 7.4) and centrifuged at $100,000g_{av}$ for 60 minutes at 4°C. The resulting supernatant was used for the determination of NOS activity.

The measurement of NOS activity was based on the quantification of $[{}^{3}H]$ citrulline converted from $[{}^{3}H]$ arginine, as described by Brand *et al.*, (1995). In brief, following removal of endogenous arginine by cation exchange, 100 µl of cytosol was added to reaction buffer. The reaction buffer contained (f.c.):-

100 mM HEPES, pH 7.5,
100 μM NADPH,
1 mM calcium chloride,
1 mM magnesium acetate,
10 μg/ml calmodulin,
50 μM tetrahydrobiopterin,
1 mM dithiothreitol,
5 μM FAD,
5 μM FMN,
100 μM L-arginine and 1 μCi/ml [³H] arginine.

After 15 minutes the reaction was terminated by addition of 50 µl of trichloroacetic acid (1.5 M) followed by 1 ml of HEPES (1.5 M) buffer, pH 6.0. Arginine was then removed from the reaction mixture by ion exchange chromatography. At this pH citrulline is neutral, is not retained by the column and therefore passes through the column and can be quantified in the eluent. NOS activity in this assay is therefore defined as the counts/minute of citrulline in a test incubate as compared to an appropriate blank. Blank incubations in this case either had sample ommitted or the NOS inhibitor NNA included (100 µM f.c). The specific activity of 50 µl of reaction buffer was measured under the conditions present on the day of assay. This specific activity value of counts per micromole of arginine incorporated allowed the activity of NOS to be calculated.

The calculation used for each test incubation was therefore as follows:-

counts in test incubation x 1.25 (1.25 ml of incubation, but only 1 ml added to scintillant)

specific radioactivity x time (min) x mg protein in sample

The activities of NOS measured and displayed in the results section are therefore given as pmol/minute/mg protein.

5.2.5 Total Antioxidant Status

The total antioxidant status was measured in synaptosomes with glucose and oxygen supplied, from both 10 day old pups and adults. Synaptosomes (approximately 2 mg/ml) were freeze-thawed three times and homogenised in a tight glass-glass homogeniser, to release all of the synaptosomal contents. After pelleting the membranes (14,000g, 5 mins), the supernatant was assayed for antioxidant status. This is a functional assay which measures the capacity of a sample to scavenge free radicals generated from the reaction between metmyoglobin and hydrogen peroxide. If unscavenged, these radicals react with 2.2'- azino-bis-3-ethylbenzo-thiaoline-6sulphonic acid (ABTS) to form a chromophore which absorbs at 734 nm. The assay was carried out as in the original method (Miller et al., 1992). In brief, 2.5 μ M metmyoglobin was activated by 75 μ M H₂O₂, and mixed with 150 μ M ABTS, all in phosphate buffered saline (pH 7.4) which had been purged with nitrogen to minimise the amount of dissolved oxygen. To this reaction mixture was added 10 μ l of sample or the α -tocopherol analogue Trolox as calibration standard, and the absorbance at 734nm was recorded. This method was configured to run on a Cobas Fara automated machine using a multi-point standard curve. This machine is routinely used for clinical samples in the Clinical Biochemistry Department of the Institute of Neurology. Results were automatically calculated as μM from the Trolox standard curve. These results were then expressed against a protein baseline so that final data was given in nmoles/mg protein.

5.2.6 Determination of glutathione status

GSH was assayed by HPLC with electrochemical detection as described by (Reed *et al.*, 1980). The HPLC system and assay was routinely maintained by Dr S J R Heales and Mr. M P Brand of the Department of Neurochemistry, Institute of Neurology. Following thawing of synaptosome samples, a 1:10 dilution with o-phosphoric acid, and centrifugation (15,000 g, 10 minutes) the supernatant was assayed for GSH content. GSH standards were also assayed. Results are expressed against a protein baseline as nmole/mg protein.

5.2.7 Peroxynitrite methods

5.2.7.a **Peroxynitrite synthesis**

Peroxynitrite was synthesised and kindly provided for these experiments by Dr. J. E. Barker, Department of Neurochemistry, Institute of Neurology. Synthesis was by the quench method described by Hughes and Nicklin (1970). In brief peroxynitrite was synthesised by rapid mixing of an ice-cold 50 ml solution of 50 mM sodium nitrite with 50 ml of 50 mM hydrogen chloride. A beaker containing 25 ml of 1 M hydrogen chloride is added to the reaction to produce peroxynitrous acid. This reaction was then rapidly quenched with 25 ml of 1.5 M sodium hydroxide after only a delay of two seconds, thus forming the peroxynitrite anion. The peroxynitrite thus formed is stable in this alkaline solution and can be kept in the freezer for a number of months.

5.2.7.b Assay of peroxynitrite

Peroxynitrite concentration was assayed by diluting the defrosted stock between 100- and 1000 fold into 1 M sodium hydroxide (NaOH) and measuring the increase in absorbance at 302 nm on a spectrophotometer. A 1 ml disposable cuvette was filled with 1 ml of 1 M NaOH and the cuvette was blanked at 302 nm. Then between 1 and 10 μ l of peroxynitrite stock was accurately added to the cuvette and mixed. The concentration of the stock was calculated using the Beer-Lambert Law, A = ϵ cl. The extinction coefficient is 1670 M⁻¹ cm⁻¹ at 302 nm (Hughes and Nicklin, 1968). As a control, the same volume of peroxynitrite was added to the buffer used for incubation

of synaptosomes with peroxynitrite in these experiments. The absorbance decreased to that of the buffer alone after a few seconds because peroxynitrite decomposes rapidly at neutral pH.

5.2.7.c Exposure time and peroxynitrite concentration

Although there is a natural tendency to express the toxicity of a compound in terms of its initial concentration, it is important with peroxynitrite to express its toxicity as a function of net exposure (ie. the area under a concentration verses time curve). Peroxynitrite decomposes rapidly at physiological pH, with a half life of about one second in a phosphate based buffer (Beckman and Crow, 1992), and will completely decompose in a few seconds. The net exposure to peroxynitrite can be calculated from the pseudo-first-order kinetics of peroxynitrite decomposition. An initial bolus addition of peroxynitrite can be calculated to be equivalent to exposure to a specific concentration of peroxynitrite for a specific time period. Integration of the following equation:-

 $[ONOO^{-}]_{t} = [ONOO^{-}]_{0} q^{-k_{t}}$ equation 5.2.7

will allow a bolus concentration of ONOO⁻ to be calculated which is equivalent to a lower concentration for a specific time period.

Studies detailed in section 5.3.2 show that approximately 2 nmoles/mg protein cumulative production of nitrite and nitrate occurred in synaptosomes during a 15 minute period of reoxygenation. This would equate to a concentration of 2 μ M per mg protein over 15 minutes and thus 130 nM steady state nitric oxide being produced per mg of protein. Nitric oxide and superoxide react to form ONOO⁻ in a 1:1 stoichiometry. Typically, the synaptosome incubations used in the exposure experiments contained 9-10 mg protein. Therefore to allow an estimated ONOO⁻ 100 nM concentration exposure per mg protein approximately 1 μ M steady state concentration of peroxynitrite would need to be applied. From equation 5.2.7 it can be calculated that to achieve the equivalent exposure of 1 μ M ONOO⁻ for 15 minutes a bolus addition of 1 mM ONOO⁻ would be necessary. Therefore in these experiments a 1 mM initial bolus concentration of ONOO⁻ was added to synaptosome incubations of approximately 10 mg/ml protein.

5.2.7.d Synaptosome incubation with peroxynitrite

Synaptosomes were prepared as detailed in section 2.2.2. for adults and 2.2.3 for day 10 pups. Synaptosomes were then incubated in HEPES buffered Hanks Balanced Salt Solution (HBSS) at 30 °C for 5 minutes before 1 mM ONOO⁻ in NaOH was added, and synaptosomes were further incubated for 15 minutes. For those control incubations not containing ONOO⁻ an equivalent amount of NaOH was added. There was no pH change in the incubations as a result of ONOO⁻ or NaOH addition.

HBSS was used for the incubations because ONOO⁻ reacts with components within the incubation buffer normally used for synaptosomes, ie. Kreb's Phosphate Buffer (KPB), and forms adducts which can donate nitric oxide in a series of reactions (Moro *et al.*, 1995). Therefore it would be difficult to dissociate the effects of ONOO⁻ from the effects of other reaction products on synaptosomal function. Control incubations of synaptosomes incubated in KPB were always performed to ensure that there was no significant differences in synaptosomal function as a result of a change in incubation medium. Control incubations were also performed in which ONOO⁻ was added to the incubation buffer, followed by synaptosomes some 15 minutes later, to ensure that any effects seen were directly due to ONOO⁻ exposure and not due to the decomposition products of ONOO⁻.

Following incubation with or without ONOO⁻, synaptosomes were pelleted (10,000 g), washed with warmed (37°C) KPB containing glucose and oxygen, and were further incubated in this KPB for 30 minutes at 37°C with oxygen supplied. At the end of this incubation period synaptosomes were again pelleted and supernatants and pellets were snap frozen in liquid nitrogen. Samples were stored at -80°C until day of assay which was within one month of storage. In the case of measurement of ATP concentrations, samples were extracted at the end of the 30 minute incubation period as detailed in section 4.2.7 and neutralised samples were stored at -80°C until

day of assay.

5.2.8 Other assays and procedures

Mitochondrial respiratory chain complexes I - V were assayed as described in sections 4.2.3 - 4.2.6 respectively.

The concentration of ATP in the peroxynitrite treated and control synaptosomes was determined as described in section 4.2.7 of this thesis.

5.2.9 Statistical analysis

The effects of peroxynitrite treatment on the parameters measured were tested using one-way analysis of variance (ANOVA) (Sokal and Rohlf, 1981). Differences between groups were tested using the Least Square Difference (LSD) test (Sokal and Rohlf, 1981).

RESULTS

5.3 Results

5.3.1 The activity of Nitric Oxide Synthase (NOS) in adult and immature brain

(Figure 5.4)

In 60 day old adult rats the total brain NOS activity, measured in homogenates, was significantly (P<0.01) higher than in the brain from 10 day old rat pups. This pattern of higher activity in the adult brain was the same in the measurements of NOS activity in cytosol from synaptosomes prepared from these age rats. The NOS activity in the cytosol from adult synaptosomes was significantly (P<0.01) higher than the NOS activity measured in the cytosol from synaptosomes prepared from synaptosomes prepared from the NOS activity measured in the cytosol from synaptosomes prepared from synaptosomes prepared from PND 10 rats.

5.3.2 The production of nitrite + nitrate from adult and immature synaptosomes.

(Figure 5.5)

The concentration of $NO_2^{-} + NO_3^{-}$ from synaptosomes, which reflected the concentration of NO, was significantly lower in pups compared to adults (p<0.01). A period of glucose and oxygen deprivation for 15 or 30 minutes followed by reintroduction of oxygen (akin to ischaemia/reoxygenation) caused a significant (P<0.01) increase in $NO_2^{-} + NO_3^{-}$ production when compared with control in the adult synaptosomes. There was no significant difference from control in the case of the synaptosomes prepared from PND 10 rat pups.

To validate that the increase in $NO_2^- + NO_3^-$ seen in adult synaptosomes was due to an increased activity of NOS and the concurrent production of NO, the NOS inhibitor N'.nitro-arginine (NNA) was included in some of the ischaemic incubations. NNA (100 μ M final concentration) prevented the significant increase in $NO_2^- + NO_3^$ during ischaemia-reperfusion as shown in table 5.1.



Legend for Fig. 5.4

Nitric oxide synthase activity was measured in adult and pup brain homogenates and synaptosomes as described in 5.2.4. Results are expressed as mean \pm SEM

n = 3 separate preparations, measurements performed in triplicate.

a represents significant difference (p<0.01) from adult value.





Legend for Fig. 5.5

Nitrite and nitrate concentration was measured in adult and pup synaptosomes as described in 5.2.3.

Results are expressed as mean + SEM

n = at least 4 separate preparations, measurements performed in duplicate.

a represents significant difference (P<0.01) from adult value

** represents significant difference (P<0.01) from control value

Table 5.1

Validation that the increased $NO_2^+ + NO_3^-$ production from adult synaptosomes during 30 minutes of ischaemia/reoxygenation is due to increased NOS activity.

	NO2 + NO3 (nmoles/mg Protein)
control (adult)	$1.15 \pm 0.11 \ (n = 6)$
ischaemia/reperfusion (adult)	$2.27 \pm 0.15 \ (n = 6)$
ischaemia/reperfusion + NNA (adult)	$1.23 \pm 0.11 \ (n = 3)$
control + ionomycin (adult)	$1.60 \pm 0.14 \ (n = 3)$

Results = mean \pm s.e.m. of between 3 and 6 separate experiments, with samples in duplicate.

To further validate that the production of $NO_2^- + NO_3^-$ from the synaptosomes reflected the calcium-dependent nNOS activity the calcium ionophore *ionomycin* was added to control synaptomes with oxygen and glucose freely available. Addition of ionomycin to control incubations increased the $NO_2^- + NO_3^-$ production by approximately 40% in the adult (Table 5.1). In the PND 10 pup synaptosomes the increase was similar (138% of control value; control = 0.49 ± 0.03 nmol/mg P; control+ inomycin = 0.68 ± 0.07 nmol/mg P, n = 3), indicating that the assay reflected calcium-dependent NOS activity in both of these synaptosome preparations.

5.3.3 Synaptosome antioxidant defence.

5.3.3.a Total antioxidant status

(Table 5.2)

The inherent total antioxidant capacity of the adult and PND pup synaptosomes under normal conditions was similar, and there was no significant difference between these two groups, as shown in table 5.2.

Table 5.2

Total antioxidant status in adult and pup synaptsomes

Total antioxidant capacity (µmoles/mg protein)		
adult	рир	
$0.11 \pm 0.01 \ (n=9)$	0.12 ± 0.01 (<i>n</i> =7)	

Results = mean \pm s.e.m. of between 7 and 9 separate experiments, with samples in duplicate.

5.3.3.b Glutathione status

(Table 5.3 and Figure 5.6)

The glutathione concentration in synaptosomes under control conditions was significantly (P<0.01) higher in pup synaptosomes than in adult synaptosomes. As shown in figure 5.6, under conditions of metabolic stress there was no significant change in glutathione status. However, in neonatal synaptosomes ischaemia but not hypoxia caused the glutathione status to significantly (P<0.01) increase.

Table 5.3

Glutathione status in adult and pup synaptsomes

Glutathione concentration (nmoles/mg protein)		
adult	рир	
3.35 ± 0.2 (<i>n</i> =6)	6.00 ± 0.45 (<i>n</i> =7)	

Results = mean \pm s.e.m. of between 7 and 9 separate experiments, with samples in duplicate.



Legend for Fig. 5.6

Glutathione concentration was measured in adult and pup synaptosomes as described in 5.2.6.

Results are expressed as mean <u>+</u> SEM

n = 7 separate preparations for adult and 9 for pups, measurements performed in duplicate

a represents significant difference (P<0.01) from adult value

** represents significant difference (P<0.01) from control value

5.3.4 <u>ONOO- treatment and the activity of the mitochondrial respiratory chain</u> enzymes.

5.3.4.a Complex I

(Figure 5.7)

For both adult and postnatal day 10 pup synaptosomes complex I activity in the synaptosomal mitochondria remained unaffected by ONOO- treatment. Incubation with ONOO-, followed by a thirty minute recovery period without the presence of ONOO- caused no irreversible decrease in complex I activity, when compared with control activities. As with all of the mitochondrial enzymes tested, there was no significant difference between the appropriate controls used which contained the same concentration of NaOH as the peroxynitrite treated samples and similar controls which contained only KPB.

5.3.4.b Complex II-III

(Figure 5.8)

In adult synaptosomes, the activity of Complex II-III became significantly (P<0.01) decreased following the ONOO- treatment when compared to controls.

Unlike the situation in the adult synaptosomes, the activity of Complex II-III remained unaffected by ONOO- treatment in the immature synaptosomes from PND pups.

5.3.4.c Complex IV

(Figure 5.9)

Incubation with ONOO- followed by a thirty minute recovery period without ONOO- present led to a significant (p<0.05) decrease in the activity of Complex IV in the adult synaptosomes. Therefore it seems that as in the case of Complex II-III activity in the adult synaptosomes, ONOO- is either directly damaging Complex IV and decreasing its activity, or ONOO- is initiating a process which during the thirty minute incubation is having a detrimental effect on these particular mitochondrial enzymes.



Legend for Fig. 5.7

Complex I activity was measured in adult and pup synaptosomes as described in 4.2.3., following peroxynitrite treatment as detailed in 5.2.7.

Results are expressed as mean + SEM

n = 5 separate preparations, measurements performed in duplicate.





Legend for Fig. 5.8.

Complex II-III activity was measured in adult and pup synaptosomes as described in 4.2.4., following peroxynitrite treatment as detailed in 5.2.7. Results are expressed as mean \pm SEM

n = 6 separate preparations, measurements performed in duplicate.

** represents significant difference (P<0.01) from control value





Legend for Fig. 5.9

Complex IV activity was measured in adult and pup synaptosomes as described in 4.2.5., following peroxynitrite treatment as detailed in 5.2.7. Results are expressed as mean \pm SEM

n = 6 separate preparations, measurements performed in duplicate.

* represents significant difference (P<0.05) from control value

In the synaptosomes from PND 10 pups there was no significant decrease in the activity of complex IV following ONOO- treatment. Therefore it appears that either the immature synaptic mitochondrial complex IV is less susceptible to direct ONOO- inhibition or that the mechanisms of inhibition that may be initiated by ONOO- treatment in the adult are not taking place in the immature nerve terminal or there is an inherent resistance to damage via these processes in the immature nerve terminal.

5.3.4.d Complex V

(Figure 5.10)

Complex V activity remained unaffected by ONOO- treatment in both the adult and immature synaptosomes.

5.3.5 ONOO- treatment and intrasynaptosomal ATP levels.

(Figure 5.11)

Following treatement with ONOO- the ATP levels in the adult synaptsomes had significantly (P<0.01) decreased when compared with the control values. The decrease in ATP concentration was of approximately 48% in the adult synaptosomes under these conditions. In the PND 10 pup synaptosomes the same treatment with ONOO- led to no significant change in intrasynaptosomal ATP levels when compared with the control values. In the control synaptosome preparations there was no significant difference between ATP levels in the two different age rat brains used for the preparations.

5.3.6 ONOO- treatment and synaptosome integrity.

In both the adult and PND 10 rat preparations there was no significant release of lactate dehydrogenase (LDH) from the synaptosomes. Release of LDH was less than 10% in both adult and pup synaptosome preparations, thus indicating that synaptosome integrity was maintained following the ONOO- treatment in both groups.



Legend for Fig. 5.10

Complex V activity was measured in adult and pup synaptosomes as described in 4.2.6., following peroxynitrite treatment as detailed in 5.2.7. Results are expressed as mean \pm SEM

n = 6 separate preparations, measurements performed in duplicate.



FIGURE 5.11

Legend for Fig. 5.11

ATP concentration was measured in adult and pup synaptosomes as described in 4.2.7., following peroxynitrite treatment as detailed in 5.2.7 Results are expressed as mean % of control values + SEM Control values were 1.7 ± 0.1 nmoles/mg protein for adults and 1.8 ± 0.1 nmoles/mg protein for pups n = 4 separate preparations, measurements performed in duplicate. ** represents significant difference (P<0.01) from control.

5.3.7 Glutamate present in the extrasynaptosomal medium following ONOOtreatment.

(Figure 5.12)

In the adult synaptosome preparations there was a significantly (P<0.05) increased amount of glutamate in the extrasynaptosomal medium following ONOO-treatment. There was no significant change from control values in the pup synaptosomes under these conditions.



Legend for Fig. 5.12

Glutamate concentration in the extrasynaptosomal medium of adult and pup synaptosome preparations was measured as described in 3.2.4., following peroxynitrite treatment as detailed in 5.2.7.

Results are expressed as mean <u>+</u> SEM

n = 6 separate preparations, measurements performed in duplicate.

* represents significant difference (P<0.05) from control value

a represents significant difference (P<0.05) from adult value under the same condition

DISCUSSION

5.4 Discussion

The data presented in this study indicate that there are distinct differences between adult and neonatal nerve terminals in some of the processes concerned with oxidative damage and protection. Furthermore, that under conditions of metabolic stress there are differences in the response of some oxidative processes in neonatal as compared with adult nerve terminals. When oxidative stress is imposed on neonatal and adult nerve terminals by the addition of an exogenous oxidising species there are fundamental differences in the dysfunction seen in the different age preparations.

5.4.1 Synaptosomal Nitric Oxide (NO) production

5.4.1.a Nitric Oxide Synthase (NOS) activity

Adult brain homogenates and, more specifically, adult nerve terminals exhibit significantly higher NOS activity than immature brain. This data is in agreement with previous findings that NOS activity in cytosolic and particulate fractions from rat brain cerebellum increases with age (Matsumoto *et al.*, 1993). In rat pups it appears that the activity of NOS in the nerve terminal from immature brain would be low during the initial stages of ischaemia/reperfusion. This is due to a combination of the low intrinsic activity of NOS in immature brain coupled with the previously discussed data, from chapter three, demonstrating no increase in synaptosomal calcium during ischaemia. This data, concerning calcium homeostasis in the neonatal nerve terminals is of key importance in NOS chemistry because the neuronal form of NOS is known to be calcium-calmodulin dependent (Knowles and Moncada, 1994).

The higher intrinsic NOS activity of adult nerve terminals coupled with a significant increase in cytosolic calcium (as presented and discussed in chapter three) during ischaemia would lead to the hypothesis that significantly higher production of NO occurs in these nerve terminals, as compared with those in the post-ischaemic neonatal brain.

5.4.1.b Nitrite and Nitrate production

In adult nerve terminals nitrite and nitrate production during a thirty minute ischaemic episode was significantly increased over control values. These observations confirmed the previous hypothesis that NO production would, at least initially, be significantly increased in post-ischaemic adult nerve terminals. The nitrite and nitrate was confirmed to be due to the production of NO from nerve terminals by the use of an NO inhibitor which attenuated the post-ischaemic nitrite and nitrate production.

In contrast to adult nerve terminals, there was no detectable increase in NO production from neonatal nerve terminals. Thus, the hypothesis of differences in the amount of NO production from post-ischaemic neonatal as compared with adult nerve terminals is supported by these observations. The differences in NO production from adult and neonatal nerve terminals suggests that NO production by nerve terminals does not play an important part of the mechanism of damage in the neonatal brain during the immediate stages of reperfusion after ischaemia.

Less research, in the area of NO and oxidative stress, has been conducted on the neonatal brain than on the adult. More specifically, the data presented here is the first report in literature of a comparative study of NO production from post-ischaemic adult and neonatal nerve terminals (Keelan *et al.*, 1996). Although less work has been carried out on the neonatal brain, some of that conducted with *in vivo* neonatal models has suggested that NOS inhibitors can ameliorate damage following focal stroke (Trifiletti, 1992). The *in vitro* model used here is more relevant to global ischaemia, and in addition there are no blood vessels present thus allowing the neuronal NOS to be studied effectively in isolation from the endothelial and inducible forms. Hence, although some reports in literature would implicate a neuroprotective role for NOS inhibition during neonatal ischaemia it is possible, and in fact likely in view of the data presented here, that deleterious NO production during ischaemia/reperfusion does not occur in the nerve terminal in neonatal brain.

5.4.1.c Consequences of increased NO production in post-ischaemic adult nerve terminals

This study reports a significantly increased production of NO from postischaemic adult nerve terminals. It is known from literature that when oxygen is resupplied to nerve terminals during reoxygenation large amounts of reactive oxygen species are produced, which includes O_2^- , OH and H_2O_2 (Kirsch, 1987; Braughler and Hall, 1989; Floyd, 1990). Mitochondria are not only an important cellular source of oxygen radicals, but are also a target of free radical attack (Radi *et al.*, 1994) and loss of mitochondrial function has often been seen following ischaemia/reperfusion (see Phillis, 1994; Sims and Zaidan, 1995) During initial reperfusion the co-production of NO and O_2^- can lead to the production of ONOO- from a reaction between these two species. ONOO- is a potent oxidising agent which has been demonstrated to specifically inhibit mitochondrial complex II-III and complex IV activities in neuronal cultures (Bolaños *et al.*, 1994). Interestingly, it is this same pattern of complex activity inhibition which is seen in adult synaptosomes following ischaemia/reperfusion in the studies reported in this thesis (chapter 4.)

In post-ischaemic adult nerve terminals increased NO production could lead to diffusion of NO to mitochondria and "site-specific" formation of ONOO- due to reactions of NO with mitochondrially derived O_2^- . Alternatively, ONOO- could be formed by reaction of NO and O_2^- in the cytosol followed by ONOO- diffusion to mitochondria because ONOO- is capable of long distance diffusion (Beckman, 1991). N**0** is also capable of reacting with molecular oxygen to form the nitrosodioxyl radical ('ONOO) which could diffuse to mitochondria and yield ONOO- due to electron leakage from mitochondria. The increased production of NO from adult nerve terminals on reoxygenation coupled with the pattern of mitochondrial complex inhibition seen in reperfused adult nerve terminals implicates a role for ONOO- in ischaemic/reperfusion damage in the adult nerve terminal. The distinct lack of NO production from neonatal post-ischaemic nerve terminals on initial reperfusion.

Interestingly, no decrease in mitochondrial respiratory chain activities was apparent in reperfused neonatal nerve terminals.

The differences in NO production from immature and adult nerve terminals reported here could be significant in terms of the resistance of the neonatal brain to ischaemia/reperfusion injury.

5.4.2 Synaptosomal antioxidant defence

5.4.2.a Total antioxidant status

Synaptic oxidative stress would occur when the production of oxygen derived damaging species exceeds the capacity of the oxidative defence mechanisms in the nerve terminal. Therefore it is important to assess not only the potential differences in production of ROS from adult as compared with neonatal ischaemic nerve terminals, but also to compare antioxidant defence between these two groups.

The data presented in this thesis in section 5.3 shows that the total soluble antioxidant capacity of adult nerve terminals did not differ from immature nerve terminals. This observation correlates with the developmental profile of the major membrane bound antioxidant alpha-tocopherol which remains constant as the brain matures (Goss-Sampson, 1987).

From the data presented in this thesis, one can take a ratio of:-

antioxidant status: NO production

as an index of oxidative stress. A low ratio would indicate a larger degree of oxidative stress. In the adult nerve terminal the ratio is lower than in the neonatal nerve terminal, and hence there is a larger degree of oxidative stress. The lower ratio in the adult is a reflection of an increased NO production which is not compensated for by an increased antioxidant status.
In the neonatal nerve terminal this ratio is much higher than in the adult nerve terminals, thus reflecting a lower degree of oxidative stress. Under the conditions of metabolic stress used here it is therefore suggested that the immature nerve terminal is less likely to suffer from NO related oxidative stress then the adult nerve terminal.

5.4.2.b Nerve terminal glutathione status

A relationship between glutathione (GSH) concentration and mitochondrial respiratory chain function has been suggested in a number of studies. In neurones a decreased GSH status led to a decrease in the activity of a number of mitochondrial respiratory chain complexes (Heales et al., 1995). Depletion of mitochondrial GSH induced lethal injury in cultured cardiomyocytes (Dhanboora and Babson, 1992). Depletion of mitochondrial but not cytosolic GSH killed hepatocytes via loss of ATP (Redegeld et al., 1992), and depletion of mitochondrial GSH has been found to induce the opening of the transition pore which destroys the mitochondrial membrane potential (Beatrice et al., 1984). In cultured neurones ONOO- exposure leads to a decrease in GSH (Bolaños et al., 1995), along with a decrease in mitochondrial complex II-III and complex IV activities (Bolaños et al., 1995) and cell death as indicated by LDH leakage. In contrast, astrocytes did not exhibit any significant loss of mitochondrial respiratory chain activity or LDH leakage following ONOOexposure (Bolaños et al., 1995). The concentration of GSH in astrocytes is double that of neurones (Bolaños et al., 1995) which is likely to be due to the approximate eightfold higher activity of γ -glutamylcysteine synthetase (a key enzyme in GSH synthesis) in astrocytes as compared with neurones (Makar et al., 1994), and which may account for the greater resistance to ONOO- attack in astrocytes. Indeed, GSH directly inactivates ONOO- (Barker et al., 1996).

The study presented in this thesis shows that under normal control conditions the glutathione status of immature nerve terminals is much higher than that of adult nerve terminals. In light of the afore mentioned differential susceptibility of neurones as compared with astrocytes (Bolaños *et al.*, 1995), this would suggest that neonatal nerve terminals are less susceptible to ONOO- exposure than adult nerve terminals.

This is indeed the case as reflected by mitochondrial function following ONOOexposure and which is discussed in section 5.4.3.

Although the levels of soluble total antioxidant capacity did not differ between adult and neonatal nerve terminals there is a distinct difference in the glutathione status of these two age preparations. This could be accounted for by other soluble antioxidant defence systems having a developmental profile in which their levels increased with development of the nerve terminal. Mavelli *et al.*, (1978) have reported a trend in increase of superoxide dismutase activity with brain development. Although Nanda and Collard (1995) have found that total brain GSH levels increase between postnatal day 10 and adult rat brain the data presented here is in contrast to that. Glutathione has been found to be enriched in synaptosomes (Reichelt and Fonnum, 1969) and therefore whole brain GSH measurement may not accurately reflect developmental trends in nerve terminals. The data here specifically measures GSH levels in synaptosomes and is more directly relevant to the susceptibility of the nerve terminal to oxidative stress.

At the end of thirty minutes of metabolic stress, such as ischaemia and hypoxia, there is a distinct difference in the glutathione status of adult as compared with neonatal synaptosomes. Adult nerve terminals do not show any significant change in GSH concentration under either ischaemia or hypoxia. Ischaemic neonatal nerve terminals exhibit a significant increase in GSH concentration although this pattern is not evident under hypoxic conditions. It is possible that GSH concentrations are increased in neonatal ischaemic nerve terminals alone because the mechanisms which lead to an increase in synthesis are not able to function under the other conditions. Alternatively, it is possible that an initial response to metabolic stress in both adult and neonatal nerve terminals is an increase in GSH levels. In the adult nerve terminals this higher level of GSH may begin to deplete as free radicals are produced and therefore react with the GSH. The lack of significant increase in GSH in the adult nerve terminals seen here could be a reflection of a higher rate of GSH depletion in these nerve terminals.

The data presented here do not enable any further insight as to differences in mechanisms connected with GSH synthesis or depletion in adult as compared with neonatal nerve terminals under metabolic stress. However, it is reported that in the adult brain, ischaemia is associated with GSH depletion (Mizui *et al.*, 1992) whereas a direct comparison between adult and neonatal GSH status following ischaemia has not previously been reported. It would be of interest to further probe whether there are fundamental differences in response of the neonatal nerve terminal which enables GSH levels to be significantly increased under ischaemia and hence lead to protection of mitochondrial function.

5.4.3 <u>Synaptosomal mitochondiral respiratory chain enzyme activity following</u> <u>ONOO- exposure</u>

5.4.3.a Adult synaptosomes

This work demonstrates that following a bolus exposure to ONOO- (which would equate to a 15 minute exposure to 1 μ M concentration) and a recovery incubation period of thirty minutes mitochondrial function was compromised in adult synaptosomes. Synaptosomal ATP levels were significantly decreased under these conditions. This decrease in ATP status correlated with a significant decrease in the activity of complex II-III and complex IV. All other complex activities remained unaffected. This pattern of damage in neuronal mitochondria exposed to ONOO- has been previously reported (Bolaños *et al.*, 1995) although ATP levels were not measured in this particular study. Bolaños *et al.*, (1994, 1995) have shown the lack of a direct effect of ONOO- on complex IV activity in isolated mitochondria, but an inhibition of complex IV activity in cultured neurones. This would suggest that intracellular processes are involved in the damage which occurs to complex IV following ONOO- exposure. The data presented in this thesis is in agreement that complex IV activity is significantly decreased after ONOO- exposure when mitochondria are in a cytosolic environment in the nerve terminal.

Complex IV activity correlates directly with the concentration of cardiolipin, a phospholipid susceptible to lipid peroxidation (Soussi *et al.*, 1990). Because ONOOmay initiate lipid peroxidation (Beckman *et al.*, 1990; Radi *et al.*, 1991) it is possible that this process is responsible for the observed inactivation of complex IV in intact cells and synaptosomes.

5.4.3.b Neonatal synaptosomes

Unlike adult synaptosomes, ONOO- exposure did not lead to compromised mitochondrial function in neonatal nerve terminals. There was no significant change in activity of any of the respiratory chain enzymes and ATP levels were not significantly different from control values. These results show that neonatal nerve terminals are highly resistant to exogenous ONOO-, whereas adult nerve terminals are more susceptible. It is plausible that the increased resistance of neonatal nerve terminals is connected with the higher GSH concentration in these nerve terminals as compared with adults. Following the implication that ONOO- may be involved in ischaemia/reperfusion damage the results of this study provide evidence in favour of a role for NO and ONOO- in the pathogenesis of neonatal brain to neuronal ischaemia/reperfusion injury may involve a decreased susceptibility to ONOO-mediated mitochondrial damage.

5.4.4 Effects of ONOO- exposure on extrasynaptosomal glutamate concentrations

The data presented here shows differences in the glutamate homeostasis of adult nerve terminals as compared with neonatal nerve terminals following ONOO-exposure. It has been found that ONOO- inhibits the reuptake of glutamate in reconstituted liposomes (Trotti *et al.*, 1996). In adult nerve terminals the effects of ONOO- on mitochondrial function are such that energy failure occurs. Under such conditions of energy failure the Ca²⁺-dependent release of glutamate is inhibited (Kauppinen *et al.*, 1988) but the Ca²⁺-independent glutamate release, due to reversal of the Na⁺-dependent uptake pathway, increases (Levi *et al..*, 1993; Kauppinen, 1994).

An increased release of glutamate from adult nerve terminals coupled with a decreased uptake due to the effects of ONOO- on glutamate transporters (Trotti *et al.*, 1996) would combine to cause a significantly increased concentration of glutamate in the extrasynaptosomal medium. In neonatal nerve terminals energy status is not compromised following ONOO- exposure and therefore increased glutamate release may not occur. This data can give no further insight however as to whether fundamental differences exist in the effects of ONOO- on glutamate homeostasis *per se* (ie. effects on reuptake) between adult and neonatal nerve terminals. However, this data does show that under the conditions of oxidative stress imposed on the two age nerve terminals increased glutamate would be present in the synaptosomal cleft, implicating a potential role for excitotoxic mechanisms to occur in the adult but not the neonate.

CHAPTER 6 DISCUSSION AND CONCLUSIONS

"Science is always wrong. It never solves a problem without

creating ten more"

George Bernard Shaw

<u>Chapter 6</u>

CONCLUSIONS

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CHAPTER 6

CONCLUSIONS

This thesis is based on the well documented observation that young, immature mammals show a heightened resistance to episodes of ischaemia in comparison with fully matured adults. The question underlying the thesis was whether this heightened resistance could be explained by differences in the response of neonatal and adult brain nerve terminals, during ischaemia/reperfusion.

6.1 Aims of the thesis

6.1.1 A valid model

The first aim of the work involved in this thesis was to characterise a valid model of neonatal and adult nerve terminals which could be subjected to conditions relevant to ischaemia and reperfusion. This aim was realised, as discussed in chapter 2, and postnatal day 10 rat pup synaptosomes were compared with adult rat synaptosomes throughout the studies in this thesis.

6.1.2 A comparison between different age nerve terminals

The main aim of this thesis was to examine a select number of nerve terminal activities and to directly compare/contrast any changes in these nerve terminal activities between adult and neonatal nerve terminals under conditions of metabolic stress. Review of the current literature concerned with ischaemia and reperfusion led to the following distinct areas being investigated in neonatal and adult nerve terminals under conditions of metabolic stress:-

calcium homeostasis, glutamate homeostasis, mitochondrial function, oxidative stress.

Table 6.1

Comparing and contrasting specific responses of adult and neonatal synaptosomes to conditions of combined oxygen and glucose lack.

 \checkmark ...refers to a response, mechanism or biochemical state that changed under conditions of combined oxygen and glucose lack, (or reperfusion where detailed).

 \times ...refers to a response, mechanism or biochemical state which remained unchanged.

	ADULT	NEONATE
[Ca ²⁺] in the synaptosomal cytosol	/	×
extrasynaptosomal glutamate concentration	1	×
complex I activity during ischaemia	\checkmark	J
complex I activity after reperfusion	×	×
complex II-III activity during ischaemia	×	
complex II-III activity after reperfusion	1	×
complex IV activity during ischaemia	×	×
complex IV activity after reperfusion	1	×
ATP concentration	1	1
NO concentration	1	×
GSH concentration	×	1

Table 6.2

Comparing and contrasting adult and neonatal synaptosomes.

different ...refers to a response, mechanism or biochemical state that was different between the two age groups of synaptosomes.

same...refers to a response, mechanism or biochemical state that was the same in the two age groups of synaptosomes.

	status between age groups
Resting cytosolic calcium concentration	different
Nitric oxide synthase activity	different
Total antioxidant status	same
GSH status	different

Conclusions

There were a number of distinct differences between adult as compared to immature nerve terminals in terms of the afore mentioned biochemical areas. Differences in each of these areas have been thoroughly discussed in sections 3.4, 4.4 and 5.4. To enable a clear comparison between adult and immature nerve terminal table 6.1 details the areas where responses were different under conditions of combined oxygen and glucose lack. The shaded responses indicate an area where the response of the neonatal nerve terminal was different to the adult nerve terminal. During the investigations it became clear that there were a number of fundamental differences in adult and neonatal nerve terminals under normal conditions that may play a part in the different responses during ischaemia. These differences are detailed in table 6.2.

6.1.2.a Ischaemic adult nerve terminals

It became clear that under conditions of combined oxygen and glucose lack the cytosolic Ca²⁺ concentration significantly increased in adult nerve terminals. This was assumed to be connected with the decrease in available ATP, which would compromise the usual Ca^{2+} buffering capability of the nerve terminal. The decreased energy status was also assumed to be linked to the altered glutamate homeostasis in the adult nerve terminals such that there was a significant increase in the concentration of extrasynaptosomal glutamate. Following these initial observations it was hypothesised that the increased Ca^{2+} concentration would increase the activity of the calcium dependent enzyme nitric oxide synthase in the nerve terminal, so that upon restoration of oxygen during reperfusion an increase in nitric oxide production would occur. This was confirmed by measuring nitrite and nitrate production and it was found there was a significant increase in nitric oxide production following ischaemia/reperfusion. The increased glutamate in the extrasynaptosomal medium was found to have a limited influence on the increase in cytosolic Ca²⁺ concentration probably through glutamate receptors on the nerve terminal membrane, however it did cause some of the increase in calcium and therefore in an indirect manner could play a role in the increased nitric oxide production.

Conclusions

It was hypothesised at this point in the study that if nitric oxide was being produced during initial reperfusion this had the potential for causing inhibition of mitochondrial function. It was therefore thought that nitric oxide may inhibit mitochondrial function so that ATP production would be affected, and this may in part explain why ATP levels were still significantly decreased after 30 minutes of reperfusion in the adult nerve terminals, even though oxygen and glucose levels were restored. In the adult nerve terminals it was found that following 30 minutes of reperfusion after metabolic stress there was a significant decrease in the activities of complex II-III and complex IV.

It also became clear that during ischaemia itself there was a different pattern of complex activity inhibition from that found in the neonatal nerve terminal. In both neonatal and adult nerve terminals complex I activity was significantly inhibited and therefore the flux of reducing equivalents (from NADH) through this complex would be reduced. However, unlike the neonatal nerve terminals, complex II-II activity was not affected by oxygen and glucose lack so that potentially the influx of reducing equivalents through this complex could still occur to the same extent as in control conditions. Complex IV and complex V activities were also not affected. In these conditions, where the terminal electron acceptor - oxygen - was not present, it is suggested that this pattern of complex activities may mean that the mitochondrial respiratory chain would be in a more highly reduced state in the adult than in the neonate. Reducing equivalents could be fed into the chain via complex II but the chain could not become oxidised by oxygen. Under conditions of ischoemia where the electron transport chain components would pile up under a more reduced state the opportunity for a burst of spontaneous oxidation upon reperfusion and the production of reactive oxygen species could increase. It is supposed that the univalent reduction of oxygen might actually increase under such reduced conditions, and it has been suggested that upon reperfusion after an ischaemic or hypoxic incident there would be a high production of the superoxide radical.

Conclusions

The state of the adult nerve terminals at the end of ischaemia included increased nitric oxide synthase activity and a possibly more reduced mitochondrial respiratory chain. It was, therefore, further hypothesised that concurrent production of superoxide and nitric oxide could lead to peroxynitrite formation during initial reperfusion in the adult nerve terminals.

Therefore these adult nerve terminals were directly exposed to peroxynitrite to assess whether the patterns of mitochondrial complex damage following such exposure would mimic those patterns of mitochondrial complex damage seen following a thirty minute period of reperfusion after ischaemia. The same pattern of complex II-III and complex IV inhibition was seen following peroxynitrite exposure. Furthermore such complex inhibition led to a decreased ability of the nerve terminals to generate sufficient ATP to fully support energy requiring processes and a decrease in ATP concentration occurred.

Following these observations it was concluded that in adult nerve terminals the production of peroxynitrite during reperfusion after an ischaemic event may play a role in producing inhibition of mitochondrial function. Such mitochondrial inhibition could lead to a long term reduction in the ability of the nerve terminal to produce ATP.

In light of these observations, it is possible that when much of the normal nerve terminal function becomes restored during extended reperfusion and the energy demands of the synapse increase the normal homeostatic mechanisms of the terminal may once again become compromised due to ATP depletion. Under such conditions damaging biochemical mechanisms including increased calcium, excitotoxic levels of glutamate and increased free radical production could once again be initiated. Ultimately, neuronal death may occur some hours after the initial ischaemic insult although oxygen and glucose are freely available to the cells at this time.

6.1.2.b Ischaemic neonatal nerve terminals

Unlike the situation in adult nerve terminals there was no increase in cytosolic Ca^{2+} concentration in the neonate during oxygen and glucose lack. The decrease in ATP was similar in both adult and neonatal nerve terminals under these conditions, which suggested that in neonatal nerve terminals a simple direct link between decreased ATP levels causing increased cytosolic calcium concentration did not exist. Although the decrease in energy status was similar in both age nerve terminals, there was no significant increase in the concentration of extrasynaptosomal glutamate in the neonatal nerve terminals.

These observations suggested that calcium and glutamate homeostasis in the nerve change as the brain develops.

It was hypothesised that the lack of Ca^{2+} increase in the neonatal nerve terminal would lead to a lower activity of nitric oxide synthase upon initial reperfusion in these terminals, as opposed to adults. It was possible, however, that the calcium dependency of this enzyme could be different between adult and neonatal preparations, indicating that this hypothesis may not hold. Nitrite and nitrate measurements confirmed that upon initial reperfusion the activity of nitric oxide synthase in neonatal nerve terminals was such that no increase in nitric oxide was apparent.

Under conditions of oxygen and glucose lack complex I and complex II-III activities were significantly inhibited in the neonatal nerve terminals. The flux of reducing equivalents into the mitochondrial respiratory chain may possibly, therefore, be inhibited to a larger degree than in the adult nerve terminals. It is, therefore, suggested that less production of free radicals may occur from the respiratory chain during initial reperfusion in the neonate because the individual components are less highly reduced than in the adult nerve terminals.

In the neonatal nerve terminals there was a much greater restoration of ATP levels following 30 minutes of reperfusion than in the case of the adult. Mitochondrial function was not found to be compromised in the neonate, in so far as the activities

Conclusions

of all the complexes were at control levels. It was, therefore, conlcuded that increased production of free radicals during ischaemia/reperfusion was the primary cause of mitochondrial dysfunction in the adult nerve terminals. Furthermore, that the key to the increased recovery of energy status seen in neonatal nerve terminals was a lack of increased free radical production during ischaemia/reperfusion.

Direct exposure of neonatal nerve terminals to peroxynitrite caused no mitochondrial dysfunction in that complex activities were not affected and the ability to generate ATP was not compromised. It thus became apparent that neonatal nerve terminals have an inherently greater resistance to oxidative stress than adult nerve terminals. Although the total soluble antioxidant capacity of both age nerve terminals is not significantly different, the glutathione status of neonatal nerve terminals is significantly higher than adults. Furthermore, this study showed that under ischaemic conditions the glutathione status of neonatal nerve terminals significantly increased, whereas, there was no change in the adults. It is, therefore, suggested that glutathione is an important and pivotal antioxidant in nerve terminals. The increased resistance of neonates to neuronal death following ischaemia and initial reperfusion may be concerned with the glutathione status, amount of oxidative stress and mitochondrial function of synapses.

6.1.3 A comparison of possible interplay between nerve terminal activities in adults and neonates.

In adult nerve terminals, comparison of the effects of oxygen lack and combined oxygen and glucose lack can allow some insight into the interplay between a number of biochemical processes during ischaemia/reperfusion. Both hypoxia and ischaemia led to the same pattern of mitochondrial complex damage and similar lack of recovery of ATP in the adult nerve terminals. In light of the effects of imposed oxidative stress (in the form of peroxynitrite exposure) on adult nerve terminal mitochondrial function, this would suggest that the degree of oxidative stress is similar in both hypoxic and ischaemic adult nerve terminals. The glutathione status is indeed similar in hypoxic and ischaemic adult nerve terminals.

Conclusions

At the end of a 30 minute incubation period of either hypoxia or ischaemia adult nerve terminals exhibited the same pattern of inhibition of mitochondrial respiratory chain complexes. Thus the redox state of the respiratory chain and free radical production from it could plausibly be the same in both hypoxic and ischaemic conditions. It is therefore suggested that under hypoxic and ischaemic conditions mitochondrial dysfunction is similar in adult nerve terminals. There are, however, apparent differences in some of the nerve terminal activities. The cytosolic calcium concentration and extrasynaptosomal glutamate concentration differ markedly between hypoxic and ischaemic adult nerve terminals.

A lack of increase in cytosolic calcium and extrasynaptosomal glutamate concentration in the hypoxic adult nerve terminals is also seen in neonatal nerve terminals under all conditions of metabolic stress. However, no obvious mitochondrial dysfunction is apparent in the neonatal nerve terminals.

From all of these observations the following conclusions have been drawn:-

1 The primary difference between adult and neonatal nerve terminals under conditions of metabolic stress is the functional state of mitochondria after a period of reperfusion/reoxygenation.

2 Increased cytosolic calcium levels and increased extrasynaptosomal glutamate concentrations may not be the primary causative factors of mitochondrial complex damage, and it is suggested that other factors are involved.

3 In adult nerve terminals ischaemic conditions cause a loss of calcium and glutamate homeostasis which is not seen under hypoxic conditions. However, under both conditions respiratory chain complexes become inhibited, suggesting that factors not including cytosolic and glutamate homeostasis must be equally affected by hypoxic and ischaemic conditions in adult nerve terminals.

Conclusions

Chapter 6

6.2 Suggestions for further work

In all good scientific research, a series of experiments will raise further avenues of investigation, and even questions that remain to be answered. The studies conducted and presented in this thesis do raise a number of ideas, concerns and questions which, if time had permitted, would have been ideal to have explored further. If these studies were to be taken further the author would suggest the following:-

1 Further investigation of the mechanisms of mitochondrial respiratory chain complex inhibition by addition of known free radical scavengers such as vitamin E and mannitol to the adult synaptosome to see if there was attenuation to the complex inhibition.

2 Inhibition of synaptosomal nitric oxide synthase activity by known inhibitors and/or increased scavenging of superoxide by addition of exogenous superoxide dismutase to probe whether NO, O_2^{-} and ONOO- play a part in the mitochondrial respiratory chain complex activity observed in adult nerve terminals.

3 Manipulation of the glutathione status of adult and neonatal nerve terminals by increasing and decreasing levels to assess whether there is a link between nerve terminal glutathione status and mitochondrial complex inhibition during ischaemia/reperfusion.

4 Investigation of changes in mitochondrial calcium changes during ischaemia/reperfusion in adult and neonatal nerve terminals. This would be to assess whether there appears to be a more direct link between mitochondrial calcium levels and mitochondrial dysfunction than there appears to be between cytosolic calcium levels and mitochondrial dysfunction.

5 A series of investigations to assess the consequences for long term synaptic function of the apparent mitochondrial dysfunction in adult nerve terminals following

Conclusions

ischaemia and short term reperfusion. Synaptosomes have a finite life span of a few hours and therefore an alternative model would be necessary for such studies, such as mature cultured neurones or *in vivo* ischaemia and long term reperfusion followed by synaptosome isolation.

6 Investigation into whether there are later changes in neonatal nerve terminals following more long term reperfusion which have not been observed in the short term studies in this thesis and which may play a role in delayed neuronal death in the neonate.

6.3 Main conclusion

The increased resistance of the neonatal brain to ischaemia/reperfusion is well documented. The studies presented in this thesis have shown that there are a number of differences in the response of neonatal as compared with adult nerve terminals to conditions of *in vitro* ischaemia. The increased resistance of neonatal brain to ischaemia/reperfusion may at least in part be concerned with an increased resistance of neonatal nerve terminals to conditions of oxidative stress. In adult nerve terminals *in vitro* ischaemia leads to inhibition of mitochondrial respiratory chain complexes and a lower recovery of ATP levels than in the neonate. Neonatal nerve terminals exhibit an increase in glutathione status during ischaemia and no apparent mitochondrial complex inhibition following 30 minutes of reperfusion. The impact of these short term differences in nerve terminal status in long term post-ischaemic neuronal activity and function is unknown. Such observations warrant further study.



CHAPTER 7

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APPENDIX PUBLICATIONS RESULTING FROM THIS STUDY

"Education's real challenge is to produce men and women who know how to think; and knowing how, do it; and having done it, voice their opinions".

Henry T. Heald. The New York Times Magazine

Intrasynaptosomal Free Calcium Concentration During Rat Brain Development: Effects of Hypoxia, Aglycaemia, and Ischaemia

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Abstract: The effects of hypoxia, aglycaemia, and hypoxia-aglycaemia on intrasynaptosomal free Ca2+ concentration ([Ca²⁺]_i) have been investigated in rat brain synaptosomes prepared from animals aged 5, 10, 15, 20, 25, and 60 days. After 60 min of hypoxia there was no significant difference, when compared with controls, in basal [Ca²⁺] or [Ca²⁺] following depolarisation in all of the ages studied. Following 60 min of aglycaemia there was no significant difference from controls in [Ca2+], of synaptosomes prepared from pups of ≤20 days, although a significant rise in [Ca²⁺], was seen in preparations from animals >20 days old. Sixty minutes of hypoxia-aglycaemia led to a significant rise in [Ca2+] only in preparations from animals 15-60 days old. With both aglycaemia and hypoxia-aglycaemia a progressive increase in the magnitude of the rise in [Ca²⁺], was seen with development. These data suggest increases in [Ca²⁺], in adult nerve terminals following prolonged aglycaemia and hypoxiaaglycaemia but no change following prolonged hypoxia. In contrast, no significant changes in [Ca2+], values were apparent in neonatal nerve terminals under any of these conditions. In control synaptosomes with glucose and oxygen freely available, a decrease in resting and depolarised [Ca2+] during development was seen, suggesting a change in calcium homeostasis within the nerve terminal as the brain develops. It is suggested that the mechanism underlying the relative resistance to ischaemic damage of neonatal brain as compared with adult brain may be related to the regulation of calcium at the nerve ending. Key Words: Brain-Synaptosomes-Calcium-Hypoxia-Ischaemia-Development.

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During the first weeks of life, the brain undergoes considerable changes in composition (Dobbing and Sands, 1979) and development of enzyme systems that are concerned with energy metabolism (Clark, 1990). Associated with this brain maturation is an increase in the sensitivity to periods of oxygen and fuel deprivation (Volpe, 1990). The greater resistance of neonatal as compared with adult mammals to episodes of hypoxia and ischaemia was observed many decades ago (LeGallois, 1813). The extent of this resistance is striking, illustrated in one study by a survival time of 25 min for newborn rat pups in 100% nitrogen compared with only 1 min for adult rats (Duffy et al., 1975). Following on from such observations, many workers have studied the biochemical mechanisms instigated by lack of oxygen and substrate (Rosenthal, 1986; Siesjö et al., 1989; Choi and Rothman, 1990; Gluckman and Williams, 1992). What has not yet been fully elucidated is the exact sequence of events that lead to cell death and which biochemical changes are the critical ones. The extremely rapid time-course of energy failure and resultant cell death following even brief periods of hypoxia-ischaemia in the adult further complicate such research.

Few studies have focused on the investigation of the fundamental differences between postischaemic biochemical changes in neonatal as compared with adult synapses (Haddad and Jiang, 1993). However, such parallel studies could provide insight into what lies behind the resistance of neonatal nerves to oxygen and fuel lack if it is due to inherently different biochemical properties. Alternatively, such studies would allow a longer interval for looking at energy failure and subsequent biochemical changes if resistance is merely a reflection of greater conservation of ATP levels by the neonatal brain (Kass and Lipton, 1989), which leads to a greater likelihood of recovery when the brain is reperfused.

It is clear that mechanisms of cell death involve other processes apart from energy failure and extend beyond changes in glucose and energy metabolism (Volpe, 1990). There is good evidence for a pivotal role of increased intracellular calcium content in adult ischaemic neuronal cell death (Siesjö, 1981). Indeed,

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Abbreviations used: $[Ca^{2+}]_i$, intrasynaptosomal free Ca^{2+} concentration; fura-2/AM, fura-2 acetoxymethyl ester; LDH, lactate dehydrogenase.

the wealth of experimental data showing elevations of intracellular calcium levels after ischaemia has led to the "Ca²⁺ hypothesis," which predicts that cellular Ca²⁺ overload occurs during ischaemia and induces a cascade of events that lead to cell death (reviewed by Siesjö et al., 1989; Haddad and Jiang, 1993). In a comparative study using brain slices (Bickler et al., 1993) there was less calcium accumulation in neonatal brain than in adult brain during hypoxia.

We wanted to focus further on the correlation among brain development, cellular ATP maintenance, and overall neuronal calcium levels in different conditions of oxygen and/or glucose deprivation. Synaptosomes were chosen as a model to allow the study of events occurring at the nerve terminal alone as distinct from the neuronal cell body or surrounding glial cells. Synaptosomes can be prepared from rats at different stages of development and are energetically viable in the appropriate medium, maintaining plasma and mitochondrial membrane potential, ATP/ADP ratios, and the correct ionic concentrations across membranes (Nicholls, 1993).

Previous work with adult brain synaptosomes (Boakye et al., 1991) or brain slice preparations (Bachelard et al., 1993) revealed differences in Ca^{2+} homeostasis following a hypoxic insult compared with a combined hypoxic-aglycaemic insult. It was therefore decided to study changes in intracellular Ca^{2+} concentrations in synaptosomes prepared from brains of rats of different ages (5–50 days postpartum) following hypoxic, aglycaemic, and hypoxic-aglycaemic insults and to compare responses in the neonatal and adult preparations.

MATERIALS AND METHODS

Materials

The animals used in this study were rat pups of mixed sex (eight to 12 animals per litter) 5, 10, 15, 20, and 25 days old from adult female Wistar rats and were allowed food and water ad libitum. Adult male Wistar rats 60 days old were also used. All rats were obtained from B and K Universal (Aldborough, Hull, U.K.)

Fura-2 acetoxymethyl ester (fura-2/AM) was purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). ATP luciferin-luciferase monitoring reagent and standard were BioOrbit (Turku, Finland) products, purchased from Labtech, U.K. (Uckfield, East Sussex, U.K.). Ficoll was from Pharmacia (Uppsala, Sweden) and was dialysed against distilled water before use. Glutamate dehydrogenase (EC 1.4.13) was from Sigma and was dialysed against phosphate buffer (100 mM, pH 7.3) to remove contaminating glutamate. All other chemicals were of analytical grade made up in deionised double-distilled water and were purchased from either BDH Ltd. (Dagenham, Essex, U.K.) or Sigma.

Synaptosome preparation

Brains were rapidly removed and placed in isolation medium (320 mM sucrose, 1 mM K₂EDTA, and 10 mM Tris-HCl, pH 7.4 at 4°C) within 30–60 s. Purified and crude synaptosomes were prepared essentially by the method of Booth and Clark (1978). In brief, 1 volume of scissorchopped forebrains was homogenised in 9 volumes of isola-

tion medium at 4°C. The homogenate was centrifuged at 1,500 g_{av} for 3 min using a Beckman JA-20 rotor at 4°C, and the resultant supernatant was centrifuged at 17,400 g_{av} for 10 min. In experiments using crude synaptosomes, care was taken to resuspend these from the resultant pellet and leave behind the central brown mitochondrial pellet. When purified synaptosomes were used, these were produced by density centrifugation on a Ficoll gradient, as in the original method for adults (Booth and Clark, 1978) and by a slight modification of the gradient for younger age pups (Almeida et al., 1995). It is of utmost importance that any functional differences observed between synaptosomes from adults and neonates are not due to inherent differences in the preparations. Therefore, the density gradient was altered to 10/6/ 4% Ficoll for all pups <21 days. This gave a comparable integrity and purity to the adult preparation (<8% free mitochondria and <20% total contamination for all preparations) as determined by marker enzymes detailed in the original method (Booth and Clark, 1978). All synaptosomes were pelleted (14,000 g_{av} , 10 min) in a modified Krebs phosphate buffer (141 mM NaCl, 5 mM KCl, 1.3 mM MgSO₄, 10 mM Na₂HPO₄, 10 mM glucose, and 1.2 mM CaCl₂, pH 7.4) that had previously been gassed with pure oxygen (oxygen concentration = 23 μ l of O₂/ml, 932 μ M, 37°C). Synaptosome pellets were kept on ice until use, which was always within 1 h.

Incubation conditions for hypoxia/aglycaemia

Synaptosomes were initially rendered hypoxic by resuspension in modified Krebs phosphate buffer that was pregassed with pure nitrogen at 37°C for 1 h (oxygen concentration = 2.64 μ l of O₂/ml, 103 μ M, 37°C, 11% of normoxic controls). Synaptosomes were then incubated in a shaking water bath (37°C) for 60 min in vials that were capped and carrying a gassing line (delivering nitrogen gas) suspended above each incubation. Evaporation was <2% using this method. In all experiments synaptosomes were initially hypoxic following resuspension in this buffer and became anoxic by 15 min owing to their active respiration and hence consumption of the remaining oxygen. These incubation conditions are therefore best described as hypoxia/anoxia. Synaptosomes were made ischaemic using this protocol but with glucose omitted from the buffer. Aglycaemic conditions were created using essentially the same protocol as ischaemic conditions but with gassing with pure oxygen. For all experiments normoxic controls were carried out similarly but using pure oxygen and glucose-containing buffer. Synaptosomal integrity was not compromised using this protocol. Measurement of lactate dehydrogenase (LDH) activity (Clark and Nicklas, 1970) in both experimental and control synaptosome preparations showed <15% leakage of LDH after a 60-min incubation in all conditions, with no significant differences between synaptosomes prepared from animals of different ages. A 60-min incubation period was chosen to try and reflect a sufficiently severe insult to affect neonatal energy status.

Intrasynaptosomal calcium measurement

The fluorescent indicator fura-2 was used to determine the intrasynaptosomal calcium concentration. The synaptosomes were loaded with the ester form of this indicator in the dark during the final 30 min of the 60-min incubation period described previously. Protein concentration during loading was ~ 5 mg/ml, and the fura-2/AM final concentration was 5 μ M dissolved in anhydrous dimethyl sulphoxide. The 30-

min loading period also allowed the internalized fura-2/AM to be hydrolysed to the Ca²⁺-sensitive free acid form, which was established by comparison of an excitation scan (340-400 nm with 510 nm emission) of loaded samples against a scan of the free fura acid (potassium salt). Autofluorescence was allowed for by performing identical incubations containing synaptosome suspensions in the different buffers and 0.5% dimethyl sulphoxide without fura-2/AM. Fura-2 loading was terminated by a rapid 10-fold dilution with either the control Krebs buffer (gassed with oxygen and kept at 37°C throughout the experiment) for the control or aglycaemic samples or with hypoxic buffer (11% oxygen, 37°C) for the hypoxic/anoxic and ischaemic samples. All diluted samples were centrifuged at 10,000 gav for 2 min in 1.5ml aliquots. The fluorescent probe was eliminated from the extrasynaptosomal medium, immediately before measurements were performed, by washing the pellet three times with the appropriate Krebs buffer at 37°C. Pellets were resuspended in either control or hypoxic buffer immediately before fluorescence measurement. Preliminary experiments showed that extensive reoxygenation of the hypoxic/anoxic and ischaemic samples did not occur during these manipulations or the fluorescence measurements. The maximal amount of oxygen available to these samples throughout was 13% oxygen tension as determined by an oxygen electrode. To determine the cytosolic free Ca²⁺ concentration, fluorescence was measured in synaptosomal preparations (final concentration, 2 mg/ml) using a Perkin-Elmer model LS50B luminescence spectrometer, at 340 and 380 nm excitation wavelengths and 510 nm emission wavelength. The instrument was operated using the ratiometric method to eliminate any inconsistencies in dye loading or leakage (Grynkiewicz et al., 1985) in synaptosomes from different age rats. Synaptosomes were depolarised by addition of 50 mM (final concentration) potassium chloride. Calibration of the fluorescent signals was performed at the end of each individual experiment by adding Triton X-100 (final concentration, 0.5%) in the presence of 1.2 mM CaCl₂ to obtain R_{max} , followed by 30 mM (final concentration) EGTA (adjusted to pH 8.0 with NaOH) to obtain R_{\min} (Komulainen and Bondy, 1987). The autofluorescence of the synaptosomes in the absence of dye was routinely subtracted from the total fluorescence. The cytosolic free Ca^{2+} concentration was calculated using a K_D of 224 nM for the Ca^{2+} -fura-2 complex (Grynkiewicz et al., 1985).

Protein content determinations

Protein concentrations were measured using the method of Lowry et al. (1951), with bovine serum albumin as the standard.

ATP level measurements

ATP was acid-extracted from synaptosomal suspensions using 3.5% (final concentration) ice-cold perchloric acid. Extracts were neutralized with 0.7 M K₂HPO₄ (final concentration), kept on ice for 30 min, and then pelleted (10,000 g_{av} for 2 min). Resultant supernatants were snap-frozen in liquid nitrogen and kept frozen at -80° C until the time of assay. ATP concentration was measured with a luciferin– luciferase bioluminescence assay (Thore, 1979) at 30°C on an LKB Wallac model 1251 Luminometer.

Removal of glutamate from incubation mixtures

To assess the potential role of presynaptic metabotropic glutamate receptors in possible calcium level changes in the



FIG. 1. Changes in resting (\bigcirc) and depolarised (\bullet) calcium concentrations with increasing age of the rats used for synaptosome preparation. Data are mean \pm SEM (bars) values (n = 6 separate preparations).

conditions used, it was necessary to remove any released endogenous glutamate from the incubations. For this purpose NADP⁺ (final concentration, 2 mM) and 75 U of glutamate dehydrogenase were added to relevant incubation mixtures.

Statistical analysis

Results are expressed as mean \pm SEM values of the number of different experiments indicated in the legends (n). Three-way ANOVA was used to analyse all the data. When the difference among the group means was statistically significant, a least square difference test was performed, and a result of p < 0.05 was taken to be significant.

RESULTS

Calcium levels during development

Figure 1 shows the developmental changes of resting and depolarised intrasynaptosomal calcium levels under control conditions of normoxia and normoglycaemia. Resting and depolarised calcium levels decrease with the age of the rat. Statistical analysis of the data revealed that results obtained from animals 5-25 days old were significantly different from those obtained from mature 60-day-old brain (p < 0.01). To ensure that the higher values of intrasynaptosomal free Ca^{2+} concentration ($[Ca^{2+}]_i$) in synaptosomes from younger-age animals were not due to a higher incidence of damaged synaptosomes, LDH activity was measured as a marker of synaptosomal integrity. The LDH release following a 1-h normoxic incubation was <10% of total LDH activity in all ages, with no significant difference between adult and neonatal preparations, thus indicating that synaptosomal integrity was similar for all ages. Addition of N, N, N', N'-tetrakis(2pyridylmethyl)ethylenediamine, a permeant heavy metal chelator (Ashley et al., 1984), did not modify the signals in samples prepared from either immature



FIG. 2. Comparison of the changes in resting calcium content compared with control values (expressed as a percentage of the control) following hypoxia, aglycaemia, and hypoxia-aglycaemia in synaptosomes from different ages. Results are expressed with respect to control values for the appropriate age incubation under normal oxygen and glucose conditions as mean \pm SEM (bars) percentages (n > 4 separate preparations for each age and each condition). *p < 0.05. Control values were 453, 436, 458, 415, 395, and 260 n*M* for animals 5, 10, 15, 20, 25, and 60 days old, respectively, as shown in Fig. 1.

(10-day-old) or adult (60-day-old) animals, indicating there was no overestimation of values due to the presence of high concentrations of trace metals (Ashley et al., 1984).

Potential differences in autofluorescence of immature and adult samples were accounted for because autofluorescence was routinely checked and subtracted from final fluorescence in all samples. Fura-2/AM loading was also assessed to ensure there were no differences across the ages. This was done by taking R_{max} as an indicator of fura-2 loaded and converted to the free acid form from the ester. There were no significant differences in the amount of fura-2 loaded into the synaptosomes throughout development, and loading was assessed to be ~80 ± 7% (n = 3) confined to the cytosol by differentially permeabilising the synaptosome plasma membrane with 0.1% (vol/vol) digitonin and then releasing the remaining fura-2 with 0.5% Triton-X100.

Responses to glucose and oxygen deprivation

The effects of hypoxia/anoxia, aglycaemia, and ischaemia on resting (Fig. 2) and depolarised (Fig. 3) $[Ca^{2+}]_i$ are presented. Control (normoxic) values are shown as 100% (horizontal line), and results from the metabolic deprivations have been expressed as a percentage of the control. The calcium data clearly present a difference in response with increasing age in two of the metabolic deprivations (aglycaemia and

ischaemia) but not in the third (hypoxia/anoxia). Therefore, ATP levels were measured in the condition showing the most dramatic differences between adults and neonates (ischaemia) and compared with the condition showing no differences between the ages (hypoxia/anoxia). ATP levels were measured in 10- (Fig. 4a) and 60-day-old (Fig. 4b) preparations during control conditions and also hypoxia/anoxia and ischaemia. Thus, energy levels between synaptosome preparations from adults and pups could be compared in a condition where there was no difference in calcium response with a condition in which there was a marked difference in calcium response.

Hypoxia/anoxia. There was no significant change in resting or depolarised $[Ca^{2+}]_i$ following 60 min of hypoxia in all of the different age animals studied.

ATP concentrations were measured in immature (10-day-old) and adult (60-day-old) hypoxic synaptosomes and showed that ATP levels did not significantly fall over the 60 min of hypoxia in the immature preparations. ATP levels did, however, fall significantly after 10 min of hypoxia in the adult to 55% of the control level, and at 60 min ATP levels were 46% of the control level. The fall in ATP content to only approximately half of the control value reflects the fact that substrate (glucose) is not limiting and also that 11% oxygen is still available in the media, thus reflecting a mild hypoxic insult rather than anoxia (total



FIG. 3. Comparison of the changes in depolarised calcium content compared with control values (expressed as a percentage of the control) following hypoxia, aglycaemia, and hypoxia-aglycaemia in synaptosomes from different ages. Results are expressed as indicated in Fig. 2 as mean \pm SEM (bars) percentages (n > 4 separate preparations for each age and each condition). *p < 0.05. Control values were 606, 554, 586, 533, 509, and 380 n*M* for animals 5, 10, 15, 20, 15, and 60 days old, respectively.



FIG. 4. Changes in synaptosome ATP concentrations over time during control, hypoxia, or hypoxia-aglycaemia conditions: (a) day 10 synaptosomes and (b) adult synaptosomes. Data are mean \pm SEM (bars) values (n = 4 separate experiments). **p* < 0.05.

lack of oxygen) and the ability to allow maintenance of some ATP.

It is evident that the changes in ATP levels do not correlate with the overall change in $[Ca^{2+}]_i$ following hypoxia because significant changes in the ATP in the adult preparations are not accompanied by a significant rise in intrasynaptosomal Ca^{2+} levels.

Aglycaemia. A lack of glucose in the incubation medium led to significant (p < 0.01) increases in both basal and depolarised $[Ca^{2+}]_i$ in 25- and 60-day-old animals, when compared with controls. A slightly higher $[Ca^{2+}]_i$ was apparent in preparations from ≤ 20 -day-old animals, but these were not significant.

Ischaemia (hypoxia/anoxia-aglycaemia). In preparations from 5- and 10-day-old pups a change in $[Ca^{2+}]_i$ was notably absent under these conditions. The combination of a lack of oxygen and glucose caused a markedly larger increase in $[Ca^{2+}]_i$ than merely a

lack of glucose alone in preparations from animals that were 15–60 days old. In synaptosomes from animals \geq 15 days old the combined insult led to a significant (p < 0.01) increase in [Ca²⁺]_i compared with the control. There is an obvious change in the severity of the rise in calcium level as age increases from 15 to 25 days, in both resting and depolarised conditions. The rise in resting calcium level following ischaemia continues to increase from 25 (279%) to 60 days (306%) but not for depolarised conditions, where the levels are very similar for both 25- and 60-day-old rats (265 and 250%, respectively). In all ages, the addition of KCI led to a significant (p < 0.05) increase in [Ca²⁺]_i compared with the resting levels.

ATP levels were measured in immature (day 10) and adult (day 60) synaptosomes. In both age groups, ATP content had fallen dramatically and significantly (p < 0.01) over the 60-min period to 27 and 29%, respectively. The neonatal synaptosomes did maintain a higher level of ATP for a slightly longer interval, however, with levels first falling to <60% after 10 min, whereas levels were <45% in the first 5 min of the insult in the adult synaptosomes.

DISCUSSION

This work demonstrates an age-dependent decrease in calcium levels measured in rat brain synaptosomes obtained from animals 5-60 days old in both resting and depolarised conditions. The resting $[Ca^{2+}]_i$ reported here for adults (60-day-old rats) is comparable with values measured previously (100-300 nM) in synaptosomes obtained from adult rat brain (Boakye et al., 1991; Nicholls, 1993; Erecińska et al., 1994). It has been shown that damaged nerve terminals give rise to spuriously high resting [Ca²⁺]_i (Fontana and Blaustein, 1995). However, the low and consistent values of LDH release from preparations from rats of all ages used suggest that this is not the reason for the age-dependent decrease in [Ca²⁺]_i. In addition, synaptosomes at all ages under normoxic conditions exhibit a rapid increase in calcium content following depolarisation by KCl, which also indicates that they are "healthy" preparations able to maintain normal ion gradients. ATP concentrations measured over a 60-min period in the control synaptosomes in both immature (postnatal day 10) and adult (postnatal day 60) preparations fall within the published range of values (Erecińska et al., 1994) and show that energy levels were well maintained throughout the incubation period, thus also indicating that all the preparations were metabolically active.

The resting calcium concentration within synaptosomes is tightly regulated. There is a steady cycling of calcium across the plasma membrane of the synaptosome, and maintenance of resting calcium levels involves balance between a constitutive inward leak from the extracellular fluid and the export of the ion by an energy-requiring plasma membrane Ca^{2+} -ATPase (Miller, 1991). A $3Na^+/Ca^{2+}$ exchanger is also present on the synaptosomal membrane, although its activity at resting $[Ca^{2+}]_i$ is very low and it probably contributes minimally to maintenance of $[Ca^{2+}]_i$ levels (Miller, 1991).

In adult cells, resting cytosolic Ca²⁺ concentration is $0.1-0.3 \mu M$, and the set point at which mitochondria start taking up calcium is $1 \mu M$, with the resting intramitochondrial calcium concentration being 50-200 μM (Rizzuto et al., 1994), although little information is available for synaptic mitochondria from immature brain.

In relation to the resting calcium concentration in synaptosomes from immature brain, it may be that in the rat nerve terminal calcium homeostasis changes with development owing to increases in activity of the ion transporters such as the Ca²⁺-ATPase. Certainly, many enzymes associated with brain energy metabolism (Dobbing and Sands, 1979) and some of the individual complexes of the synaptic mitochondrial respiratory chain (Almeida et al., 1995) undergo considerable increases in activity during the course of normal brain development. Muller et al. (1993) have demonstrated increases in the activities of ATPase-ADPases in synaptosomes from rat cerebral cortex, attaining adult levels at 21 days postpartum. There is also a considerable increase in Na⁺,K⁺-ATPase activity during the first 4 weeks of development (Abdel-Latif et al., 1967). The activity of the Na^+/Ca^{2+} exchanger also increases between day 1 and 6 in cultures of primary neurons (Michaelis et al., 1994).

In neuronal cultures, the process of neurone development, neurite extension, and the motility of growth cones requires a close regulation of $[Ca^{2+}]_i$ in the range 100-300 nM. Such cultures (many of which are primary cultures from neonatal brain) often require an elevated $[Ca^{2+}]_i$ for optimal growth and neurite expression. These elevations can be created either by activation of Ca²⁺-transporting NMDA receptors or by partial depolarisation with KCl, and such partial depolarising conditions have been shown to remove partially a requirement for nerve growth factor (Solem et al., 1995). If $[Ca^{2+}]$ deviates from the intracellular level of 100-300 nM, then growth and motility cease, and the neurones may begin a process of programmed cell death (apoptosis) (Nicholls, 1994). Mailleux et al. (1993) suggested a change in calcium homeostasis during development, following studies showing that synaptogenesis corresponded with an increase in mRNA content for the inositol 1,4,5-trisphosphate receptor (which is concerned with mobilisation of intracellular calcium stores). All of these observations suggest that the developmental decrease in Ca²⁺ concentration reported in the present study is a reflection of a more complex regulation of calcium fluxes during development and that higher levels of $[Ca^{2+}]_i$ may be necessary in young nerve terminals, for normal growth and development, than in mature nerve terminals, where the levels of $[Ca^{2+}]_i$ measured in the synaptosomes from immature brain would probably be pathological.

Oxygen and glucose deprivation

The same conditions of glucose and oxygen deprivation were imposed on synaptosomes from immature and adult brain to enable direct comparison of the effects on the energy status and calcium homeostasis. The ATP data suggest that synaptosomes from immature brain can maintain their energy levels under the hypoxic conditions used, probably owing to the availability of glucose. ATP levels did fall to 50% of the control values in the adult synaptosomes, however, even though the same amount of glucose was available as to synaptosomes from immature brain. This may reflect an increased requirement for energy by adult synaptosomes or a change in their capabilities to synthesize ATP by aerobic/anaerobic mechanisms as compared with the synaptosomes from neonatal brain. In the rat it has been shown that the neonatal brain is capable of metabolising both glucose and ketone bodies for energy provision (Clark, 1990) and that the facility for full oxidative metabolism does not develop until postweaning (Almeida et al., 1995). Thus, a transient deficit in oxygen supply may be less detrimental to the energy supply for the neonatal brain than is the case for the fully matured adult.

The lack of increase in $[Ca^{2+}]_i$ over the 60-min hypoxic insult in the synaptosomes from immature brain is probably due to the maintenance of ATP levels in these conditions and hence the maintenance of normal calcium homeostasis. In the adult situation, however, although a drop in ATP content did occur, there was still no increase in $[Ca^{2+}]_i$. This could reflect the maintenance of ATP above a "critical threshold" in the adult synaptosomes, such that intracellular calcium homeostasis was not compromised. A further possibility is that the fall in ATP levels after 10 min did lead to an increase in $[Ca^{2+}]_i$ but that over the following interval the partly ATP-depleted hypoxic synaptosomes reestablished a calcium gradient. In relation to this, Carroll et al. (1992) found with PC12 cells that a chemical hypoxia reduced ATP concentrations by 92% and that after 10 min $[Ca^{2+}]_i$ increased 2.5-fold above the control, but that after 30 min the calcium gradient was reestablished and that at this interval and also at 60 min $[Ca^{2+}]_i$ was equivalent in hypoxic and control cells. In a study comparing the effects of anoxia on preweanling and adult rat CA1 neurones, an increase in fluorescence due to calcium was seen in the adult neurones, followed by a decrease after 10 min even though the neurones were still anoxic (Friedman and Haddad, 1993). Such observations involving hypoxic adult neurones undergoing an increase in Ca² concentration as ATP levels decline raise the question: do prehypoxic levels of calcium become reestablished, possibly as calcium is sequestered into intracellular stores such as mitochondria, even though ATP levels remain depleted? However, this present study focuses on a direct comparison of responses between nerve terminals at different stages of maturity, and there is no difference in calcium levels after prolonged hypoxia/ anoxia at any stage of maturity.

In the present study, a combination of both oxygen and substrate deprivation led to a decline in ATP levels of the same extent in both adult and immature synaptosomes. However, this was not reflected in similar changes in cytosolic calcium levels. Only in more mature preparations (>10 days) did the combined insult cause a significant increase in cytosolic calcium levels. This is in agreement with a study of chemical hypoxia (using brain slices in the presence of cyanide), where a similar developmental profile of calcium levels was observed (Bickler et al., 1993). The authors showed that only brain slices from animals > 2 weeks old showed rapid decreases in ATP levels and rapid increases in intracellular calcium during hypoxia. In addition, after a 20-min exposure to hypoxia, there was a much smaller rise in intracellular calcium in brain slices from animals <14 days old.

As the major increases in $[Ca^{2+}]_i$ were observed following hypoxia-aglycaemia in synaptosomes from older-age rats only, the possible involvement of presynaptic metabotropic glutamate receptors were assessed in 60-day-old rats. The enzymatic removal of any glutamate released under the conditions used (and hence prevention of metabotropic receptor involvement) attenuated the $[Ca^{2+}]_i$ rise seen after hypoxiaaglycaemia in these adult synaptosomes. However, the $[Ca^{2+}]_i$ was still significantly high when compared with controls (232% of the control after removal of glutamate). Thus, stimulation of glutamate receptors plays only a limited role in the calcium increases observed in the adult synaptosomes. Confirmation of the differences between glutamate and calcium homeostasis in adult and neonatal brain was given by glutamate release experiments (J. K., unpublished data), where there was increased release of glutamate from adult synaptosomes during hypoxia-aglycaemia compared with the control but no increase in these conditions in synaptosomes from neonates.

In summary, the differences in $[Ca^{2+}]_i$ measured in hypoxia and hypoxia-aglycaemia in the adult nerve terminals suggest that calcium content changes in adult brain ischaemia are not due merely to a lack of oxygen. In addition, these data suggest that neuronal damage due to a lack of oxygen involves different mechanisms in relation to calcium metabolism than those initiated by a combined lack of oxygen and substrate.

The data from immature nerve terminals suggest that resistance of neonatal neurones to hypoxia could be due to a prolonged maintenance of energy levels. However, this seems to be an unlikely explanation for the resistance to prolonged ischaemia as ATP levels decline similarly in the adult and immature synaptosome. It seems more likely that the discrepancy in changes in cytoslic calcium levels in the immature and adult nerve terminal following ischaemia plays a role in the resistance of neonates to ischaemic insult. Whether these variations are due to fundamental differences in calcium influx or storage and release from intracellular stores or whether they are due to an increased calciumbuffering ability of the neonatal nerve terminals (owing perhaps to increased mitochondrial uptake) merits further investigation and may give important information concerning the different ways we should treat neonatal and adult stroke patients.

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Nitric Oxide and Antioxidant Status in Glucose and Oxygen Deprived Neonatal and Adult Rat Brain Synaptosomes

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Nitric oxide (NO) has been implicated in the process of cerebral ischemia/reperfusion injury. We have examined the production of NO, as reflected by nitrite $(NO_2^-) + nitrate (NO_3^-)$ accumulation, from synaptosomes isolated from neonatal or adult rat brain and subjected to a period of glucose and oxygen deprivation. There was a significant increase in the amount of $NO_2^- + NO_3^-$ production from adult synaptosomes under these conditions, whereas there was no difference compared to control in the production of $NO_2^- + NO_3^-$ from the neonatal synaptosomes. The total antioxidant status of the synaptosomes at these different stages of brain development was found to be the same. These data suggest that the vulnerability of the adult brain to ischemia/reperfusion injury may be associated with the production of NO^o from nerve terminals. The ratios of antioxidant capacity to NO^o production under such conditions have been shown here to be different between the neonatal and adult nerve terminals. Thus the well documented resistance of neonatal brain to ischemia/reperfusion injury may involve the neonatal nerve terminal being under less oxidative stress than the adult.

KEY WORDS: Nitric oxide (NO), brain, synaptosomes, ischemia/reperfusion, neonatal, antioxidant.

INTRODUCTION

The adult mammalian brain is known to be vulnerable to damage during episodes of ischemia and reperfusion such as those experienced during a stroke or following cardiac arrest. It is well documented that the neonatal brain is more tolerant to such periods of ischemia/reperfusion (1), however the basis of this resistance is not fully understood. The biochemical mechanisms of neuronal injury that occur during an ischemic period and subsequent reperfusion are incompletely defined both in the neonate and adult.

² Department of Clinical Biochemistry, National Hospital for Neurology and Neurosurgery, Queen Square, London, WC1N 3BG, England. Ischemia and subsequent reperfusion favour the increased formation of free radicals such as superoxide (O_2^{-}) from sites including the mitochondrial respiratory chain (2), and the enzymatic action of xanthine oxidase (3). The conversion of superoxide by the enzyme superoxide dismutase produces hydrogen peroxide which is able to diffuse across cell membranes and in the presence of iron forms the highly reactive hydroxyl radical (OH). Another free radical, nitric oxide (NO), has recently received much attention in studies of normal and postischemic events (2,4). NO has been postulated to be a prime mediator (2,4) of neuronal injury, possibly as a result of peroxynitrite (ONOO⁻) formation, particularly following stroke (4). However the precise mechanism of that injury is not known.

In view of the known relative resistance of the immature brain to ischemia/reperfusion, we decided to investigate the response of nerve terminals (synaptosomes) from immature and adult rat brain to a period of glucose

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and oxygen deprivation (akin to ischemia) followed by reintroduction of oxygen (reperfusion). Previous work using these preparations has shown that as a result of lack of oxygen and glucose adult synaptosomes demonstrate a significant increase in cytosolic calcium concentration which did not occur in the synaptosomes prepared from immature brain (5). Because the activity of the neuronal form of nitric oxide synthase (nNOS) is known to be calcium-calmodulin dependent (6), we have focused on the NO^o production from synaptosomes prepared from both neonatal and adult rats, and their inherent ability to cope with free radical production, i.e. their total antioxidant capacity.

EXPERIMENTAL PROCEDURE

Synaptosome Preparation. 10 day old Wistar rat pups, and adult male Wistar rats age 60 days (B and K Universal, Aldborough, England) were used for this study. Brains were rapidly removed and placed in isolation medium (320 mM sucrose, 1 mM K+EDTA, 10 mM Tris-HCl, pH 7.4 at 4°C) within 30-60 seconds. Purified synaptosomes were prepared from 4 adult rats per experiment by the method of Booth and Clark (7), or from 8-12 rat pups using essentially the same method but with a slight modification of the density gradient. (5) In brief, 1 volume of scissor-chopped forebrains were homogenised in nine volumes of isolation medium at 4°C. The homogenate was centrifuged at 1,500 g_{av} , for 3 min using a Beckman JA-20 rotor at 4°C, and the resultant supernatant was centrifuged at 17,400 g_{av} for 10 min. Purified synaptosomes were produced by density gradient centrifugation using Ficoll, as in the original method for adults (7), and by a slight modification of the gradient for younger age pups (5). It is of utmost importance that any functional differences observed between synaptosomes from adults and neonates are not due to inherent differences in the preparations. Therefore the density gradient was altered to 10%, 6%, 4% Ficoll for all pups younger than 21 days. This gave a comparable integrity and purity to the adult preparation (<8% free mitochondria and <20% total contamination for all preparations) as determined by marker enzymes detailed in the original method (7). All synaptosomes were pelleted (14,000 gav, 10 min) in a modified Kreb's phosphate buffer (KPB) (141 mM NaCl, 5 mM KCl, 1.3 mM MgSO₄, 10 mM Na₂HPO₄, 10 mM glucose, 1.2 mM CaCl₂, pH 7.4) which had been equilibrated with air (maximum oxygen concentration in KPB = 195 μ M O₂ at 37°C).

Incubation Conditions for Glucose and Oxygen Deprivation. Synaptosomes were deprived of oxygen and glucose by resuspension in a modified Kreb's phosphate buffer which did not contain glucose and which had been depleted of oxygen by the addition of the reducing agent sodium dithionite (0.1 mM) and continuous bubbling with 100% nitrogen gas. This is a widely accepted protocol for rendering physiological buffers anoxic (8). Synaptosomes were then incubated in a shaking water bath (37°C) for 15 or 30 minutes in vials which were capped and carrying a gassing line (delivering nitrogen gas) suspended above each incubation. This protocol was classed as in vitro ischemia. For all experiments normoxic controls were carried out similarly but using buffer containing 10 mM glucose, and gassing lines carrying pure oxygen. All incubations contained 100 µM arginine final concentration (f.c.) so that this substrate for Nitric Oxide Synthase (NOS) did not become limiting. Synaptosome integrity was not compromised using this protocol. Measurement of lactate dehydrogenase activity (LDH) (9) in the supernatant of pelleted synaptosome preparations, showed that less than 10% of total activity of this cytosolic protein had leaked from the intact synaptosomes. After the incubation period, synaptosomes were pelleted (4,000 g_{av} , 1 minute), 700 µl KPB removed and replaced with 700 µl of control KPB (which had been equilibrated with pure oxygen and kept at 37°C), to allow reintroduction of oxygen into the ischemic incubations. Synaptosomes were further incubated with access to pure oxygen for 15 minutes before pelleting (14,000 g_{av} , 3 minutes).

ATP Measurements. ATP was acid extracted from synaptosomal suspensions using 3.5% (final concentration) ice-cold perchloric acid. Extracts were neutralized with 0.7 M K₂HPO₄ (final concentration), kept on ice for 30 minutes and then pelleted (10,000 g_{sv} for 2 minutes). Resultant supernatants were snap frozen in liquid nitrogen and kept frozen at -80°C until the time of assay. ATP concentration was measured with a luciferin-luciferase bioluminescence assay (10), at 30°C on a 1251 LKB Wallac Luminometer.

Nitrite+Nitrate Determination. NO production from the synaptosomes was measured by assaying the stable end products of NO degradation in aqueous solution-nitrite (NO₂⁻) and nitrate (NO₃⁻) (6), as the half-life of NO⁻ is very short (11). NO₂⁻ + NO₃⁻ released from the intact synaptosomes were measured in the supernatant of the synaptosomal incubations by a colorimetric procedure using the Griess reagent as described by Green et al. (12), which has been previously used with synaptosomes (13). To validate that the assay was reflecting NO production the calcium ionophore, ionomycin (10 µM f.c.), was added to control synaptosomes to establish whether $NO_2^- + NO_3^$ production would adequately reflect an increased calcium concentration and subsequent increased activity of NOS and thus NO production. Applications of the known NOS inhibitor N'. nitro-arginine (NNA) were made to the ischemic incubations (100 μ M f.c.) (11) to assess whether increased $NO_2^- + NO_3^-$ production was due to increased NOS activity.

Nitric Oxide Synthase Activity. NOS activity was determined in brain cytosolic fractions prepared from 10 and 60 day old rats. Whole brain was homogenised (25% w/v) in isolation buffer (320 mM sucrose, 10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) and centrifuged at 100,000 g_{av} for 60 minutes at 4°C. The resulting supernatant was used for the determination of NOS activity. Measurement of NOS activity is based on the quantification of [3H]citrulline converted from [3H]arginine, as described by Brand et al. [14]. In brief, following removal of endogenous arginine by cation exchange, 100 μl of cytosol was added to 100 µl reaction buffer containing (f.c.) 100 mM Hepes, pH 7.5, 100 µM NADPH, 1 mM calcium chloride, 1 mM magnesium acetate, 10 µg/ml calmodulin, 50 µM tetrahydrobiopterin, 1 mM dithiothreitol, 5 µM FAD, 5 µM FMN, 100 µM L-arginine and 1 µCi/ml [3H]arginine. After 15 minutes the reaction was terminated by addition of 50 µl of trichloroacetic acid (1.5 M) followed by 1 ml of Hepes (1.5 M) buffer, pH 6.0. Arginine was then removed from the reaction mixture by ion exchange chromatography and the amount of labeled citrulline present was determined by liquid scintillation counting.

Antioxidant Status. Total antioxidant status was measured in synaptosomes with glucose and oxygen supplied, from both 10 day old pups and adults. Synaptosomes (approximately 2 mg/ml) were freezethawed three times and homogenised in a tight glass-glass homogenizer, to release all the synaptosomal contents. After pelleting the membranes (14,000 g_{av} , 5 mins), the supernatant was assayed for an-

Table I. Concentration of ATP in Adult and Pup Synaptosomes After 15 or 30 Minutes of Hypoxia-Aglycaemia

	ATP Concentration (nmol/mg protein)	
Conditions	Pup Synaptosomes	Adult Synaptosomes
Control,	1.31 ± 0.15	1.73 ± 0.15
15 mins	(n=4)	(n=5)
Hypoxia-aglycaemia,	0.18 ± 0.02^{a}	0.24 ± 0.03^{a}
15 mins	(n=4)	(n=5)
Control,	1.28 ± 0.27	1.68 ± 0.05
30 mins	(n=4)	(n=5)
Hypoxia-aglycaemia,	0.19 ± 0.02^{a}	0.23 ± 0.02^{a}
30 mins	(n=4)	(n=5)

Control values for the appropriate incubation periods (i.e., 15 or 30 minutes) are also shown.

 $^{a}P < 0.01$ compared with appropriate control.



Fig. 1. Bar graph showing nitric oxide synthase (NOS) activity, as determined by [3H]citrulline formed from [3H]arginine, in whole brain homogenates from neonates (10 day old rat pups) and adults (60 day old rats). Results are expressed as mean \pm SEM, n = 3 separate synaptosome preparations, measurements performed in triplicate. ** represents significant (P < 0.01) difference from neonatal value.

tioxidant status. This is a functional assay which measures the capacity of a sample to scavenge free radicals generated from the reaction between metmyoglobin and hydrogen peroxide. If unscavenged, these radicals react with 2.2'-azino-bis-3-ethylbenzo-thiaoline-6-sulphonic acid (ABTS) to form a chromophore which absorbs at 734 nm. The assay was carried out as in the original method [15].

Protein Measurement. Protein concentrations were determined according to Lowry et al, [16].

Statistical Analysis. Differences in the means of separate experiments were tested by analysis of variance (ANOVA), followed by the least significant difference multiple range test. All results are expressed as the mean \pm standard error of the mean (SEM).

Table II. Concentration of NO_2^- + NO_3^- Released from Adult and Pup Synaptosomes After 15 or 30 Minutes of Hypoxia-Aglycaemia Followed by 15 Minutes Reperfusion with Oxygen Supplied

	Nitrite + Nitrate Concentration (nmol/mg protein)	
Conditions	Pup Synaptosomes	Adult Synaptosomes
Control, for	0.50 ± 0.04^{a}	1.15 ± 0.11
15 mins + 15 mins oxygen	(n=4)	(n=6)
Hypoxia-aglycaemia, for	0.47 ± 0.02^{a}	2.24 ± 0.12^{b}
15 mins + 15 mins oxygen	(n=4)	(n=6)
Control, for	0.49 ± 0.03^{a}	1.10 ± 0.14
30 mins + 15 mins oxygen	(n=4)	(n=6)
Hypoxia-aglycaemia, for	0.49 ± 0.03^{a}	2.27 ± 0.15^{b}
30 mins + 15 mins oxygen	(n=4)	(n=6)

Control values for the appropriate incubation periods (i.e., 15 or 30 minutes followed by 15 minutes reperfusion with oxygen) are also shown

^{*a*}P < 0.01 compared to adult.

 $^{b}P < 0.01$ compared with appropriate adult control.

RESULTS

Synaptosomal hypoxia-aglycaemia (oxygen and glucose deprivation) was confirmed by analysis of ATP. These data are detailed in Table I. Results show that both the adult and neonatal synaptosomes experienced a relatively severe degree of hypoxia-aglycaemia because ATP levels fell to 13-15% of controls by 15 minutes in both types of preparation.

In 60 day old adult rats the total brain NOS activity was significantly (P < 0.01) higher than in 10 day old rat pups (Fig. 1). This was also reflected in measurements of NOS activity in cytosol from synaptosomes prepared from these ages (pup value was 65% of adult value. ie. pup = 74 \pm 1 pmol/min/mg, n = 3, adult = $113 \pm 4 \text{ pmol/min/mg}, n = 3, P < 0.01$).

The production of NO[•] from synaptosomes, reflected by the $NO_2 + NO_3$ concentration was also significantly lower in pups compared to adults (Table II). This data therefore suggests an increase in NOS activity and NO[•] formation as the brain matures. A period of glucose and oxygen deprivation for 15 or 30 minutes followed by reintroduction of oxygen (akin to ischaemia/reperfusion) caused a marked increase in NO[•] production from the adult synaptosomes (Table I), but no increase in the synaptosomes prepared from pups. The increase in $NO_2^- + NO_3^-$ seen in adult synaptosomes was prevented by the addition of the NOS inhibitor NNA to the incubation, (hypoxia-aglycaemia + NNA, $30 \text{ min} = 1.23 \pm 0.11 \text{ nmol/mg protein}, n = 3$). Addition of ionomycin to control incubations increased the
Table III. Total Antioxidant Status of Adult and Pup Synaptosomes

Total antioxidant capacity (µmol/mg protein)		
Adult		Pup
0.11 ± 0.01 (n=9)		0.12 ± 0.01 (n=7)

 $NO_2^- + NO_3^-$ production by 40%, indicating that the assay reflected calcium-dependent NOS activity.

The total antioxidant status of immature and adult synaptosomes were not statistically different (Table III), suggesting that the adult and immature brain have similar capabilities for scavenging free radicals.

DISCUSSION

In this study, we have provided evidence to suggest that the activity of cytosolic nitric oxide synthase (NOS) in rat brain homogenates and synaptosomes is much lower in the immature brain than in the fully matured adult brain. This data is in agreement with previous findings that NOS activity in rat brain cerebellum cytosolic and particulate fractions increases with age (17). In pups it appears that the activity of NOS in the nerve terminal from immature brain would be low during the initial stages of ischaemia/reperfusion. This is due to a combination of the low intrinsic activity of NOS in immature brain, coupled with previous data demonstrating no increase in cytosolic calcium during glucose and oxygen deprivation (5), which would hence keep the NOS activity low under these conditions. This is confirmed by our $NO_2^- + NO_3^-$ results after hypoxia-aglycaemia and oxygen restoration, where there is no significant difference in NO production compared to control levels in the nerve terminals from immature brain. This observation suggests that NO[•] production by nerve terminals does not feature as an important part of the mechanism of damage in the neonatal brain during the immediate stages of reperfusion after ischaemia.

In a number of in vivo studies it has been reported that brain damage after ischaemia/reperfusion in can be ameliorated by administration of NOS inhibitors, suggesting a role for NO in the pathogenesis of cerebral damage in the brain (4). However there are also a number of contradictory reports concerning the possible neuroprotective effect of NOS inhibitors in ischaemia/reperfusion injury. These differences may reflect the various experimental procedures and models used but the findings of these and other studies are generally supportive of an involvement of reactive oxygen species in adult ischaemia/reperfusion injury (2). Less work has been done on the neonatal brain specifically although some in vivo models have suggested that NOS inhibitors can ameliorate damage following focal stroke (18). The model that we have used here is more relevant to global ischaemia and there are no blood vessels present, thus allowing the neuronal NOS to be studied effectively in isolation from the endothelial and inducible forms.

Recent work on developing brain showed that adult brain slices recovering from glucose and oxygen deprivation suffered a significant decrease in energy metabolites when NO was generated in the system from an NO donor, but in neonatal slices under the same conditions NO did not decrease the energy metabolites at all (19). We show here that there is significant increased production of NO from adult nerve terminals after glucose and oxygen deprivation followed by oxygen restoration. Such NO produced during ischaemia/reperfusion could react with superoxide (O_2^-) to form the peroxynitrite anion (ONOO⁻), which is thought to be the agent mainly responsible for nitric-oxide mediated neurotoxicity [20]. Intact neurones, in contrast to astrocytes, have been shown to be especially susceptible to ONOO-, possibly due to their relative paucity of antioxidants [21]. Inhibition of the mitochondrial respiratory chain enzymes succinate cytochrome-c reductase and cytochrome c oxidase were demonstrated after ONOO- exposure and significant neuronal death occurred [21]. NO[•] production and subsequent mitochondrial damage, probably mediated through ONOO-, could therefore play a part in ischaemia/reperfusion neuronal death in the adult brain.

Although there are significant differences in the amount of NO⁻ production from immature and adult nerve terminals, the inherent ability of the brain to cope with NO/ONOO- and other reactive species may be an important determinant of whether damage will occur. Our data show that the antioxidant status of the nerve terminal does not change with development, at least in the membrane free fraction studied here. However, this observation correlates with the developmental profile of the major membrane bound antioxidant alpha-tocopherol which remains constant as the brain matures [22]. Thus the increased production from the adult as compared with the immature animal may not compensated for by an increased capacity of the nerve terminal to scavenge free radicals. In this respect therefore the immature brain is less likely to suffer from NO⁻ related oxidative stress than the adult brain because of the differences in the ratio of antioxidant status: NO production in the adult and neonate.

Nitric Oxide and Antioxidant Status in Ischemic Neonatal and Adult Synaptosomes

The overall development of the brain is complex. It is known that the oxygen consumption of the neonatal brain is lower than that of the adult brain, as are the resting cellular membrane potentials and EEG activity [23]. Many enzymes of the glycolytic pathway have been shown to change with development [24], and the activities of many of the complexes of the mitochondrial respiratory chain increase as the brain matures [25], [26]. There are complicated developmental profiles of a number of receptors such as the NMDA receptor in brain [27], and nerve terminal calcium homeostasis has shown to change as the brain develops [5]. All of these changes and others such as anti-oxidant status means that a variety of mechanisms may be involved in the resistance of neonatal brain to ischaemia/reperfusion. However, brain function is centred at the synapse and the data we present here may play an important part in this resistance.

In summary, the differences in NO[•] production from immature and mature nerve terminals that we report here could be significant in light of the vulnerability of elderly brain and the resistance of neonatal brain to ischaemia/reperfusion injury. The results of this study provide evidence in favour of a role for NO[•] in the pathogenesis of neuronal ischemia/reperfusion injury in the adult brain, and suggest that the well documented resistance of neonatal brain to ischemia/reperfusion may involve a relative lack of NO[•] production from the immature nerve terminals in comparison with adults.

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Differences in the amount of glutamate released by neonatal and adult synaptosomes under conditions of *in vitro* ischaemia/reperfusion.

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Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system. The excessive release of glutamate and exaggerated stimulation of post-synaptic glutamate receptors are involved in the process known as excitotoxicity. Excitotoxicity has been widely implicated in neuronal damage following an ischaemic episode and the ensuing reperfusion [1]. The neonatal brain is known to be more resistant to ischaemia and reperfusion (I/R), although all of the mechanisms involved in this resistance are not fully understood.

The aim of this study was to determine whether there was a difference in the amount of glutamate released from adult as compared with 10 day old rat synaptosomes under the following conditions:

i) during a 30 minute ischaemic period (no oxygen and glucose in medium)

ii) during a 30 minute incubation period following a single exposure to 1 mM peroxynitrite (ONOO⁻) which is a highly reactive oxidising agent implicated in oxidative stress and neuronal death during I/R.

Synaptosomes were prepared from whole brains of either 4 male Wistar adult rats, or a litter of 8 - 12 rat pups, age 10 days. Preparation of synaptosomes was using a discontinuous Ficoll gradient as in the original method [2], or slightly modified in the case of the pups [3]. Protein was determined by the method of Lowry et al. [4]. Incubation medium contained 141 mM NaCl, 5 mM KCl, 10 mM Na2HPO4, 1.3 mM MgSO4, 1.2 mM CaCl2 and 10 mM glucose. Synaptosomes were incubated at 37°C in 1 ml of the incubation medium for 30 minutes with pure oxygen supplied via a gassing manifold. For ischaemic conditions glucose was omitted from the buffer and pure nitrogen replaced the oxygen. For peroxynitrite treatment 1 mM ONOO⁻in NaOH was added to synaptosomes resuspended in HBSS/HEPES. After 5 minutes the synaptosomes were pelleted, washed, resuspended in 1 ml of control incubation medium and incubated at 37°C for 30 minutes with oxygen and glucose supplied. For all experimental conditions, synaptosomes were pelleted after the 30 minutes and supernatants were snap frozen and stored at -80°C until assaying for glutamate.

Glutamate was determined in the supernatants by a fluorometric assay using a Perkin-Elmer LS-B50 luminescence spectrometer. To a stirred cuvette containing incubation medium, 1.25 mM NADP⁺ and 50 U glutamate dehydrogenase 50-200 μ l of the supernatant was added and the fluorescence of NADPH was followed at 340 nm excitation and 460 nm emission. Concentration of glutamate was determined by use of a standard curve of 1-15 nmoles, r=0.999. A standard addition of 5 nmoles of glutamate was added at the end of each sample analysis to ensure that saturation of substrates had not occurred. Results are expressed as mean \pm SEM of the number of different experiments indicated in the table(n). Statistical analysis was performed using analysis of variance (ANOVA) and the least square difference multiple range test. Significance was assumed when P<0.05.

Table 1. Glutamate (nmoles/mg protein) present in the extrasynaptosomal medium after 30 minutes incubation at 37°C. Results are means \pm SEM from 4 separate experiments.

Condition	Adult	Neonate
control	4.50 ± 0.65	5.54 ± 0.81
15 mins ischemia	13.66 ± 2.49 a	6.05 ± 1.40 Å
30 mins ischemia	9.75 ± 0.41 a	4.85 ± 1.09 Å

b = P < 0.01 compared to control which own age. b = P < 0.01 compared to adult under same condition.

Table 2. Glutamate (nmoles/mg protein) present in the extrasynaptosomal medium after 30 minutes incubation following $ONOO^-$ treatment. Results are means \pm SEM from 6 separate experiments.

Condition	Adult	Neonate
control	3.38 ± 0.33	4.34 ± 0.50
1 mM ONOO [.]	6.34 ± 0.51 <i>a</i>	4.32 ± 0.67 b

P < 0.05 compared to control within own age. P<0.05 compared to adult under same condition. LDH release was less than 10% in all preparations (adult and neonate) throughout all incubations showing that increased membrane permeability is not involved in the increased concentrations of glutamate in the extrasynaptosomal medium. The results presented in tables 1 and 2 indicate that under conditions of oxygen and glucose lack there is a significant increased amount of glutamate in the extrasynaptosomal medium. This could be due to a combination of increased release and decreased re-uptake or due to one of these processes alone. The relevance of these data is that during ischaemia in the adult there would be an increased amount of glutamate available to postsynaptic glutamate receptors. The data further suggest that peroxynitrite attack also leads to an increased availability of glutamate to post-synaptic receptors in the adult brain. The immature brain does not show this phenomenon in either ischaemia or peroxynitrite attack. These data suggest that differences in glutamate homeostasis between the adult and the neonate may have a role in the increased resistance of the immature brain to ischaemia/reperfusion.

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AND FINALLY.....

"We must not let science hypnotise us into believing that simply by sitting in front of desks and drawing boards and instruments all day, we are contributing to the character of man. Man must balance science with other qualities of life, qualities of body and spirit as well as those of mind - qualities he cannot develop when he lets mechanics and luxury insulate him too greatly from the earth to which he was born. Only by placing man above the value of his productsmeasuring scientific accomplishments by their effect on man himself.....can we retrieve the qualities of mankind."

Charles A Lindbergh.