Astrocyte-neurone communication following an ischaemic insult

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Abstract

Ischaemia results from the cessation of blood flow to all or part of the brain, causing a rapid depletion of O_2 and impairment of oxidative phosphorylation. Bioenergetic failure follows within minutes, which precipitates directly immediate neuronal death. Upon reperfusion, despite the return of energy substrates, further neuronal death can occur up to several days later, depending on the severity of the initial insult. Astrocytes have been shown to be considerably more resistant to ischaemia/reperfusion injury than neurones and may play either a neuroprotective role or indeed exacerbate the neuronal injury. We have explored the possible interactions between astrocytes and neurones following ischaemia using an *in vitro* model of ischaemia/reperfusion injury, as a controlled environment that lends itself more easily to manipulation of the numerous variables involved in such an insult. As such we have produced an oxygen glucose deprivation model. We have constructed a chamber in which O_2 can be lowered to a concentration of 1μM. We have further developed a primary cortical neuronal culture that is >99% pure and which can survive to at least 10 days *in vitro*. We have additionally established a novel system for the co-culture of astrocytes and neurones in order to study the communication between these cells in a manner that can allow the complete separation of one cell-type from another. Astrocytes cultured alone do not exhibit signs of cell death during reperfusion of 24hrs duration following ischaemia of up to 2hrs whereas neuron cultures show profound cell death following an ischaemic period of only 15mins. We have co-cultured neurones, which have been subjected to a 15min ischemic insult, with either non-insulted astrocytes or astrocyte conditioned medium during the reperfusion stage. Results show that both astrocytes and astrocyte-conditioned medium enhance neuronal survival. We have finally investigated possible mechanisms to account for this.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propoinate
AO	Antioxidants
AraC	Cytosine arabinofuranoside
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CCD	Charge-Coupled Device
CICR	Ca^{2+} induced Ca^{2+} release
Cys	Cysteine
DAG	Diacylglycerol
DAPI	4,6-diamidino-2-phenylindole HCL
DIV	Day *in vitro*
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
EAAT	Excitatory amino acid transporters
EBSS	Earle's balanced salt solution
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FBS	Foetal Bovine Serum
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FITC	Fluorescein-isothiocyanate
Fura-2 AM	Fura-2 pentakis (acetoxymethyl) ester
GFAP	Glial fibrillary acid protein
GlDH	Glutamate dehydrogenase
Gln	Glutamine
Glu	Glutamate
GR	Glutathione reductase
GSH	Glutathione
GSHPx	Glutathione peroxidase
GSSG	Glutathione disulphide

HBSS	Hanks' buffered salt solution
HPLC	High performance liquid chromatography
IGluR	Ionotropic glutamate receptor
INT	Iodonitrotetrazolium chloride
IP_3	Inositol-1,4,5-trisphosphate
Lac	Lactate
LDH	Lactate dehydrogenase
MEM	Minimum Essential Medium
mGluR	Metabotropic glutamate receptor
NAD	Nicotinamide adenine dinucleotide oxidised form
NADH	Nicotinamide adenine dinucleotide reduced form
NB	Neurobasal medium
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NR	Nitrate reductase
OGD	Oxygen glucose deprivation
P/L	Poly-D-lysine
P/O	Poly-L-Ornithine
PI	Propidium iodide
Rh123	Rhodamine 123
ROS	Reactive Oxygen species
SNARE	Soluble NSF attachment protein receptor
SOD	Superoxide dismutase
SVCT	Sodium-dependent vitamin c transporter
T/E	Trypsin/EDTA
TBS	Tris buffered saline
TCA	Tricarboxylic acid cycle
TMRM	Tetramethyl rhodamine methyl ester
Tub	Beta III tubulin
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
γGluCys	γ-glutamyl-cysteine
$\Delta\psi_m$	Mitochondrial membrane potential

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Chapter 1

Introduction

1.1 Cerebral ischaemia

1.1.1 What is ischaemia?

To answer the question as simply as possible, ischaemia describes a situation where blood flow to an area of the brain is halted, thereby cutting the supply of vital nutrients and O_2 to that area whilst also preventing the removal of waste products. As with every cell in the body, the cells that make up the brain generate and use ATP as their main energy source. But, more so than many other parts of the body, the brain is extremely dependent on the aerobic oxidation of glucose for the generation of its ATP. To put this in context, the brain's O_2 requirement accounts for around 20% of total consumption yet it accounts for only 2% of body mass. However, there is a huge disparity between the amount of O_2 and glucose required by the brain and the amount stored by the brain which is very little (Fiskum 1986). A perturbation in blood flow has the immediate effect of interrupting energy production by oxidative phosphorylation and leaves the brain with only a less than four-minute window of ATP usage (Fiskum 1983) after which the effects to the individual can be devastating and long lasting.

1.1.2 Causes of ischaemia

Cerebral ischaemia can occur due to a wide variety of disease states and insults such as stroke, head injury or cardiac arrest. Although the aetiology may vary, the cellular events are very similar and the insult generally falls in to either of two categories, focal or global. *Global ischaemia* describes the situation in which the blood supply to the entire brain is terminated, such as may happen following a cardiac arrest. In *focal ischaemia* only a particular brain area is effected which may be the case following the occlusion of specific blood vessels due for example to clot formation. The induction of ischaemia in laboratory animals is based upon reproducing either of these two events.

Focal ischaemia is usually induced by the occlusion of the middle cerebral artery (MCA). This is generally done in one of three ways. A ligature can be applied to the appropriate artery, blood can be induced to

coagulate chemically, or drugs that have vasoconstrictive properties can be injected in to the animal. Recently the 'macrosphere model' has been introduced in which spheres of 0.3-0.4mm diameter are placed inside the MCA to cause a blockage (Gerriets et al 2003). Particles of smaller size but greater number have also been used to specifically block major arteries (Oshikawa et al 2004). Global ischaemia can be induced by the occlusion of two or more of the main vessels supplying the brain, usually branches of the carotid artery. This is normally carried out by injecting a hypertensive agent coupled with partially occluding the feeder artery (Fig 1.1).

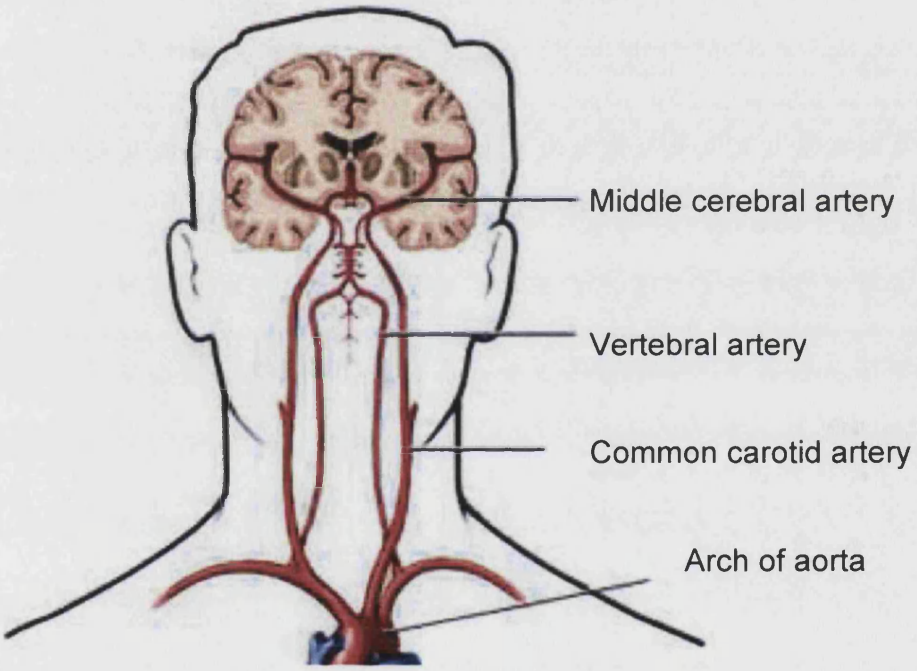


Fig 1.1 Main arteries of the brain. The figure illustrates the main arteries of the brain, which are often targets of occlusion. Adapted from http://stroke.upmc.com/Overview.htm.

1.1.3 Patterns of ischaemic damage

The development of ischaemic damage can be divided into two phases, one mediated directly by early events during the ischaemic period itself and one mediated by knock on events such as the production of free radicals (both described later). An ischaemic insult may be permanent or it may be followed by a period of reperfusion during which blood flow is

returned to the injured area. Most injury, and most of the mediators of secondary damage, is observed during the reperfusion period. However, precisely how well the brain survives is mostly dependent upon the length of the ischaemic period.

Delayed death is a particular feature of global ischaemia. A sublethal global insult (generally less than 30mins) will result in immediate bioenergetic failure and loss of ion homeostasis in the entire brain. At this point not all cells are affected equally and it is observed that cell death occurs chiefly in neuronal populations (Lipton 1999). Reperfusion stimulates an apparent return to normal function, which is only briefly sustained in certain 'vulnerable' regions of the brain. Here, death is seen to occur several days later concomitant with a secondary energy failure, but in a cell specific manner (Horn et al 1992). This phenomenon, called delayed neuronal death, was first described in pyramidal cells of the CA1 hippocampal area (Kirino 1982) but has since been observed in other cell groups, such as the CA2 and CA3 hippocampal regions and layers of the cortex, as a function of insult length and severity (Pulsinelli et al 1982, Cervos-Navarro et al 1991). Precisely how this phenomenon occurs is a topic of much research.

Although classical 'delayed death' is associated with global insults, focal ischaemia too is marked by cell death for considerable periods of time following ischaemia. Severe focal ischaemia (60-120mins) usually results in the development of a core region consisting of damaged cells of all types. This area represents the part of the brain formerly supplied by the occluded vessel and the mass of damage is called the infarct. The surrounding area, the penumbra, shows a graded decrease in cell loss with distance from the core and these cells can die up to several hours after the insult. The longer the ischaemic period however, the greater is the spread of the infarct during reperfusion (Memezawa et al 1992). There is generally a sharp division between the mass of damaged cells at the core and the surrounding area, as the glia present swell and proliferate to wall off the area in a process called anisomorphic gliosis

(Liberto et al 2004). Very brief focal insults (15mins) are similar to global insults in that they can produce a smattering of vulnerable dead cells in the core rather than a huge generalised infarct (Li et al 1995).

1.1.4 Type of ischaemic cell death

The measurement of cell death during and after ischaemia is an important benchmark of ischaemic damage, so much so that the strength of an ischaemic insult and the efficacy of therapeutics is gauged by the level and type of death induced. The initial *in vivo* studies on cell death established two distinct classifications, apoptosis and necrosis, to reflect the observation that physiological and pathological cell death, respectively, appeared morphologically different from each other. Apoptosis was first described by Kerr *et al* in 1972, according to a number of structural features. The dying cell separates from its neighbours, undergoes condensation of the cytosol and of nuclear chromatin, nuclear fragmentation, plasma membrane blebbing, cell shrinkage and ultimately phagocytosis, leaving no evidence of its previous existence (Wyllie 1997, Carmody et al 1998). In sharp contrast to this, cells undergoing necrosis suffer severe membrane disruption, swell and lyse, often triggering a damaging immune response (Nicotera et al 1999).

Apoptosis is a controlled energy dependent process, which highlights the fact that the apoptotic cell is trying to remove itself with minimum damage to its surroundings. It is often called a 'programmed' cell death, as there are a number of dedicated signalling cascades activated by the process involving transcription of nuclear factors, release of signalling molecules and the activation of specific proteases. Most importantly, up to a point, apoptosis is not necessarily an all or nothing event and so inhibitors of apoptosis have been shown to prevent cell death. Particularly, inhibitors of caspases, the proteases responsible for effecting death (Wyllie 1997).

Necrosis on the other hand lacks the surgical removal observed following the apoptotic dissection of a cell. Instead, there is a marked drop in ATP

level such that ionic gradients can no longer be maintained. This is followed by membrane depolarisation, Ca^{2+} entry and the activation of lipases and proteases, all of which culminates in severe membrane disruption and the leakage of the cell's content, as mentioned previously (Nicotera et al 1999).

Death observed in an infarcted area during the ischaemic insult has been described as necrotic. Apoptosis is an energy requiring process and there is insufficient energy to support it under extreme ischaemic conditions. On the other hand death during the reperfusion phase and delayed neuronal death in particular, are often described as being apoptotic due to evidence of the activation of caspases (Love 2003) but the issue is muddied somewhat.

It has become clear that ischaemic cell death does not fall neatly into either category, as morphological and biochemical characteristics of each can be found following both global and focal insults (Graham et al 2001, Lipton 1999). Two schools of thought have thus emerged on this topic. First that necrosis and apoptosis form the poles between which there is a broad continuum of death type (Hou et al 2002, Martin et al 1998) and second that necrosis masks an underlying apoptotic process (Benchoua et al 2001, Gwag et al 1995).

1.1.5 Early events of ischaemia

Here we describe the events that occur in direct response to the lack of blood flow to a particular area. These events are common to focal and global insults and are not in themselves damaging but will trigger a cascade of damaging events that will precipitate cell death if blood supply is not returned in a timely manner.

1.1.5.1 Inhibition of electron transport

As alluded to previously, the brain produces most of its ATP by the process of oxidative phosphorylation, which takes place in the mitochondrion. To summarise, glucose is metabolised in the cytoplasm

to pyruvate, which is then transported into the mitochondrion where it is oxidised to CO_2 and H_2O. Reducing equivalents are removed from the sequential degradation of glucose molecules and passed via the electron transport chain to awaiting O_2 molecules. The energy released in sending these equivalents from molecules with positive redox potential to negative redox potential (O_2), is used to generate a proton gradient across the inner mitochondrial membrane. The electro-chemical energy stored in the gradient is used to drive the generation of ATP from ADP and Pi. Under normal conditions it is estimated that over 95% of ATP is synthesised in neurones via oxidative phosphorylation, with very little lactate produced (fig 1.2).

Clearly, the immediate effect of ischaemia is to inhibit the activity of the electron transport chain and ATP production by oxidative phosphorylation. There is only enough O_2 stored in the vasculature to support the generation of ATP via oxidative phosphorylation for a few seconds (Hansen AJ 1985) however it takes around 90 secs of ischaemia for ATP to fall to its lowest levels (Katsura *et al* 1993). This is mainly because neurones have stores of phosphocreatine from which ATP can be produced.

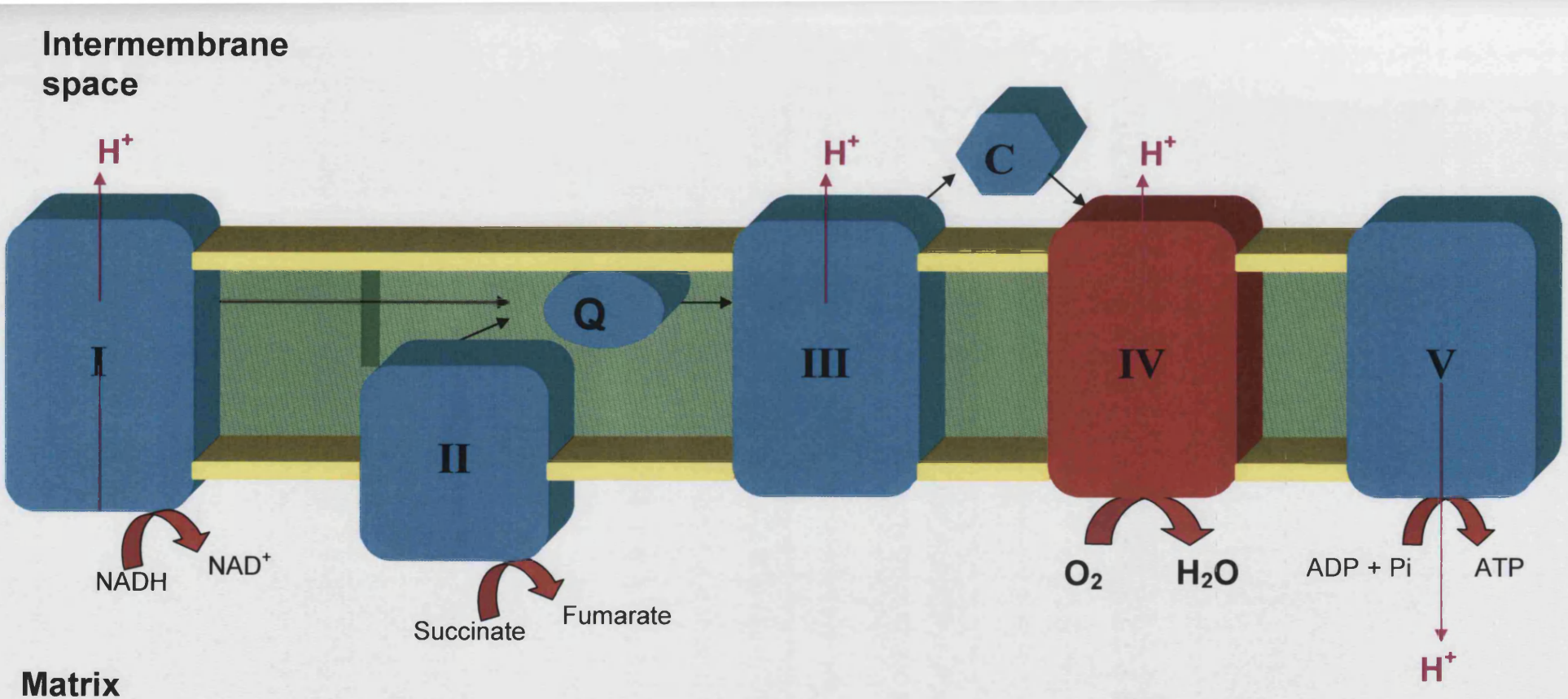


Fig 1.2 Electron transfer chain. Reducing equivalents are passed from NADH and succinate to complex I and II respectively. Electrons are then transferred to co-enzyme Q (Q) which transports electrons through the lipid bilayer to complex III. Electrons are then transferred to cytochrone C (C) which moves along the outside of the inner membrane to complex IV. Complex IV then catalyses the reduction of molecular O_2. Protons (H^+) are extruded to the inner membrane space at complexes I, III and IV. The gradient created by the extrusion of H^+ is used to drive the synthesis of ATP at complex V. Lack of O_2 prevents electron flow at complex IV, highlighted in red. *(Based on principles explained in Lehninger et al).*

1.1.5.2 Loss of ion homeostasis

Loss of ATP is first seen as a disturbance in active ion transport and thus membrane polarisation. Ion pumping consumes around 50% of ATP produced at rest but this percentage rises during increased activity (Hansen 1985). Different cells have been shown to exhibit either a hyperpolarisation or a depolarisation at around 1min of ischaemia, depending on the dominant conductances in the cell type. However, most neurones do exhibit an increase in K^+ conductance leading to a slow rise in extracellular [K^+] (Martin et al 1994). When ATP levels fall sufficiently low the 'anoxic depolarisation' follows. This is suggested to result principally from a failure of the Na^+/K^+ ATPase at the plasma membrane (Rossi et al 2000). This enzyme not only consumes the largest amount of ATP but also is very sensitive to ATP decrease due to its very low apparent Km for ATP (0.4-0.7mM), the regulatory and catalytic sites both bind ATP with the regulatory site having the lower apparent Km (Erecinska et al 2001). The anoxic depolarisation therefore sees all neurones in the effected area collectively depolarise rapidly and significantly at around 4mins of ischaemia (Hansen 1985). At this time there are large reductions in extracellular Ca^{2+}, Na^+ and Cl^- ions.

1.1.5.3 Acidosis

At 15secs of ischaemia, intracellular pH has been shown to drop to around 6.5 (Hansen 1985). This actually precedes the anoxic depolarisation and occurs at a time when the ATP levels are still rather high. The most likely explanation for the decrease in pH is again directly related to the inhibition of oxidative phosphorylation. It is suggested that continued functioning of the glycolytic pathway results in lactate accumulation (Lipton 1999). The lactate produced would then cause the amount of HCO_3^- to be lowered and CO_2 to increase. The buffering capacity of the cell is compromised and intracellular H^+ produced cause the drop in pH (Tombaugh et al 1993).

Reperfusion returns a significant amount of function to the cell if blood flow is restored within around 30mins (Nowak et al 1985, Pulsinelli et al

1983). In fact it is thought that damage sustained during the ischaemic period is not irreversible but only becomes so as reperfusion progresses (Sims et al 2002). Because this thesis is based around the development of damage during reperfusion, the next two sections will focus largely on processes that may cause cerebral damage. Global and focal insults will be described simultaneously for ease of reference but events specific to either will be clearly demarcated, as will any references to *in vitro* studies.

1.2 Ischaemia and excitotoxicity

The process of excitotoxicity is a major point of study in the field of ischaemic research. As a result this section is designed to introduce the reader to the phenomenon and to detail evidence of the process in the evolution of ischaemic damage.

1.2.1 Glutamate in the CNS

An introduction to the world of glutamate will invariably start with a statement of the fact that it is the principal excitatory neurotransmitter in the central nervous system. And it is, but glutamate's sphere of influence extends far beyond the brain to every other cell in the body, as it is an essential component of protein synthesis and intermediary metabolism. However, despite its necessity to the maintenance of life, glutamate is in fact a very potent neurotoxin at high concentrations. Lucas and Newhouse first suggested this in 1957, following their observations that glutamate overexposure could destroy part of the mouse retina. The observation has grown in to a field of study and it has been shown time and again that glutamate release is responsible for a significant amount of neuronal death relating to cerebral ischaemia (Choi et al 1990). The toxic effects of glutamate do not stop at neuronal populations as has been demonstrated by Chen et al (2000) who show that astrocytes too can be damaged albeit by a predominantly different mechanism.

1.2.2 Glutamate release

It has been estimated that neuronal cytosolic glutamate concentration is around 5mM, that of astrocytes is around 3mM, while the extracellular space has a resting glutamate concentration of around 2μM at the very most (Nedergaard et al 2002, Anderson et al 2000). Extracellular levels are kept low by glutamate cellular uptake, which is chiefly accomplished by the Na+ dependent excitatory amino acid transporters (EAATs) of which there are five identified subtypes (EAAT1-5). EAAT1 and EAAT2 are predominantly glial whereas the other three are neuronal (O'Shea 2002). EAATs have also been found on oligodendrocytes, microglia and

endothelia (Anderson et al 2000). These transporters function by moving one molecule of glutamate, three molecules of Na^+ and one H^+ in to the cell and one K^+ out (Barbour et al 1988). The activation of the EAATs is also associated with a Cl^- conductance (Wadiche et al 1995).

During ischaemia, [glutamate] in the extracellular space has been shown to rise to 20-fold or even around 40-fold its original concentration in some experimental preparations. The immediate increase in [glutamate] is most likely due to Ca^{2+} dependent exocytosis but the bulk thereafter is due to reversal of the EAATs as the driving force for glutamate uptake in to cells (the Na^+ gradient) is diminished (Rossi et al 2000).

Feustal et al (2004) suggest that glutamate release in less severely ischaemic areas is due mainly to the activation of volume-regulated organic anion channels. This is not a likely route of release in an ischaemic core as the process is ATP dependent. Indeed, it has been shown that the inhibition of these anion channels can occur as a result of inhibiting mitochondrial respiration (Patel et al 1998), which is likely to be prevalent in the core. It could however play a part in glutamate release during the reperfusion period.

1.2.3 Receptor mediated ischaemic damage

Glutamate works as a signalling molecule by binding to two pharmacologically and functionally distinct receptor classes at the plasma membrane. These are the metabotropic glutamate receptors (mGluR) and the ionotropic glutamate receptors (iGluR). Excessive glutamate release causes over activation of these receptors. Overstimulation leads to an intracellular accumulation of ions such as Ca^{2+} and Na^+ and messengers such as inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Choi 1990) which mediate the toxic effects of glutamate.

1.2.3.1 Ionotropic receptors

The iGluRs activate ligand gated ion channels and are grouped in to three separate families named according to agonist specificity. These

comprise of the NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propoinate) and kainate receptors. Each family assumes a heteromeric structure composed of a number of subunits whose particular combination of assembly is dependent on tissue type and stage of development. NMDA receptor channels have seven subunits (NR1, NR2A-D, and two NR3) and are highly permeable to $Ca^{2+}$and Na^{+}. They also have a voltage-dependent Mg^{2+} block and require glycine as a co-agonist. The particular combination of NR1 and 2 subunits will determine not only the agonist affinity but also the sensitivity of the Mg^{2+} block and single-channel conductance. AMPA channels have four subunits (GluR1-4) and kainate channels have five (GluR5-7, KA1 and KA2) and are both permeable to Na^{+} and K^{+}(Dingledine et al 1999, Madden 2002). AMPA receptors are also permeable to Ca2+ unless there is at least one GluR2 subunit present.

The NMDA receptors have received the most attention in terms of glutamate toxicity as antagonists to this receptor class were first shown to have possible neuroprotective effects (Simon et al 1984). Goldberg and Choi (1993) showed that activation of NMDA receptors could mediate an influx of Na^{+} and Cl^{-} ions and the movement of water that mediates early cell swelling. Furthermore they showed that delayed cell death was due to Ca^{2+} entry via NMDA receptor channels. In fact it is this relationship between Ca^{2+} influx and cell death that has gained most notoriety for the NMDA receptors. Although the swelling effects related to Na^{+} are important, Ca^{2+} has been shown to be the main mediator of neuronal death (Kristian et al 1996). Rossi et al (2000) have linked glutamate release via reversed transport to the initiation of the anoxic depolarisation. In their hippocampal slice preparation, they have proposed a feed forward mechanism whereby NMDA receptor activation depolarises the cell, releasing more glutamate and K^{+}, to activate more NMDA receptors and so on.

AMPA receptors have been implicated in ischaemic neuronal death directly and indirectly. Indirectly, AMPA receptors can contribute to Ca^{2+}

influx by depolarising the membrane in a Na^+ dependent manner and thus removing the Mg^{2+}-dependent block on the NMDA receptors. Brorson et al (1994) first suggested that, at least in the cerebellum, Ca^{2+} entry via AMPA/(Kainate) channels could mediate excitotoxicity. Recent work by Liu et al (2004) suggests that the lack of expression of the NR2 subunit is responsible for selective CA1 region hippocampal cell death following transient forebrain ischaemia, as greater Ca^{2+} permeability results. Oguro et al (2004) support this idea by correlating neuronal death following OGD, at different stages of development, with NR2 expression.

Kainate receptors are far less studied than either AMPA or NMDA receptors. It is thought that kainate channels may not play a major role in glutamate excitotoxicity as they are rapidly desensitized (Wilding et al 1997) and have very limited Ca^{2+} permeability. Again, Na^+-mediated membrane depolarisation has been postulated to be the only contribution made by kainate channels towards excitotoxicity.

1.2.3.2 Metabotropic receptors

The mGluRs are G-protein coupled receptors of which there are at least eight types divided into three groups. Group I mGluRs include mGlu1 and mGlu5, which have five and two splice variants respectively. They are coupled to the $G\alpha_q$ protein and so mobilize intercellular Ca^{2+} through the activation of phospholipase C. Group II mGluRs comprise mGlu2 and 3 whereas group III includes mGlu4, 6, 7 and 8 (each with splice variants). Groups II and III are coupled to the $G\alpha_i$ protein and so act to inhibit the formation of cyclic adenosine monophosphate (cAMP) (Jingami et al 2003, Pin et al 2002, Pin et al 1995, Bruno et al 2001).

The mGluRs, specifically mGluR1 and 5, have been implicated in the neurotoxicity of glutamate but are thought to have a more modulatory role (Bruno et al 2001). Conflicting results have emerged showing that they can both attenuate and exacerbate the toxic effects mediated by the iGluRs (Pizzi et al 1996, Bruno et al1995).

1.2.4 Ca^{2+} dysregulation

One of the most salient features of ischaemia, both during the actual insult and the following reperfusion period, is a very large increase in intracellular Ca^{2+} (Lipton 1999). Ca^{2+} based cell signalling is very tightly regulated and contributes to a very wide range of cellular events from activation of proteases to secretion events to cell division. The capacity of the cell to buffer Ca^{2+} is very important in modulating the spikes and waves that constitute Ca^{2+} signalling such that they can be decoded in the correct manner. Clearly, both severe increases and decreases will derail such signalling and have pathological potential. There is a very steep gradient in Ca^{2+} concentration across the plasma membrane (mM to μM) but there are also a number of gradients existing between the cytosol and intracellular compartments such as mitochondria and the endoplasmic reticulum (ER). So, the cytosolic level and profile of Ca^{2+} is dependent on, and has influences upon, a number of sources.

The rise in intracellular Ca^{2+} during ischaemia is predominantly due to entry via NMDA receptor channels as discussed. It has been shown that intracellular Ca^{2+} stores also contribute to the cytosolic rise particularly due to Calcium-induced-calcium release (CICR) and IP_3 stimulation at the ER (Verkhratsky et al 2003).

So how does Ca^{2+} mediate cell death? Mitochondrial Ca^{2+} overload may be one of the key factors in translating the rise in cytosolic Ca^{2+} to cell damage during reperfusion at least. Under physiological conditions, uniporters in the mitochondrion can transport Ca^{2+} from the cytosol when levels are raised particularly when Ca^{2+} is released from the ER, given the close apposition of ER and mitochondrial membranes (Rizzuto et al 2000). Na^{+}/Ca^{2+} exchangers also exist to bring Ca^{2+} back to the cytosol. In fact reversal of these exchangers has been documented during hypoxia leading to a rise in mitochondrial Ca^{2+} (Griffiths et al 1998). It is unlikely that the transporter functions to increase mitochondrial Ca^{2+} during severe ischaemia, as its function requires the mitochondrial membrane potential. However, a number of groups have catalogued an

increase in mitochondrial Ca^{2+} at the onset of reperfusion suggesting an import of cytosolic Ca^{2+} as mitochondrial function returns (Zaidan et al 1994). Levels of Ca^{2+} usually return to normal following global ischaemia but there is a second large mitochondrial influx later in the reperfusion period concomitant with delayed energy failure and death in certain cells (Kristian et al 1998).

The importance of mitochondrial accumulation was suggested when inhibition of mitochondrial Ca^{2+} uptake proved to be protective during an excitotoxic insult despite a continuing accumulation of cytosolic Ca^{2+} (Stout et al 1998). Ca^{2+} uptake at its most profound is accompanied by mitochondrial membrane depolarisation and induction of the permeability transition pore, which mediates the release of a number of pro-apoptotic factors (Duchen 1999). Inside the mitochondria, Ca^{2+} activates a number of key enzymes in the TCA cycle and can thus stimulate increased metabolism. The increases in Ca^{2+} during reperfusion, when the cell is in a considerably more oxidised state, could therefore result in the overproduction of mitochondrially generated reactive oxygen species (ROS). The consequence of this will be discussed later.

In the cytosol, Ca^{2+} accumulation has potentially toxic consequences as a result of the sustained activation of a number of key enzymes: The protease calpain has been shown to cause cytoskeletal breakdown following both focal and global ischaemia (Neumar et al 2001, Liebetrau et al 2004); endonucleases have been shown to cause DNA fragmentation (Tominaga et al 1993); phospholipase A2 is associated with injury following ischaemia (Tabuchi et al 2003) due to arachidonic acid formation and an accumulation of ROS (Adibhatla et al 2003). For a more comprehensive introduction to the role of Ca^{2+} in neuronal death see Paschen (2003) and references within.

1.2.5 Na^+

NMDA receptors have been suggested to mediate cell damage via Na^+ entry (Goldberg et al 1986). Beck et al (2003) have also demonstrated

the possibility that Na^+ and indeed Cl- entry via the Na-K-Cl cotransporter isoform 1 (NKCC1) can mediate early excitotoxic damage. The accumulation of these ions would result in the marked cell swelling that is evident during ischaemia.

1.3 Ischaemia and oxidative stress

1.3.1 Oxidative stress

Oxidative stress describes the situation where the cell's anti-oxidant capacity is compromised either by the excess production of damaging oxidizing species or the down-regulation of endogenous anti-oxidant systems. Either way, the cell is incapable of inactivating oxidizing agents such as reactive oxygen species (ROS) and a host of damaging events ensues. Oxidative stress has been implicated in neuronal death following both global ischaemia (Hayashi et al 2003) and focal ischaemia (Saito et al 2004). In fact the brain is particularly vulnerable to this type of insult due to its large O_2 consumption, high levels of polyunsaturated fatty acids and a surprising dearth of antioxidant defenses compared to the rest of the body (Floyd 1999, Juurlink et al 1997).

1.3.2 Evidence of oxidative stress in ischaemia

The production of free radicals, particularly radical O_2 species, is a major contributor to oxidative stress. Areas of the brain that are severely ischaemic do not contain O_2 so ROS formation is largely a phenomenon of reperfusion. However, infiltration of ROS from penumbral regions following a focal insult may produce a certain amount of damage during the ischaemic period. The events that occur during ischaemia very nicely set the stage for ROS production during reperfusion and it is at this point that oxidative stress becomes evident and damaging. In fact ROS have been shown to elevate very rapidly, in a burst like manner, at the onset of reperfusion (Dirnagl et al 1995, Kumura et al 1996) as O_2 availability returns.

There is evidence of hydroxyl radical ($OH^{\cdot}$) formation during focal ischaemia (Ste-Marie et al 2000) and indeed evidence of a progressive increase in superoxide ion ($O_2^{-\cdot}$) during reperfusion following a focal ischaemic insult (Mori et al 1999). It has also been shown that the core of the lesion produces far more $OH^{\cdot}$ than the penumbra and as such correlates well with damage (Liu et al 2003). One of the most highly toxic

of the ROS is peroxynitrite ($ONOO^-$), which is formed when $O_2^{-\cdot}$ reacts with NO. It is extremely membrane permeable and so has the potential to spread its damaging effects a distance from its source of production.

In demonstration of the type of damage ROS can do, peroxynitrite can peroxidate lipids, hydroxylate and nitrate aromatic residues of proteins and nucleotides and can also cause DNA strand breaks (Thiyagarajan et al 2004). Evidence of increased peroxynitrite production following ischaemia has been documented in a number of laboratories and indeed its reduction has been shown to be neuroprotective (Hattori et al 2002, Thiyagarajan et al 2004a, Nagai et al 2002).

1.3.3 Origin of radical species

As mentioned previously, a number of events occur during ischaemia which prepare the way for massive ROS production at the onset of reperfusion. The production of radical species therefore, has its true origins during the ischaemic event. We will discuss just a few of these.

There are a number of mechanisms by which ROS are thought to be produced. First of all the mitochondrion is particularly adept at producing $O_2^{-\cdot}$. Under normal circumstances there is a certain amount of electron 'leak' from the respiratory chain and so molecular O_2 is not completely converted to H_2O but instead produces $O_2^{-\cdot}$ (Turrens 1997). This is thought to result from the addition of electrons via complexes I and III particularly (Kudin et al 2004). During ischaemia the complexes of the respiratory chain become very reduced and so conditions are ripe for extra 'leak' and ROS production on the return of O_2 at the start of reperfusion.

The $O_2^{-\cdot}$ anion itself is not particularly membrane permeable but H_2O_2, produced by mitochondrial manganese-superoxide dismutase (MnSOD) activity, is and so can leave mitochondria (Raha et al 2000). H_2O_2 may also be converted to $^{\cdot}OH$ and OH^- in the presence of reduced iron (Fe^{2+}) through the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

The brain contains a very large amount of iron, so is particularly disposed to the production of these extremely reactive species.

As mentioned in section 1.3.2, oxidizing species cause a significant amount of direct damage to biological molecules. Another major consequence of the induction of oxidative stress is the opening of the permeability transition pore in mitochondria and the release of cytochrome c and other pro-apoptotic factors (Chernyak et al 1996). Inhibiting the opening of the pore has in fact been shown to reduce neuronal death following global ischaemia (Abe et al 2004) and focal ischaemia (Oshikawa et al 2004).

The enzyme xanthine oxidase (XO) has also been attributed with the generation of free radicals. Normally XO exists as a dehydrogenase (XDH) and both enzyme forms convert hypoxanthine to urate. However only XO actually produces $O_2^{-\cdot}$ in the process. XDH is converted to XO during ischaemia by a proteolytic process (Lipton 1999). The substrate of the reaction hypoxanthine also accumulates during ischaemia due to adenosine monophosphate breakdown (Parkinson et al 2002) so again upon reperfusion a lot of $O_2^{-\cdot}$ can result. Ghoneim et al (2002) support the idea that the XO reaction is a contributor to ROS production, as discussed, by inhibiting the XO-XDH conversion during ischaemia.

Nitric oxide (NO) is another radical species that is generated following ischaemia. NO is produced by the nitric oxide synthase (NOS) family of enzymes from the oxidative metabolism of arginine. There are three principal isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS) and the glial inducible NOS (iNOS). All three isoforms are found to be upregulated following ischaemia (Bolanos et al 1999). NO can be directly harmful to proteins and lipids but can also produce the even more harmful $ONOO^-$ molecule.

1.3.4 Antioxidant systems and neuroprotection

The brain houses a number of systems with which to cope with the generation of oxidants. Clearly the burst in ROS production during reperfusion will heavily burden the systems in place. How efficient an antioxidant system the cell has, or how quickly a cell can turnover its antioxidants, will therefore determine the extent of damage. This is made all the more critical when the antioxidant systems themselves become targets of the radical species produced by the cell. The development of damage during ischaemia has been correlated with the failure of antioxidants and is thought to contribute to the selective vulnerability seen in the brain.

1.3.2.1 Antioxidant enzymes

As mentioned above, $O_2^{-\cdot}$ is constantly produced by mitochondria under normal physiological conditions. This is usually disposed of by superoxide dismutase (SOD), which dismutates $O_2^{-\cdot}$ to produce H_2O_2 and O_2. There are three SOD isoforms, Cu/Zn SOD (cytosolic), MnSOD (mitochondrial) and extracellular SOD. There is much evidence supporting a neuroprotective role for these enzymes following ischaemia. Fujimara et al (1999) showed, using MnSOD knockout mice that this isoform attenuates mitochondrially-mediated apoptosis following focal ischaemia. This group has also shown that the overexpession of Cu/Zn SOD is neuroprotective following the same insult (Fujimara et al 2000). SOD mimetics have also been used successfully to achieve neuroprotection at least in young rat brains following a hypoxic period (Shimizu et al 2003). There is some evidence linking the lack of upregulation of both Cu/Zn SOD and MnSOD following ischaemia to vulnerability to delayed death (Takeutchi et al 2000). Furthermore Chan et al (1998) have shown that overexpression of Cu/Zn SOD protects CA1 neurones following global ischaemia.

The H_2O_2 formed by SOD is scavenged by the enzymes catalase (CAT), found in peroxisomes, and glutathione peroxidase (GSHPx), found in the

cytosol and mitochondria, which essentially terminate the toxic effects of O_2^{-}.

CAT, which decomposes H_2O_2 to O_2 and H_2O, is expressed constitutively at comparatively low levels in neurones and so GSHPx is very important for detoxifiying H_2O_2. Chen et al (2000a) have shown that the activities of CAT, GSHPx and indeed MnSOD are all increased in late reperfusion following ischaemia. Interestingly, Homi et al (2002) have shown that vulnerable regions such as the hippocampus show a more marked decay in CAT activity than areas that are resistant, early in the reperfusion phase following global ischaemia. This may indeed contribute to selective vulnerability later on as these cells have further to go to match the general increase in ROS mentioned earlier.

GSHPx detoxifies H_2O_2 at the expense of glutathione (GSH), which is considered to be the cell's major antioxidant. The overexpression of GSHPx has been shown to be neuroprotective following ischaemia (Hoehn et al 2003, Ishibashi et al 2002). In support of these findings, the GSHPx mimetic ebselen has also been shown to reduce oxidative damage following ischaemia (Imai et al 2003). Li et al (2002) have further attributed the protective effects of spiramine T in ischaemia-reperfusion injury in part to an upregulation of GSHPx. Finally, transgenic mice deficient in cytosolic GSHPx show exaggerated injury in response to ischaemia/reperfusion (Crack et al 2001 and 2003).

1.3.4.2 Antioxidant molecules

GSH is the principal antioxidant molecule in the brain and is present at mM concentrations within the cell (Dringen 2000). It is synthesised in two steps, first, glutamate and cysteine (cys) are combined to form γ-glutamyl-cysteine (γGluCys) by the enzyme γGluCys synthetase and then glycine is added by the enzyme GSH synthetase. GSH can work to directly scavenge ROS nonenzymatically or as a substrate of GSHPx. The product is either the oxidised form of glutathione (GSSG) or protein-glutathione mixed disulfides. GSSG can then be recycled to GSH by the

enzyme glutathione reductase (GR) at the expense of NADPH (Dringen et al 2000).

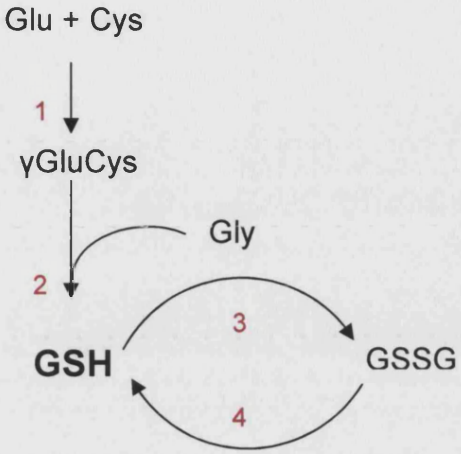


Fig 1.3 GSH metabolism. This figure highlights the main enzymes in GSH metabolism concerned with this discussion; γglutamylcysteine synthetase (1); glutathione synthetase (2); glutathione peroxidase (3); glutathione reductase (4).

The role of GSH in oxidant scavenging is supported most obviously by the recordings of its decline during the reperfusion period, when ROS are reported to flourish (Shivakumar et al 1995). In culture, the actions of ROS-generating excitotoxins have been shown to depend on intracellular GSH level (Ceccon et al 2000, Parihar et al 2003). Curiously, extracellular GSH has been shown to be neuroprotective both *in vivo* and *in vitro* (Cho et al 2003) but it has also been suggested to exacerbate ischaemia-related neuronal injury in culture by reducing the redox site of the NMDA receptor (Regan et al 1999a and b).

It has been shown that a specific reduction of brain GSH with inhibitors of its biosynthetic pathway leads to the elevation of ROS (Gupta et al 2000) and renders the brain far more sensitive to ischaemic injury (Mizui et al 1992). This has also been observed in astrocyte cultures during aglycaemia (Papadopoulos et al 1997). The corollaries of these experiments are those that show increasing cellular GSH to be protective against oxidative stress. Sola et al (1996) showed that fructose-1,6-bisphosphate is protective in a model of ischaemia and have gone on to

show that this is due to a preservation of cellular GSH as a result of NADPH being made available for the GSH reductase reaction (Vexler et al 2003). However, it should be mentioned that GSH depletion is actually neuroprotective in some situations. Vanella et al (1993) deplete GSH 24hrs before ischaemia-reperfusion and conclude it to be neuroprotective. Yang et al (1995) have produced evidence that this effect is mediated by decreased glutamate release.

We have already mentioned that mitochondria are the principal source of ROS following ischaemia. Fittingly the GSH content of this organelle is very important in determining the extent to which ROS can harm the cell. Anderson et al (2002) have observed that the development of tissue infarction following focal ischaemia is associated with changes in the mitochondrial GSH pool. They have recently added to this by showing that specific increase of mitochondrial GSH is neuroprotective in the same injury (Anderson et al 2004 a and b). Such an observation has also been made *in vitro* as Huang et al (1996) show that specific reduction of mitochondrial GSH in astrocyte cultures leaves them highly susceptible to injury. The link between mitochondrial protection and GSH levels is further strengthened by Makarov et al (2002). They show that there is a threshold level of cellular GSH below which further, ischaemia-induced depletion and mitochondrial damage is exaggerated.

Ascorbate, like GSH, is present in the brain at mM concentrations and is also assumed to be an important antioxidant. Interestingly, unlike GSH, ascorbate concentrations are greater in neurones than in astrocytes (Rice et al 1998, Berger et al 2003). Ascorbate cannot be synthesised in the brain by mammals and in fact cannot be synthesised at all by humans (Rice 2000) so it must therefore be consumed in the diet and transported into the cells of the brain. This task is primarily achieved by the Na^+ dependent vitamin C transporter 2 (SVCT2) and once inside, any oxidised ascorbate must be recycled using endogenous reducing molecules (Tsukaguchi et al 1999). Ascorbate can also be transported in heteroexchange with glutamate and via a glucose transporter, both of

which we will refer again to later (Cammack et al 1991, Rebec et al 1994, Vera et al 1993).

In its actions as an antioxidant, ascorbate can be fully oxidised to dehydroascorbate but the loss of only one of its two electrons results in the formation of the ascorbyl radical, which is itself, a marker of oxidative stress (Rice 2000). With respect to ischaemia, ascorbate release to the extracellular space has been recorded as an early event of focal ischaemia and global ischaemia-reperfusion (Crespi 1996 Yusa 2000). It has been shown to be neuroprotective through a number of experiments.

Administration of ascorbate reduced infarct size following focal ischaemia in a primate model (Henry et al 1998). Evidence suggests that its actions in preventing neuronal damage are due to free radical scavenging (Perez-Pinzon et al 1997, McGregor et al 1996). However it has also been shown that ascorbate attenuates NMDA receptor mediated insults through a direct redox modulation of the receptor (Majewska et al 1990, Bell et al 1996). However, although Perez-Pinzon (1997) demonstrate neuroprotection in a hippocampal slice, Stamford et al (1999) claim that ascorbate is not protective in the hippocampus but is in the striatum following global ischaemia. In fact, apart from having no action in some conditions ascorbate has been shown to have pro-oxidant actions in others. This story will be picked up again in section1.4.

The final antioxidant molecule we will mention is α-tocopherol (vitamin E), which is derived from vegetable oils, taken up in the intestine and distributed throughout the body where it forms part of the cell's membranes. As such it has been shown to be crucial in protecting membrane lipids from peroxidation (Herrera et al 2001). Cellular uptake of α-tocopherol is mediated, at least in culture, by the scavenger receptor class B type I (SR-B1) (Mardones et al 2002). It is a potent antioxidant with proven neuroprotective effects following cerebral ischaemia (Yamamoto et al 1983, Hara et al 1990, Tagami et al 1999, Chaudhary et al 2003, Sikorska et al 2003). Analogues of α-tocopherol have likewise been used to attenuate infarction following ischaemia (Van der Worp

1999, Cowley et al 1996). The brevity of this section is testament to the fact that α-tocopherol is not as well explored as other antioxidant molecules and indeed there is no evidence to suggest that ischaemia targets α-tocopherol uptake and distribution in any way.

1.4 Astrocytes and ischaemia

As an information-processing unit, the brain must be considered to be the sum of its parts, it cannot work efficiently (or at all) unless all its constituent cells are present. As such, neurones are no longer considered to be the sole components of networks in the brain, even if fast synaptic transmission is the accepted tool of brain function, as it has become abundantly evident that each cell type has evolved its own specialized function that contributes to the regulation and fine-tuning of each and every processed signal. The death, or indeed damage of glia will affect neurones and fast synaptic signalling and it is with this in mind that the following section will be presented. Only the role of astrocytes will be described, as these are the best-characterised glia to date with respect to ischaemia-reperfusion. We will begin however by illustrating the critical role astrocytes play in the brain in the form of a case study of the glutamate system.

1.4.1 Astrocyte-neurone partnership

Astrocytes and neurones are intimately linked by the glutamate system on a number of levels and it is the study of this system that has uncovered the true importance of astrocytes. First of all astrocyte EAATs are the main scavengers of glutamate from the synaptic cleft (Anderson et al 2000) and so play an important role in the modulation and fine tuning of synaptic transmission. Pfrieger et al (1997) and Oliet et al (2001) have neatly demonstrated the importance of glial presence to the efficacy of synaptic transmission and specifically the role played by glutamate clearance in this process. Further to this, astrocyte glutamate uptake is also important to the regulation of 'spillover' and thus intersynaptic crosstalk (Barbour et al 1997, Mitchell et al 2000, Piet et al 2004).

Beyond a direct role in synaptic transmission glutamate forms a metabolic link between astrocytes and neurones. Glutamate is produced in the mitochondrial matrix following the transamination of α-ketoglutarate, a TCA-cycle intermediate, by the action of glutamate dehydrogenase. This, and the neurotransmitter pool, can be subsequently used to produce glutamine in a reaction catalysed by glutamine synthetase. These two amino acids are essential in providing nitrogen for the synthesis of proteins, nucleic acids and a host of other molecules. However neurones lack the enzyme glutamine synthetase and so do not have the capacity to generate their own glutamine. They therefore rely on astrocytes to convert their glutamate to glutamine and export it back to the neurone (Hertz et al 1999) (fig 1.4).

Glutamate uptake has been shown to alter astrocyte metabolism. Transport of neuronal derived glutamate by astrocytes has been suggested to increase glycolytic rate and the subsequent release of lactate for neurones to use as fuel, at least *in vitro* (Pellerin et al 1994, Magistretti et al 1999). The relevance of this 'lactate-shuttle' hypothesis as it is called is however hotly debated as it has yet to be shown that such a system functions *in vivo* (See Dienel et al 2004 and Pellerin et al 2004 for a detailed argument).

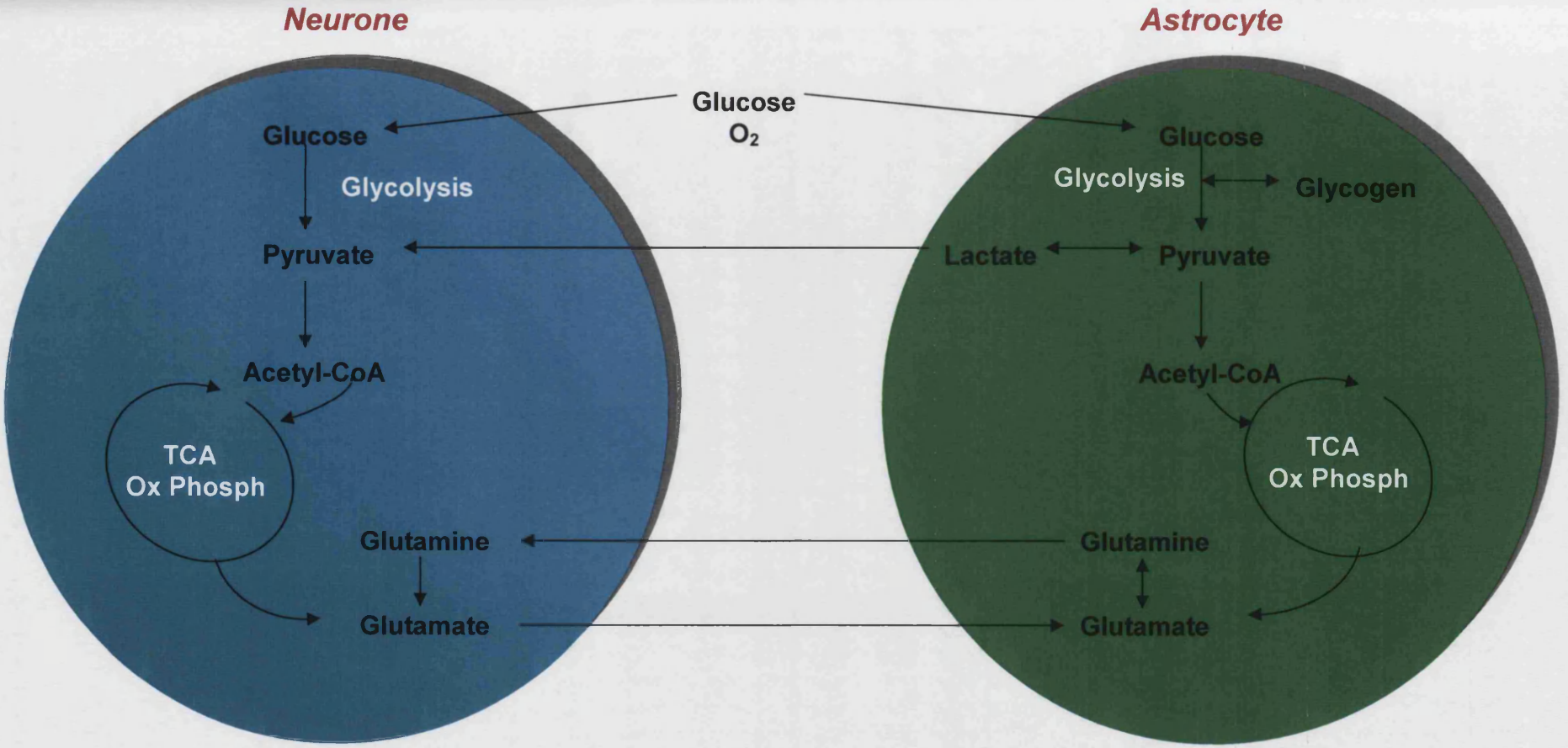


Fig1.4 Likely metabolic coupling. This figure highlights the points of metabolic exchange between astrocytes and neurones. Both cells synthesise ATP in the mitochondrion through oxidative phosphorylation. Both cells also produce glutamate via the tricarboxylic acid cycle (TCA) however only astrocytes can synthesise glutamine, which they shuttle to neurones. Included is the transfer from astrocytes to neurones, which has been shown in culture. (*Based on principles in Hertz et al 1999*).

The story does not stop simply with the uptake of glutamate. Astrocytes have been shown to express a number of glutamate receptor subunits (Nedergaard et al 2002). In fact Cornell-Bell et al (1990) showed for the first time that applied glutamate was capable of inducing Ca2+ waves in astrocytes, an observation that was repeated later with neuron derived glutamate *in vitro* (Dani et al 1992, Porter et al 1996) and most recently *in vivo* (Hirase et al 2004). Interestingly, a consequence of these Ca^{2+} waves is the release by astrocytes of glutamate, amongst other molecules, that can feedback to the neuron (Parpura et al 1994, Pasti et al 1997). The idea of a feedback system is elegantly demonstrated by Dziedzic et al (2003) in the neuroendocrine system. They have suggested that neuronal glutamatergic signaling stimulates an erbB-related back signal from the astrocyte resulting in the release of luteinizing hormone-releasing hormone (LHRH) from neurones.

The precise mechanisms of glutamate release from astrocytes remain to be fully elucidated. We have just described the existence of a Ca^{2+} dependent glutamate release and it has been suggested that a vesicle-mediated mechanism, similar to neurones may be at least partly responsible. In fact Bezzi et al (2004) have recently established, quite convincingly that astrocytes house glutamate vesicles containing vesicular glutamate transporters (VGLUTs) that undergo SNARE (soluble NSF attachment protein receptors) dependent fusion and glutamate release. Release has also been shown to occur through volume-sensitive organic anion channels (VSOACs), which also allow the passage of aspartate and taurine down their concentration gradients (Kimelberg et al 1990, Feustal et al 2004) and which may or may not be Ca^{2+} related. It has recently been suggested that glutamate may be released through connexin hemichannels (Ye et al 2003). As has previously been discussed glutamate is also released due to reverse operation of the Na^{+} glutamate transporters during ischaemia but no role has yet been evidenced for this mechanism to exist under normal physiological conditions (Barbour et al 1998, Rossi et al 2000).

1.4.2 Fate of astroglia

Ischaemic cell death is not the preserve of neuronal populations, as astrocytes and other glia have also been shown to die following ischaemic insults. Liu et al (1999) report astrocyte death to precede neuronal death during permanent focal ischaemia. Petito et al (1998) showed that not only are glia susceptible to ischaemic damage but that oligodendrocytes are even more susceptible than neurones in certain regions. Microglia on the other hand have been reported to show a high degree of resistance to ischaemia thought to be on a par with astrocytes (Yenari et al 2001). Loss of glia will directly affect neurones, for example oligodendrocyte death will result in demyelination and defective synaptic transmission (Dewar et al 2003, Tanaka et al 2001). The spotlight is often shone on microglia and macrophages because of the neurotoxic substances they secrete during and after ischaemia (Banati et al 1993, Mabuchi et al 2000).

Astrocytes have been shown to be far more resilient than neurones to ischaemic insults in culture (Almeida et al 2002), furthermore two of the most interesting phenomena of ischaemia, delayed death and selective vulnerability, were largely considered to be sole features of neurones. These facts coupled with the long held opinion that astrocytes (and glia generally) were inconsequential bystander cells in the brain led to glia being ignored for quite a while. However, astrocytes too exhibit a regional and type-specific vulnerability. Zhao et al (2000) show, in culture, that hippocampal astrocytes are more sensitive than cortical astrocytes to O_2-glucose deprivation (OGD). Whereas Lukaszevicz et al (2002) show that protoplasmic astrocytes are more prone to ischaemic injury than fibrous astrocytes.

It was mentioned in section 1.1.3 that glia respond to severe focal ischaemia in a process called anisomorphic gliosis. This results in the formation of a 'glial scar', the wall that surrounds the infarct, and the release of a number of neurotoxic molecules as the glia become 'activated'. As part of the glial scar activated astrocytes are more

correctly called reactive astrocytes although this terminology isn't widely acknowledged. The appearance of activated/reactive astrocytes is one of the most salient features of ischaemia (Gabryel et al 2001). These astrocytes exhibit marked hypertrophy and proliferation and show an increase in the cytoskeletal proteins GFAP and vimentin (Petito et al 1990, Kunkler et al 1997). Astrocyte processes also become elongated particularly in the area surrounding the infarction (Kajihara et al 2001). Although reactive gliosis is attributed with the production of a number of neurotoxic molecules, reactive astrocytes may not in themselves be harmful, as reactive microglia appear to be the major source of neurotoxins (Liberto et al 2004). In less severe ischaemia, there is also a general astrocyte upregulation of antioxidant enzymes such as SOD and GSHPx and a host of growth and signaling factors (Liu et al 1993 and see Gabryel et al 2001 for extensive list). Such a reaction is considered beneficial to surrounding neurones and this type of astrocyte is considered to be activated (Liberto et al 2004).

1.4.3 The role of gap junctions

Astrocytes signal extensively between themselves and can in fact be considered to exist as part of a glial syncytium as they are intimately connected to both other astrocytes and oligodendroglia via gap junctions (Dermietzel et al 1993, Altevogt et al 2004). Gap junctions are composed of protein channels, which directly link the cytoplasm of one cell with that of another allowing the passage of ions and molecules of up to 1kDa such as Ca^{2+} and inositol trisphosphate. Each channel is composed of two connecting channels called connexons, one contributed by either cell. Each connexon is then made of connexins, which belong to a family of highly related proteins. Connexins show a regional expression pattern with Cx43 being particularly prominent in astrocytes (Nagy et al 2001). Neurones too have been shown to express the connexin proteins that make up the gap junction channels (Rash et al 2000) and there is evidence that they can form gap junctions with themselves and with astrocytes (Duan et al 2004, Nedergaard 1994).

An illustration of the extent to which astrocytes are capable of communicating is seen in Ca^{2+} mediated signaling. We have already mentioned the findings that Ca^{2+} waves have been shown to spread through astrocyte layers in culture and indeed in vivo. This has been shown to occur via gap junctions directly (Charles 1998) but also indirectly via the release of ATP through hemichannels (half a gap junction, leading to the extracellular space) and subsequent activation of purinergic receptors (Stout et al 2002).

Gap junctions have roles in processes other than signalling for example the maintenance of ion homeostasis. Astrocytes are very important in maintaining and restoring extracellular ion concentrations after sustained neuronal activity. They are involved in limiting extracellular $[K^+]$ and redistributing it through gap junctions to other cells in a process called spatial buffering (Karwoski et al 1989). Extracellular $[H^+]$ and thereby pH is also controlled by astrocyte uptake (Deitmer et al 1996).

Ion uptake and redistribution via gap junctions is not always considered to be a positive step. For example, it has been suggested to contribute to ischaemic spreading depression in focal ischaemia (Nedergaard et al 1993). This is a process whereby depolarization of neurones spreads in a wave-like manner from the ischaemic core. The Ca^{2+} ion has received particular attention in this regard due to the multiplicity of intracellular processes it is involved in. There is evidence for and against the spread of Ca^{2+} waves through astrocytes as a mechanism for propagating spreading depression. Peters et al (2003) seem to have resolved this by showing that the velocity of the Ca^{2+} wave through astrocytes is dependent on spreading depression but that it is propagated in a different manner.

The role of astrocyte gap junctions in ischaemic injury is somewhat of a grey area. Gap junction communication can be disrupted by high levels of Ca^{2+}, low pH and the generation of ROS conditions (Martinez et al

2000), which abound after ischaemia. But what effect does this have on cell survival?

Lian et al (2004a and b) support the view that astrocytes are involved in regulating K^+ and Ca^{2+} concentrations during spreading depression but they claim that astrocyte dysfunction, and the inability to redistribute ions, exacerbates neuronal damage to this type of insult. Nakase et al (2003) also demonstrate increased penumbral damage in ischaemic mice deficient in connexin43, a major astrocyte gap junction protein. Similarly Blanc et al (1998) have shown that astrocyte gap junctions reduced neuronal vulnerability to oxidative stress in culture. However, Lin et al (1998) argue that gap junctions provide a route by which pro-death signals can be propagated from one cell to another implicating them in the production of secondary injury following ischaemia. In support of this hypothesis Frantseva et al (2002) show in hippocampal slices that knockdown of both astrocyte and neuronal connexin subtypes inhibits ischaemic cell death. With respect to gap junctions in other cell types, Oguro et al (2001) link an increase in neuron and oligodendrocyte specific connexins with selective neuronal resistance following global ischaemia.

Clearly the contribution of gap junctions to ischaemic injury is a complicated one. Contreras et al (2002) show that, in culture, ischaemia-like conditions promote the opening of hemichannels. This may marry the previous observations, as the proportion of full channels versus hemichannels that are open could vary under different conditions in different cell types and thus produce different injury profiles.

1.4.4 Antioxidant support

Another fine example of how heavily neurones, and indeed some other brain cells rely on astrocytes, is in the role they play as part of the brain's antioxidant defence system. As mentioned previously, the generation of ROS is a major cause of cell damage following ischaemia. The burden largely falls upon astrocytes as to whether the outcome of a ROS insult is

positive or negative. This is due to the intimate redox coupling between astrocytes and neurones centered on the molecules GSH and ascorbate.

We have shown that GSH is protective against ischaemic neuronal damage but what part do astrocytes play? First of all astrocytes are thought to have a more efficient GSH system than neurones. Culture studies suggest that not only do they have much higher levels of GSH immediately available but also much higher levels of the crucial enzymes involved in GSH metabolism (Wilson 1997, Makar et al 1994). For example, Almeida et al (2002) have shown that astrocytes in culture could use their glucose from their glycogen stores to generate NADPH, via the pentose phosphate pathway, in order to recycle GSH during O_2-glucose deprivation, rendering them more resistant than neurones.

Astrocytes are therefore more competent to deal with ROS but also support ROS scavenging in neurones especially as ascorbate, the dominant antioxidant in neurones, is not synthesized by the neurones. It is conceivable that astrocytes may relieve neurones of some stress by offering themselves as an alternate target for ROS attack, using their endogenous GSH directly (Chen et al 2001) or indeed GSH released from the astrocytes may itself act as a scavenger extra-cellularly (Cho et al 2003).

Astrocytes do in fact support the levels of GSH found in neurones (Dringen et al 2000) and it has been shown that cultured neurones have very low levels of GSH unless they are in coculture with astrocytes (Sagara et al 1993). Dringen et al (1999) have suggested that astrocytes in culture release GSH, which is cleaved by the ectoenzyme γ-glutamyl transpeptidase to produce Cys-Gly, which is then used by neurones to make GSH under some conditions. Wang et al (2000) suggest that the released GSH is used to generate Cysteine from Cystine and is then taken up by neurones. In either event, neurones cultured with astrocytes are far more resistant to attack by ROS than neurons cultured alone (Tanaka et al 1999). Going one step further astrocytes, which have been

depleted of GSH, lose their ability to help co-cultured neurones (Chen et al 2001).

The GSH system also plays a part in the recycling of ascorbate to form a single redox cycling unit. Astrocytes in culture have been shown to recycle dehydroascorbic acid to ascorbate, in a GSH dependent manner. But more importantly it is suggested that they can then release this ascorbate to the extracellular space for neurones to take up (Siushansian et al 1997, Wilson 1997). Dehydroascorbate treatment has been shown to reduce ischaemic injury in vivo following a focal insult (Huang et al 2001) but dehydroascorbate cannot itself scavenge free radicals, it must be converted to ascorbate first. This would therefore suggest an important role for astrocytes in shuttling converted dehydroascorbate to neurones, as ascorbate, to elicit the beneficial effects.

The role of astrocytes is further strengthened by Wilson et al (2000) who provide evidence that astrocytes in culture release ascorbate via glutamate activated ascorbate channels in response to application of extracellular glutamate. The implication is that such a system exists to prevent oxidative stress during physiological glutamate signalling, and further during excitotoxicity.

When it is working properly, the astrocyte-neurone redox system is clearly effective at maintaining neuronal viability. However, the oxidative stress that follows ischaemia can sour the relationship and astrocytes can exacerbate neuronal damage. GSH depletion in mixed cultures has actually been shown to affect glial GSH levels more than neuron levels (Keelan et al 2001). Furthermore Mytilineou et al (1999) have shown that glia actually mediate neuronal injury following GSH depletion in culture.

With respect to ascorbate, it is at this point that we can rejoin the recounting of ascorbate as a pro-oxidant. Song et al (2001) suggest that, in vitro, dehydroascorbate uptake is toxic to neurones cultured on their own. It is thought to be transported in to the cell via the glucose

transporter GLUT-1 (Patel et al 2001); once inside it is converted to ascorbate but this process uses up endogenous antioxidants and so excess uptake leads to oxidative stress (Song et al 2002). Oxidative stress reduces the ability of astrocytes to take up dehydroascorbate (Daskalopoulos et al 2002), which leaves more around for neuronal uptake. Interestingly, very high levels of ascorbate have been shown to disrupt glucose transporters (Patel et al 2001), which may have adverse effects on astrocyte metabolism during ischaemia.

See figure 1.5 for an illustration of astrocyte-neurone redox coupling.

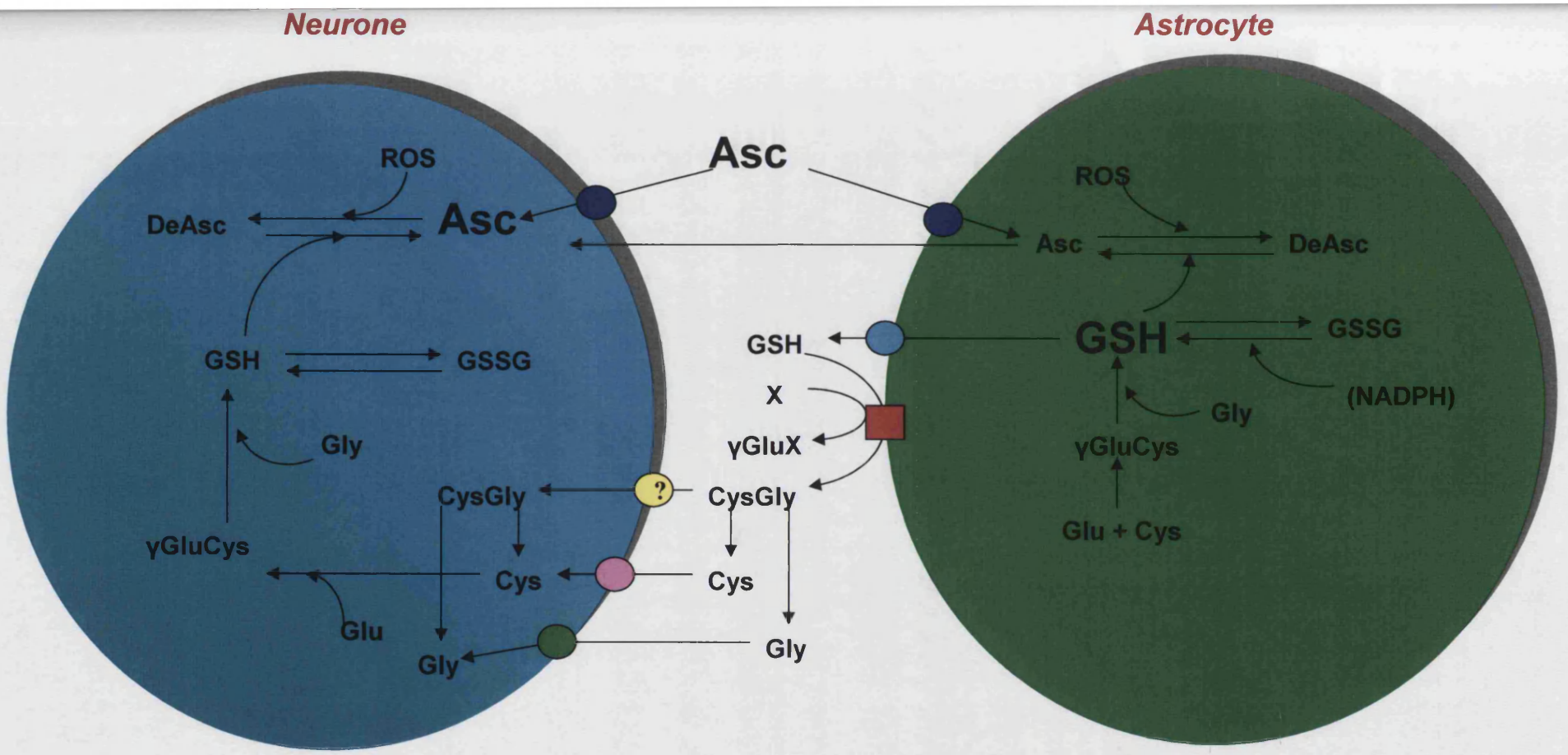


Fig 1.5 Redox coupling. This figure is an overview of the fate of antioxidant cycling between astrocytes and neurones. Ascorbate (Asc) is taken up by astrocytes and neurones by SVCT2, and oxidised to dehydroascorbate (DeAsc). Dehydroascorbate can be recycled to ascorbate at the expense of GSH. Astrocytes release both ascorbate and GSH for eventual neuronal uptake. GSH is released via MRP proteins and is cleaved by γglutamyl transpeptidase. Cys-gly is cleaved by neuronal PEPT2. Included is the point at which pentose phosphate-derived NADPH fits in to GSH synthesis. (*Based on principles in Dringen et al 2000)*

1.4.5 Secretion of other neuromodulatory substances

We have already seen the bi-directional communication between astrocytes and neurones from the point of view of just one signaling system, glutamate. Astrocytes do however release a whole host of other molecules that modulate neuron function and indeed the function of other cell types. These include neurotrophins such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), both on neuronal demand (Galv-Roperh et al 1997, Mizuta et al 2001, Wu et al 2004); inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor (TNF) (Acarin et al 2000); and finally chemokines such as erythropoietin (Epo) (Ruscher et al 2002). Astrocytes have also been shown to release molecules such as cholesterol, which reportedly affects neuronal glutamate transport (Canolle et al 2004).

Ischaemia also increases the astrocyte production of cytokines such as tumour necrosis factors, interleukins and interferons as they become activated (Dong et al 2001). The ultimate effects on neuronal survival of this reaction is mixed. IL-6 for example is suggested to be neuroprotective against excitotoxic insults (Pizzi et al 2004) and Matsuda et al (1996) demonstrated its ability to prevent CA1 neuronal loss following ischaemia. However, another pro-inflammatory cytokine TNF-α is suggested to mediate neuronal death following ischaemia and excitotoxic insults (Liao et al 2002, Yu et al 2002). It remains to be determined precisely what role specific astrocyte release of these cytokines has *in vivo.*

One of the key features of astrocytes' response to ischaemia is to induce expression of iNOS and thus release NO (Endoh et al 1993). As mentioned, NO is implicated in neuronal death following ischaemia. In fact astrocyte-derived NO from iNOS upregulation is suggested to have significant neurotoxic effects in culture following O_2-glucose deprivation (Hewett et al 1996). Furthermore, the expression of iNOS coincides with delayed neuronal death and as such this has been proposed as one of

the factors implicated in the occurrence of this phenomenon (Yamashita et al 2000).

1.5 Modelling ischaemia

An ischaemic insult can be modelled both *in vivo* and *in vitro* in a variety of different ways. We will concentrate on the detail of simulating ischaemia *in vitro* given that this is the thrust of the current thesis. For a comprehensive review on the use of whole animal systems, please see Lipton 1999.

1.5.1 Terminology

Ischaemia/reperfusion injury is a field of study that has been explored at many levels *in vivo*. When using an *in vitro* model however, protocol dictates that the insult be called oxygen-glucose deprivation (OGD)/reoxygenation, which neatly breaks the task of establishing the model into three parts. Oxygen must reach acceptably low levels, metabolic substrates must be eliminated and finally, the system must be returned to 'normal' conditions. A point to note is the use of the term 'glucose deprivation'. It has become the generic term for substrate deprivation as most groups actually deprive cells of glucose, amino acids, serum and any other metabolisable factors.

1.5.2 Simulating ischaemia

1.5.2.1 Oxygen deprivation

Without doubt, the most challenging part in simulating ischaemia is in the creation of a hypoxic environment. Hypoxia in cell culture is usually achieved by replacing atmospheric O_2 with an inert gas mixture within an airtight chamber. Typically combinations of 95%N_2/5%CO_2 or 85%N_2/5%CO_2/10%H_2 are used. Commercial units are available from companies such as Forma scientific and Don Whitely scientific, which consist of dedicated incubators equipped with temperature controlling, O_2 sensing and O_2 scavenging systems. Some units have glove box access, which allows cultures to be manipulated within the anoxic environment. In such cases, the anoxic gas mixture can be efficiently bubbled through the culture media to remove O_2. Excess O_2 is then often mopped up by kits, which exploit the reaction between O_2 and H_2 in the presence of a Pd

catalyst (GasPak, Beckton Dickinson) or the reaction between activated carbon and ascorbic acid (Anaerogen, Oxoid Ltd). These anaerobic chambers can be very expensive to buy and maintain and so a number of alternative approaches have been devised. Furthermore, the replacement of O_2 with N_2 is a relatively slow process in terms of producing a decline in ATP similar to *in vivo* hypoxia.

Some groups have bypassed the necessity for a dedicated airtight incubator by substituting N_2 with Ar (Kusumoto et al 1996, Meloni et al 2001). Because of its high molecular mass, Ar not only replaces O_2 far more quickly than N_2 but it eliminates the requirement for a sealed chamber. Such a system is therefore ideal for real time analysis using, for example imaging systems. However, as with most alternatives there is a down side, Ar will replace not only O_2 but also CO_2, which will upset the bicarbonate buffer system.

1.5.2.2 Chemical ischaemia

The elimination of environmental O_2 can be completely bypassed. Elements of ischaemic damage can be simulated effectively by using metabolic inhibitors in lieu of O_2 deprivation. This is generally done at either the level of the mitochondrial respiratory chain or the glycolytic pathway. Salts of CN^- have been used to inhibit the cytochrome aa3 complex (Erkkila et al 2003) and Antimycin A has been used to inhibit electron flow between cytochrome b and c1 (Jeong et al 2003) thus causing a decline in ATP similar to that observed in the absence of O_2 *in vivo*. Glycolysis can be blocked through the use of iodoacetate, an inhibitor of glyceraldehyde dehydrogenase (Uto et al 1995). A popular approach has been to use 2-deoxy glucose which is an inhibitor of glycogen phosphorylase when metabolised to 2-deoxy-glucose-6-phosphate (Dringen et al 1993).

The inhibition of respiratory chain elements results in a rapid decline in ATP similar to the falls recorded *in vivo*, however there is evidence that the cell may react in a fundamentally different manner to inhibitors of

oxidative phosphorylation and so the method is not as physiologically relevant as O_2 replacement (Shin et al 2003). Furthermore, the chemicals used tend to have complicating side effects, for example, iodoacetate inactivates thiol-containing enzymes. Also, it is slightly more difficult to remove the inhibition and make sure these agents have been eliminated if studying reperfusion injury.

1.5.2.3 Substrate deprivation

Anoxia can be combined with the deprivation of metabolic substrates to fully achieve ischaemia. This may involve the simple removal of glucose, pyruvate, and any other compounds capable of feeding into the glycolytic pathway and citric acid cycle, from the incubating media (Pauwels et al 1985) or it may involve the removal of all trophic support, such as growth factor-containing serum, as well (Xu et al 2001, Goldberg et al 1993).

1.5.2.4 O_2 detection

If a model of hypoxia is to be used based on the withdrawal of O_2, it is important to be able to measure the levels of O_2 within the system of study. There are a number of options available, some of which suit different experimental set-ups better than others. A common approach has been to determine O_2 concentrations polarographically which gives online real-time measurements of O_2 tensions. Some companies have produced miniature electrodes specifically for dipping into fluids (eg. MI-730 from Microelectrodes inc., Licox MCB from Harvard Apparatus), others have produced a more traditional arrangement where the solution of study is placed in the electrode chamber (eg. 782 Oxygen meter from Strathkelvin Instruments) which can be housed within an anaerobic incubator with little in the way of accompanying cables.

A complete departure from the use of O_2 electrodes is the use of immunohistochemistry based on the binding of nitroimidazole compounds to hypoxic tissue and cells (Raleigh et al 2001, Raleigh et al 1999, Arteel et al 1998). Such compounds are reductively activated and bind to macromolecules at low O_2 tensions. This chemistry was initially used to

track hypoxia related radiation resistance within tumours, but Koch et al (2002) have developed methods for measuring hypoxia within both culture and intact tissue based on EF5 (a nitroimidazole) binding. Although extremely accurate, this method gives a snap shot rather than a continuous assessment of O_2 concentrations.

It is desirable to precisely quantify the amount of O_2 present in a given system, yet it is not always practical to do so on every experimental run. For this reason some groups turn to the use of chemical indicators that will produce a simple colour change upon the elimination of O_2. This approach is common practice amongst microbiologists working with anaerobic bacteria. Popular examples would be the GasPak methylene blue system from Beckton Dickinson or the Oxoid resazurin based indicator.

1.5.3 Ischaemia in culture

1.5.3.1 Existing culture systems

Tissue culture models now provide a means to dramatically reduce the number of variables inherent in the study of neuroscience and crucially, allow for the delineation of cellular and molecular events. In essence, culture techniques allow the individual threads of biological processes to be teased apart, manipulated and reassembled into a coherent understanding of brain function.

Organotypic slice cultures are a popular tool with which to create a model of ischaemia in culture. They effectively offer themselves as the next step down from animal models of ischaemia in terms of complexity, as the cartography of the brain is largely retained without the involvement of factors such as blood pressure and body temperature (Strasser et al 1995, Pringle et al 1997, Laake et al 1999). Particularly, hippocampal and neocortical slice cultures exhibit the phenomena of delayed death and regional vulnerability (Bernaudin et al 1998), important aspects of ischaemia research.

Dissociated cell cultures take pride of place in the study of ischaemia in terms of model simplicity. Glia and neurone rich cultures from the neocortex (Goldberg et al 1993), striatum (Xu et al 2001), hippocampus (Khaspekov et al 1998) and cerebellum (Scorziello et al 2001) have all been used extensively to model O_2-glucose deprivation. These cultures can be highly pure for neurones (Dugan et al 1995, Meloni et al 2002) or astrocytes (Zhao et al 2000) or contain a mixture of neurones and glia (Goldberg et al 1993).

1.5.3.2 Advantages of dissociated cell cultures

Ischaemia has profoundly differing effects on different brain cell types from different regions. For example, in response to a global ischemic insult, neurones of the CA1 hippocampal sub-field are most vulnerable to death (Cervos-Navarro et al 1991). There is a significant body of research into the effects of ischaemia on whole brain preparations and slice systems, particularly in identifying vulnerable and resistant cell populations (Lipton 1999). Their chief advantage is in the maintenance of the brain's architecture and a close approximation of the global consequences of ischaemia. However, much along the lines of the Heisenberg uncertainty principle, maintaining the networks of the brain means that only a limited amount of knowledge can be gained from individual cell types with respect to molecular and biochemical processes. To gain such information it is therefore more logical to create ischaemia within a homogenous cell population.

An important advantage of dissociated cultures over *in vivo* models and sometimes slice systems is the ease with which the cellular environment can be manipulated. Body temperature, blood flow and the vasculature generally become very important parameters during *in vivo* ischaemia. For example, a change in temperature of just 2°C can have a dramatic effect on injury outcome (Dietrich et al 1990) and so must be tightly controlled. There is considerable literature on the contribution of vascular damage, blood brain barrier permeability and blood flow to ischaemic damage (Ayata et al 2002, Croll et al 2001), illustrating that there are an

incredible amount of components to consider when analysing ischaemic injury. Furthermore, when considering the application of drugs, delivery across the blood brain barrier to the correct place and at the most effective concentration, can be a problem for in vivo models (Partridge 2002). By using cell cultures one can, not only control factors such as temperature more easily, but also the application of drugs is greatly simplified.

1.5.3.3 Relevance of dissociated cultures

There is a vast amount of literature showing that the regional and cellular vulnerabilities to ischaemia found within the intact brain also exist in dissociated cell cultures: Scorziello et al (2001) show differential vulnerability of cortical and cerebellar neurone cultures deprived of O_2 and glucose; Xu et al (2001) show hippocampal astrocytes to be more sensitive to O_2-glucose deprivation than cortical astrocytes; Meloni et al (2001) have succeeded in modelling delayed neuronal death in culture. This clearly illustrates that an important component of ischaemic damage is dependent upon the cell's identity and thus justifies the elimination of the compounding factors mentioned above.

1.5.3.4 Building a more complex model

Pure astrocyte and neurone cultures can be viewed as building blocks from which more complicated models can be constructed. They can be used to determine the components of cell injury that are a property of the cell itself. Or, by adding any combination of metabolites, toxins or other signalling elements directly to the media it can be determined how much cell survival depends on extrinsic factors. This is popularly done for example when studying the effects of glutamate toxicity (Ahlemeyer et al 2003, Sinor et al 2000). More importantly, monocultures can be built into a coculture structure, thus replicating the cellular conversations occurring in vivo. Often, it is necessary for a number of signalling elements to pass to and fro between cell types before the critical signal is received (Kelic et al 2001).

1.5.3.5 Existing Coculture models

Some coculture models are based simply upon the idea of seeding one cell type on top of another (Drukarch et al 1998) or on creating a heterogenous mixture (Goldberg et al 1993) such that different cells are in physical contact but as a consequence, inseparable. As such these methods are ideal for imaging studies where cell types can be identified and assayed on an individual basis (Keelan et al 2001), but not for biochemical study. A popular approach to achieving separability of cultures is to grow one cell type on a porous membrane that can be suspended over another cultured in tissue culture plates (Stewart et al 2000, Kelleher et al 1996). In this instance, the porous membrane lies between the cell populations and so acts as a barrier to the diffusion of some molecules. The distance between the cells is also stretched to an average of 2mm. One group in particular has defied the barrier and indeed distance elements by placing the cell supporting membrane directly onto the awaiting cells in the culture plate such that the cells are facing each other (Chen et al 2001).

Another approach is to grow one group of cells on coverslips, which are placed directly into the culture wells and on to the awaiting cells (Ohgoh et al 2000). This may however cause some stress to the underlying cells. Some groups have inverted the coverslips so that, again the cells are facing each other, and allowed them to rest on paraffin end feet, creating a space between the cells (Bartlett et al 1984, Lamigeon et al 2001). A huge advantage of coculture systems is in being able to treat one cell without the other and then allowing the cells to interact (Chen et al 2001, Tamatani et al 1998).

We must end this introduction by acknowledging the fact that culture systems such as those described, do allow a large amount of controllability, however what is gained in controllability is lost in reproducing the complexity of the whole system. Clearly, a balance must be reached according to the questions being asked by what one is studying.

1.6 Objectives

The main objective of this thesis is to test the hypothesis that 'astrocyte-derived factors are protective to neurones which have been exposed to an oxygen glucose deprivation/reoxygenation injury'. We have divided the thesis into two main sections each with its own objectives in order to test this hypothesis. The first section involves the establishment of a working model of Oxygen glucose deprivation/reoxygenation using cultured neurones and astrocytes. This section also includes the steps by which we arrived at a protocol for the reproduction of progressive neuronal death in the reoxygenation phase. The second section directly examines the role of astrocytes in eliciting neuroprotection during reoxygenation.

Chapter 2

Materials and Methods

2.1 Materials

Earle's balanced salt solution (EBSS), Hanks' buffered salt solution (HBSS), antibiotic-antimycotic solution (10,000 units/ml penicillin, 10mg/ml streptomycin, 25µg/ml amphotericin B), Dulbecco's modified Eagle's medium (DMEM; without glucose, sodium-pyruvate, sodium bicarbonate and phenol red), L-glutamine, bovine serum albumin (BSA), deoxyribonuclease type 1 (DNAse, EC 3.1.21.1; from bovine pancreas), trypsin (EC 3.4.21.4, from bovine pancreas), trypsin-EDTA (5g porcine trypsin and 2g EDTA/L), cytosine arabinofuranoside (AraC), 4-trifluoromethoxyphenylhydrazone (FCCP), trypan blue, poly-L-ornithine (P/O, molecular mass 30,000 – 70,000 g/mol), poly-D-lysine (P/L, molecular mass 300,000 g/mol), propidium iodide (PI), sodium bicarbonate solution (7.5%), oxygen trap model 1000, iodonitrotetrazolium chloride, hydrazine hydrate, perchloric acid, β-nicotinamide adenine dinucleotide, glutathione (reduced and oxidised forms), monosodium glutamate, lactate standard (40mg/dl) and, sodium pyruvate, Botulinum Toxin (BoTox), 1-aminoindan-1,5-dicarboxylic acid (AIDA), MK801, DL-*threo*-β-benzyloxyaspartate (TBOA), 2-Methyl-6-(phenylethynyl)-pyridine (MPEP) and Anaerobic indicator (Oxoid, Basingstoke, UK) were purchased from Sigma-Aldrich Chemical Co. (Poole, UK). Hoechst33342, tetramethyl rhodamine methyl ester (TMRM), rhodamine-123 and Fura-2 acetoxymethylester (AM) were purchased from Molecular Probes Europe BV (Leiden, the Netherlands). L-Lactate dehydrogenase, glutathione reductase, glutamate dehydrogenase, and diaphorase were purchased from Roche (Lewes, UK).

B-27 supplement (with and without anti-oxidants), Neurobasal medium (NB), minimum essential medium (MEM, L-valine based), goat serum, and foetal bovine serum (FBS) were purchased from Gibco BRL (Renfrewshire, UK). The 95%N_2/5%CO_2 gas was purchased from BOC (Surrey, UK). Microscope coverglass was purchased from VWR (Poole, UK). Cell culture flasks, 6-well plates and single wells for astrocyte use

were purchased from Nagle Nunc International (Naperville, IL, USA). Six-well plates and single wells for neurone use were purchased from Costar (Corning Costar, High Wycombe, UK). Beads were purchased from The Bead Shop (London, UK). Mouse anti beta III tubulin antibody and donkey anti mouse Fluorescein-isothiocyanate (FITC) conjugated antibody were purchased from Abcam (Cambridge, UK). Donkey serum, rabbit anti GFAP antibody and Cy5 conjugated goat anti rabbit antibody were purchased from Chemicon (Hampshire, UK). Mouse anti CD11b antibody was purchased from Serotec (Oxford, UK). Citifluor was purchased from Citifluor Ltd (London, UK). Silicone sealant (Unibond, Cheshire UK) was purchased from Robert Dyas. The 782 O_2 meter (Strathkline instruments, Glasgow Scotland) was purchased from Strathkline instruments. Absolute digimatic, vernier callipers model no. CD-6"C (Mitutoyo, UK) was borrowed from th eengineerig dept, University College London.

2.2 Tissue Culture

All animals were procured from colonies maintained at the Biological Services unit of University College London and used in accordance with Home Office guidelines.

All references to 'cold' temperature solutions correspond to solutions at 4°C. All references to 'warm' temperature solutions correspond to 37°C.

2.2.1 Cortical astrocyte cultures from neonatal rat brain

2.2.1.1 Dissection

Neonatal Sprague Dawley rats were sacrificed at 0-48 hours of age by decapitation. Cerebella were carefully removed and discarded. Forebrains were placed in a dish containing Hank's buffered salt solution (HBSS) on ice. Under the dissecting microscope, cerebral hemispheres were carefully teased apart and midbrain and hippocampi removed and discarded. Meninges were then removed and also discarded. Cortices were placed into a dish containing 10ml cold **solution A** (Earle's balanced salt solution (EBSS) containing 20µg/ml DNAse and 3mg/ml BSA). Tissue was chopped until very fine using scissors and then triturated briefly with a 1ml Gilson pipette. The suspension was centrifuged at 500g for 5mins at 4°C.

The pellet was resuspended in 20ml of warm **solution B** (EBSS containing 3mg/ml BSA, 0.25mg/ml trypsin and 0.1mg/ml DNAse). The cell suspension was incubated at 37°C for approximately 12mins to allow cell dissociation. The reaction was stopped by the addition of 1ml cold foetal bovine serum (FBS). The suspension was then centrifuged at 500g for 5mins at 4°C. The pellet was resuspended in 10ml **solution A.** The cell suspension was mixed 6 times with a 1ml Gilson pipette followed by a 0.2ml Gilson pipette and allowed to settle for 3mins. Supernatant was removed and kept. This resuspension step was repeated and supernatants were pooled. The pooled suspension was centrifuged at 500g for 5mins at 4°C.

The supernatant was discarded and the pellet resuspended in culture medium (Minimum essential medium (MEM) supplemented with 10% FBS and 2mM L-glutamine). The suspension was filtered through a nylon mesh of 100µm pore size. The cortical suspensions were plated into a number of $80cm^3$ tissue culture flasks equivalent to the number of animals used. Flasks were then kept in an incubator at 37°C with humidity >90% in an atmosphere of 5%CO_2/95% air.

Medium was replaced with fresh culture medium after 24 hours and subsequently changed every 3 days. After 7 days or at such a time point that the cells reached confluency, oligodendrocytes and microglia were removed by shaking. The flasks were shaken on a Heidolph unimax 100 shaker (Lab Plant, Huddersfield, England) housed in a standard incubator, for 16hrs at 200rpm. The media containing floating cells was removed and discarded and the flask contents were harvested (section 2.2.1.2) and reseeded to double the number of flasks originally obtained. The medium was subsequently replaced every 3 days.

2.2.1.2 Harvesting of astrocyte cultures

Medium was removed and the flasks rinsed with warm HBSS. Approximately 10mls of T/E solution (HBSS containing 5mg/ml porcine trypsin and 2mg/ml ethylene diamine tetraacetic acid (EDTA)) was added to each flask. The flasks were incubated at 37°C for 5mins. The trypsinisation reaction was then stopped with the addition of 1ml cold FBS to each flask. The cell suspensions were removed from each flask and pooled. Flasks were further rinsed with HBSS, which was also pooled with the cell suspensions. The resulting suspension was centrifuged at 500g for 5mins at 4°C. The pellet was then re-suspended in 10mls of medium.

Astrocytes which had been seeded in 6-well plates were harvested upon completion of each experiment in a similar manner. Supernatant was removed and wells were washed with 1ml warm HBSS. The HBSS was

then removed and 1ml of trypsin was added to each well. The reaction was stopped by the addition of 100µl cold FBS. The astrocytes were then removed with the aid of a cell scraper and the wells washed once again with HBSS. The resulting cell suspension was centrifuged at 500g for 5mins at 4°C. The supernatant was removed and the pellet was resuspended in 300µl HBSS and placed in a 1.5ml eppendorf tube. This suspension was again centrifuged at 500g for 5mins at 4°C, the supernatant was discarded and the pellet was frozen and stored at -70°C.

2.2.1.3 Seeding of astrocytes for experimentation

After 14 days, cultures had reached confluence and were again harvested. The number of viable cells in the final 10ml suspension was counted based on trypan blue exclusion. For this, a 10µl aliquot of the harvested cell suspension was taken and mixed with 40µl trypan blue and 30µl HBSS (giving a final trypan blue concentration of 0.2mM). The number of cells that excluded trypan blue were counted in a 10µl aliquot of this mixture placed on a haemocytometer using a phase contrast, inverted-microscope. The cell density was subsequently adjusted such that there were 10^6 cells/ml medium and 1ml of this suspension was added to each well of a 6-well plate (section 2.2.1.4). Wells were maintained for a further 24 hours to allow cells to settle.

For experiments involving imaging, cells were seeded onto coated glass coverslips (25mm, thickness 1) (section 2.2.1.4). For this 100µl of the 10^6cells/ml suspension was placed in the middle of the slip and incubated at 37°C in an atmosphere of 5%CO_2/95% air for 1 hour in order to allow cells to settle. This was followed by the addition of 2ml media (described above). Cells were used for experimentation 24 hours later.

2.2.1.4 Coating of surfaces for astrocyte seeding.

Plastic 6-well plates were coated with poly-L-ornithine (P/O). Approximately 1ml of a 0.01% P/O solution was placed into each well and allowed to incubate at 37°C for 1 hour. The solution was then removed and the well rinsed twice with sterile distilled H_2O. Each plate was subsequently placed in front of a UV lamp for 20mins after which they were ready for use.

Glass coverslips were coated with poly-D-lysine (P/L). Coverslips were baked in an oven at 160°C for 2 hours and allowed to cool. Approximately 300µl of 0.01% P/L solution was added to the centre of each coverslip. They were then left at room temperature for 30mins after which time the P/L was removed and the coverslips washed twice with sterile distilled H_2O. All coverslips were allowed to dry and were then placed in front of a UV lamp for 20mins.

2.2.1.5 Note on astrocyte care

We have found that astrocyte growth rate and health can be greatly improved by tapping the flasks sharply against the flow unit surface just prior to media change. This appears to aid in the clearance of dying cells and toxins.

2.2.2 Primary cortical neurone cultures from foetal rat brains

2.2.2.1 Dissection

Pregnant Sprague Dawley rats at 17 days of gestation were sacrificed by cervical dislocation. Foetuses were carefully extracted and placed in a Petri dish containing cold EBSS. Foetal heads were cut with scissors, so as to expose the brain, and the cerebellum was removed and discarded. Cortices were carefully removed and rolled gently on filter paper (which had been previously soaked with ethanol, allowed to dry, and exposed to UV light for 20mins) in order to remove meninges.

The tissue was then placed in a Petri dish containing 10ml **solution C** (Neurobasal medium (NB) containing 3mg/ml BSA and 24µg/ml DNAse) and chopped until very fine with scissors. This suspension was then placed in a Falcon tube and allowed to settle for two minutes under the influence of gravity. The supernatant was removed and 10ml **solution D** (NB containing 3.25mg/ml BSA, 24µg/ml DNAse and 0.25mg/ml trypsin) was added. The cell suspension was incubated at 37°C for 15mins. The reaction was stopped by the addition of 1ml of cold FBS. The suspension was allowed to settle for 2 mins and the supernatant was removed. 12ml of **solution C** was added and the suspension was triturated gently 4 times using a 1ml Gilson pipette followed by a 0.2ml Gilson pipette. The cell suspension was allowed to settle for 4 mins after which time the supernatant was removed and placed in another Falcon tube. This resuspension step was repeated and supernatants pooled.

The pooled suspension was centrifuged at 500g for 5mins at 4°C. The supernatant was discarded and the pellet resuspended in 3ml of NB supplemented with 2% B27 with antioxidants (B27+AO), 25µM glutamate and 2mM glutamine. The suspension was filtered through a nylon mesh of 100µm pore size. The number of viable cells was counted based on trypan blue exclusion (section 2.2.1.3) and the cell concentration adjusted to 1million/ml with media. 100µl of cell suspension was added directly to the centre of treated coverslips (section 2.2.2.2) and incubated at 37°C in an atmosphere of 5%CO_2/95% air for 1 hour to allow cells to settle. After this time 2ml of supplemented NB was added carefully to each well. The cells were subsequently housed in an incubator at 37°C in an atmosphere of 5%CO_2/95% air at >90% humidity.

Cell medium was half changed on day 3 by replacement of 1ml of old medium with 1 ml of astrocyte conditioned NB (section 2.2.2.3) supplemented with 20µM cytosine arabinofuranoside (AraC) giving a final concentration of 10µM. AraC is added to prevent glial proliferation. Media was half changed again on day 7 with astrocyte conditioned NB. Neurones were used for experimentation on DIV10.

2.2.2.2 Preparation of coverslips for neuronal cultures

Glass coverslips were soaked overnight in 20% nitric acid. They were removed, washed three times with fresh distilled deionised H_2O and dried in acetone. They were then sterilised by baking in the oven for 2hours at 160°C and allowed to cool.

Coverslips were placed in 6-well plates or Petri dishes and coated with 300µl of 0.01% P/L for 1 hour at room temperature. The P/L was removed and the coverslips washed three times with sterile distilled H_2O. Coverslips were allowed to dry and were then placed on front of a UV light for 20mins. 100µl NB containing 2% B27, 5% horse serum, 5% FBS, 2mM glutamine and 62.5µM glutamate was added to the centre of the coverslips and incubated at 37°C in an atmosphere of 5%CO_2/95% air for 3 hours. Cell suspensions were added directly to this solution.

2.2.2.3 Astrocyte conditioned neurobasal medium

NB containing 2% B27 (+AO) and 2mM glutamine, was placed on astrocyte cultures, aged between DIV13 and DIV15, for 24 hours. The media was then removed and centrifuged at 500g for 5mins at 4°C, to remove cell debris. Conditioned media was frozen at -80°C and stored for up to two weeks.

2.2.2.4 Harvesting of neurone cultures

Neurones were harvested such that the cells from three coverslips were combined in one pellet. Each coverslip was placed immediately in to 1ml of HBSS containing 5mg/ml porcine trypsin and 2mg/ml ethylene diamine tetraacetic acid (EDTA) (T/E). The plates were incubated at 37°C for 5mins. The trypsinisation reaction was then stopped with the addition of 100µl cold FBS to each well. A cell scraper was used to remove all remaining neurones. The wells were washed with 1ml HBSS, which was then added to the cell suspension. This was then centrifuged at 500g for 5mins at 4°C. The supernatant was removed and the pellet was resuspended in 300µl HBSS and placed in a 1.5ml eppendorf tube. This

suspension was again centrifuged at 500g for 5mins at 4°C, the supernatant was discarded and the pellet was frozen in liquid nitrogen and stored at -70°C.

2.2.3 Co-culture of Cortical neurones and astrocytes

Neurones grown on glass coverslips were used on DIV10 for co-culture. Astrocytes were seeded on modified 6-well plates (Costar) at DIV13. The 6-well plates were altered as follows.

Four size 11 glass beads (cleaned and sterilised in the same manner as glass coverslips) were placed on the underside of each well of a 6-well plate to form a square of dimensions 10x10mm. Reproducibility was achieved through the use of a template, which was placed on the back of the 6-well plate. A soldering iron was then applied to the top of each bead causing the bead to heat and sink through the surface of the plastic well. The bead was allowed to sink through until its outer surface was on the same plane as the outer surface of the 6-well plate. The soldering iron was removed and forceps were quickly applied to hold the bead in place until the plastic hardened. The distance between the coverslip and the surface of the 6-well plate was measured using an 'Absolute digimatic' vernier callipers.

Astrocytes were seeded as described (Section 2.2.1.3) and allowed to rest for 24 hours. Co-culture was achieved by inverting the coverslips bearing the neurones and placing them on the beads.

2.3 Immunocytochemistry

2.3.1 Antibodies used

Immunocytochemistry was performed on both astrocyte and neurone cultures in order to determine the cellular purity of each culture population. Antibodies against cellular markers specific for neurones, astrocytes and microglia were incubated with both cell types. The neurone specific marker is the beta III Tubulin subunit (Tub), the astrocyte marker is glial fibrillary acid protein (GFAP), both are cytoskeletal elements, and the microglial marker is the integrin CD11b.

Two different secondary antibody types were used in order to visualise primary antibody binding to the specific cellular markers. These were an anti-mouse Fluorescein-isothiocyanate (FITC)-conjugated antibody raised in goat and an anti-rabbit CY5-conjugated antibody raised in donkey. Both FITC and CY5 are fluorescent molecules, which absorb and emit light at different wavelengths and thus allow double staining of each culture to be carried out.

2.3.2 Application of antibodies

Cells on glass coverslips were rinsed with Tris buffered saline (TBS) and fixed by the addition of ice-cold methanol for 10 mins, on ice. Methanol was removed and the cells were rinsed twice for 5 mins with TBS and then treated with 4mM sodium deoxycholate for 10 mins. This step permeabilises the cells allowing access for antibodies.

Cells were then rinsed twice more for 5 mins in TBS containing 0.025% Triton X-100, to reduce surface tension and allow reagents to cover the entire cell preparation. The cells were blocked in 10% normal serum (from the animal in which the secondary antibody was raised) with 1%BSA in TBS at room temperature for 2 hrs. The normal sera used were goat (for Tub and CD11b stains) and donkey (for GFAP stains). Blocking solution was removed and primary antibody applied at suitable concentrations overnight at 4°C. Astrocytes were incubated with rabbit

anti-GFAP antibody (1:1000 with TBS/1%BSA) and mouse anti-cd11b antibody (1:1000 in TBS/1%BSA). Neurones were incubated with rabbit anti-GFAP antibody (1:1000 in TBS/1%BSA) and mouse anti-Tub antibody (1:500 in TBS/1%BSA).

Cells were washed twice for 5 mins with TBS and then incubated with a solution containing FITC-conjugated secondary antibody (1:500) and a CY5-conjugated secondary antibody (1:500) for 1 hour at room temperature. The cells were again rinsed twice for 5 mins with TBS and then incubated with 0.01% 4,6-diamidino-2-phenylindole HCL (DAPI) for 10mins followed by a final rinse with TBS. DAPI is a nuclear stain that allows all cells present to be visualised. The coverslips were then mounted on glass microscope slides as follows. 10µl of citifluor was placed in the middle of the slide and the coverslip placed carefully, cells facing down, onto it. Citifluor does not dry and so coverslips were held in place by dabbing small patches of nail varnish (60seconds, silver bullet, Rimmel London) along the edge.

Negative controls were carried out by the omission of primary antibody, secondary antibody or the omission of cultures. Single stains were also carried out in order to discount the possibility of the antibodies producing a signal by interfering with each other. Stains were visualised using confocal microscopy.

2.3.3 Imaging immunocytochemical stains

Immunocytochemical stains were visualised using confocal microscopy (section 2.6.2). The 364nm line of the argon UV laser was used to excite DAPI, the 488nm line of the argon laser was used to excite FITC and the 633nm helium neon laser was used to excite Cy5. DAPI, FITC and Cy5 emit blue, green and red light respectively which were collected on three separate channels using appropriate filters and mirrors.

2.3.4 Quantifying immunocytochemical stain

A number of fields were chosen from each coverslip and imaged. The number of nuclei visible in each field was taken as the total number of cells present. Positively stained cells were counted as those whose nucleus had a surrounding stain. Purity was therefore expressed as the percentage of positively stained cells present with respect to the total number of nuclei.

2.4 Oxygen-glucose deprivation/reoxygenation

All experiments were carried out such that control and test cells were derived from the same culture. Control cells were handled in a manner as similar as possible to test cells, the only difference being the actual insult given.

2.4.1 O_2 deprivation

To establish hypoxia, cells (astrocytes and/or neurones) were placed in an airtight glass desiccator with specially modified lid. This was then housed in an incubator at a temperature of 37°C. Humidity was maintained by the inclusion of H_2O at the base of the desiccator. Air was removed by flushing the desiccator through with 5%CO_2/95%N_2. This gas mixture was first of all passed through an O_2 trap to remove trace O_2 from the stream.

The chamber was prepared before each experiment by flushing with gas for 15mins after which the cells were added and the chamber flushed again for approximately 60mins. Lack of O_2 was indicated by a resazurin soaked pad, which turns from pink to white when low O_2 concentration is reached. This change marked the start of the hypoxic time period.

The effectiveness of the resazurin pad was checked by measuring O_2 level in the chamber using a Clark-type oxygen electrode (Section 2.7). The electrode was placed within the chamber. The accompanying leads to the data storage unit were accommodated by cutting channels in the lid of the chamber and sealing the leads in with silicone sealant. Measurements were made whilst the chamber was gassed continuously, noting the length of time for O_2 levels to reach their lowest. Temperature was monitored by placing a medical precision thermometer, (Ellab,Copenhagan Denmark) in the chamber at the same time. The leakiness of the chamber to O_2 was determined by continuing the measurement of O_2 after the cessation of gas flow.

2.4.2 O_2/glucose deprivation

2.4.2.1 Astrocyte O_2/glucose deprivation

To establish the O_2/glucose deprivation (OGD) injury, astrocytes were first rinsed with warm HBSS and then incubated in 1ml DMEM without glutamine, glucose, pyruvate or serum supplement. They were then placed in the above chamber to achieve hypoxic conditions. Control cells were rinsed with warm HBSS and 1ml of DMEM supplemented with 2mM glutamine, 5.5mM glucose, 1mM sodium pyruvate and 2% B27 (-AO) was added. Cells were incubated at 37°C in an atmosphere of 5%CO_2/95% air at >90% humidity for the duration of the OGD period.

2.4.2.2 *Neurone O_2/glucose deprivation*

Neurones grown on coverslips were subjected to OGD by removal of the coverslip from the growth media and placing it directly into a well containing 2ml of DMEM without glutamine, glucose, pyruvate or serum supplement. This was then placed in the hypoxia chamber as described in section 2.4.1. Similarly, control coverslips were placed in wells containing 2ml of DMEM supplemented with 2mM glutamine, 5.5mM glucose, 1mM sodium pyruvate and 2% B27 (-AO), and incubated at 37°C in an atmosphere of 5%CO_2/95% air at >90% humidity for the duration of the ischaemic period.

2.4.3 Reoxygenation

2.4.3.1 Astrocyte reoxygenation

Reoxygenation was achieved by replacing both test and control media with DMEM supplemented with 2mM glutamine, 5.5mM glucose, 1mM pyruvate and 2% B27 (-AO) following the OGD period. Cells were then housed in an incubator at 37°C in an atmosphere of 5%CO_2/95% air at >90% humidity.

2.4.3.2 Neurone reoxygenation

Reoxygenation was achieved by placing each coverslip (test and control) in a well containing 2ml of Neurobasal media supplemented with 2% B27 (-AO) and 2mM glutamine. Both control and test neurones were then

placed in an incubator at 37°C in an atmosphere of 5%CO_2/95% air at >90% humidity.

For some experiments neurones were reoxygenated in astrocyte conditioned media. This conditioned media was made using the method described in section 2.2.2.3 with the exception that B27 without anti-oxidants was used.

2.4.3.3 Co-culture reoxygenation

Some experiments involved insulting neurones, as described above, and allowing reperfusion to occur in the presence of astrocytes in co-culture conditions was as described in section 2.2.3. Reoxygenation was achieved by adding 2ml of NB (2% B27(-AO), 2mM glutamine) to each of the astrocyte wells 15mins prior to the addition of the neuronal coverslips. The coverslips were then inverted and placed carefully on the beads over the astrocytes.

2.5. Cell survival

2.5.1 LDH assay

2.5.1.1 Principle

Lactate dehydrogenase (LDH, NAD^+ oxidoreductase EC 1.1.1.27) catalyses the reversible oxidation of lactate to form pyruvate.

$$\text{Lactate} + NAD^+ \xleftrightarrow{\text{LDH}} \text{pyruvate} + NADH + H^+$$

This enzyme is predominantly cytosolic and its release from cells into the surrounding media was used as a measure of plasma membrane leakage and hence as a measure of cell death.

2.5.1.2 Protocol

The assay was carried out using astrocyte cultures from 6-well plates as follows. 50µl of supernatant was removed and snap-frozen in liquid N_2. This represents the extra-cellular fraction. 10µl of 10% triton X-100 was added to each well to solubilise cell membranes and release LDH. The solubilised sample (total) was collected and also snap-frozen in liquid N_2. Samples were later defrosted in a water bath at 37°C just prior to use.

The enzyme activity was measured using a Uvikon-XL spectrophotometer (BioTek Instruments Ltd). The decrease in absorbance at 340nm due to the oxidation of nicotinamide adenine dinucleotide (NADH) was monitored as a measure of the reduction of pyruvate to lactate by LDH. The assay was performed at a temperature of 30°C. The reaction mixture contained 100mM potassium phosphate buffer at pH 7.5, 0.345mM sodium pyruvate and 8mM disodium NADH. The reaction was carried out in a 1ml cuvette and initiated by the addition of 33µl of sample. Absorbance changes were read against a blank cuvette containing all components of the reaction mixture without any sample. LDH activity in the extra-cellular fraction was expressed as a percentage of total LDH activity.

2.5.1.2 Assay validation

The linear limits of the assay were ascertained by using pure lactate dehydrogenase (LDH) enzyme directly in the assay at various different concentrations. LDH was diluted to specific concentrations according to protein content, using NB medium. There was a linear increase in assay rate with LDH concentration from 0.005µg/ml to 5µg/ml. Between 0.005µg/ml and 0.1µg/ml R^2=0.9992, between 0.1µg/ml and 5µg/ml R^2=1 (Fig2.1).

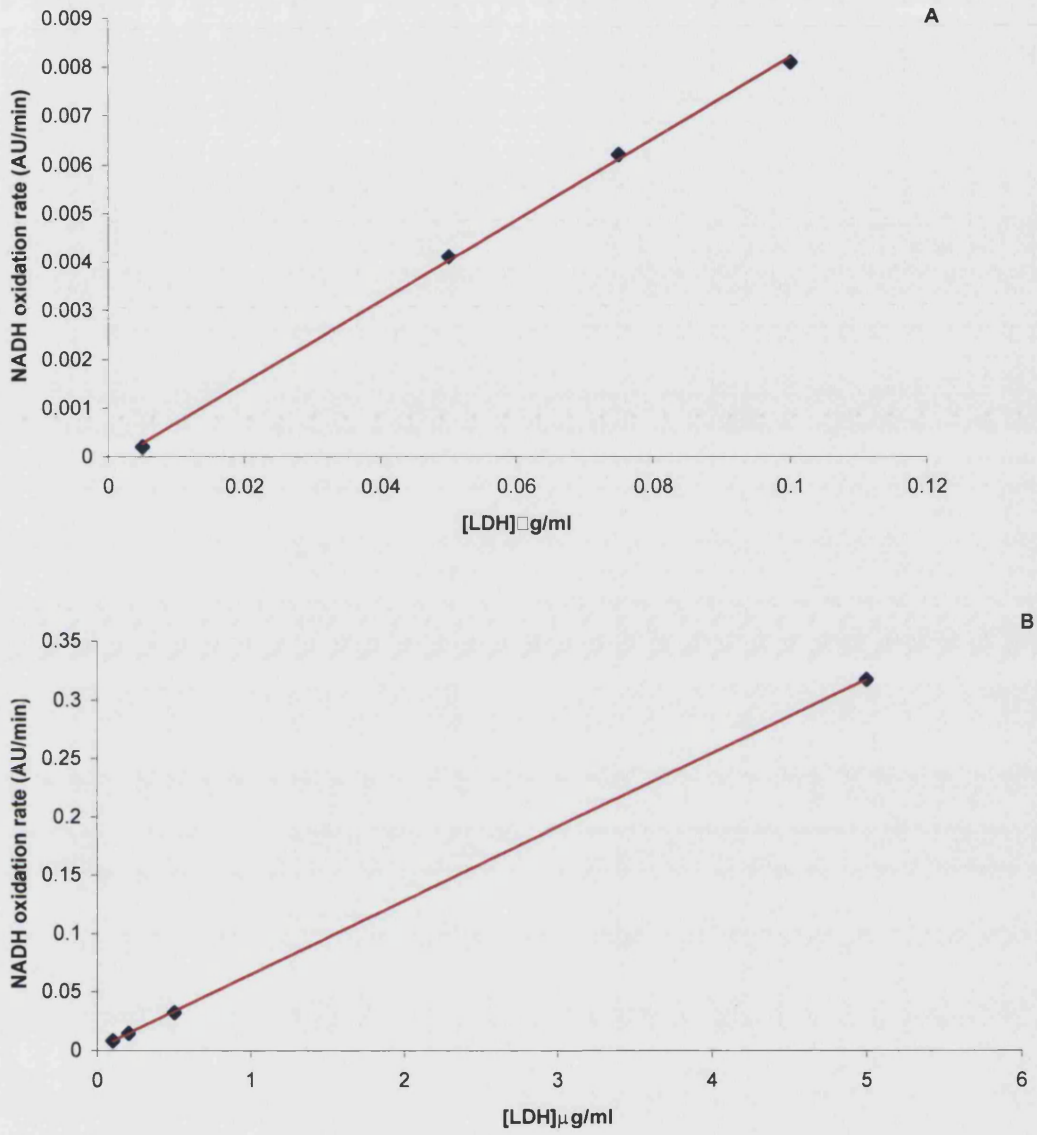


Fig 2.1 LDH activity. The graph illustrates the rate of NADH oxidation by different concentrations of LDH, expressed as absorbance units/min. Linear trend lines have been fitted to the points obtained. A) illustrates LDH concentrations from 0.005μg/ml to 0.1μg/ml, B) illustrates LDH concentrations from 0.1μg/ml to 5μg/ml.

2.5.2 Dye exclusion assay

Neuronal cell death was quantified by assessing the integrity of the plasma membrane, and the shape of the neuronal nucleus. This was accomplished through the use of vital dye uptake and a subsequent

analysis of nuclear colour and shape in order to distinguish between apoptotic and necrotic cell death.

2.5.2.1 Dye application

Cells were loaded for 20mins with 10μM Propidium Iodide (PI) and 4.5μM Hoechst 33342 in HEPES buffered salt solution. Both PI and Hoechst are fluorescent molecules that target nuclear DNA. PI is cell impermeant and is thus excluded from cells with an intact plasma membrane. Hoechst is cell permeant and stains all nuclei present. Only dead cells will take up PI however, cell death by apoptosis does not always involve early disruption of membranes therefore the use of Hoechst allows apoptotic nuclei to be identified according to nuclear morphology only.

2.5.2.2 Dye visualisation

Nuclei were observed using fluorescence microscopy (section 2.6.3). Hoechst has an excitation peak at 360nm and an emission peak at 460nm **(blue colour)** whereas PI has an excitation peak at 530nm and an emission peak at 615nm **(red colour).** Both dyes were visualised by eye following the passage of emitted light through a 390nm dichroic mirror and 420nm long pass filter.

2.5.2.3 Counting nuclei

Nuclei from 10 regions of interest (ROI) were counted according to shape and colour and assigned to one of five groups as shown below. These groups allow the nuclei to be counted as live, early apoptotic, late apoptotic or necrotic.

Group I (**live**)- Nuclei with blue fluorescence only, rounded appearance, and clear homogenous chromatin structures.

Group II (**early apoptotic**)- Blue nuclei, smaller than group I, some chromatin condensation and rounded shape maintained.

Group III (**late apoptotic**)- Small, homogenous bright blue nuclei, no clear chromatin structures, fragmented appearance.

Group IV (**necrotic**)- Red nuclei with appearance as in group I.

Group V(**late apoptotic**)- Red nuclei with appearance as in group III

Only nuclei that could be clearly fitted into these groups were considered.

Cell survival was quantified by expressing the number of nuclei in group I and group II as a percentage of the total number of nuclei present. Group II was considered as a live group as it was thought that these cells may be capable of being rescued. Necrotic nuclei were quantified by expressing the number of nuclei in group IV as a percentage of the total number in groups III, IV and V. Apoptotic nuclei were quantified by expressing the total number of nuclei in groups III and V as a percentage of the total number of nuclei in groups III, IV and V.

2.6 Microscopy

2.6.1 Phase-contrast light microscopy

Cells were photographed using a digital camera (Nikon Coolpix950) mounted over a standard phase-contrast microscope (Nikon TMS) equipped with 20x and 10x air objective lenses. Digital images were stored and processed using Adobe Photoshop technology.

2.6.2 Confocal microscopy

Confocal microscopy was performed using a Zeiss 510 laser scanning confocal microscope equipped with x20 (air) and x40 and x63 (oil immersion), quartz objective lenses (NA 0.75, 1.3 and 1.4, respectively). A photomultiplier tube detected emitted light. Images were analysed using the Zeiss LSM image browser.

2.6.3 Fluorescence microscopy

Fluorescence microscopy for manual observation of fluorescence was performed using a Leitz Fluovert FU inverted fluorescence microscope (Leica, Wetzlar, Germany), equipped with a 40x objective lens.

2.6.4 Digital fluorescence microscopy

2.6.4.1 Image acquisition

Experiments were carried out using an inverted epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a 20x fluorite objective. Excitation light was emitted from a xenon arc lamp and was passed through 10nm band pass filters centred at 340 and 380nm housed in a computer-controlled filter wheel (Cairn Research, Kent, UK). Emitted fluorescence was reflected through a 515nm long-pass filter to a frame transfer cooled CCD camera (Hamamatsu 4880). Data was collected at 10 to 15sec intervals using the Acquisition Manager software package (Kinetic Imaging) and analysed using Lucida Analyse 6 (Kinetic imaging, Liverpool, UK).

2.6.4.2 Measuring intracellular Ca^{2+} levels

Cells were loaded for 40mins at room temperature with 5μM Fura-2 AM and 0.005% pluronic (a mild detergent to aid access of the dye to the cell) in recording media: HEPES buffered salt solution composed of mM: 156 NaCl, 3 KCl, 2 $MgSO_4.7H_2O$, 1.25 KH_2PO_4, 10 glucose, 10 HEPES, 2 $CaCl_2$ adjusted to pH 7.35 with NaOH.

Fura-2 AM is a high affinity, intracellular calcium indicator that is ratiometric and UV light–excitable. Upon binding Ca^{2+}, fura-2 exhibits an absorption shift from 340 to 380 nm. Emission is detected at 510nm. Ratioing the 340 and 380nm signal eliminates error produced by uneven dye loading, dye leakage and photobleaching. Fig 2.2 is an example of how the spectral characteristics of Fura-2 change with Ca^{2+} binding.

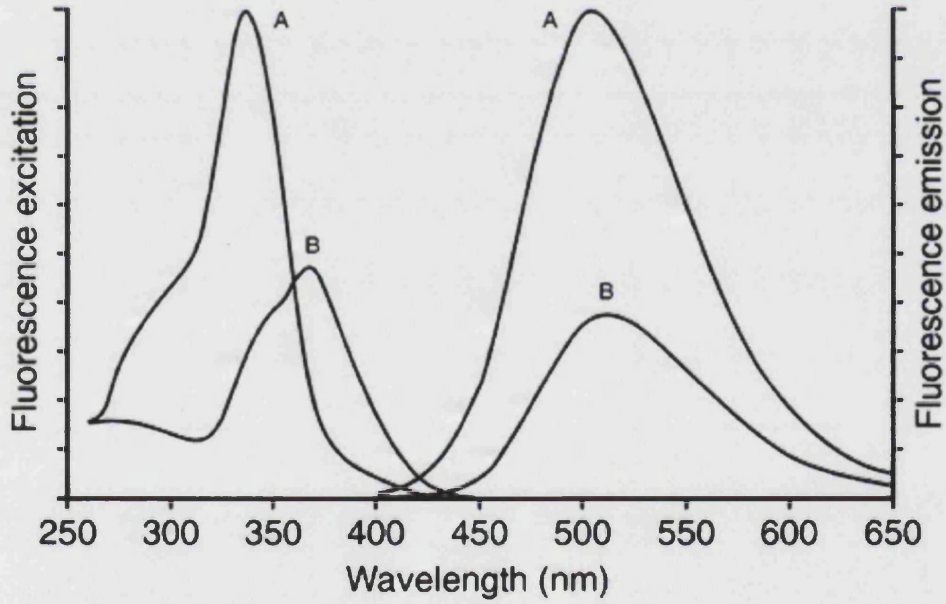


Fig 2.2 Fura-2/Ca^{2+} spectra. Fluorescence excitation (detected at 510 nm) and emission (excited at 340 nm) spectra of Ca^{2+}-saturated (A) and Ca^{2+}-free (B) fura-2 in pH 7.2 buffer. *From molecular probes website* http://www.probes.com/servlets/spectra?fileid=1200ca.

Cells were washed a number of times after loading to remove excess dye. The cellular Ca^{2+} response to glutamate was determined by

exchanging the recording medium with medium containing 100μM glutamate.

2.7 Oxygen Consumption

2.7.1 Electrode calibration

The 782 oxygen meter (Strathkelvin Instruments, Glasgow) was used to monitor O_2 levels. Data was recorded using the on-board datalogging facility and analysed by uploading to the accompanying software package on PC. The meter was calibrated according to manufacturer's instructions. The zero O_2 level was obtained by adding 50µl of a solution containing 2% sodium sulphite in sodium borate. Maximum O_2 concentration was registered by adding 50µl of respiration buffer (HBSS containing 2mM $CaCl_2$, 20mM HEPES). This solution had been fully oxygenated by vigorous shaking for 20mins and was estimated to contain O_2 at a concentration of 200µM (calibration chart provided by Strathkline Instruments). Both solutions were warmed to 37°C and all measurements were carried out within an incubator at this temperature also.

2.7.2 Measuring O_2 during hypoxia

The hypoxia chamber was modified such that the O_2 electrode could be sealed within it whilst remaining connected to the accompanying recording meter. This was done, by cutting a suitable area of glass from the lid, reshaping it with epoxy resin to the width of the attached wires and finally sealing the wires in with silicone sealant. O_2 levels were then followed as air was evacuated from the chamber (section 2.4.1).

2.7.3 O_2 measurements in an astrocyte suspension

Astrocytes were harvested and resuspended in respiration buffer (section 2.7.1). They were counted and brought to a concentration of 100,000cells/ml buffer (section 2.2.1.3). 50µl of this suspension was placed in the O_2 meter chamber.

2.8 Quantitation of Chemical Compounds

2.8.1 Protein

2.8.1.1 Principle

Protein content was determined using the Bio-Rad protein assay kit (Bio-Rad, Buckinghamshire, UK) and is derived from the method of Lowry et al (1951). The assay is based on the chemistry between tryptophan and the phenolic group of tyrosine residues within proteins, and alkaline copper tartrate solution and Folin-Ciocalteau reagent (consisting of sodium tungstate, molybdate and phosphate). The cuprous ion resulting from the protein-copper complex reduces the Folin reagent to produce a solution that has an absorbance maximum at 750nm. The absorbance is proportional to the protein content of the sample.

2.8.1.2 Protocol

The assay was carried out in triplicate. Standards of bovine serum albumin (BSA) in H_2O were made in the range from 5-200µg protein.ml^{-1}. 10µl of sample were diluted in 200µl of H_2O. 100µl of the alkaline copper tartrate solution and 800µl of the Folin reagent were added to each sample and standard. The reaction mixture was placed in the dark at room temperature for 20mins. Absorbance was then read at 750nm using a Uvikon 940 spectrophotometer (Kontron instruments Ltd. Watford, UK). Standard curves were linear within the range tested.

2.8.2 Sample deproteination

For some assays samples were deproteinated by acid extraction using perchloric acid (PCA). To every 1ml of sample, 250µl of PCA (24%) was added. The mixture was placed on ice over a magnetic stirrer and allowed to stir for 15mins. The resulting solution was then centrifuged at 740g for 10mins at 4°C. The supernatant was removed and its volume recorded. The pH was then measured using a pH meter and brought to pH 5.5-6.0 with buffer solution (0.417M triethanolamine, 2M K_2CO_3 in H_2O). The volume was again noted and the solution was centrifuged at

120g for 10mins at 4°C. The supernatant was kept and the pellet discarded.

2.8.3 L-Glutamate

2.8.3.1 Principle

L-Glutamate concentration was determined by the method of Beutlar and Michal (1974) and is based on the use of glutamate dehydrogenase (GlDH, EC. 1.4.1.3), diaphorase (NADH:lipoamide oxidoreductase, EC. 1.6.4.3) and tetrazolium salts, with minor modifications. L-Glutamate reacts with nicotinamide adenine dinucleotide (NAD^+) to produce 2-oxoglutarate, reduced nicotinamide adenine dinucleotide (NADH) and an ammonium ion (1). In the presence of diaphorase, the NADH produced reacts with iodonitrotetrazolium chloride (INT) to produce an orange formazan product (2), which can then be measured spectrophotometrically at 492nm. The reaction proceeds stoichiometrically and thus the concentration of glutamate is determined from the extinction coefficient of INT using the Beer-Lambert law.

1. L-glutamate + NAD^+ + H_2O $\xleftrightarrow{\text{GlDH}}$ 2-oxoglutarate + NADH + NH_4^+

2. NADH + INT + H^+ $\xrightarrow{\text{Diaphorase}}$ NAD^+ + Formazan

2.8.3.2 Protocol

The assay was carried out using deproteinated sample (2.8.1). The assay was performed at a temperature of 25°C in a 1ml cuvette. The reaction mixture contained final concentrations of: triethanolamine phosphate buffer (57mM TRA, 14mM potassium phosphate, pH 8.6), 0.38mM NAD, 0.068mM INT, and 14.3U/ml diaphorase. Upon addition of 143µl of sample to the reaction mixture, the cuvette was placed in a Uvikon 940 spectrophotometer (Kontron instruments Ltd. Watford, UK) and absorbance was continuously read at 492nm. After a flat baseline was reached (around 2 minutes), 14U/ml of GlDH was added to the mixture and absorbance read continuously for a further 30 minutes.

Recording was stopped once a flat line had been reached and the maximum absorbance was noted. Absorbance changes were read against a blank cuvette containing all components of the reaction mixture with deproteinated sample medium instead of sample.

The extinction coefficient of the INT solution must be determined with every new solution. This is done by measuring the absorbance change in a standard glutamate solution made in H_2O (136µM final concentration).

2.8.3.3 Assay validation

Three separate standard curves were constructed in the range 1-8µM, 10-60µM and 70-136µM glutamate in NB. The assay was found to be linear in the range from 1µM to 60µM glutamate but this was lost in the final curve. Fig 2.3 is a combination of the standard curves using 1-60µM Glutamate, R^2=0.9967. Samples found to exceed 60µM were diluted and re-assayed.

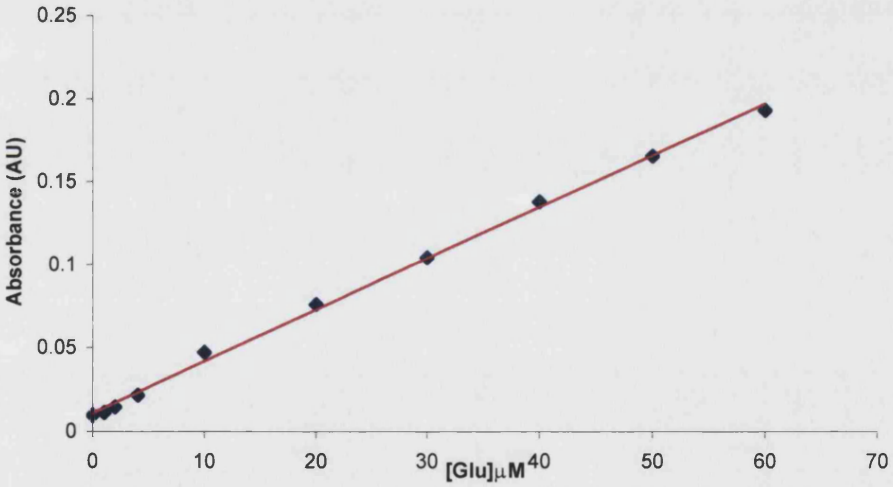


Fig 2.3 Glutamate standard curve. The graph shows the absorbance values of glutamate standards made in deproteinated Neurobasal medium from 1-60µM. A linear trendline has been fitted to the data.

2.8.4 L-Lactate

2.8.4.1 Principle

L-Lactate was measured according to the method of Gutman and Wahlfeld (1974) using lactate dehydrogenase (LDH, L-lactate:NAD oxidoreductase, EC.1.1.1.27) and nicotinamide adenine dinucleotide (NAD). LDH catalyses the reduction of NAD^+ by lactate to produce reduced nicotinamide adenine dinucleotide (NADH) (1). NADH formation is measured spectrophotometrically at 340nm. The concentration of lactate is determined from the extinction coefficient of NADH using the Beer-Lambert law.

1. L-lactate + $NAD^+ \xleftrightarrow{LDH}$ pyruvate + NADH + H^+

2.8.4.2 Protocol

The assay was carried out using deproteinated sample (2.8.1). The assay was performed at a temperature of 25°C in a 1ml cuvette. The reaction mixture contained final concentrations of: hydrazine/glycine buffer(0.43M gly, 0.34M hydrazine in H_2O, pH 9.0) and 2.75mM NAD. Upon addition of 200µl of sample to the reaction mixture, the cuvette was placed in a Uvikon 940 spectrophotometer (Kontron instruments Ltd. Watford, UK) and absorbance was continuously read at 340nm. After a flat baseline was reached (around 2 minutes), 19U/ml of LDH was added to the mixture and absorbance read continuously for a further 30-60 minutes. Recording was stopped once a flat line had been reached and the maximum absorbance was noted. Absorbance changes were read against a blank cuvette containing all components of the reaction mixture with deproteinated sample medium instead of sample.

2.8.4.3 Assay validation

A standard curve was constructed from a lactate standard solution, in the range 0-40µM in NB medium. The assay was found to be linear in this range, R^2=0.9945 (Fig 2.4). Samples found to exceed 40µM were diluted and re-assayed.

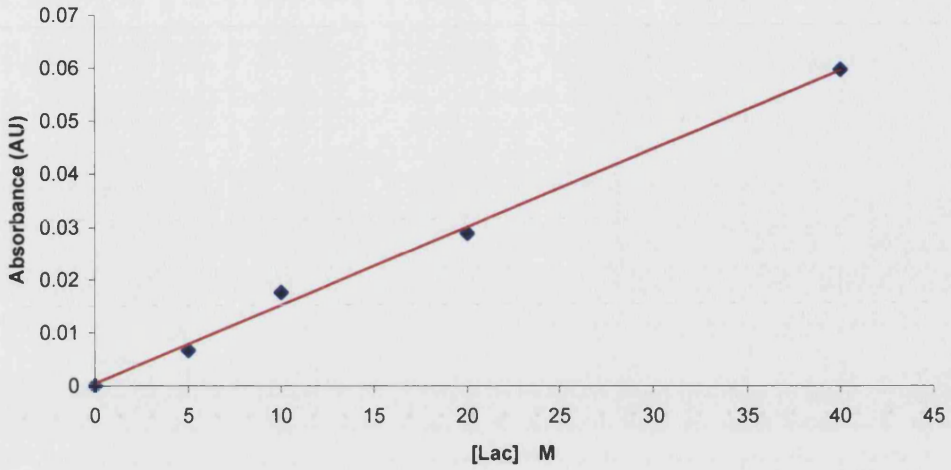


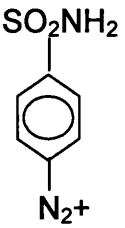
Fig 2.4 Lactate standard curve. The graph shows the absorbance values of lactate standards made in deproteinated Neurobasal medium from 1-60μM. A linear trendline has been fitted to the data.

2.8.5 Nitrate and Nitrite

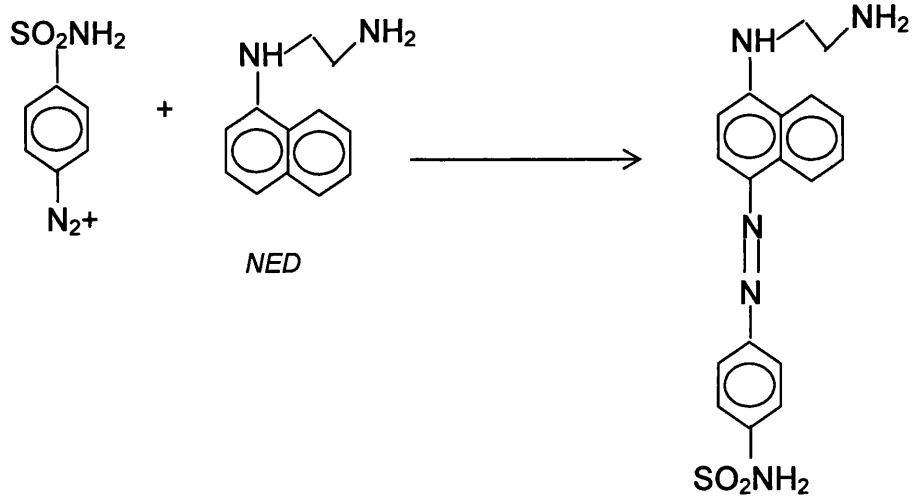
2.8.5.1 Principle

Nitrite (NO_2^-) is measured according to the method of Green et al (1982), as a measure of nitric oxide (NO) formation. NO_2^- is one of two primary, stable and non-volatile breakdown products of NO. The other is NO_3^- (Nitrate). The assay is done in 2 parts; the first part involves the conversion of all NO_3^- present to NO_2^- using nitrate reductase (NR, EC 1.6.6.2), reaction 1 below. The second part involves the addition of Griess reagent for the detection of nitrite, reactions 2 and 3 below.

1. $NO_3^- \xrightarrow{NR} NO_2^-$

2. $NO_2^- + SO_2NH_2\text{-}C_6H_4\text{-}NH_2 \xrightarrow{H^+}$ 

Sulfanilamide

3. 

NED

Azo Product

The Griess reagent system relies on a chemical reaction which uses sulphanilamide and N-(1-Naphthyl)ethylenediamine dihydrochloride (NED) under acidic conditions, to convert NO_2^- into a deep purple azo compound. Photometric determination of the absorbance due to this azo compound accurately determines NO_2^- concentration. NADPH is essential for the nitrate reductase reaction however, it interferes with the

chemistry of the Griess reagents and so excess NADPH must be removed. It is scavenged by the reaction between lactate dehydrogenase (LDH) and sodium pyruvate.

2.8.5.2 Protocol

Standards of sodium nitrite and sodium nitrate were made in neurobasal medium (NB) at concentrations of 5-50µM. A 50µl aliquot of each standard and sample was pipetted in triplicate on to a 96-well plate. To each well, 10µl of NR (0.1umits.ml^{-1}, final concentration) and 10µl of NADPH (100µM, final concentration) were added. The plate was then incubated for 30mins at 37°C. Upon completion of the reaction, 10µl each of LDH (100U.ml^{-1}, final concentration) and sodium pyruvate (100mM, final concentration) were added and the plate was incubated again for a further 15mins at 37°C.

Griess reagent was prepared by mixing equal volumes of NED (0.1% (w/v) in H_2O) and sulphanilamide (1% in 5% (w/v) conc H_3PO_4). 100µl of Griess reagent was then added to each well and the plates were left in the dark at room temperature for 15mins. The absorbance of each well was read at 540nm in a spectrophotometric plate reader (SpectraMax Plus®, Molecular Devices, Wokingham UK) against blank wells without sample or standard. The nitrite standard curve was used to determine the nitrite concentration of each sample well. In theory both curves should be identical but in practice, small discrepancies are to be expected. An assay was accepted only if conversion of nitrate to nitrite was greater than 95%, as judged by a comparison between both nitrite and nitrate standard curves. Fig 2.5 shows that the standard curve was found to be linear in the range 0-50µM, R^2> 0.98 for both lines. Comparison of slopes shows that >95% conversion is achieved.

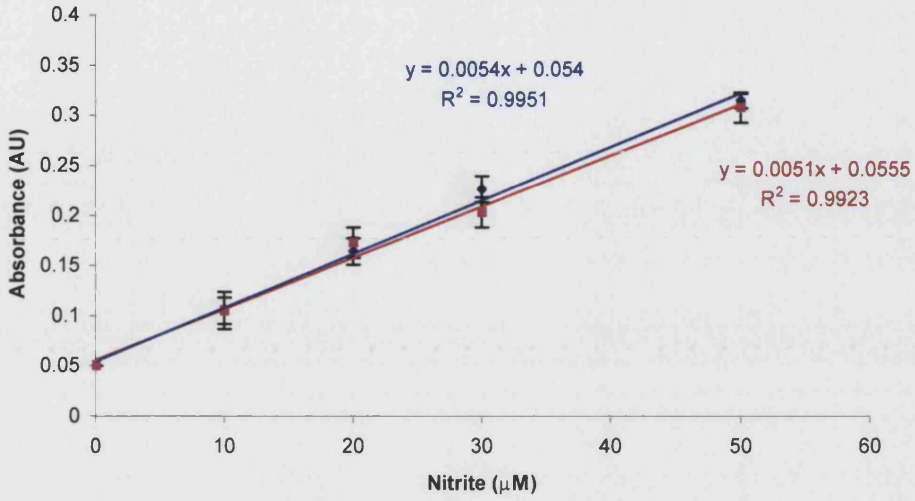


Fig 2.5 Griess assay standard curves. The graph shows the linear regression lines for the absorbance of both the nitrite standards (Blue line) and the reduced nitrate standards (Red line). The equations show that R^2 values for both nitrite (Blue equation) and nitrate (Red equation) exceed 0.98. Mean ± SEM, n=3.

2.8.6 Glutathione (GSH)

GSH from test samples was isolated and quantified using a reverse-phase high performance liquid chromatography (HPLC) column and a coulometric electrochemical detector respectively. The method is derived from that of Riederer et al (1989).

2.8.6.1 HPLC System

The HPLC system consisted of the following. An autosampler (Model 360, Kontron instruments, Watford, UK) was used to inject 20µl of sample on to a guard column (dimensions 3mm x 10mm) to protect the main column from possible contamination. Sample was then resolved by passing through an analytical column (dimensions 4.6mm x 250mm). Both columns were packed with 5µm octadecasilyl (Techsphere, HPLC technology, Macclesfield, UK). Column temperature was held at 30°C by a column block heater (Model 7970, Jones Chromatography, Mid. Glamorgan, UK). The mobile phase consisted of 15mM orthophosphoric

acid (OPA) pH 2.5, which had been prepared in ultrapure H_2O purified to an electrical resistence of 18MΩ.cm^{-1}(Purelab Maxima, Elga, Derbyshire, UK). Flow rate was maintained at 0.75ml/min by a pump (Jasco PU-1580, Great Dunmow, UK). Upon column separation, sample GSH was detected using a dual-electrode coulometric analytical cell made of porous graphite (Model 5010, ESA Analytical, Aylesbury, UK). A control module (Model 5100A, ESA Analytical, Aylesbury, UK) maintained the upstream electrode at a potential of +100mV to screen out molecules of lower oxidation potential than GSH whereas the downstream electrode was maintained at a potential of +520mV. The current at the downstream electrode is proportional to the amount of GSH present and was recorded by an integrator (Chromjet Ch-1, Thermo Separation Products, Florida, USA) at a chart speed of 0.25cm.min^{-1}.

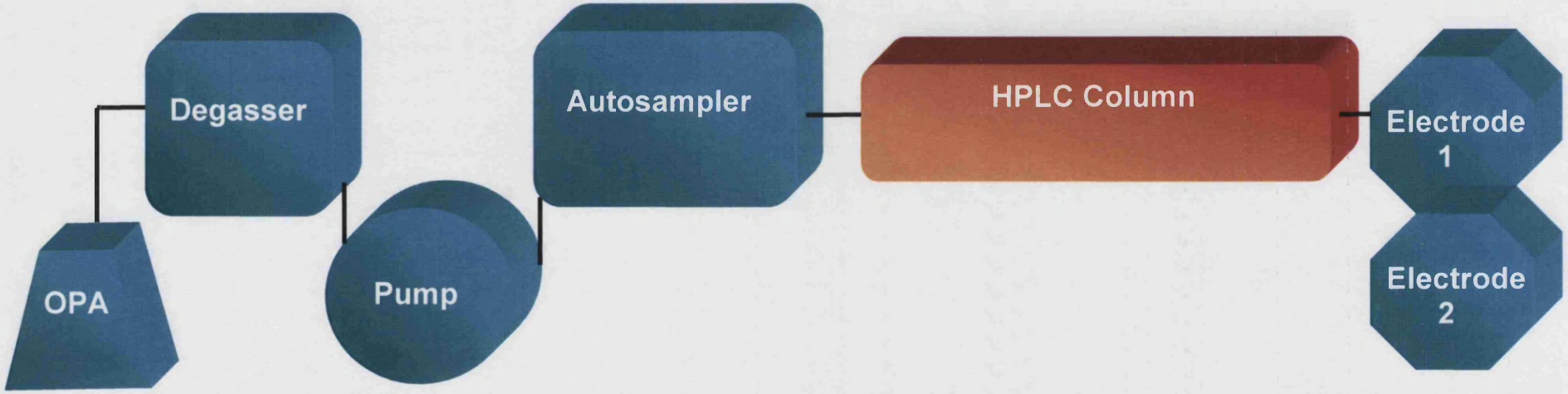


Fig 2.5 HPLC setup. The black lines represent the flow of mobile phase (orthophosphoric acid) and sample as they travel through the HPLC and on to the electrochemical detector.

2.8.6.2 Determination of Potential for GSH detection

GSH standards, at a concentration of 5μM (GSH in 15mM OPA, pH 2.6) were injected on to the HPLC system at various downstream electrode potentials in order to ascertain the optimal potential for the determination of GSH. The voltamogram generated is illustrated in Fig2.7 and illustrates that a plateau was reached at around +530mV.

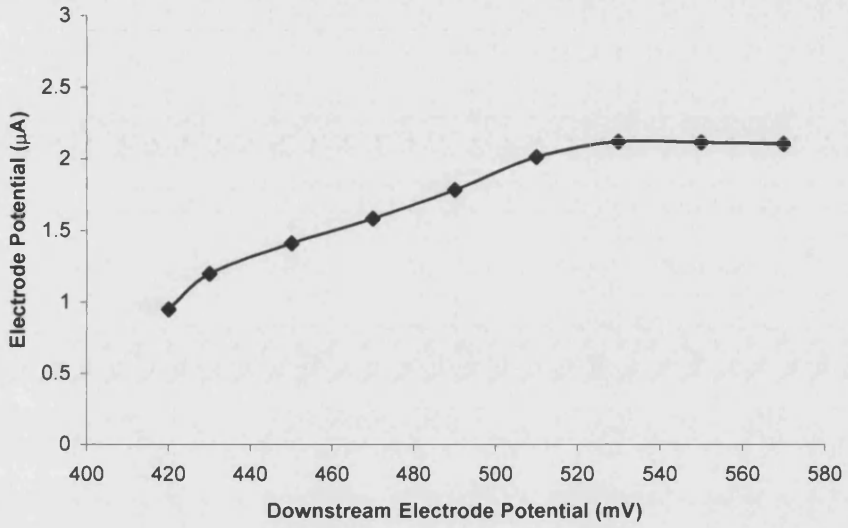


Fig 2.7 GSH Voltamogram. 5μM GSH standards were separated by HPLC and detected at various downstream electrode potentials. Upstream potential remained at +100mV, downstream potential was varied between +420mV and +570mV.

2.8.6.3 Sample and standard preparation

GSH standards were prepared in 15mM OPA (which had been prepared in ultrapure H_2O), at concentrations between 0.5 and 10μM. These were frozen at -70°C until used. Cell pellets suspended in HBSS were diluted further with HBSS if necessary. The cell suspension was then mixed 1:1 (vol/vol) with 15mM OPA to extract GSH. The suspension was then centrifuged at 500g for 5mins at 4°C to precipitate out protein. The supernatant was removed and stored at -70°C. Cell culture medium was similarly treated. Medium was mixed 1:1 (vol/vol) with 15mM OPA and centrifuged at 500g for 5mins at 4°C. The supernatant was removed and stored at -70°C. All cell suspension samples were stable for at least one month when stored frozen. Medium samples were not stable beyond 2 weeks.

2.8.6.4 Sample handling

Acidified pellets, medium and standards were defrosted and 200µl was sealed in Chromacol autosampler vials. Such samples were rotated automatically using the cell changer of the autosampler and each was automatically injected every 15mins. Samples containing cell culture medium were placed on the autosampler and manually injected one at a time. Medium samples took 2hours to pass through the column due to late eluting peaks and so could not be allowed to remain in a queue for column injection. This is due to the fact that GSH degrades at room temperature in medium/OPA mix (Fig 2.8).

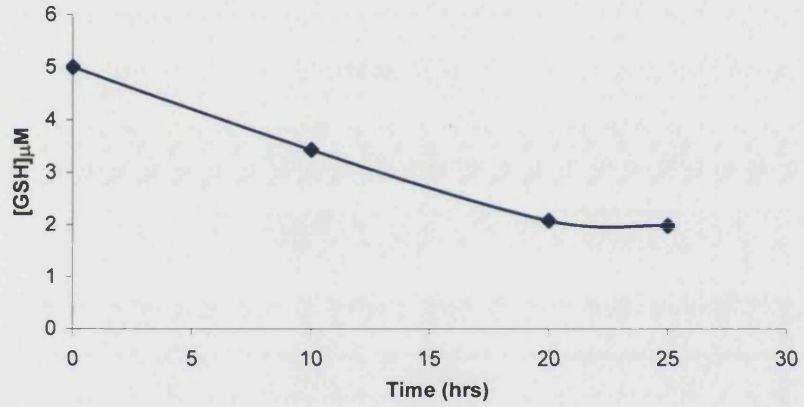


Fig 2.8 GSH degradation at room temperature. A 5µM GSH standard was repeatedly injected on to the HPLC column using the automatic function of the autosampler unit. GSH concentration was reduced to 2µM after 20hours.

The current generated by each sample was converted to GSH concentration by comparing peak height with a standard GSH curve. Fig 2.9 shows that electrochemical detection was linear from 0.5 to 10µM, R^2=0.991. The retention time for GSH on the HPLC column was 6.6±0.1 mins (mean±SD) (Fig 2.10). Previous to acid extraction, a sample of the cell suspension was removed for protein determination (section 2.8.1). GSH concentration was expressed as nmol/mg protein for cell suspensions and µM for medium samples.

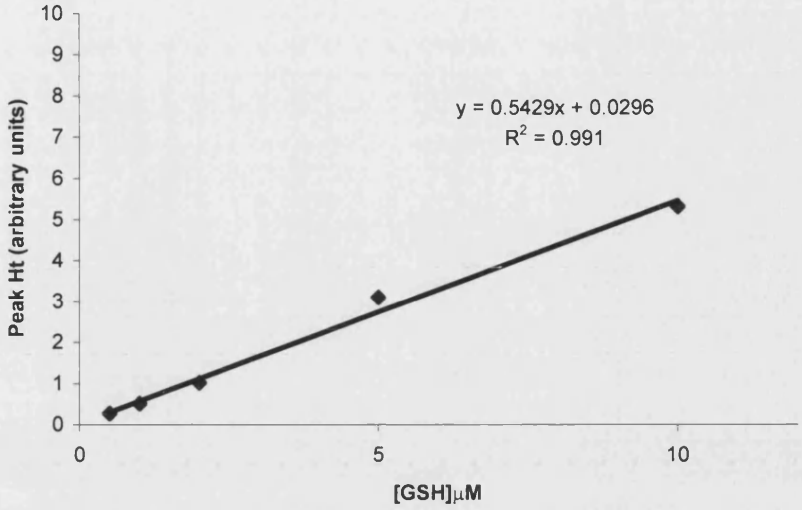


Fig 2.9 GSH Standard Curve. Glutathione standards at concentrations of 0.5, 1, 2, 5 and 10μM were injected on to the HPLC column. The points on the graph represent each sample, linear regression was performed using Microsoft excel.

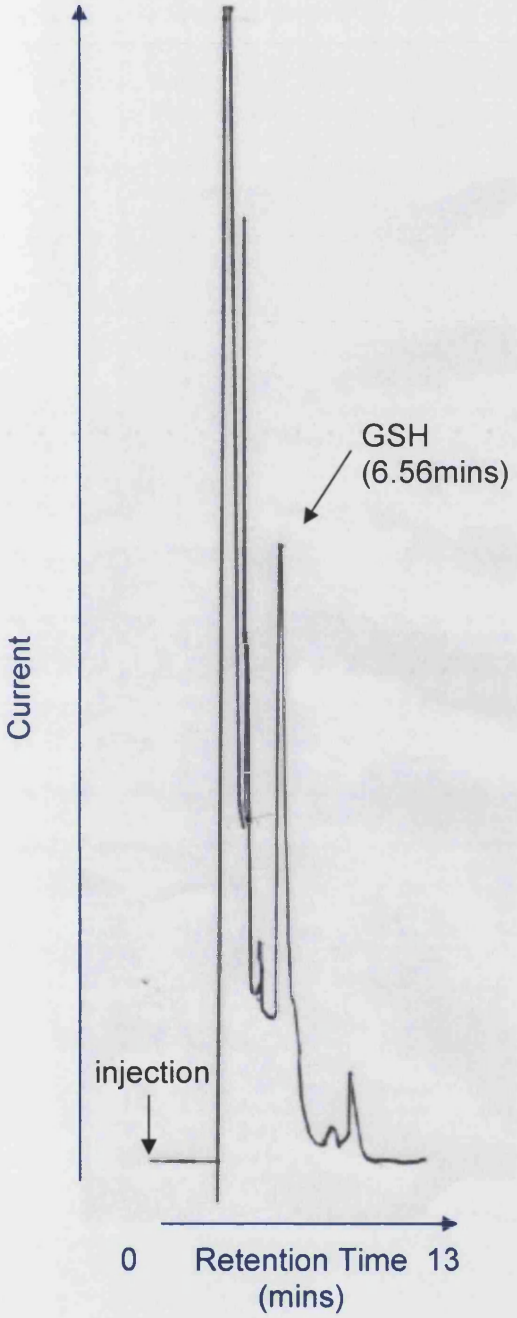


Fig 2.10 Chromatogram of Neurobasal sample. The figure is a representative chromatogram of neurobasal medium that had been removed from a layer of astrocytes.

2.9 Statistics

Statistical analysis was carried out using Microsoft excel. Student's t-test for paired samples was used, accepting $p<0.05$ as significant.

Chapter 3

'Establishing a tissue culture environment for the study of oxygen glucose deprivation/reoxygenation related injury'

3.1 Introduction

As detailed in chapter 1, a variety of models for the study of ischaemia exist. In order to answer questions of biological significance in the most accurate and informative manner, it is crucial to choose an appropriate model to the questions being asked. Because we are interested in tapping in to the complicated messaging systems that exist specifically between astrocytes and neurones we have chosen to take an approach that limits and controls most variables. We have therefore modelled ischaemia/reperfusion in dissociated cell cultures.

It has been necessary for us to produce a method both for the culture of primary cortical rat neurones and for the induction of hypoxia in these cells before studies on cell behaviour following oxygen glucose deprivation could be initiated. The following details our reasoning for this.

In the optimisation of the neuronal culture protocol we wished to meet three crucial criteria. Firstly, we wanted a culture of high purity in order to reduce noise in the gathering of biochemical data. That is to say, we did not want the response of one cell type to overwhelm the detection of a response from another cell type. Furthermore it has been documented on numerous occasions that the presence of contaminating cells affects the physiology and viability of neurones (Ahlemeyer et al 2002). We wanted to distil the insult given, to a response related entirely to the neurones themselves and not other cell types.

Secondly, we wanted to maintain a culture that would live until at least DIV10 in order that protein expression patterns within each cell would more closely parallel an *in vivo* insult. Chihab et al (1998) have shown how mature and immature neurones react differently to hypoxia. This is particularly important with respect to NMDA receptor sub-unit expression. A major focus of ischaemia research is excitotoxicity, which is mediated largely through NMDA receptor activation (Arundine et al 2003), yet the appropriate subunits are not expressed in culture until at least DIV7

(Mizuta et al 1998). In a similar vein, it has been shown that synapse development begins and rapidly proceeds through the second week of culture (Lin et al 2002). Neurones rely heavily upon the supply of signalling/nutritive factors from other cell types and so it is difficult to grow long-lived *and* pure neurones in culture. It is common to achieve longevity by the use of a glial feeder layer (Rogers et al 1997) yet this reduces purity. Media supplementation with survival factors such as brain derived neurotrophic factor has been used (Negishi et al 2003). However, as shown by Dai et al (2003) the efficient survival of neurones is dependent upon a number of factors, identified and unknown, working in concert.

Thirdly, we wanted to establish growth of the culture on glass coverslips in order to carry out imaging studies of Ca^{2+} dynamics and immunocytochemistry to characterise the neurones and establish neuronal viability and identity.

The existing method for the growth of neuronal cultures within our laboratory led to a final product that had to be used on DIV6, was around 85% pure and could only be grown on plastic tissue culture plates. Clearly this did not meet our needs and so we had to seek an alternative approach.

We have arrived at the current culture method by focussing and improving on three crucial areas within the culture preparation namely, 1) the dissection and dissociation of cells from foetal brains, 2) the seeding of cells on suitable surfaces for culture, and finally 3) the use of appropriate feeding media. This method has evolved by piecing together information from a number of sources and observations.

With respect to the establishment of hypoxia, the introduction chapter details the complexities involved. We have chosen again to limit complicating factors by aiming to induce an ischaemic insult by the removal of metabolic support and not by the direct chemical inhibition of

respiratory elements. We wanted to achieve OGD in a manner that would strike a balance between efficiency in terms of the speed of O_2 removal, ease of use, reproducibility and cost effectiveness.

3.2 Aim

This chapter has two central aims. The first aim is to develop a pure cortical neuronal culture and the means with which to coculture neurones with pure cortical astrocytes. The second aim is to derive a method for the induction of hypoxia in cultured cells.

3.3 Methods

3.3.1 Cell culture

Cultures of rat cortical neurones and astrocytes were used and prepared as described in section 2.2.

3.3.2 Cell death

Cell death was quantified in neurones using the method detailed in section 2.5.2 based on nuclear dye exclusion. Astrocytes cell death was quantified using the LDH release assay as described in section 2.5.1.

3.3.3 Hypoxia measurement

O_2 concentration within the glass desiccating chamber was measured using the 782 oxygen meter (Strathkelvin Instruments, Glasgow) as described in section 2.7.

3.3.4 Immunocytochemistry

Immunocytochemistry was performed on neurone and astrocyte cultures using the method detailed in section 2.3.

3.4 Evolution of methodology

3.4.1 Cortical neurone culture

We began with a method for the preparation of cortical neurone cultures from rat brain devised by Almeida et al (1998) and used routinely in our laboratory. Briefly, cells dissected from foetal rat brains were fed with FBS supplemented DMEM for 3 days followed by Horse serum supplemented DMEM for the rest of the culture life. However, as mentioned previously, this method is optimised for neuronal growth up to DIV6 for cells that were seeded on plastic dishes. The basic approach to the dissection was kept, most importantly the use of embryonic neurones as a starting point.

We also attempted to isolate cortical neurones from postnatal day 1 (P1) Sprague Dawley rats by this same method, but found them to be far more over run with glia (by phase contrast microscopy investigation). It should be mentioned that there are methods, which can be used to purify postnatal cultures such as immunopanning (Barres et al 1988). We chose to concentrate on the embryonic neurones rather than try different techniques ad infinitum with the P1 animals.

3.4.1.1 Trituration

We found that there was a considerable amount of debris within the culture wells and so we hypothesised that cytotoxic agents released during trituration were limiting cell growth. Our first step was therefore to try and limit tissue damage during the trituration process in an effort to reach a compromise between culture yield and viability. We have found that a maximum yield of healthy cells was achieved by using 4 strokes of the 1ml Gilson followed by 4 strokes of the 0.2ml Gilson on each repeat of the step. This limits the mechanical damage caused to the cells upon repeated pipetting.

3.4.1.2 Seeding surface

The choice of seeding surface is vital for adhesion, growth and differentiation. The original method stipulated the use of plastic 6-well plates however, we needed to seed on a surface that would allow us to image neurones using fluorescence microscopy, for reasons mentioned previously. However a) the plastic at the base of the 6-well plates is quite thick and is therefore outside the working distance of the objective lenses used in our microscopes and b) the plates are composed of polystyrene, which is not conducive to fluorescence studies.

We came across Thermanox coverslips (Nunc, Rochester NY), which overcome these problems of imaging. They are made from a solvent resistant plastic material that allows for fluorescence studies and are more likely to encourage cell attachment due to the plastic composition. However we were unable to grow cells on their surface. At this point we speculate that other aspects that had yet to be sorted out in the protocol were probably responsible for this. We therefore, turned to the use of glass coverslips.

Glass cover slips were cleaned to remove oils and grit, and coated with poly-lysine (P/L). Basic amino-acid polymers, such as poly-ornithine (P/O) and P/L, apply a charge to the surface acting as an adhesive for cell surface proteins. This provides a means by which to anchor the cells and to aid in the extension of processes as the culture matures. This latter point is important to remember given the fact that the neurones have been sheared of axons and dendrites during tissue and cell dissociation and therefore must form processes afresh. We have tested a number of concentrations of P/O and P/L, in combination with various incubation conditions. We used concentrations ranging from 0.1 to 0.001%, for time periods ranging from 30mins to 24 hours whilst being incubated in a humidified atmosphere at 37°C or simply resting in a flow unit at room temperature. Higher concentrations and longer incubation periods resulted in no cell adhesion whereas the lower concentrations resulted in little cell adhesion by DIV2. We found that 0.01% P/L that has been very

well mixed at room temperature gave the highest yield of healthy cells when allowed to sit on the coverslips for 30mins.

3.4.1.3 Feeding medium constituents

We have found that these neurones preferred a particular media preparation. We departed from the use of DMEM and turned instead to Neurobasal A medium (NB) throughout the preparation of this culture (Brewer et al 1995). According to the manufacturer's guidelines, NB supplemented with 2mM glutamine and 2%B27 provides optimised conditions for long-term neuronal survival (Gibco, NB product sheet) and is sufficient to maintain cultures for up to four weeks in some instances.

In order to sustain cell growth beyond DIV2 the seeding surface was coated with modified NB (Section 2.2.2.2). We saw that a number of groups use this approach for preparing the seeding surface (Harms et al 2000, Meloni et al 2002) and so we devised our own concoction based upon what we felt was most beneficial to the neurones. The mixture of equilibrated nutrients awaiting the cell suspension provides a rich environment in which the neurones can recover from the trauma of tissue/cell dissociation. Without this treatment, neurones on glass tended to form dense clumps connected by long phase bright processes. Fig 3.1 is representative of the condition reached by neurone cultures when conditions were imperfect.

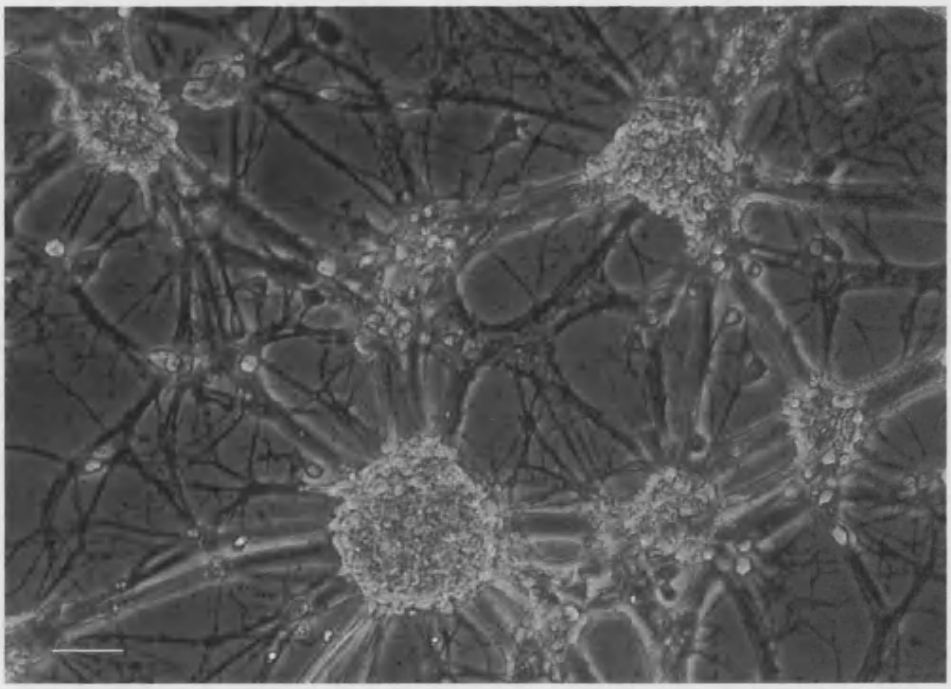


Fig 3.1 Unhealthy neurones. Phase contrast image of cortical neurones in an unsuitable growth environment. Cell bodies associate to form large balls connected by threads of neuritic processes. 10X objective lens, scale bar represents 80µm.

Traditionally foetal bovine serum (FBS) and horse serum (HS) have been used in neuronal culture medium to promote growth and differentiation respectively. Animal sera contain a plethora of trophic/nutritive factors, however we found that the presence of sera in the staple media resulted in an increase in the number of glia. The small concentrations used in the coating media are clearly adequate to have a positive effect on neuronal survival and so we use them in the coating media only.

Glutamine is extremely important in providing carbon to the TCA cycle of cultured cells and is also an important supplier of organic nitrogen. B27 is a serum supplement containing a combination of amino acids, vitamins and anti-oxidants designed to function in conjunction with NB. Its advantage over animal sera lies in the fact that its components can be defined and controlled. NB does not contain the excitatory amino acids glutamate and aspartate partly in order to limit the instances of excitotoxicity during later culture life and also to limit the effects of excess

glutamate on receptor desensitisation. However, glutamate is an important nutritive factor in aiding neurite outgrowth in early culture life. Although the B27 supplement has a certain amount of glutamate (25μM), it was necessary to add extra glutamate to the media to a final concentration of 50μM until DIV3. It may be the case in this instance that the cells have a heavier reliance on glutamine, which is catered for by the extra glutamate.

We have found through immunocytochemical analysis (Section2.3), that neurones at DIV10 contained a high proportion of astrocytes when fed only with the manufacturer's formula (Table 3.3). We therefore included cytosine arabinofuranoside (AraC) in the feeding media, as per many other protocols (Almeida et al 1998) at DIV3 to a final concentration of 10μM. AraC is a nucleotide analogue that inhibits DNA replication in dividing cells. Because glia continue to proliferate but neurones do not, Ara-C selectively kills glia.

However, we observed a dramatic drop in viability by DIV7 (observed under phase contrast microscopy). Cells became detached from their surface and the remainders formed clumps (Fig3.1). We hypothesised that this was due to the lack of glial support and not directly due to the addition of Ara-C. We therefore overcame the problem by the use of astrocyte conditioned NB, from DIV3, under the premise that we could compensate for glial absence by providing essential glia derived trophic/protective factors directly. Cultures were fed with conditioned media on DIV3 and DIV7 and used for experimentation on DIV10. All feeds were carried out by half-changing the media, as the cells died within 24 hours of total media change. It appears that a total change may disturb trophic support. Table 3.1 summarises the entire feeding protocol according to essential modifications.

Day In Culture	Conditioned medium	Glutamate	Ara-C
DIV 0	-	+	-
DIV3	+	-	+
DIV7	+	-	-

Table 3.1 Protocol for feeding of neuronal cell cultures. Medium used contained (+) or did not contain (-) elements in heading.

3.4.2 Coculture of astrocytes and neurones

A knock on effect of developing the current neurone preparation was that our choice for coculture technique became limited. Our lab routinely uses cell culture inserts. Basically neurones are grown on the bottom of a dish and astrocytes are suspended above them on a membrane that sits in a plastic holder. Neurones had to be grown on glass and so cell culture inserts could not be used efficiently. We found that, because the coverslips were significantly higher than the base of the culture dishes, the neurones were effectively suffocated. It became clear that the neurone containing coverslips would have to be inverted over the astrocyte cultures somehow.

We did not want to risk damage to the neurone layer by placing the coverslips directly on to the astrocytes. We decided that the space between the two cultures had to be at a minimum with as little impedance to diffusion of solutes as possible. Furthermore the space had to be highly reproducible from one preparation to the next. We first of all tried to dimple the seeding surface of the culture plates by applying a heated soldering iron. The idea was that heating the plastic would cause a raised doughnut shape upon which we could rest a coverslip. In our hands this did not work very well and the height was far from reproducible. The use of glass beads in the culture dish fills all our criteria nicely with the addition that they are inert and easily sterilised.

We initially soldered three beads in to the seeding surface of the 6-well plates on which we placed the coverslips. However, it was difficult to create a reproducible height above the surface and leaks were quite common. This was due to the fact that the walls of the wells made it awkward to place and control the soldering iron. Furthermore, the coverslips did not balance well on three points. We therefore turned to using four beads but melted them in to the outside bottom of the 6-well plate instead. This gave us much better control over the height of the beads with the added benefit that the sterility of the system was maintained more carefully. We also created a template for the placement of the beads to ensure that each coverslip was in exactly the same position from well to well (Fig 3.2).

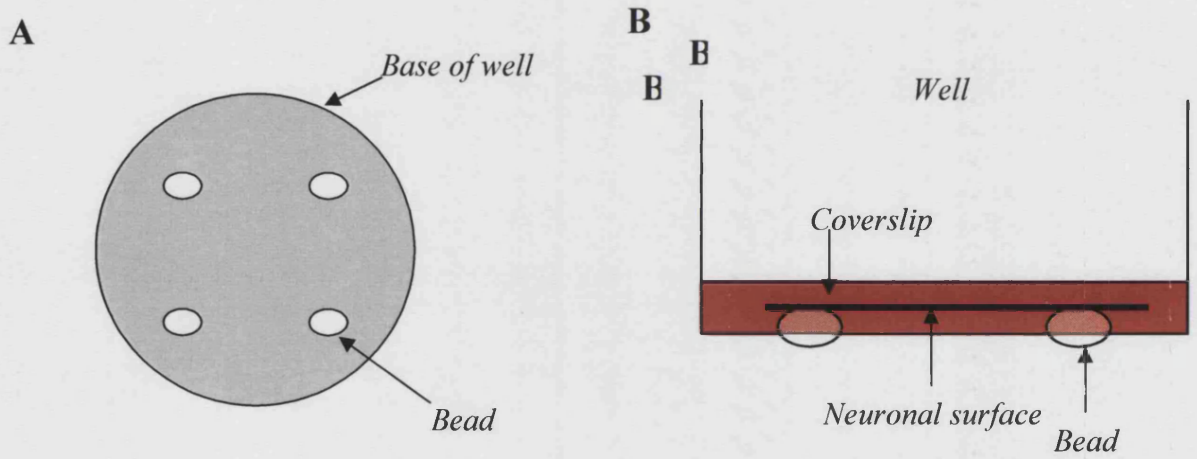


Fig 3.2 Co-culture system. **A** is an overhead view showing the position of the glass beads in a well of a 6-well plate. **B** is a side view showing the glass beads infused in the plastic well. The coverslip is resting on the raised surface with neurones facing the astrocytes lying on the bottom of the plate.

3.4.3 Creating a hypoxic environment

The first step in creating a hypoxic environment for cell cultures was to find a way of housing the cells during the insult and almost as importantly, to find a suitable method to indicate low O_2 levels. We initially used a Perspex box for the purpose of housing cells (Fig 3.3), which was sealed within a temperature-controlled incubator. We included a resazurin soaked pad as an indicator of hypoxia (section 2.4.1).

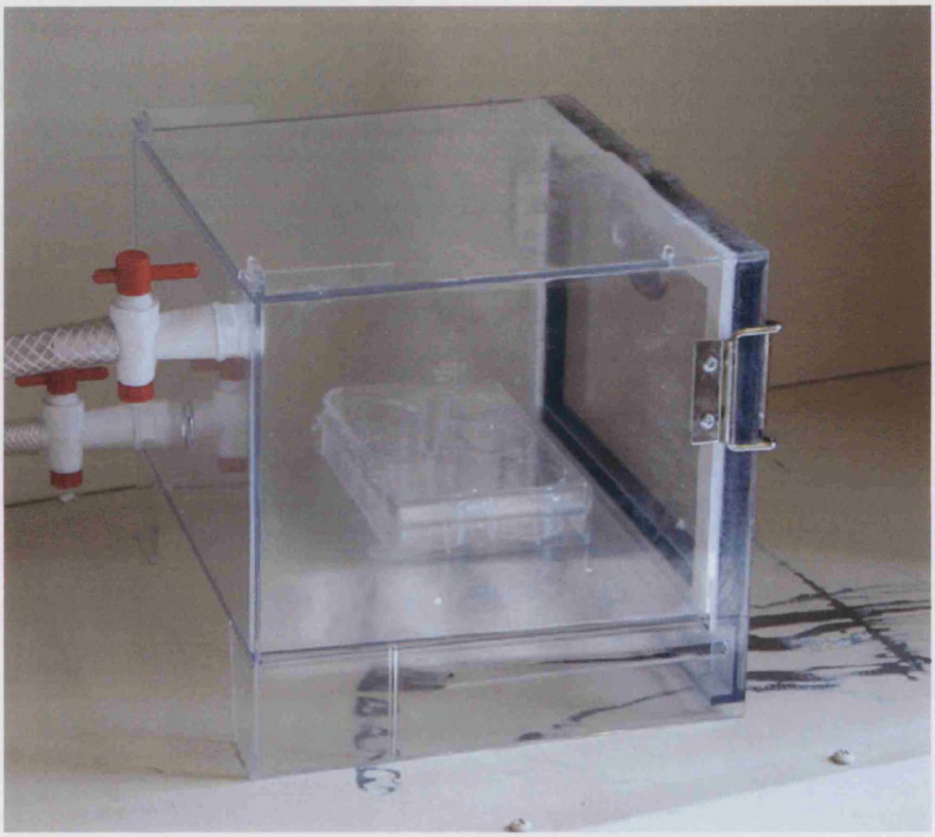


Fig 3.3 Perspex box. The box is housed within an incubator. The Box contains tubes for entry and exit of gas (left hand side of diagram), and a 6-well plate as a means with which to perceive the dimensions of the apparatus.

It quickly became apparent that the perspex was far too permeable to O_2 to efficiently induce hypoxia. We therefore turned to the use of a glass desiccating chamber using a modified lid (constructed by Radleys, Essex, UK) for inclusion of gas inlet/outlet ports (Fig 3.4), housed within an incubator.

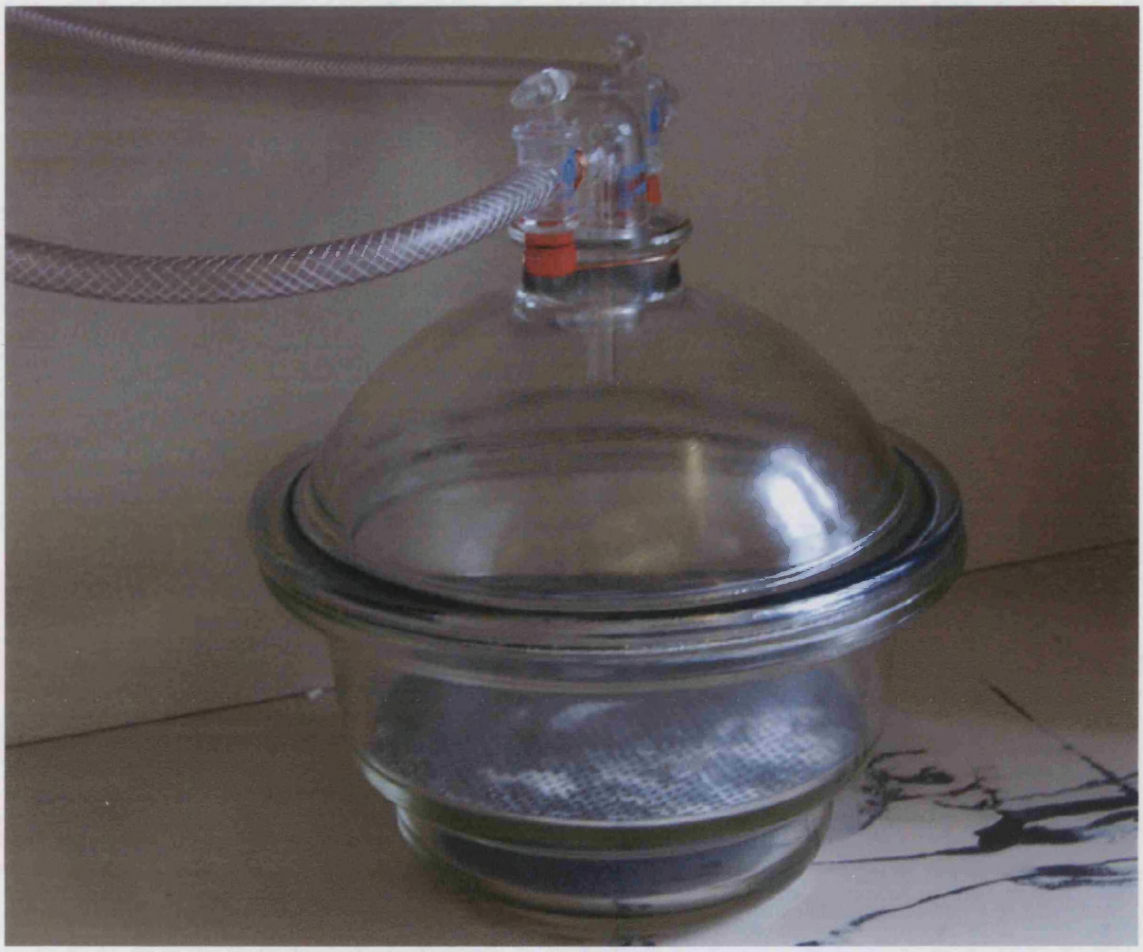


Fig 3.4 Glass desiccator. The modified lid is seen at the top complete with tubing for passage of gases, the base in filled with H_2O to achieve a humid atmosphere.

We achieved hypoxia by flushing the glass chamber with a mixture of 95%N_2/5%CO_2, that had been passed through an O_2 trap to remove trace O_2 from the stream. We also tested a number of chemical systems to limit the amount of gassing time necessary. These are summarised in Table 3.2. As none of these methods made a significant difference, they were not used for the final protocol.

Method	*Description*	*Outcome*
Glucose oxidase	Oxidation of glucose eliminates O_2, catalase used to eliminate H_2O_2 byproduct.	O_2 not sufficiently low.
Na_2CO_3/K_2HPO_4	Ascorbate oxidises at alkali pH, using O_2.	O_2 not sufficiently low.
$Na_2S_2O_4/KOH/Na_2CO_3$	$Na_2S_2O_4$ is a reducing agent at alkali pH. Na_2CO_3 used to prevent CO_2 depletion.	O_2 removed but culture media very alkali.
Powdered Fe^{2+}	Binds to O_2.	O_2 not sufficiently low.

Table 3.2 Chemical scavenging mechanisms. Summary of the methods used in conjunction with the flushing of the chamber with gas.

3.4.4 Hypoxia in cultured cells

There were a number of extra factors to consider when making cell cultures hypoxic. First of all, the culture dishes tend to store a certain amount of O_2 (Meloni et al 2001, Munns et al 2002). Also, the H_2O at the base of the chamber was capable of releasing O_2. To circumvent these potential sources of O_2, we gassed the chamber, and where possible, the culture dishes before the experiment began. The culture media itself is another potential O_2 source. Most groups tend to bubble the media with an anoxic gas mixture. However, we felt that this was an unnecessary step because in the length of time it took to prepare the cells for the chamber, the O_2 would have rapidly returned.

3.4.5 Completing OGD/reoxygenation

With hypoxia established we completed the simulation of ischaemia by using Dulbecco's modified Eagle's medium (DMEM) deprived of essential

energy substrates rather than a balanced salt solution suggested by the method of Goldberg (1993). The intention was to limit the number of variables being removed from the system. For astrocytes we simply washed the culture and exchanged the media however the neurone culture did not react well to this procedure. We therefore decided to quickly move the coverslips from one condition to the next.

We originally used complete DMEM for the reoxygenation media but again this resulted in high mortality in the control neurone culture, specifically 32±4.95% (mean±SEM) cell death after 24hours of reoxygenation (measured as described in section 2.5.2). We therefore used the NB/B27 combination but this time without the antioxidants so that there would be no masking of potential neurotoxic effects. The medium was also devoid of phenol red as it interferes with a number of biochemical assays used.

We have used relatively high glucose concentrations in the control and reperfusion media. Both contain 2.5mM, which is typical of concentrations required by neurones in primary culture. We opted for this concentration rather than a more physiologically accurate amount, as we did not want to add another stress variable to the equation. Because we used this concentration with neurone cultures, we matched it in astrocyte cultures.

3.5 Model validation

3.5.1 A pure neurone culture?

The homogeneity of the cortical neurone cell culture was assessed by means of immunocytochemistry (Section 2.3) and gross morphology under phase contrast conditions (Section 2.6.1). Immunocytochemistry showed the cortical neurone cultures to be almost completely devoid of astrocytes and microglia by DIV10. Table 3.3 highlights this fact and shows how the alternative method of excluding Ara-C affects the purity of the neuronal culture.

Method	*GFAP+*	*Cd11b*	*Tub+*	*Orphan*
NB only	12%	none	85%	3%
NB + AraC	1%	none	99%	none

Table 3.3 Immunocytochemistry. Neuronal cultures were stained with antibodies as described in section (2.3). GFAP is the astrocyte marker, cd11b the microglial marker and Tub the neuronal marker. Orphan represents nuclei present that did not have a surrounding stain.

Confocal images show the specificity with which the anti-tub antibody stains neuronal processes and the intricate network of connections formed by these. Fig 3.5 shows the interconnecting neuronal processes.

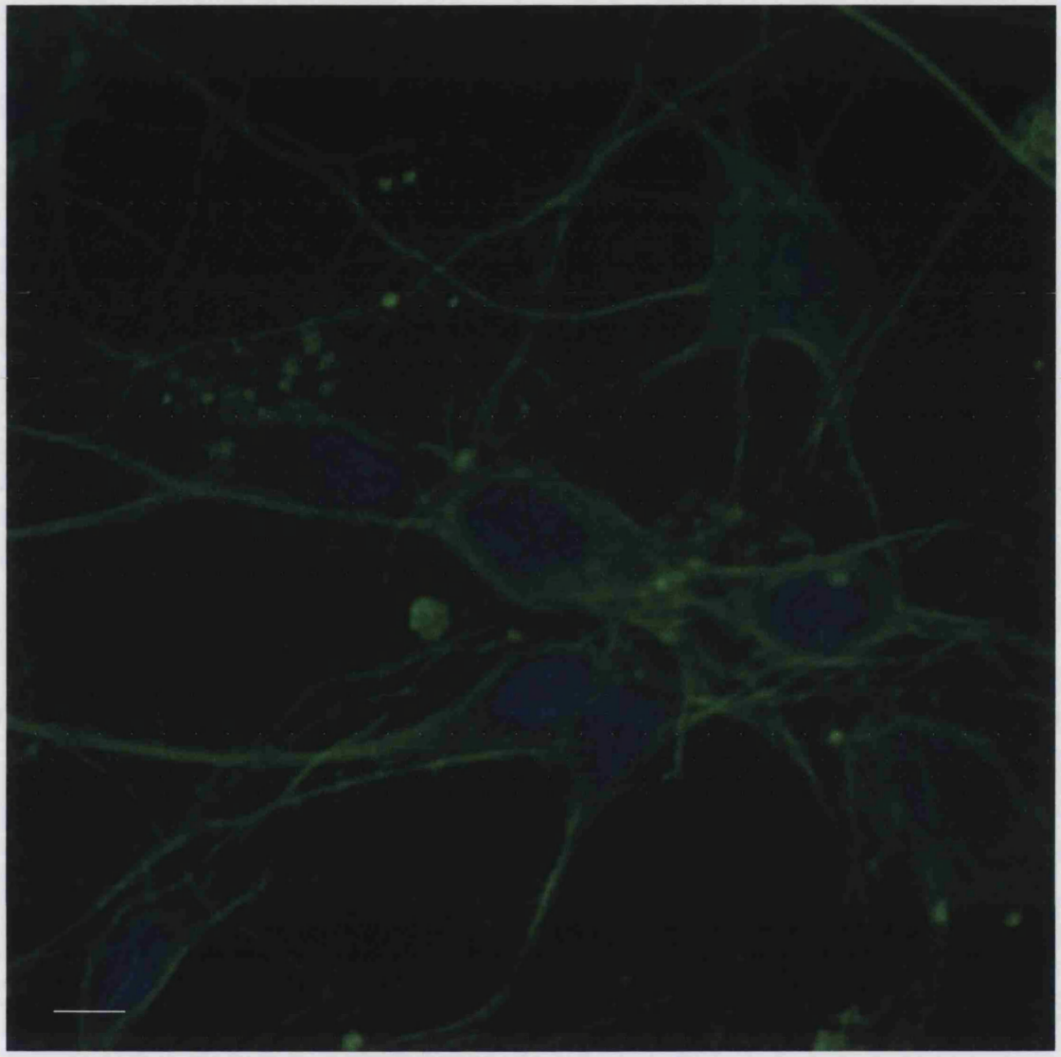


Fig 3.5 Immunocytochemical stain of DIV 10 cortical neurones. Image taken using confocal microscopy. Green colour represents the anti-tubulin stain and the blue colour represents DAPI stained nuclear DNA. 63x objective, scale bar represents 10μm.

Astrocytes, which have been detected on occasion in these cultures, appear quite spikey, resembling thick tree branches (Fig 3.6). This is in stark contrast to the more delicate neuritic processes and to the flat, rounded appearance of astrocytes in pure astrocyte cultures of the same age.

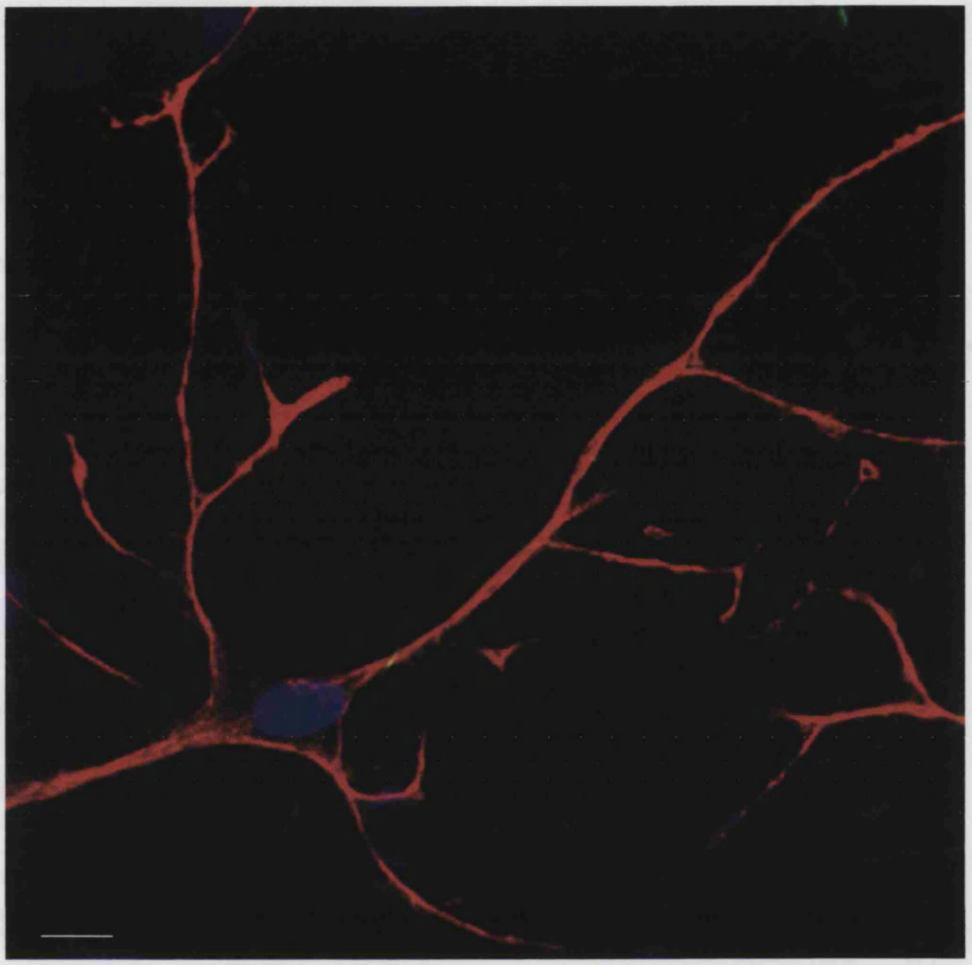


Fig 3.6 Immunocytochemical stain of DIV10 cortical astrocytes in a neuronal culture. Image taken using confocal microscopy. Red colour represents the anti-GFAP stain and the blue colour represents DAPI stained nuclear DNA. 40x objective, scale bar represents 20μm.

Using phase contrast microscopy, neuronal soma can be seen to have a morphology that varies between pyramidal and slightly oblong, with a small dark nucleus at the centre. The culture was relatively dense and was marked by an intricate network of processes (Fig 3.7). There were no obvious glia present.

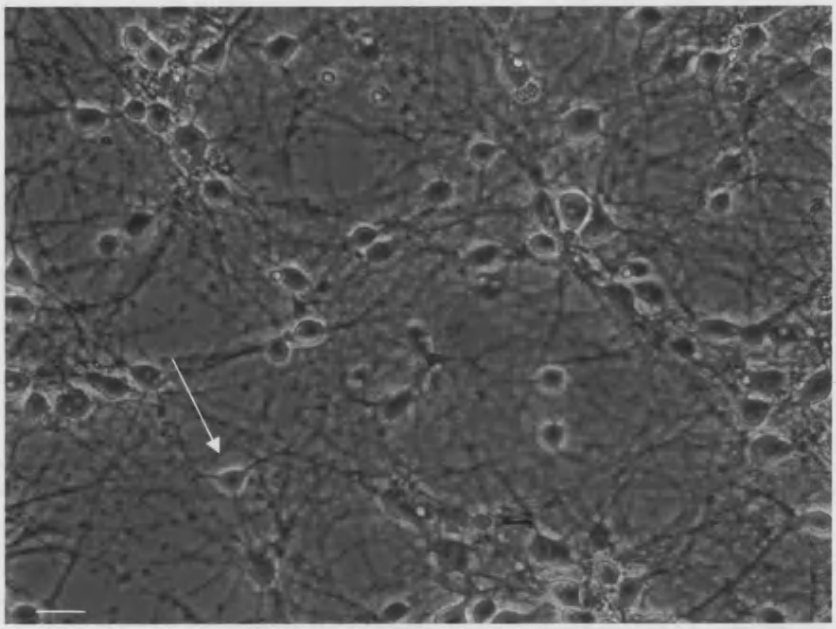


Fig 3.7 Primary cortical neurones. Phase contrast image of neurones taken at DIV10. Neuronal soma can be clearly identified as almost spherical objects with extended neurites. The arrow is pointing to a typical neurone. 20X objective lens, scale bar represents 40µm.

3.5.2 Gross morphology of neurones through development

The morphology of the neurone culture was recorded as it developed from the original dissection to DIV10 (Fig 3.8). On DIV1, cells have successfully adhered to the growth surface and have begun to extend processes. By DIV3, just prior to AraC treatment, these processes have made contact with neighbouring cells to form the beginning of a network. At this point also, it is possible to see some astrocytes as cells that are flat, large and less phase dark than the neurones. As the culture advances to DIV10, the most notable feature is the increasing density of neurites, which is consistent with the timing of synapse formation (Lin et al 2002). The neuronal soma itself does not appear to get significantly bigger from DIV3, although there is some loss of phase dark character and a roundening of shape.

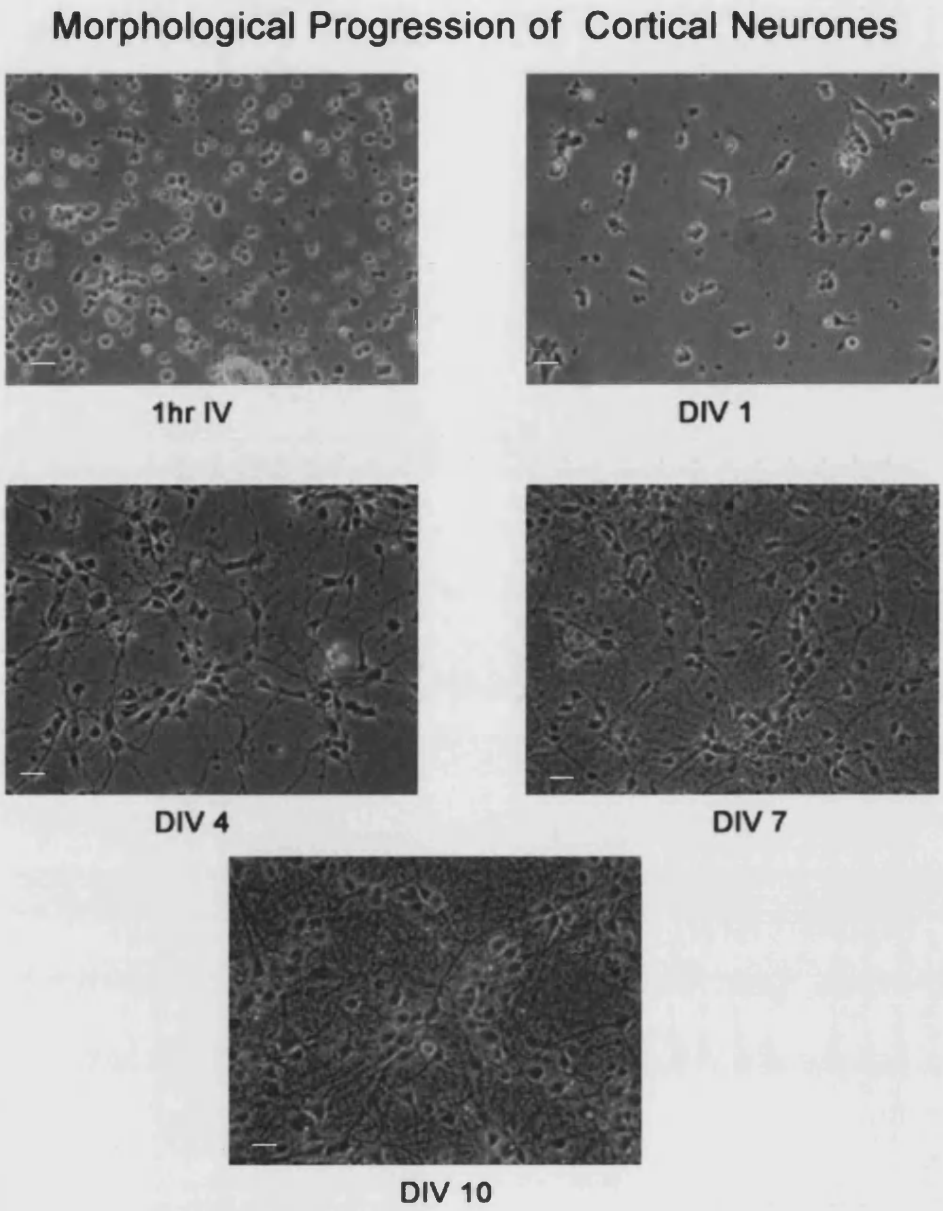


Fig 3.8 Morphology of cortical neurones in culture. Images taken at 1 hour, 1 day, 4 days, 7 days and 10 days *in vitro*. 20X objective lens, scale bar represents 40µm.

3.5.3 Health of neurone culture

The number of live cells in the culture as a whole was assessed at DIV7 and DIV10 by analysis of nuclear morphology (section 2.5.2). It was found that the culture was 94.6±4.52% populated with live cells at DIV7 (mean ± SEM, n=3) and 90.6±3.65% at DIV10 (mean ± SEM, n=6) at DIV10. The cultures were closely monitored for loss of adhesion and the appearance of cells in the culture medium, by phase contrast microscopy. By visual inspection, there was no significant cell loss between DIV4 (following Ara-C treatment) and DIV10.

To confirm viability and identity of the cells we monitored intracellular Ca^{2+} dynamics in response to an application of 100μM glutamate (section 2.6.2.1). Fig 3.9 is representative of a typical experiment and demonstrates how all cells monitored showed a rise in Ca^{2+} concentration following the application of glutamate. Cells exposed to vehicle (buffer solution only) did not show any response.

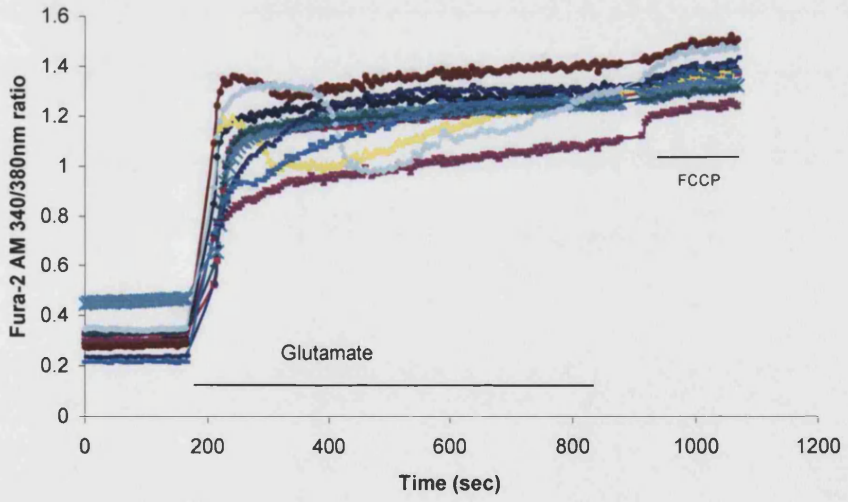


Fig 3.9 Ca^{2+} dynamics in cortical neurones. Change in intracellular Ca^{2+} concentration in response to the application of 100μM glu. The lines represent the response from individual neurones within a single field of vision.

We finally wished to determine how amenable this neuronal prep was to experimentation. We moved a number of coverslips from one well

containing NB to another. The culture contained 85% live cells immediately after moving and 82%, 24hours later.

3.5.4 Efficacy of bead system

The distance between each coverslip and the surface of the 6-well plate was measured using 'Absolute digimatic' vernier callipers and found to be 0.55±0.009mm (mean ± SD, n=24). We compared the viability of neurones inverted over the beads, with neurones sitting right side up in the base of a 6-well plate. Cell death was measured as described in section2.5.2. Fig 3.10 shows that there is no statistically significant difference in the level of cell death between the two methods at each time point.

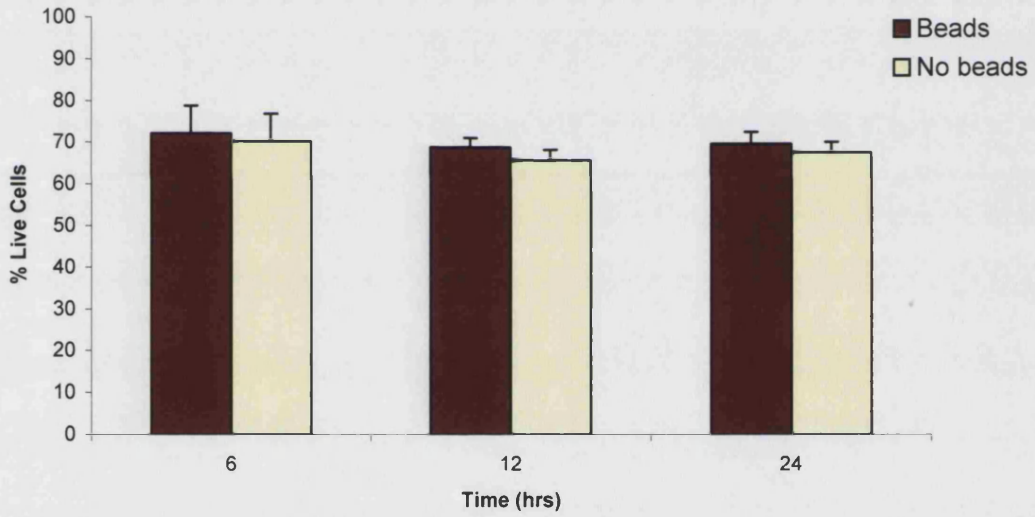


Fig 3.10 Bead system. Comparison between inverted and 'right side up' neurones. Neurones were assessed according to nuclear morphology at 6, 12 and 24 hours following placement of the coverslips either over beads (■) or in the bottom of a tissue culture plate (□). Mean ± SEM, n=4.

We have also observed that the health of the neurone culture, as judged by phase contrast microscopy, is greater when in closer apposition to the astrocyte layer. This was observed by allowing 2 beads (of the 4) to be placed further back in to the plastic, thereby creating a graded decrease in distance between the 2 cell types.

3.5.5 Confirming hypoxia

An O_2 meter was used within the glass desiccator in order to measure the O_2 concentration (section 2.7). The O_2 concentration of respiration buffer on its own was first measured and found to reach 1µM after 60mins of gassing (Fig 3.11). Over a period of 12 hours (after the end of the gassing period), the concentration of O_2 did not rise significantly, indicating the system to be airtight. The colour change of the resazurin pad mirrored these results. As the O_2 meter is far too large to place in the chamber for all experiments, the pad was used to confirm hypoxia on all subsequent occasions. The O_2 concentration within a suspension of astrocytes in respiration buffer was then measured and found to reach 0 µM by 60mins of gassing, reflecting O_2 consumption by the cells. The suspension was at a concentration that would reflect that of cells present in an area of the culture wells equivalent to the area of the O_2 electrode.

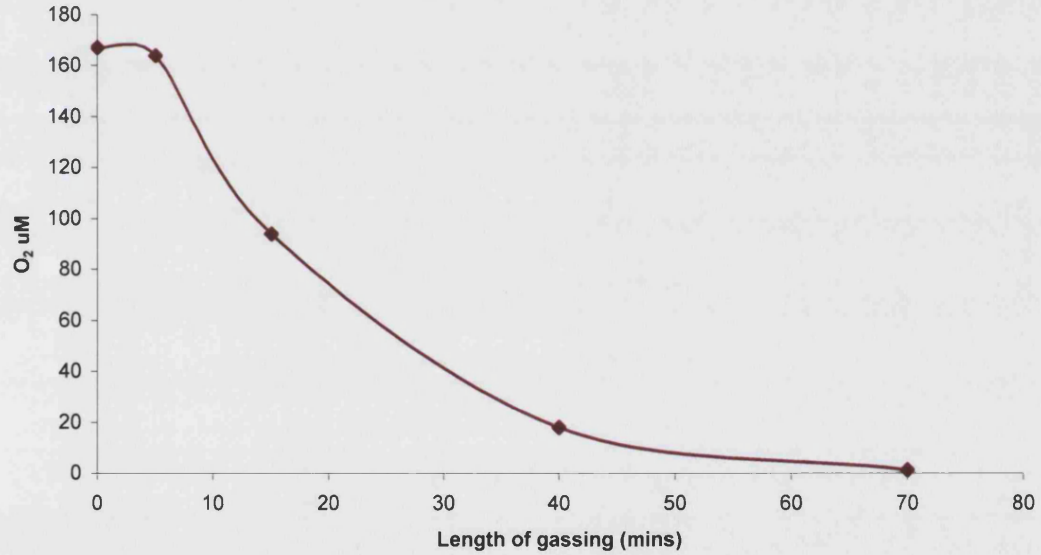


Fig 3.11 O_2 depletion. Oxygen removal from glass desiccator. O_2 concentration was measured using the 782 O_2 meter as 95%N_2/5%CO_2 was flushed through the chamber.

In order to confirm that suitably hypoxic conditions were being reached within the cell culture dishes, and that cells were in fact being made ischaemic and not just suffering from substrate deprivation, we deprived cortical astrocytes of essential metabolic substrates only. Astrocytes were deprived of glutamine, glucose and pyruvate as per an ischaemic insult but were incubated in 95% air. As can be seen in Fig 3.12 LDH release was extremely low even at 24 hours there was no statistically significant difference between control and test cells.

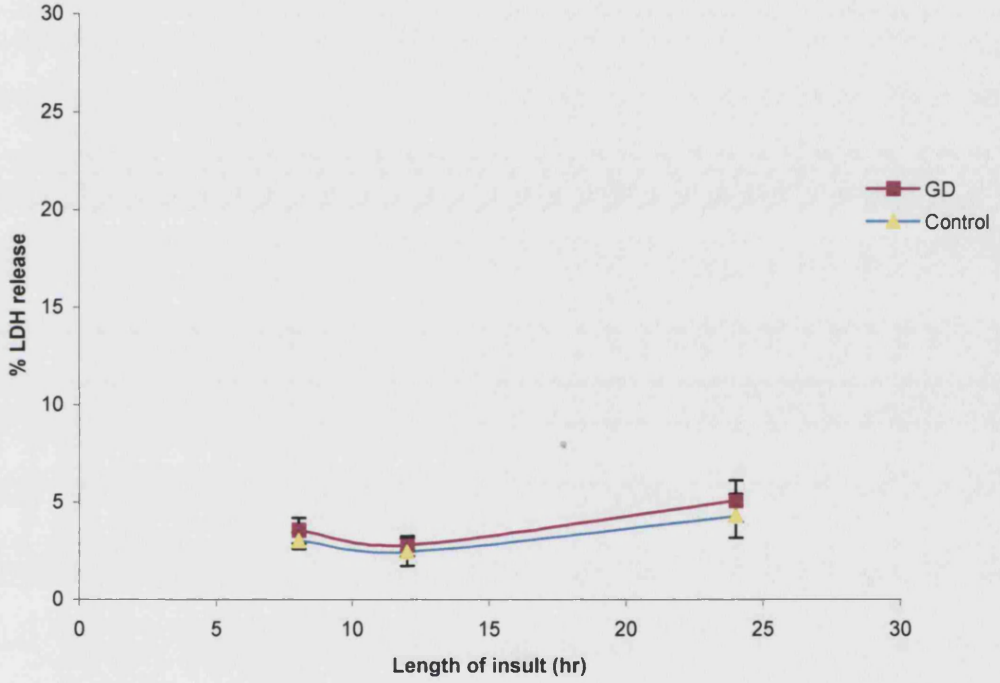


Fig 3.12 Astrocytes subjected to glucose deprivation. The (pink line) represents astrocytes deprived of glutamine, glucose, pyruvate and serum supplement, the (light blue line) represents control cells. Data analysed using a paired t-test with $p<0.05$ significance, n=4.

3.5.6 Stability of temperature

Temperature was measured during gassing and throughout the ischaemic period. The temperature was found to drop from 37°C to 36°C over the 60mins of gassing but returned to 37°C within 10mins of the start of ischaemia.

3.6 Discussion

3.6.1 Neurone culture

We have set out to build a culture that is highly homogenous for rat cortical neurones. The results show that this has indeed been achieved. We have observed that astrocytes in this culture exhibit a branching morphology, on the rare occasions that they have actually been detected. Ahlemeyer et al (2002 and 2003) suggest that this is due most likely to activation by the addition of AraC. However, Perez-Capote et al (2004) describe the same morphology in their mixed neurone-glia cultures, which have not been exposed to Ara-C. It is more likely therefore that the stellate morphology arises from either a) diffusible factors from neurones or b) physical contact between the two cell types.

Our viability studies showed that there was little cell death within the cultures when assessed at DIV10. The figure of 10% dead cells could be interpreted in a different manner because the counting method we use discounts floating material. It could be that 10% cells are dying each day from the time of plating in which case massive cell death has occurred by DIV 10. In dispute of this theory we have observed, by phase contrast inspection, that there is very little debris within the cell culture dishes as the growth *in vitro* continued from DIV 1. Indeed the clarity of the images in fig 3.8 is proof of this. It would appear therefore that the dead cells counted refer to an accumulation of dead cells from the time of plating to the time of counting at DIV10, and is minimal.

The cells that are alive at DIV 10 are also viable. We have shown this by looking at the response of the neurone cultures to an application of 100μM glutamate. There was a clear and consistent rise in intracellular Ca^{2+} concentration amongst nearly all neurones present in the field of vision. Further to confirming the health of the neurone culture, the response to glutamate in most cells analysed, also confirms that the cells are in fact neurones as Ca^{2+} levels in astrocytes do not respond to glutamate.

Perhaps the most intriguing part of the current method is in the use of astrocyte-conditioned media to promote the survival of neurones. However, this will be discussed in detail in chapter 5.

3.6.2 Co-culture

We have also produced a novel co-culture system, which we have shown to be effective in combining astrocytes and neurones. The method is highly reproducible, as judged by the error in measurements of bead height and is also a considerable improvement on the distance achieved by other methods (detailed in chapter 1).

The reproducibility of the distance is an important aspect of co-culture as we have seen that neuronal health is enhanced when neurones are closer to the astrocyte layer. Variability in height would inevitably lead to variability in results. The most logical reason for this observation would be a reduction in dilution of compounds being released by either cell type. This is always a problem with cell culture studies, when relating them to more physiological situations. The volume of medium above the cell cultures, vastly exceeds the volume of fluid normally bathing a cell, and thus dilutes anything being released.

We have not actively carried out a detailed analysis of the relationship between inter-culture distance and neurone viability. However, in theory, our co-culture system is perfectly set up to do this. The beads can be infused in the plastic to achieve much smaller or larger bumps but importantly this can be done in a reproducible manner.

3.6.3 Limits of neurone culture

It is not clear how closely this neurone preparation mimics the behaviour of cortical neurones of similar age *in vivo*. A crucial difference between this preparation and neurones growing in vivo is the absence of myelinating glia. It is unlikely that there are oligodendrocytes present and so the neurone axons most probably lack a myelin sheath, which will affect fast synaptic transmission. Neurones in culture have been shown

to form synaptic connections but with highly inefficient results in terms of synaptic activity (Pfrieger et al 1997). Furthermore, it must be kept in mind that there is a significantly greater volume above the cultures than the equivalent compartment *in vivo.* As mentioned previously, this could have a diluting effect on signalling factors.

As with all monocultures, it is clear therefore that what we have developed is a tool that has to be used with careful consideration as to the nature of the specific questions being asked.

3.6.4 Hypoxia in culture

The results show quite clearly that O_2 is being eliminated efficiently from the glass chamber with little adverse effect on temperature. Temperature change can effect ischaemic outcome (Adachi et al 1998) particularly when elements such as NMDA receptor activity is involved (Lipton 1999). We had to deduce O_2 levels in the culture wells, as our detection system could not measure O_2 in culture wells directly. Firstly, the cell suspension was deprived of O_2 within the same time frame as respiration buffer only. Low levels were maintained over many hours. Because the O_2 probe consisted of a relatively large box, with a lot of potential storage space for O_2, we can assume that the length of time for O_2 evacuation was actually over-estimated.

Secondly, astrocytes that had been deprived of glucose only, did not suffer significant cell death following a 24hour substrate free period. It has been shown that astrocytes, which have been deprived of substrate or O_2 separately, take significantly longer to exhibit cell death than astrocytes given the combined insult (Pauwels et al 1985, Hertz et al 1995). Our results agree with these observations and confirm that hypoxia had been induced.

3.6.5 Points to note

A possible problem with the current model is the length of time it takes to achieve hypoxia. *In vivo*, the interruption of blood supply causes a

dramatic decline in O_2 as trace amounts of the gas are quickly consumed by the mass of tissue surrounding the infarct area. However, this sharp decline is difficult to achieve *in vitro.* Because cytochrome oxidase has an apparent Km of 0.1μM for O_2, mitochondrial respiration can continue at very low levels of O_2 (Clark et al 1976). Therefore, the cultured cells may receive a graded insult rather than an acute burst which may possibly allow time for adaptation.

The time taken to establish hypoxia can be reduced by using an anaerobic incubator with a glove box system (described in introduction), as this set up allows for media to be evacuated of O_2 in a more efficient manner. However these systems are expensive and as such were inaccessible for our uses. Furthermore, although the bulk O_2 can be removed from the system quickly there would still be sufficient O_2 in the vicinity of the cells, and particularly cytochrome oxidase, to support a graded reduction in respiration rather than a precipitous decline.

We could have used the approach of using metabolic inhibitors to more rapidly effect hypoxia but, in keeping with our main aim, we wanted to create a simple method. Inherent in this simplicity is the existence of as few variables as possible. As alluded to previously, chemical ischaemia is not very clear-cut in terms of the processes it effects within the cell and more importantly these chemicals may linger during reoxygenation.

3.7 Conclusions

We have met the two central aims of this chapter and successfully created a culture system with which to carry out oxygen glucose deprivation. The neurone preparation is 1) relatively long lived, 2) pure and 3) robust. Likewise, the coculture system has no adverse effects on neuronal character that we could determine and allows for the efficient exchange of information between two different cell populations. Our method for the removal of O_2 from cultured cells is simple and efficient. It is effective and reproducible, keeping complicating factors to a minimum.

Chapter 4

'The effect of oxygen glucose deprivation/reoxygenation on the survival of cortical neurones and astrocytes in culture'

4.1 Introduction

Having established a culture system and the means with which to make it hypoxic, we now want to combine these techniques to do two things. First of all we want to produce a model of neuronal reperfusion injury *in vitro*. And secondly we want to characterise our model of O_2 glucose deprivation with reference to existing models.

We hypothesise that the length of the OGD period determines the pattern of cell death in the reoxygenation period such that the progressive/delayed neuronal death seen in parts of the brain during reperfusion can be reproduced. Some groups have used pharmacological intervention to imitate aspects of ischaemic neuronal injury (Meloni et al 2001, Uto et al 1995, Gwag et al 1995). Although such models do succeed in producing features of *in vivo* cell death during reperfusion, we believe it is more prudent to keep the insult as pure as possible as complicating variables such as NMDA receptor blockade, a popular approach, may have effects beyond the scope of the true insult.

It is quite clear that the ischaemia/reperfusion insult involves the interplay of many different facets of cell signalling. It would therefore seem more logical to establish a model based on the insult-inducing parameters soley, rather than to establish a model based on a single pathway of the insult-inducing parameters. For this reason we will attempt to produce a steady increase in neuronal death, by varying the time of the insult only, without interfering directly with the cell's signalling pathways. We have chosen to model a progressive neuronal loss as this mirrors focal penumbral damage and parts of global ischaemic damage (see chapter 1 for description). By its nature, death after the ischaemic insult suggests a possibility for therapeutic intervention and so is worth exploring.

With respect to characterising our method of OGD, it is important to be able to compare and contrast results from one model to the next in order to make sense of data and ask the most appropriate questions. With this

in mind, we will characterise this model using two related parameters. Firstly we will look at the temporal profile of cell death induction in both astrocytes and neurones individually. Secondly we will make a comparison between the two cell types. Dissociated cell culture models highlight quite effectively the fact that astrocytes survive OGD of significantly greater lengths than neurones, although the time course for injury induction varies between one model and the next (discussed later) (Wang et al 2002, Goldberg et al 1993). It is therefore important for us to ask if such a difference exists in this model.

4.2 Aim

The aims of this chapter are:

1) To create a model of reperfusion injury in cortical neurone cultures from foetal rat brain.
2) To ascertain the temporal parameters of cell death of both cortical astrocytes and neurons, in response to OGD.

4.3 Methods

4.3.1 Cell culture

Cultures of rat cortical neurones and astrocytes were used and prepared as described in section 2.2.

4.3.2 O_2 glucose deprivation/reoxygenation

Neurones and astrocytes were subjected to OGD/reoxygenation as described in section 2.4.

4.3.3 Cell death

Cell death was quantified in neuronal cultures according to the method detailed in section 2.5.2. It was not necessary to immunolabel the cells being counted as previous immunocytochemistry showed the population to be devoid of other cell types. Astrocyte cell death was quantified using the LDH release assay as described in section 2.5.1.

4.3.4 Microscopy

The health of neurone and astrocyte cultures was monitored by visual inspection using phase contrast microscopy as detailed in section 2.6.1.

4.3.5 Immunocytochemistry

Immunocytochemistry was performed on neurone and astrocyte cultures using the method detailed in section 2.3.

4.3 Results

4.3.1 Characterisation of neurone culture

See chapter 3.

4.3.2 Characterisation of astrocyte culture

4.3.2.1 Purity

The cortical astrocyte cultures were found to be completely devoid of neurones and microglia, as determined by immunocytochemistry. Almost all nuclei could be assigned to a surrounding GFAP stain, accounting for 99% of nuclei present. A very strong positive stain was achieved through use of the anti-GFAP/Cy5 combination (Fig 4.1).

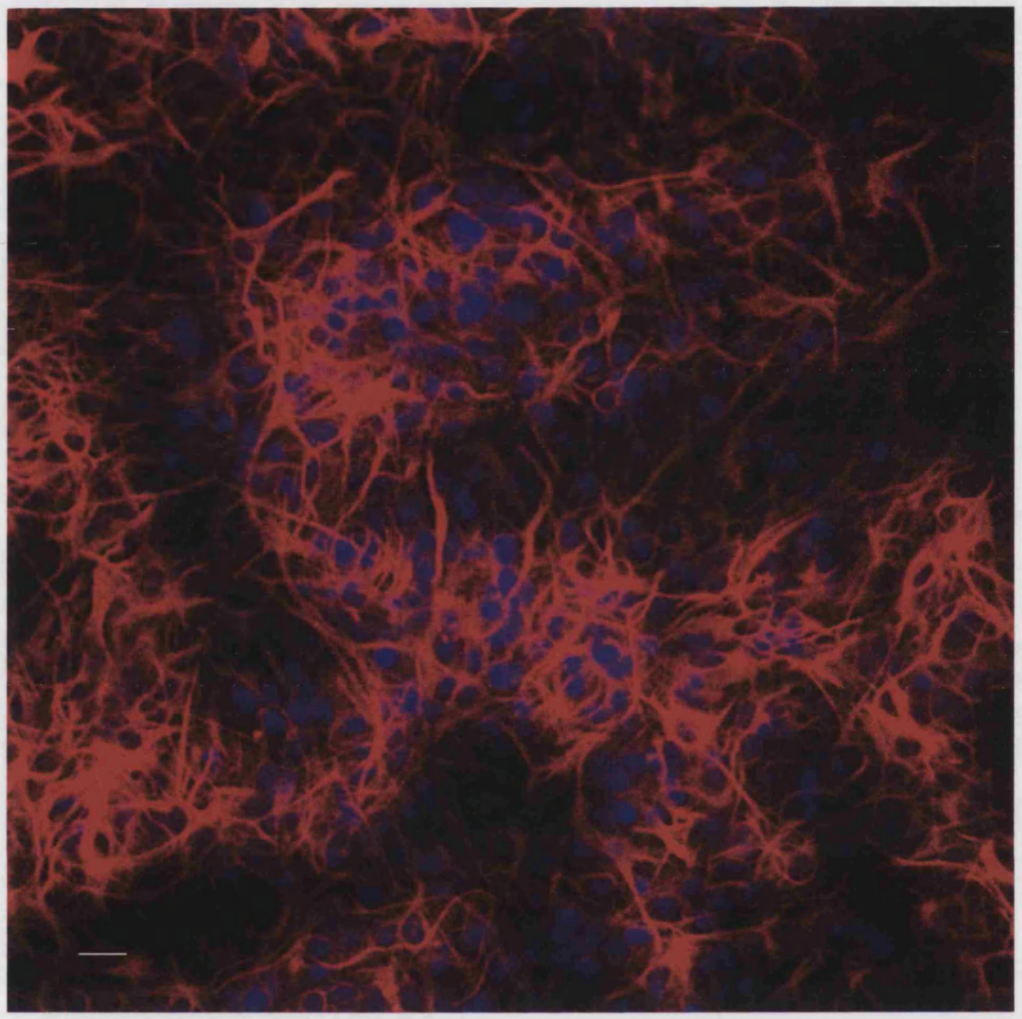


Fig 4.1 Cortical astrocytes. Immunocytochemical stain of DIV14 cortical astrocytes. The confocal image is a z stack reconstruction, dark patches represent undulations beyond the limits of the plane acquired. Red colour represents the anti-GFAP stain and the blue colour represents DAPI stained nuclear DNA. 20x objective, scale bar represents 20μm

4.3.2.2 Morphology

The astrocyte monolayer has a flat cobblestone-like appearance under phase contrast conditions. The culture appears quite homogenous. The individual cell soma appear more phase bright than neurones with a nucleus that is difficult to discern from the cytosol (Fig 4.2).

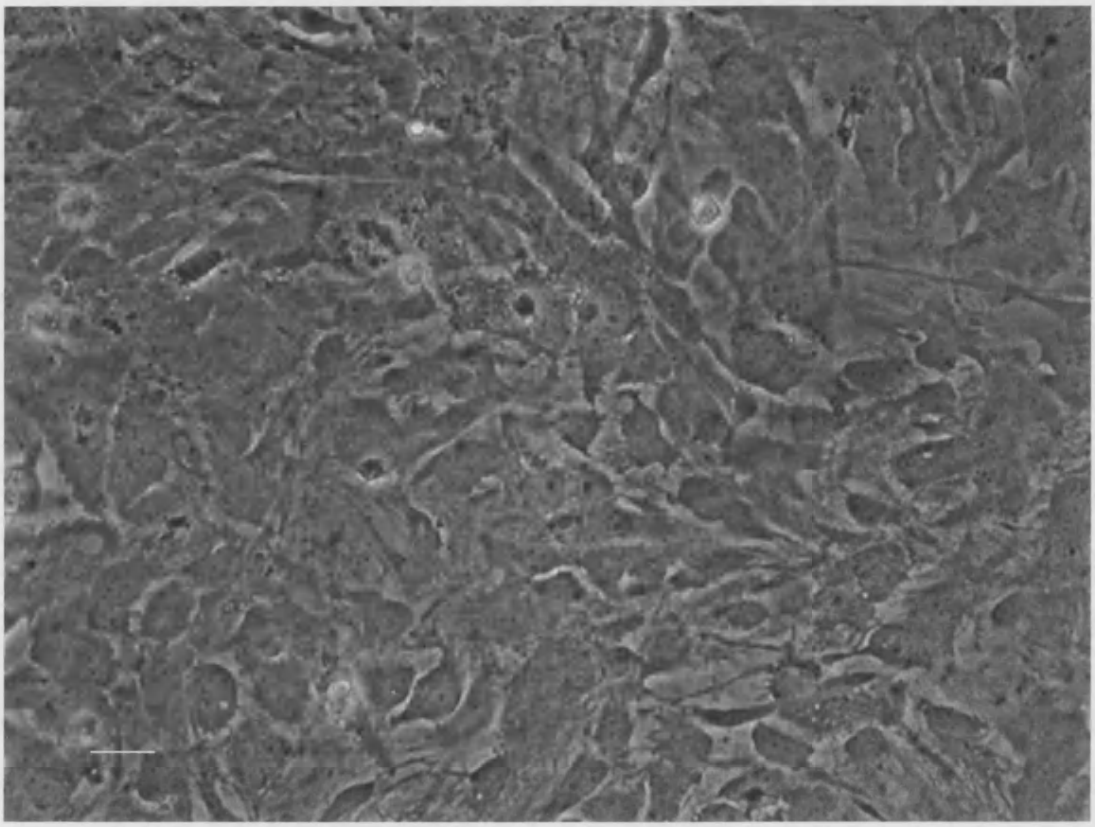


Fig 4.2 Cortical astrocytes. Phase contrast image of DIV14 cortical astrocytes. 10x objective, scale bar represents 40μm.

4.3.3 Effect of OGD/reoxygenation on neurone culture

4.3.3.1 Neuronal death increases with insult severity

Cortical neurones were subjected to OGD for 15, 30, 60 and 120mins and were then allowed to recover for up to 24hours. Cell death was quantified at defined time points during re-oxygenation by analysis of nuclear morphology (section 2.5.2). Fig 4.3 summarises the data obtained following 15, 60 and 120mins of OGD, showing the proportion of live cells in the whole cell population for each condition. The data for 30mins OGD are not shown, as we did not carry out enough experiments with which to judge statistical significance. To recapitulate, the total live cell count is the sum of the nuclei that fell in to the live and early apoptotic categories.

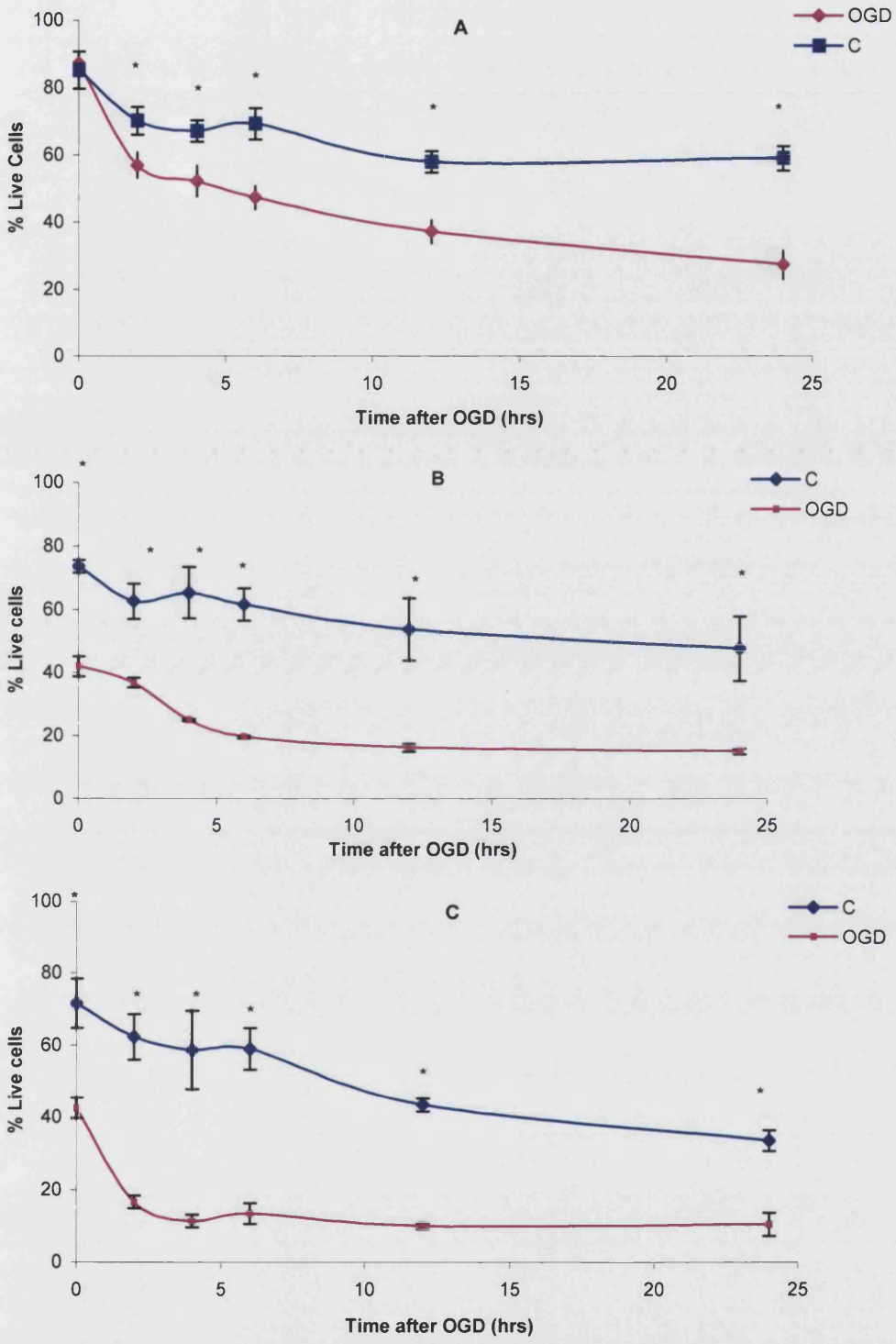


Fig 4.3 OGD and re-oxygenation in cortical neurone cultures. Cortical neurones were subjected to an OGD insult of either 15mins (graph A), 60min (graph B) or 120mins (graph C) and allowed to recover for a 24 hour period. The graph shows the percentage of live cells present in the total cell population. The pink line represents neurones subjected to OGD, the blue line represents control neurones. Data, mean ± SEM, analysed using a t-test with $p<0.05$, (*) marks significant difference between control and OGD groups, n=4 for B and C, n=7 for A.

An insult of 15mins OGD is less severe than an insult of 60 and 120mins OGD. There are two facets to the current data that illustrate this point quite clearly. These are the values obtained for live cell numbers at the start of the recovery period (ie end of the OGD period) and the rate of neuronal decline during the recovery period.

It can be seen when looking at T0, the time point at the end of the OGD period, that there is an immediate and considerable amount of cell death in OGD cultures of the 60 and 120mins conditions (Live cells, 42±2.8% 60mins, 42±3.2% 120mins). However there are far more live cells in the 15mins OGD culture (86±3.2%). The 15min T0 was found to be significantly different to both the 60 and 120mins T0 at $p<0.005$.

There was a significant difference in the number of live cells between control and OGD cultures at each time point following OGD of 60 or 120mins ($p<0.05$). This contrasts with the data obtained following 15min OGD. Here, control and OGD values were almost identical at the onset of the recovery period (86±3.2% for OGD and 85±7.1% for control cultures) but significantly different there after ($p<0.05$).

Neuronal death proceeded throughout recovery but with a different rate profile between the 15, 60 and 120mins conditions. Fig 4.4 shows that the 60 and 120mins condition dropped to a plateau level of cell death within 6hours of the end of the OGD period. In contrast to this, the 15mins condition declined at a steadier rate without reaching a plateau, but reaching the same final values as the 120 and 60mins conditions. The results are expressed as a fraction of the control values to account for cell death within the culture, not directly related to the OGD insult and to eliminate variability between separate cultures. It can be seen in both fig 4.3 and 4.4 that there is also a slight but still significant difference in the initial death rate between the 60 and 120 mins conditions. The 120mins condition drops more quickly than the 60mins condition between 0 and 6 hours of recovery. The number of live cells in the 15min

condition was still significantly greater at 2hours of recovery than the 60 and 120mins conditions at 0hours ($p<0.05$).

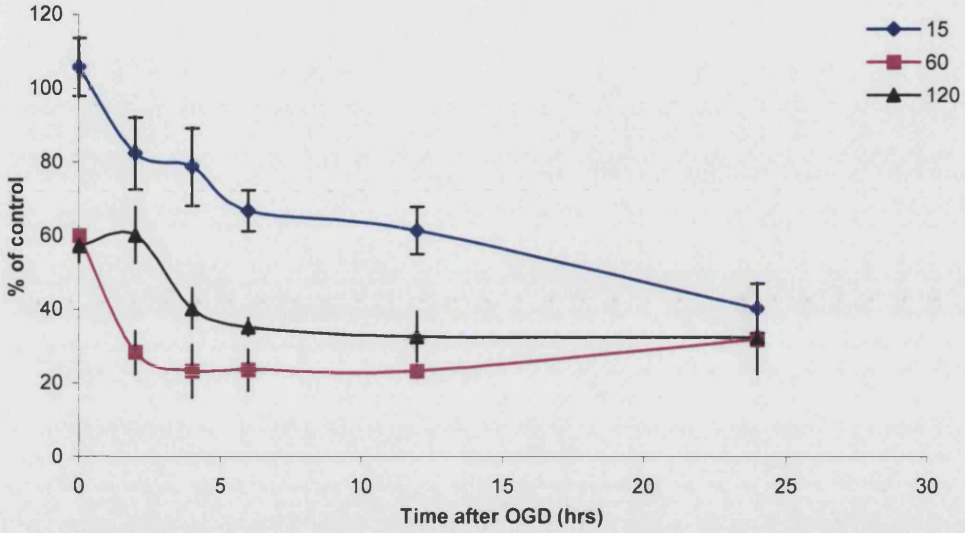


Fig 4.4 Rate of cell death in cortical neurone cultures. Cortical neurones were subjected to an OGD insult of 15mins (blue line), 60min (black line) or 120mins (pink line) and allowed to recover for a 24-hour period. The graph shows the level of live cells in the cultures subjected to OGD expressed as a percentage of the live cells in the control cultures. Data, mean ± SEM, n=4.

We also noted that cell death seemed more severe in areas of the coverslip in which there was a high density of cells. As much as possible, coverslip areas were counted in a random manner such that the overall counts reflect both sparse and densely populated areas.

4.3.3.2 Nuclear morphology

The nuclear stains showed that there was a marked difference in nuclear size between control and OGD neurones across the different insults. Essentially, nuclei that were counted as 'live' were smaller than their control counterparts, yet retained the homogenous light blue appearance. There also appeared to be more nuclei per field in the OGD cultures despite a similar plating density. (Fig 4.5). It was found that, although OGD nuclei could be counted in one group with respect to the appearance of the nuclear DNA, they were in fact hovering on the border

with another group when compared with controls. Standardisation of group assignments therefore became difficult (see discussion).

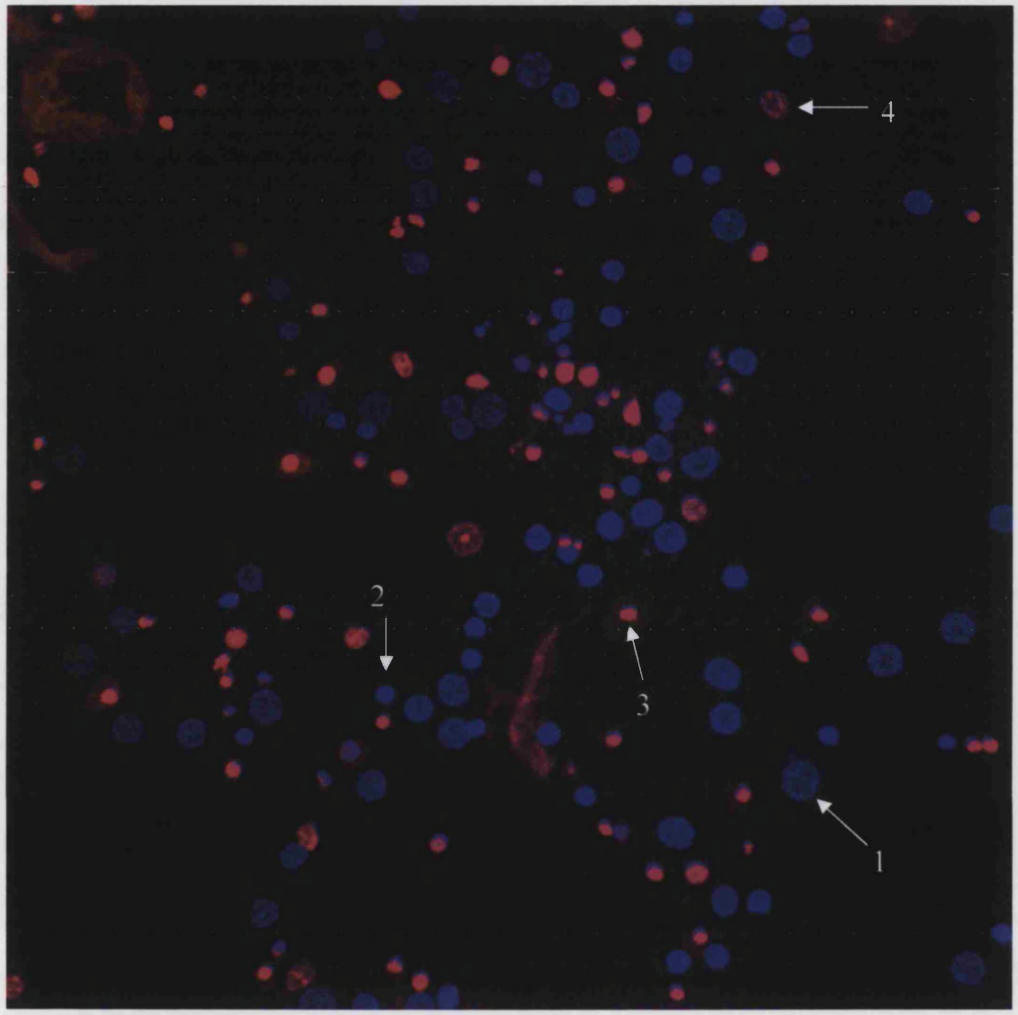


Fig 4.5 Neurones stained with fluorescent dyes targeting nuclear DNA. Blue colour corresponds to Hoechst, red colour corresponds to Propidium Iodide. Arrow 1 points to live healthy nucleus. Arrows 2 and 3 point to apoptotic nuclei. Arrow 4 points to necrotic nuclei.

4.3.3.3 Neuronal death is predominantly necrotic

Dead and dying neuronal cells were identified as being necrotic or apoptotic (criteria detailed in section 2.5.2). Table 4.1 illustrates the spread of cell death type during the recovery phase following insults of 15, 60 and 120mins duration. We have only shown data for necrosis here, as obviously, apoptosis constitutes the remaining fraction of cell death type. There is no significant difference between control and OGD groups with respect to cell death type. At each time point from each

injury group (ie. 15, 60 or 120mins insult), the same proportion of necrosis (and therefore apoptosis) was observed in the control and test cells.

Necrotic cells, % of total dead cells						
Time (hrs)	15mins		60mins		120mins	
	C	OGD	C	OGD	C	OGD
0	34±16.1	36±24.1	78±12.9	72±9.2	71±6.5	77±1.1
2	63±10.9	64±8.7	48±7.9	75±6.9	80±2.9	80±7.9
4	63±7.2	54±13.6	45±11.7	67±8.6	64±10.9	81±4.6
6	69±2.9	71±6.8	59±7.9	56±17.1	74±11.1	76±6.0
12	64±13.8	54±17	61±1.2	75±10.1	60.6±16.4	69±10.9
24	60±17.7	56±18.9	69±3.0	73±4.3	84±3.2	77±8.5

Table 4.1 Cell death type. This table shows the amount of necrotic death within the neurone cultures expressed as a percentage of the total amount of dead cells. Data, mean±SEM, n=4 or 5.

The table shows that necrosis was the dominant form of death type during recovery at most time points. However, there was less necrosis and more apoptosis observed at T0 of the 15mins insult group.

4.3.3.4 Gross morphology

The gross morphology of the cortical neurones was also assessed by phase contrast microscopy (section 2.6.1). There was an obvious difference between control and OGD neurone cultures from the outset of re-oxygenation. The soma of OGD cells were more swollen than control and showed a more phase bright character (Fig 4.6). The figure clearly shows that the OGD culture has far fewer recognisable cells. There appeared also to be more remnants of broken cells such that the culture as a whole assumed quite a grainy appearance.

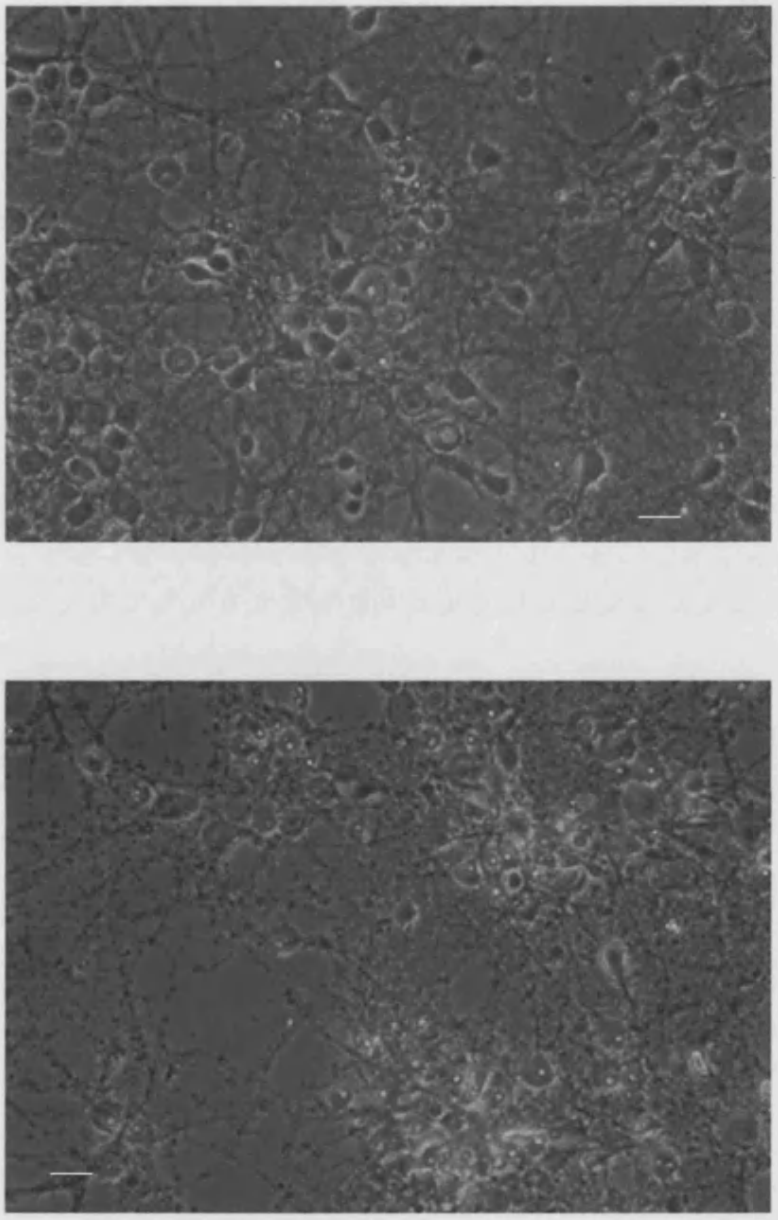


Fig 4.6 Neurone death during reoxygenation. Phase contrast images of neurones following 20hrs of re-oxygenation after a 15min OGD period. The upper panel shows control neurones, the lower shows OGD neurones. 20x objective lens, scale bar represents 30μm.

Neurite breakdown was a salient feature of ischaemic cultures from 12 hours of re-oxygenation (Fig 4.7). As reperfusion continued, control

cultures assumed this morphology yet, in general, the cell bodies remain more phase dark and the cultures show less neurite breakdown.

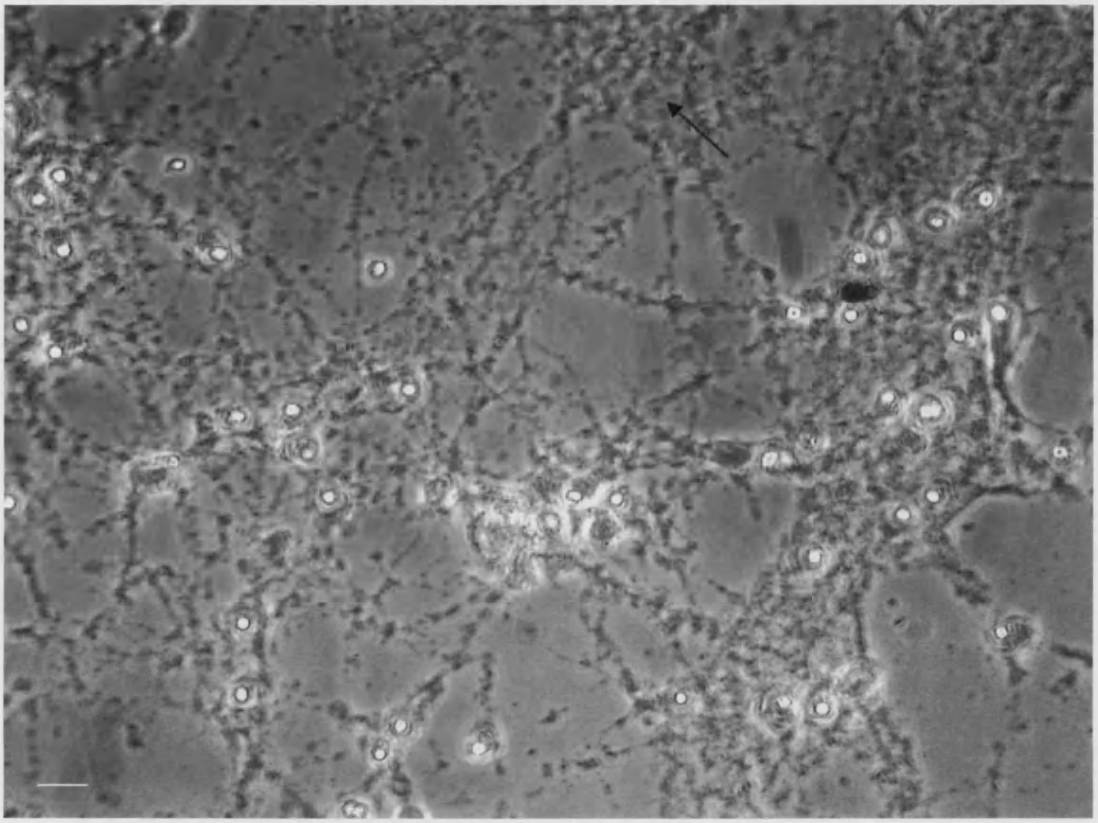


Fig 4.7 Morphology of neurites following OGD. Phase contrast image of neurones at 24hrs of re-oxygenation following a 15min OGD insult. Neurites are very grainy in appearance (arrow) and no longer appear to form connections with each other. 20x objective lens, scale bar represents 30μm.

4.3.4 Effect of OGD/reoxygenation on astrocyte culture

4.3.4.1 Astrocyte death increases with insult severity

Astrocytes were subjected to OGD for various lengths of time from 0hr to 20hr and viability was assessed by means of the LDH release assay (section 2.5.1) Fig 4.8 shows that significant LDH release was observed immediately after 8hours of OGD.

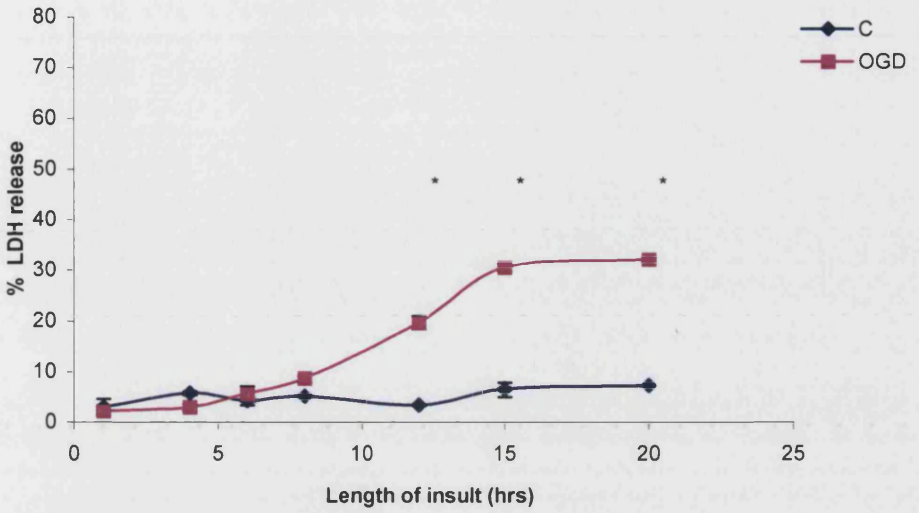


Fig 4.8 OGD in cortical astrocytes. LDH release following OGD of various lengths in cortical astrocytes, expressed as fraction of total. Blue line represents values from control cells, pink line represents values from cells subjected to OGD. Data, mean ± SEM, n=7, $p<0.05$ significance, '*' marks significant points between control and OGD cells.

Astrocytes were also subjected to OGD of 30, 60 and 120mins and allowed to re-oxygenate for up to 24hrs. There was no significant cell death during re-oxygenation following insults of these lengths. Fig 4.9 illustrates this point taking the case of a 60min OGD insult.

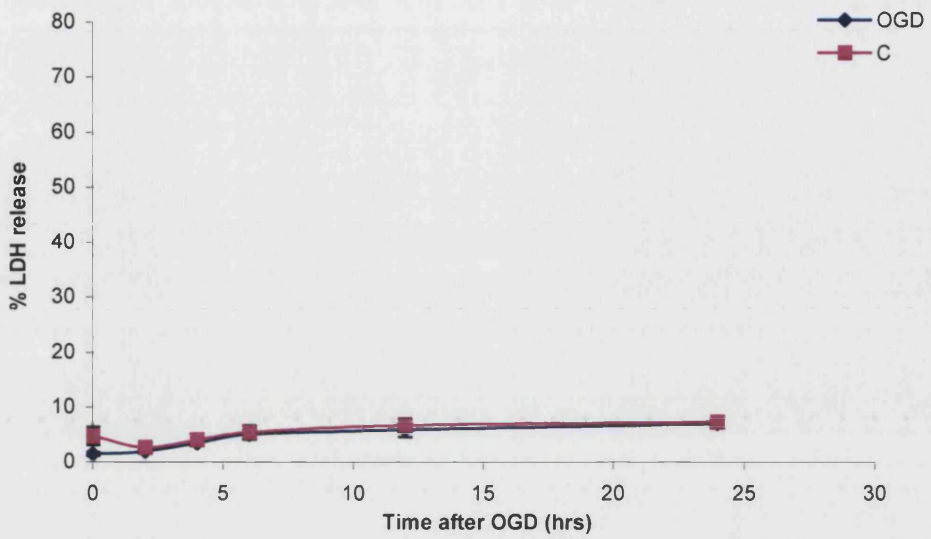


Fig 4.9 60min OGD with reoxygenation in cortical astrocytes. LDH release during a 24 hour recovery period following OGD of 60min duration in cortical astrocytes. Pink line represents control cells, blue line represents cells subjected to OGD. Data, mean ± SEM, n=5.

4.3.4.2 Morphology of astrocytes

Phase contrast microscopy of the astrocytes reflected the results of the LDH release assay in that no morphological changes were seen in the cultures until between 6 and 8hrs of OGD was achieved. At this point, single cells began to detach from the surface of the plate and the nucleus became more prominent, surrounded by a darkened cytoplasm. Fig 4.10 illustrates this morphology

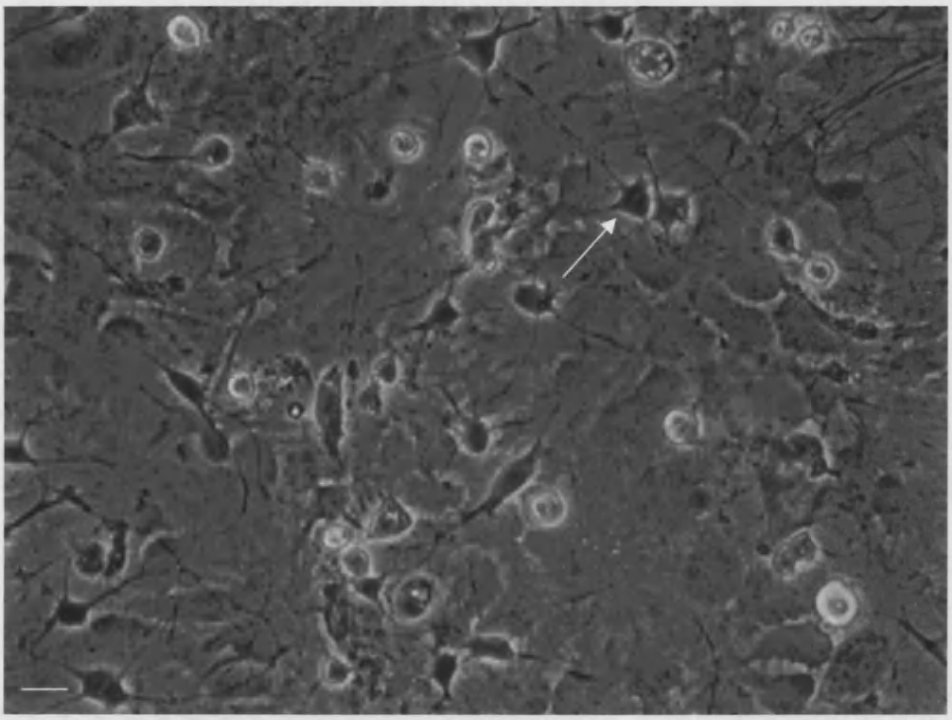


Fig 4.10 Astrocyte morphology following OGD. Phase contrast image of cortical astrocytes taken after 8 hours of OGD. The arrow points to an astrocyte whose morphology has become altered. 20X objective lens, scale bar represents 40µm.

4.4 Discussion

4.4.1 Choice of cell death assay

Cell death is a common endpoint for the determination of OGD induced damage giving information of both a qualitative and quantitative nature. In the latter case, quantifying dead cells is a clean method for statistical analysis. In the former, determining precisely which route a cell chooses to take towards its demise may provide an opportunity for therapeutic intervention. We were originally interested in looking at the type of neuronal death yet we only wanted to quantify astrocyte death as a means of establishing an injury end point.

For astrocyte cultures we chose to measure LDH release which has been shown to be an accurate indicator of culture viability (Koh et al 1987). This is generally not a viable option for neuronal cultures as they traditionally contain a small amount of glia and glia contain more LDH than neurones which could potentially mask neuronal results. Also the LDH assay cannot distinguish between apoptosis and necrosis. We therefore turned to a dye exclusion type assay (Juurlink et al 1993). Dye exclusion as we use it, was not an option for astrocytes, as they would have had to be grown on glass coverslips. We have found that, following relatively short periods of OGD, astrocytes lose adhesion to the glass surface and thus cannot be counted or observed in a similar manner to neurones. Loss of adhesion does not necessarily mean the cells are dead and so counts can be misleading.

Both assays rely on membrane integrity the compromise of which is an early indicator of cell death. The assessment of membrane integrity on its own is fairly non-specific in differentiating forms of cell death, particularly as the plasma membrane remains intact well into the apoptotic process (Smolewski et al 2002). For this reason, we incorporated an analysis of both nuclear and whole cell morphology by microscopy for neurone cultures.

4.4.2 OGD and neurones

4.4.2.1 Cell death increases with insult severity

We have shown that there is significantly more cell death in neuronal cultures immediately at the end of a 60 or 120min OGD insult when compared with cultures at the end of a 15min insult. Furthermore, cell death reaches a maximum more rapidly during recovery following insults of a greater length.

It is not in the least bit surprising that depriving neurones of essential metabolic substrates for 120 and even 60mins is more damaging than 15mins. It is well documented that the extent to which neurones die during re-oxygenation is dependent upon the length of the initial insult (Goldberg et al 1993, Wang et al 2002, Meloni et al 2001), which is also a corollary of our observations at the outset of recovery. It is however slightly surprising that there is no difference between the 60 and 120mins insults. Why should this happen?

In dealing with the OGD period itself, one could assume that there is a point of OGD duration at which maximum damage is done. We propose the idea that the insult may have a 'maximum capacity' with respect to insult severity. Because some injury takes time to develop and be observed, there would be a plateau point in cell death between the time when all immediate effects are seen and when late effects are beginning. Taking our data in to account, the beginning of this plateau is somewhere between 15 and 60mins and its end is somewhere after 120mins of OGD so that what we see at 15mins is the beginning of a steady decline. This could therefore account for the lack of distinction in initial death levels between the 60 and 120min periods.

Although there is no difference in the initial level of death between the 60 and 120min insults, there is a clear difference in the rate at which death proceeds during the recovery phase. Both insults arrive at a similar level of death by 24hours of recovery and share an exponential decay profile with respect to time. However, the 60min insult takes longer to reach its

maximum level of death. This does directly support and reflect the previously stated observations that OGD severity strongly influences the extent of cell death during recovery.

At first glance it would appear that the trend in our data with respect to insult severity and cell death marries well with the current literature however, there does seem to be some disparity in the length of time taken to induce cell death from model to model using a system similar to our own. By this we mean systems, which use simple gas exchange to induce hypoxia without metabolic inhibitors or receptor antagonists. As is shown in the results section, we have seen an average of around 78% cell death within the culture by 24hours following an insult of only 15min. Goldberg et al (1993), register little cell death with insults of less than 30mins. They report almost 90% cell death 24hrs after a 60min insult. These figures are supported by the results of Gwag et al's control groups (1995). Strangely, the progressive cell death model of Meloni et al (2001) requires an insult of at least 4hours to produce levels of cell death that rise above controls. All cultures are of a similar age to ours. The Gwag and the Goldberg models use mouse cortex whereas the Meloni model uses rat, so species difference doesn't appear to be a problem. So why does this difference in viability exist?

First of all, our system of evacuating O_2 from the chamber whilst cells are present may actually result in the cell damage beginning before the 60min gassing period has ended, thereby extending the period of ischaemia and underestimating the insult length (See section 3.6.4 and 3.6.5 for discussion). With an extended OGD length in mind, our insult may possibly more closely approximate Goldberg's or Gwag's model. In these systems a matter of 15mins difference in the OGD period seems to have a significant impact on neuronal viability.

Another explanation, and perhaps the most likely, could be that our neuronal culture is more prone to injury due to its high purity. Both the Goldberg and Gwag models use neurone cultures, which are either a

mixture of glia and neurones or neurones seeded on a glial monolayer whereas our culture consists of pure neurones. Astrocytes have a well-documented ability to aid neuronal survival and function (discussed in chapter 5). Astrocytes take significantly longer than neurones to die from OGD. At the short periods used to study neuronal survival the neuroprotective facilities of the astrocytes may be completely intact. Therefore, a neuronal culture rich in astrocytes could suffer less cell death. The Meloni model is quite pure yet shows an extraordinarily long amount of time to induce cell death despite the use of Ar instead of N_2. Only by adding 2-deoxy-glucose do they come within the time period standard of Goldberg et al. It would seem that this group may not have achieved sufficiently low levels of O_2, values were not quoted in their publications.

A third explanation could be down to the ability of plastic tissue culture vessels to store O_2. Munns et al (2002) elegantly demonstrated a dramatic difference in neurone viability depending on whether they were made anoxic in a plastic or a glass culture vessel. Essentially, as mentioned in chapter 1, plastic acts as a sink for O_2. Both the Gwag and Goldberg models used plastic vessels entirely, which would host a large concentration of O_2 to supply the neurones once initial O_2 evacuation had been completed. Our model consists of a glass coverslip resting on a plastic plate. We propose that the bulk of the plastic-stored O_2 diffuses out into the glass chamber and through the outlet pipe but is prevented from being used by the neurones due to the barrier to the plastic provided by the glass coverslip. The Munns model achieves 92% cell death following 25min OGD in a glass dish (with an extra 15mins before anoxia is achieved making it 40mins in total), which most closely resembles our model.

4.4.2.2 Time of cell death measurement

A point, which has repeatedly surfaced, is that almost all the OGD studies we have come across involve insulting neurones for a defined period of time, then letting them recover for 24 to 48hrs before cell death is

measured. Most groups cite the damage at 24hours as a measure of the OGD length and do not discuss the implications of re-oxygenation in the intervening period (reperfusion injury). Our approach has been to look at cell death in real time at points following the OGD insult up until 24hours. Clearly the former approach allows time for injury development, which may or may not be due exclusively to OGD length.

We have seen no distinction in cell death level between 60 and 120mins of injury yet the 15mins insult results in significantly more live cells at the end of OGD. By 24hours of recovery however, even the 15min insult has reached a level of death comparable to the longer OGD insults. Considering the recovery data alone, one would assume that the insults were identical when in fact the profile of death rate was quite different. The distinction between reperfusion injury and injury directly relating to an insult is therefore an important one. It is particularly important to understand this distinction in order to take advantage of a possible therapeutic window. We believe that the re-oxygenation period does contribute to cell injury and will continue to investigate the injury under this assumption.

4.4.2.3 Cell morphology in the current model

We wanted to design the current system such that it parallels, as much as possible, cell death following a global ischaemic insult and penumbral damage following a focal insult. Essentially, little neuronal death is observed immediately following hypoxia yet death occurs progressively as reperfusion proceeds. We have ascertained that OGD of 15mins produces little cell death immediately but neurones show considerable decline throughout the re-oxygenation period.

Immediately following the 15min OGD period, insulted cells were found to be slightly more swollen than control cells yet membranes were still intact and impermeable to PI, likewise the neuritic network seemed undamaged. Although phase contrast images suggested continuing cell damage from the outset of re-oxygenation, cell death (by counting) did

not reach significant levels until 6 hours of re-oxygenation, reflecting the time necessary for membrane lysis. Culture morphology mirrors previous observations (Goldberg et al 1993, Meloni et al 2001). The cellular appearance at this stage was due entirely to the OGD insult as reperfusion medium had not been introduced yet.

In our model, the early stages of re-oxygenation particularly showed that insulted cells tended to exhibit marked cytoplasmic swelling yet the nuclei either appeared normal or only slightly shrunken. Chromatin became less diffuse, within a normal nuclear shape, and then began to form irregular clumps, reminiscent of apoptotic nuclei. It was not clear if separate membrane bound chromatin bundles had formed as the method allows only nuclear DNA to be visualised and not the nuclear envelope itself. Many small spherical blobs of chromatin dominated in the late stages of reoxygenation. At this point the somata were quite phase bright and appeared 'empty'. Only when taking in to consideration both apparent nuclear and somal shape could we see that we were witnessing a cell death close to what has been observed during *in vivo* ischaemia, and described as edematous cell change, a type of necrosis (Lipton 1999). Cell swelling can be accounted for by a loss of ion homeostasis due to insufficient energy available for ion pumping at membranes. Enzymatic processes within the cell then digest its organelles giving the later appearance (Allen et al 1997).

We observed that ischaemic nuclei became considerably more shrunken than control nuclei. Most probably, due to a disturbance in ion balance between cytosol and nucleus. This caused a slight problem in determining a cut off point for cells that were early apoptotic or live so we erred on the side of labelling dubious cells as live. Nuclear shrinkage has been used in its own right as an early marker for cell death in hippocampal slices following OGD (Bonde et al 2002, Kasischke et al 2001). We may therefore have underestimated the quantity of cells that were at an early apoptotic stage. In any event this raises an interesting point, what is the cut-off point for condemning a cell? It is not at all clear

when the irreversible processes of cell death begin when just looking down a microscope.

Even if the 'correct' cut-off were assigned, another factor, which will blur the borders between cell groups, is the input of the individual doing the counting. It is one matter to fix on a cut-off criteria but it is quite another to rely on a person to rigidly stick to it on every experimental run, particularly if the cut-off criteria become difficult to spot. Demonstrating the acuity of 20:20 hindsight, It would perhaps be more useful to standardise nuclear counting by using a computer aided imaging system which could quantify staining intensity and nuclear size and shape more readily (Veltri et al 2000 for review).

Clearly, caution should be applied when using nuclear stains to determine death type. In this instance, siphoning nuclei into different counting groups based on appearance and colour could not effectively distinguish between apoptosis and necrosis. This could be due to the limited criteria chosen by us. However, it may also underline the fact that the traditional features of either necrosis or apoptosis can be found within the same population and furthermore negates the use of either term in defining, absolutely, a mode of death (as detailed in section 1.1.4).

4.4.3 Astrocyte survival

4.4.3.1 OGD and cell death

Changes in LDH release reached significant levels only after 8hrs of OGD in cortical astrocytes reaching a peak at 15hours of OGD. There was similarly, no visible injury until many hours of OGD had been imposed. In comparing these to previous observations, it is worth mentioning that we use the LDH release assay in a slightly different manner to many groups in this field. As mentioned above, we assessed LDH release, and thus astrocyte injury, immediately upon cessation of the insult, whereas almost all groups to which our insult compares, assessed LDH release 24 hours later. To re-iterate, our future work is concerned with the reperfusion phase following OGD and so it makes more sense for us to assess

damage and its causes immediately following OGD without the added factors brought by re-oxygenation. In essence, we are purifying the insult.

A number of groups have observed that most of the LDH release occurs between 4 and 8 hours of OGD (Juurlink et al 1993, Zhao et al 2000, Goldberg et al 1993). In our hands, the bulk LDH was released between 8 and 15hours of OGD. Not only is this later than previously observed but it also happened in a much slower manner. Furthermore, it appears that the total LDH released in our system was considerably lower than others. Juurlink et al (1993), show that 80% of total culture LDH is released by 9 hours whereas, even at 20hours we only see a 25% release (subtracting control values from OGD values). Other groups quote their figures as the percentage of total LDH released 24hours following the maximum OGD insult and not as a fraction of the total culture LDH. It is therefore difficult to directly compare our absolute figures.

We have established that O_2 is indeed being adequately evacuated from the glass chamber. However, there may still be O_2 remaining in the plastic culture dishes. This point was addressed in section 4.4.2.1 with respect to neuron cell death. As astrocytes do not have the experience of sitting on a glass coverslip to act as a barrier, they may well receive a supply of O_2 that supports respiration for some time in to the OGD insult. This would explain the later and more gradual release of LDH that we observe. It does not however, account for the comparatively small amount released.

A major point of difference between our culture system and that of the groups mentioned, is that our experiments were carried out when the cultures were 14days old. This was simply because it was the established day for culture use within our laboratory. The other groups used cortical cultures, which were between 3 and 6 weeks old. Papadopoulos et al (1998) show a sharp rise in LDH release (and thus astrocyte death) from

cultures aged between 15 and 22 days, following OGD of 8hours. Furthermore Rajapakse et al (2003) use cultures of 10-12 days old and show 60% viability following 12 hours of OGD. This more closely resembles our model. A number of hypotheses have been proposed as to why older cultures may be more susceptible to injury (Papadopoulus et al 1998, Pauwels et al 1998).

A far more simple explanation may be due to the assay itself. It may take a few hours for complete cell lysis to occur in response to an insult. This is the chief reason to allow a 24hour grace period before assessing damage. Again, for reasons stated in section 4.4.2.2, we did not want to do this. It is therefore more logical to accept our maximum release figures in a comparative rather than an absolute sense.

4.4.3.2 Recovery and cell death

We have assessed astrocyte cell death during a 24hour re-oxygenation period following OGD of up to 2hours. The results clearly show that there is no significant LDH release. Furthermore, there is no obvious morphological change. These observations are consistent with the current literature, when taking in to account that most groups measure acute death 24hours later (as mentioned above). Of course, lack of cell death does not necessarily mean that the astrocytes have not been affected by the OGD insult. As mentioned previously, astrocytes lose adhesion to glass coverslips long before cell death is registered on plastic dishes, showing that some physiological change is occurring in response to the insult. On a similar theme, Reichert et al (2001) demonstrate that astrocyte mitochondria become depolarized after relatively brief OGD without precipitating cell death.

4.4.4 Astrocytes are more robust than Neurones

Our results show that neurones are considerably more vulnerable to OGD than astrocytes in terms of duration necessary to cause significant cell death. Different aspects of the OGD insult have been studied to determine the underlying factors, which contribute to the observed

differential vulnerability between astrocytes and neurons. An important difference lies in the greater metabolic capacity of the astrocytes. Astrocytes express a number of enzymes, which are not expressed in neurons such as glycogen phosphorylase, glutamine synthetase and pyruvate carboxylase to name but a few (Kirchhoff et al 2001). Furthermore they have a much larger glycolytic capacity to generate ATP readily from glycogen stores during ischaemia (Ransom et al 1997). Glycogen stores are also used by astrocytes to protect against oxidative damage rather than simply generate ATP: Almeida et al (2002) elegantly showed that astrocytes have an enhanced ability to regenerate GSH by shuttling glucose through the pentose phosphate pathway.

It is not surprising that there is a disparity between astrocyte and neuronal survival. To recapitulate, the brain appears to have evolved such that different cell types have become highly specialized for different functions, most obviously, neurones for fast synaptic signaling. In an energy conserving measure, astrocytes have been assigned the burden of providing neurones with metabolic support (Vega et al 2003, Pellerin and Magistretti 2003, Pellerin et al 1994). Indeed, this is not the extent of the astrocyte's contribution to the maintenance of neuronal homeostasis, as has been described in detail in chapter 1.

Astrocytes may not die in the timely manner that neurones do, but there is a documented change in physiology following OGD that could lead to a reduction in neuronal support (Sugawara et al 2002, Reichert et al 2001). Clearly, when neurones are deprived of astrocyte support, under the extreme conditions of an ischaemic insult, they cannot cope. If neurones could be encouraged to adapt the features of astrocyte biology, that make them resistant to cell death, following an ischaemic episode, severe neuronal loss may be avoided. Similarly, astrocytes may be encouraged to boost their neuroprotective capacity. We will expand on this theme in the next chapter.

4.5 Conclusion

In answer to the most fundamental part of our aim, we have succeeded in establishing a model of reperfusion injury. In keeping with trends in the current literature, we have shown that the extent, to which neurone cultures die during reoxygenation, is dependent upon the length of the initial insult. We have further shown that astrocytes survive OGD of considerably greater lengths than neurones. By illustrating these points we have validated our model as being fit for use in the study of OGD-reoxygenation, thereby meeting the aims of this chapter.

Chapter 5

'The effect of astrocytes on neuronal survival following oxygen glucose deprivation '

5.1 Introduction

In chapter 4 we produced a model of neuronal injury in response to OGD-reoxygenation. This was done, not as an end in itself, but with the intention that the model could be used as a basis for the study of neuroprotection. In this chapter we will take a first step in this direction by looking at the effect astrocytes may have on neuronal survival.

The lives of neurones and astrocytes are intimately linked on many different levels *in vivo* and the role of astrocytes in sustaining neuronal function has already been described in chapter 1. There is much evidence to suggest that astrocytes are passively neuroprotective *in vitro* and much work has been done in characterising the basis of this neuroprotection. For example, Drukarch et al (1998) implicate astrocytes in extra-cellular ROS scavenging thus limiting oxidant attack on neurones. Dhandapani et al (2003) show that astrocyte derived growth factors prevent neuronal death due to serum deprivation. Wang et al (2001) show that astrocyte-released pyruvate attenuates cysteine-related neuronal death in culture. To reiterate, in each case the neuroprotection is a natural function of the astrocytes involved and is not a function of induction.

Because of the natural role of astrocytes in promoting neuronal survival a number of groups have targeted astrocytes in order to elicit a secondary effect of protecting neurones. For example, the stimulation of the pentose-phosphate pathway in astrocytes by the addition of fructose-1,6-bisphosphate has been shown to enhance neurone survival during hypoxia (Kelleher et al 1996); the transfection of astrocytes with the glutamic acid decarboxylase gene has been shown to increase their antioxidant capacity and ability to attenuate neuronal oxidative stress (Lamigeon et al 2001); and the overexpression of specific astrocyte genes is reportedly neuroprotective against OGD (Xu et al 1999).

Clearly, evidence abounds as to the neuroprotective abilities of astrocytes under a range of different conditions. We will therefore assess the ability of cortical astrocytes to attenuate neuronal injury as a result of OGD-reoxygenation using cell death as a measurable endpoint of the injury.

5.2 Aim

The aim of this chapter is to test the hypothesis that astrocytes are capable of exerting a protective effect on neurones, which have been subjected to OGD.

5.3 Methods

5.3.1 Cell culture

Astrocyte cultures were prepared as described in section 2.2.1 and neurone cultures were prepared as described in section 2.2.2. Co-culture was carried out as described in section 2.2.3. Astrocyte conditioned medium (con med) was prepared as described in section 2.2.2.3. All experiments were carried out in 6-well plates.

5.3.2 O_2 glucose deprivation/reoxygenation

Neurones were subjected to OGD/reoxygenation as described in section 2.4.2.

5.3.3 Cell death

Cell death was quantified in neuronal cultures according to the method detailed in section 2.5.2.

5.3.4 Griess assay

Nitrite was measured in the culture medium using the Griess assay as detailed in section 2.8.3

5.3.5 Microscopy

The health of neurone and astrocyte cultures was monitored by visual inspection using phase contrast microscopy as detailed in section 2.6.1.

5.4 Results

5.4.1 Astrocytes attenuate neuronal death during reoxygenation.

We have subjected cortical neurone cultures to OGD for 15mins and allowed them to recover for up to 24hours in the presence or absence of astrocytes (Fig 5.1). The data for neurones recovering in the absence of astrocytes were first shown in chapter 4. There was no statistically significant difference in the number of live cells between the control cultures from each group at all time points. However there was a significant difference between OGD-exposed cultures and their respective sister control cultures at 6, 12 and 24hours of recovery (marked as '**' on the graph). There were significantly more live cells at 6 and 24hrs of recovery in the OGD-exposed culture recovered in the presence of astrocytes (57.2±2.5% for 6hrs and 47.5±5.1% for 24hrs) than in the OGD-exposed culture recovered in neurobasal (NB) medium only (52.3±2.63 for 6hrs and 27.2±4.38% for 24hrs).

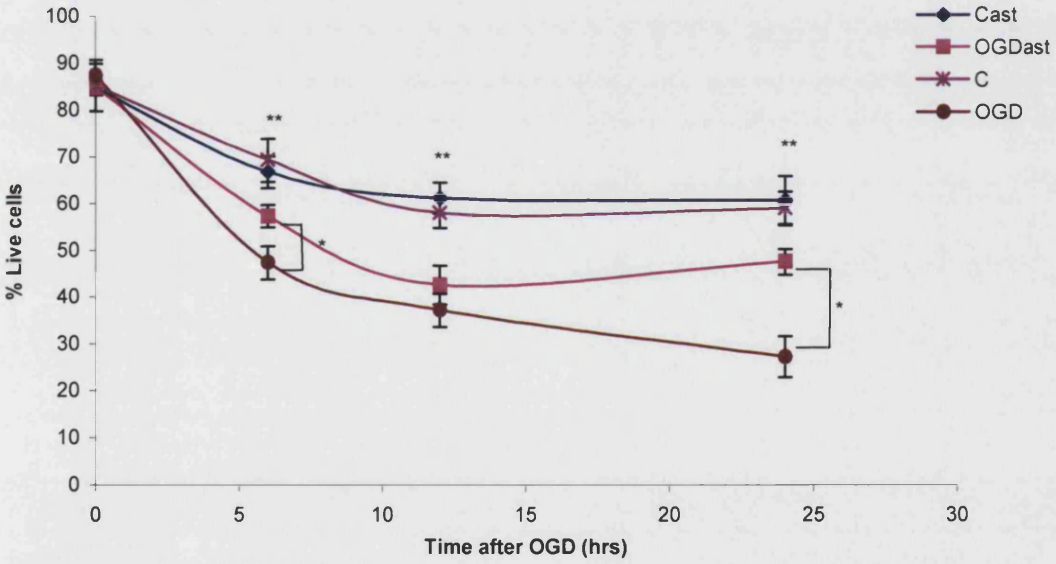


Fig 5.1 Recovery of cortical neurones in the presence of astrocytes. The graph shows the number of live cells present in neuronal cultures during recovery from 15mins OGD in the presence and absence of astrocytes. The lines (C) and (OGD) represent control and OGD-exposed neurones respectively, recovering in NB medium only. The lines (Cast) and (OGDast) represent control and OGD-exposed neurones respectively, recovering in NB medium in the presence of astrocytes. Data, mean ± SEM, n=7, '*' marks points of significant difference, '**' marks significant difference between sister cultures $p<0.05$.

5.4.2 Astrocyte-conditioned medium can reduce neuronal death to the same extent as the presence of astrocytes.

In order to determine whether or not astrocyte presence was a crucial factor, neurones subjected to OGD for 15mins were allowed to recover in the presence of astrocyte-conditioned medium (con med) for up to 24 hours (Fig 5.2). Again, the data for neurones recovering in the absence of astrocytes were first shown in chapter 4. There were significantly more live cells in the control cultures than in the OGD-exposed cultures when recovered in the presence of con med and when recovered in NB medium only, at 6,12 and 24hours of recovery (marked as '**' on the graph). Taking only the OGD-exposed cultures into consideration, there were significantly more live cells in cultures recovered in con med than in cultures recovered in NB medium only at 12hrs (51.7±2.24% and 37.2±3.5% respectively) and 24hrs (41.9±4.67 and 27.2±4.38% respectively) of recovery.

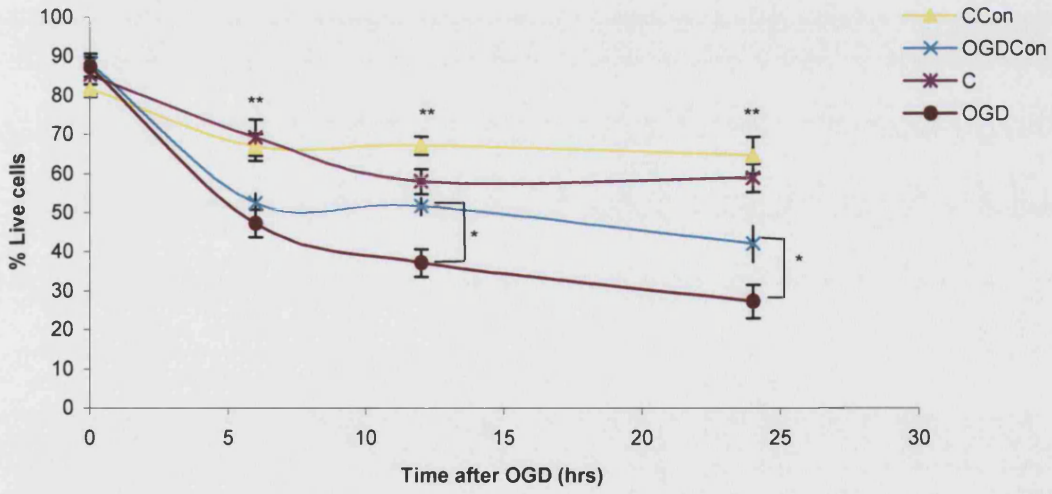


Fig 5.2 Recovery of cortical neurones in the presence of astrocyte-conditioned medium. The graph shows the number of live cells present in neuronal cultures during recovery from 15mins OGD in the presence and absence of astrocyte-conditioned medium. The lines (C) and (OGD) represent control and OGD-exposed neurones respectively, recovering in NB medium only. The lines (CCon) and (OGDCon) represent control and OGD-exposed neurones respectively, recovering in conditioned medium. Data, mean ± SEM, n=7, '*' marks points of significant difference, '**' marks significant difference between sister cultures $p<0.05$.

Fig 5.3 summarises the data obtained for OGD-exposed neurones only. Here we can see that exposure to con med actually resulted in less cell death at 12hrs than exposure to astrocytes. There is no statistically significant difference between conditioned medium-exposed and astrocyte-exposed cultures at either 6 or 24hrs of recovery.

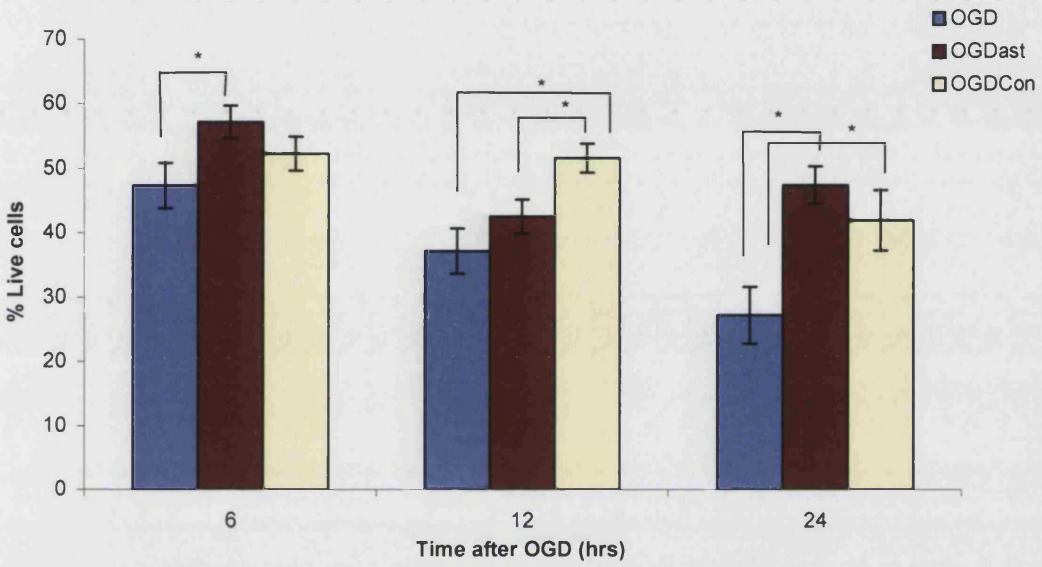


Fig 5.3 Recovery of OGD-exposed cortical neurone cultures. Cortical neurones were subjected to OGD for 15mins. The number of live cells was counted at intervals up to 24hrs of recovery. Neurones were allowed to recover in the presence of NB medium only (OGD), astrocytes (OGDast) or astrocyte-conditioned medium (OGDcon). Data, mean ± SEM, n=7, '*' marks points of statistically significant difference, $p<0.05$.

5.4.3 Astrocytes are not activated by the presence of injured neurones

We wanted to determine if astrocytes were being activated during the course of the experiments. A hallmark of the activation of astrocytes is the induction of the inducible nitric oxide synthase enzyme (iNOS). This leads to an elevation of NO. We have measured the concentration of the NO breakdown product nitrite, in the co-culture medium and in astrocyte medium alone as an index of NO production. As detailed in the methods section we have assayed for the presence of nitrite following the

conversion of nitrate to nitrite. We will therefore refer to the total nitrite detected as NOx. Fig 5.4 clearly shows that the OGD-exposed neurones do not precipitate an increase in the level of NOx, when compared with controls at any time point. Furthermore, there is a low basal level of NOx produced by astrocytes which rises to a concentration of ~10μM by 24hrs and is unaffected by the presence of neurones.

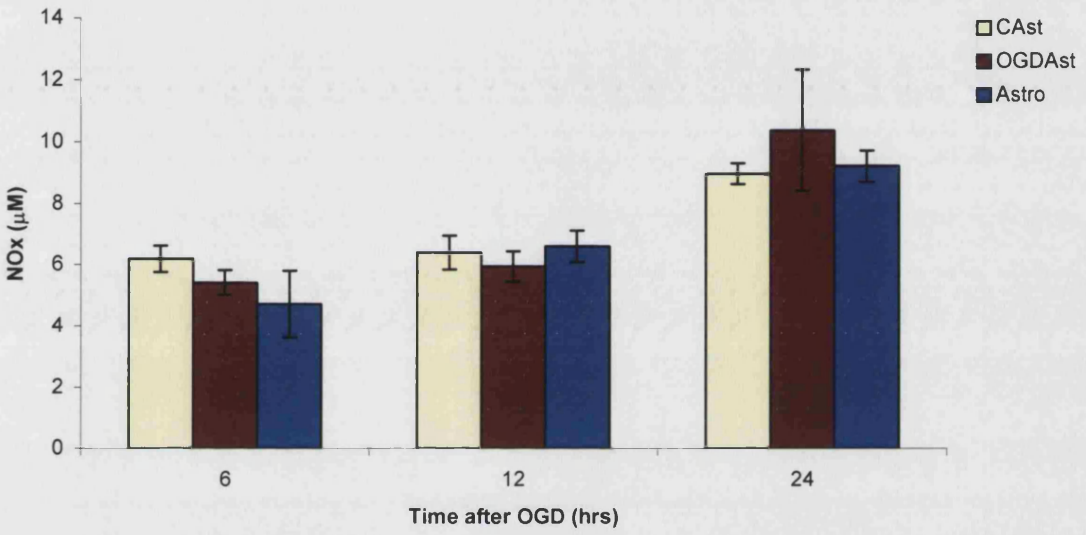


Fig 5.4 Nitrite production following OGD. Nitrite concentration in culture medium of astrocytes co-cultured with neurones and in culture medium of astrocytes only was determined. Neurones were exposed to OGD for 15mins and allowed to recover for up to 24hrs. The first two rectangles represent control neurones (CAst) and OGD-exposed (OGDAst) recovering in the presence of astrocytes. The third rectangle represents astrocytes incubated in NB medium only for 24hrs. Data, mean ± SEM, n=3.

Morphological investigation confirmed that the astrocyte cultures were healthy at all time points measured, assuming the flat cobblestone appearance described in chapter 4. Astrocytes that did not conform to this morphology at the outset of experimentation were discarded.

5.5 Discussion

We have allowed neurones, which have been insulted for 15mins, to undergo reoxygenation in the presence of cortical astrocytes and astrocyte-conditioned medium (con med). The results show that both these conditions are neuroprotective and suggest that protection is at least partly due to constitutive release of diffusible factors.

Let us consider the hypothesis that astrocytes constitutively release neuroprotective factors. There was significantly less death at T6 in the presence of astrocytes, but not at T12 when compared to neurones on their own. However, in the presence of con-med, there was no significance at T6 but there was at T12. At first glance it would appear difficult to reconcile these observations apart from considering the data to be an experimental fluke! The very fact that any neuroprotection at all was seen with con-med, is proof of constitutive release. The con-med was allowed to sit on the astrocytes for 24hours and so in theory it has a greater capacity to protect neurones immediately at the start of reoxygenation than the presence of astrocytes who are just beginning to pump out the neuroprotective factors. At T12 there is greatest survival in the con-med exposed neurones, which does reflect more neuroprotective activity in the earlier part of reoxygenation. In fact this was almost on a par with the control values for the neurones reoxygenating on their own. So why is there less death in the cultures reoxygenated with astrocytes at T6? Flukes aside the real issue may be that the neuroprotection we are seeing is arrived at by completely different mechanisms.

The use of conditioned medium removes the element of conversation yet it allows for the signalling components to be distilled and either identified or at least categorised. Many different groups have examined the effects of conditioned medium on neuronal survival with positive and negative results; Microglia conditioned medium induces apoptosis in hippocampal cultures in some circumstances (Flavin et al 1997): Astrocyte conditioned medium promotes cortical neurite outgrowth (Le et al 2002): Activated-

astrocyte conditioned medium increases hippocampal neuronal susceptibility to glutamate (Ahlemeyer et al 2003). Not forgetting of course the use of glia conditioned medium in the maintenance of primary neuronal cultures (Dugan et al 1997).

A new dimension is opened up when astrocytes are present. Yes, they may still be releasing the same factors that are present in the conditioned medium but they may also be directly asked by the neurones to release a host of other factors. More crucially, the astrocytes may be removing a neurotoxin from the environment rather than specifically releasing a neuroprotectant. We will explore the nature of the neuroprotection in the next chapter.

A particular point to note about the current work is that we are not subjecting the astrocytes to any insult (intentionally at least!). To our knowledge no group has reoxygenated OGD-exposed neurones in the presence of un-insulted astrocytes. Curiously, the incubation of insulted astrocytes with non-insulted neurones has been examined (Tamatini et al 1998). It is more usually the case that glial influence is monitored in the context of a mixed glia-neurone culture (Goldberg et al 1993), coculture (Kelleher et al 1996) or slice preparation (Schurr et al 1997) such that both cell types are present during the relatively short neuronal insult. Why do we mention this? We believe that it is worth considering the role of unchallenged astrocytes in the study of neurone-astrocyte communication and further, neuroprotection.

It is well accepted that glia are considerably more resistant than neurones however even short insults have been shown to cause some physiological change. For example, Reichert et al (2001) evidence mitochondrial depolarisation during OGD, which has no effect on ultimate astrocyte survival. Such a change in astrocyte homeostasis may however effect communication with neurones. Indeed Lian et al (2004) have shown that, *in vivo*, a drop in astrocyte ATP levels increases neurone susceptibility to injury. The change in communication is not

always negative, Ruscher et al (2002) elegantly demonstrated *in vitro*, using conditioned medium, that a brief OGD insult to astrocytes enhanced their neuroprotective abilities. A similar conclusion can be drawn *in vivo* from the work of Kitagawa et al (1990) on ischaemic tolerance. What we don't know however, is how perfectly healthy astrocytes react to and communicate with injured neurones.

The study of how OGD-exposed neurones and uninsulted astrocytes interact is of particular relevance in preventing the spread of infarction following a focal ischaemic insult. If we are to assume that cells of the peri-infarct area are 'normal' it is more accurate to study their responses to injured neurones when they themselves are in a 'normal' physiological state. This could then lead to the development of therapeutic strategies by which the natural neuronal survival-promoting pathways of astrocytes could be bolstered immediately following the ischaemic episode effectively establishing a shield against further infarction.

5.6 Conclusion

We have determined that astrocytes are capable of attenuating neuronal death following OGD in this model. We have further taken steps to characterise this neuroprotection and have shown that astrocyte-conditioned medium also significantly reduces neuronal death.

Chapter 6

'The elements of astrocyte-neurone communication'

6.1 Introduction

In chapter 5 we asked the question are astrocytes capable of rescuing neurones from OGD-reoxygenation induced cell death? We found that indeed they are. The next obvious question is how is this possible? We intend to follow three different yet related lines of investigation; 1) astrocytes are removing a neurotoxin from the environment; 2) astrocytes are providing metabolic support; 3) astrocytes are working in an antioxidant capacity. It is our intention to identify the existence of elements, which may support one or all of these hypotheses in the context of the current model so that we may arrive at a mechanism of neuronal injury.

Glutamate stands as the best candidate for a possible neurotoxin in this model. The function and consequences of glutamate as an excitotoxin have been elaborated upon section 1.2. In brief, excessive glutamate release causes over activation of NMDA receptors and a cascade of intracellular signalling that derails physiological cell function. Glutamate release has been documented as a result of ischaemia/reperfusion both in vivo (Erecinska et al 1984, Molchanova et al 2004,Taguchi et al 1996, Yang et al 2001, Mori et al 2004) and in vitro (Hauptman et al 1984, Goldberg et al 1990, Marcoli et al 2004). Elevated glutamate levels have been directly linked with ischaemic cell death (Arundine et al 2004) and inhibitors of glutamate receptors have been shown to attenuate damage following ischaemia (McIntosh et al 1989, Pohorecki et al 1990, Pellegrini-Giampietro et al 1999, Meli et al 2002).

The provision of metabolic support by astrocytes most probably comes mainly in the form of lactate. Lactate was shown to be a metabolic fuel for neurones some time ago (McIlwain 1953) and there is a significant amount of evidence suggesting its use as the preferential neuronal fuel under a variety of conditions *in vitro* (See Pellerin et al 2004 for list). It has long had a negative association with

brain ischaemia due to circumstantial evidence of its concomitant increase with falling pH and further the relationship between high preischaemic glucose values and subsequent acidosis (Siesjo et al 1996). However lactate has been shown to have beneficial effects *in vitro.*

Schurr et al (1997c) show that lactate and not glucose aids neuronal recovery following hypoxia in hippocampal slices; Cater et al (2001) show that lactate is protective during glucose deprivation and glucose-metabolism inhibition in hippocampal slice cultures; Cater et al (2003) elaborate on their previous work to show that lactate is more beneficial than glucose during reoxygenation following hypoxia in the hippocampal slice system. Cronberg et al (2004) take the matter a step further and show that high glucose can actually exacerbate injury but lactate does not.

When we introduce neuroprotection by astrocytes in to the equation we revisit the 'lactate shuttle' hypothesis (Fig 1.4). In a nutshell, this suggests that neurones release glutamate which stimulates the upregulation of glycolysis in astrocytes which then release lactate for neurones to use as metabolic fuel under conditions of increased energy demand (Magistretti et al 1999, Pellerin et al 2004). In part agreement of this Schurr et al (1997a,b,c) have shown in hippocampal slices that not only can lactate be used to enhance posthypoxic neuronal recovery but that glia are its main source. Piecing all these facts together, a case emerges for the investigation of lactate in our model system.

Finally, glutathione (GSH) represents the putative antioxidant support. In section 1.4.4 we have already detailed how astrocytes can both support neuronal GSH levels and how they actually are neuroprotective in a GSH dependent manner. Artificially increasing glutathione levels has also been shown to be neuroprotective

following ischaemia (Anderson et al 2004 a and b). We intend to look at the role of GSH both inside and outside the cell.

6.2 Aims

The aim of this chapter is to test three hypotheses for the protection of neurones by astrocytes:

1. Astrocytes remove a toxin from the neuronal environment
2. Astrocytes provide metabolic support
3. Astrocytes provide antioxidant support

6.3 Methods

6.3.1 Cell culture

Astrocyte cultures were prepared as described in section 2.2.1 and neurone cultures were prepared as described in section 2.2.2. Co-culture was carried out as described in section 2.2.3. Astrocyte conditioned medium was prepared as described in section 2.2.2.3. All experiments were carried out in 6-well plates.

All astrocytes were seeded at a concentration of $1x10^6$ cells per well. Neurones were seeded at an estimated concentration of 100,000 cells per coverslip.

6.3.2 O_2 glucose deprivation/reoxygenation

Neurones were subjected to O_2 glucose deprivation (OGD)/reoxygenation as described in 2.4.2.

6.3.3 Glutamate, lactate and GSH

6.3.3.1 Sample handling

Three coverslips of neurones were used for each of the control and OGD conditions at each time point. Three wells of astrocytes were therefore used for each of the control and OGD conditions during co-culture experiments. The medium (2ml per coverslip) above each coverslip was pooled, giving 6ml per condition. Medium form

astrocytes alone was prepared as follows. Astrocytes were incubated in neurobasal medium (NB) for 24 hours at 37°C in an atmosphere of 5%CO_2/95%air. Each well contained 2ml of NB. Samples of conditioned medium alone were similarly prepared. 2ml of conditioned NB was placed in each well and allowed to incubate at 37°C in an atmosphere of 5%CO_2/95%.

For some experiments, medium was spiked with GSH. Medium was filtered through a 20µm filter after spiking to remove any micro organisms that may have affected the growth of the cell cultures.

1ml of medium was kept for lactate and glutamate determination. This was deproteinated according to the protocol in section 2.8.2. A further 1ml was deproteinised by mixing with 1ml of the mobile phase orthophosphoric acid (OPA) and centrifuged at 500g for 5min at 4°C. Supernatant was kept for GSH determination.

Cell suspensions were prepared from astrocytes as detailed in section 2.2.1.3. Pellets were prepared from neurones by a slight modification of this protocol. Each coverslip was directly transferred to 1ml of warm trypsin solution that had been placed in a 6-well plate. For neurones a single cell suspension was made from all three coverslips used in a condition. Likewise if more than one well was used for an astrocyte condition, a pooled suspension was made. Cells were pelleted from all suspensions by centrifugation at 500g for 5min at 4°C and subsequently frozen.

6.3.3.2 Protocol

Glutamate was measured spectrophotometrically as described in section 2.8.2. All samples that had been incubated in neurobasal (NB) medium were measured against a blank containing the assay mixture and 143µl of deproteinated NB medium. Similarly, samples that had been incubated in Dulbecco's modified Eagle's medium

(DMEM) were measured against a blank containing the assay mixture and 143µl of deproteinated DMEM.

Lactate was measured spectrophotometrically as described in section 2.8.3. All samples that had been incubated in neurobasal medium (NB) were measured against a blank containing the assay mixture and 200µl of deproteinated NB. Similarly, samples that had been incubated in Dulbecco's modified Eagle's medium (DMEM) were measured against a blank containing the assay mixture and 200µl of deproteinated DMEM.

GSH was measured by HPLC as described in section 2.8.4

6.4 Results

6.4.1 Glutamate

Glutamate concentration is expressed relative to volume only. We could not accurately determine the amount of protein in the NB medium due to interference with the assay. Neither could we express glutamate against protein in the cell pellets as it became difficult to compare results using co-culture experiments. We did however seed exactly the same number of cells per well and coverslip, and have determined cell loss to be minimal before time of experimentation. Therefore, it is acceptable to express glutamate as concentration in the medium. Concentration is expressed as total glutamate minus glutamate in the NB medium only.

6.4.1.1 Glutamate levels in neurone medium increase during recovery

We wanted to ascertain the level of glutamate in the medium of neurones subjected to 15mins OGD and recovery in order to determine if there is a correlation between OGD and glutamate concentration.

Neurones were subjected to OGD of 15mins and allowed to recover for 24hours in NB medium only. Glutamate that was measured immediately at the end of the OGD period (0 hours) represents glutamate accumulated during the 15min insult. Glutamate was also measured at 6, 12 and 24 hours of recovery. Fig 6.1 shows that there is significantly more glutamate in the culture medium of OGD exposed neurones at each time point during recovery when compared with control cultures ($p<0.05$). Furthermore glutamate in the OGD exposed culture medium rose from 16.8±3.0μM at 6hours to 26.7±0.8μM at 24hours, a statistically significant increase (mean ± SEM, n=4, $p<0.05$). All OGD cultures contain significantly more glutamate at 6, 12 and 24hours than at 0hrs. Exactly the same pattern is seen in the control cultures. Control glutamate levels remained similar between 6, 12 and 24 hours.

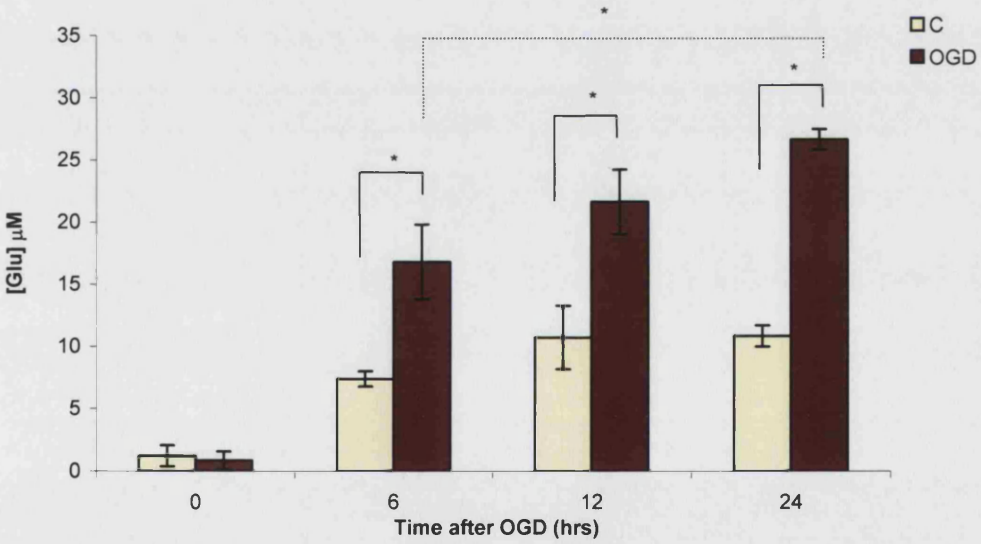


Fig 6.1 Glutamate Concentration following 15mins OGD. The graph shows glutamate concentration in the culture medium of cortical neurones following 15mins OGD minus glu in NB medium only. The 0hrs timepoint represents glutamate concentration in DMEM immediately at the end of the OGD period. The other time points correspond to glutamate concentration in NB medium up to 24 hours after the OGD insult. The cream rectangle represents control cultures and the brown rectangle represents the OGD-subjected cultures. Data, mean ± SEM, n=4. '*' represent points of statistical significance, $p<0.05$.

6.4.1.2 Glutamate concentration is lowered in the presence of astrocytes.

Neurones were subjected to OGD for 15mins and allowed to recover in the presence of astrocytes in order to investigate the possibility that co-culture with astrocytes could reduce extracellular glutamate concentration. Glutamate concentration was measured at 6, 12 and 24 hours of recovery and was also measured in NB medium of astrocytes alone. Fig 6.2 shows that there was a significant decrease in glutamate concentration in medium of co-cultured OGD-subjected neurones at all time points measured when compared with OGD-subjected neurones recovering in NB medium only ($p<0.05$). There was significantly more glutamate in the medium of control neurones recovered in NB medium only, than there was in the medium of control neurones recovered in co-culture, at 6 and 24 hours only ($p<0.05$).

There was no significant difference between control and OGD glutamate levels when neurones were recovered in co-culture at any time point nor was there a significant increase in glutamate with time. Fig 6.2 also shows that there was a significant increase in the glutamate concentration of medium incubated with astrocytes only from 6 to 24 hours. There was no significant difference in glutamate levels between the medium of astrocytes without neurones and the medium of either control or OGD-subjected neurones at any time point measured.

Data for neurones recovering in NB medium only was presented first in fig 6.1 and so statistical significance within this data is not shown again in fig 6.2.

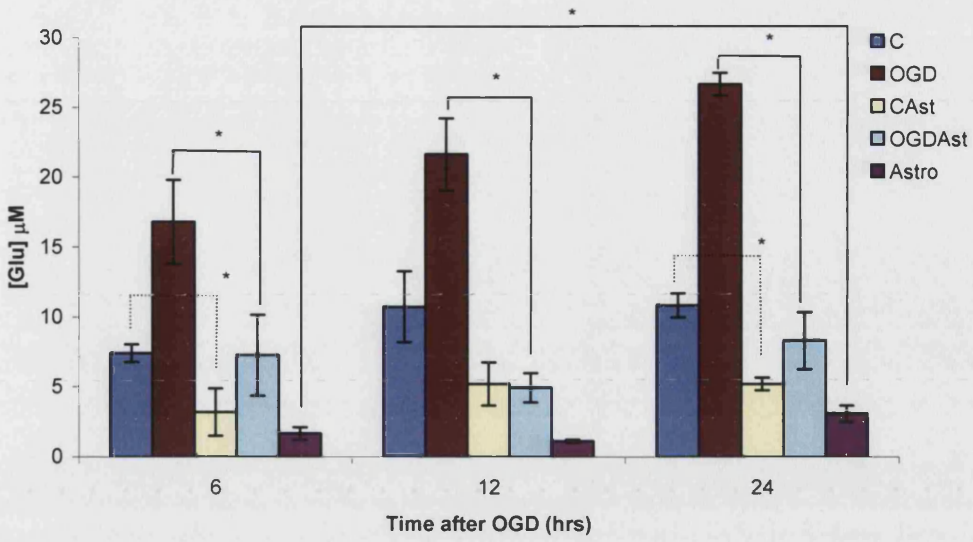


Fig 6.2 Glutamate in co-culture medium. Neurones were subjected to OGD of 15min and allowed to recover in the presence of astrocytes. The graph illustrates OGD-subjected neurones recovering in co-culture with astrocyte (OGDAst) and in NB medium alone (OGD). Control neuronal cultures were also incubated in co-culture (CAst) and in NB medium alone (C). Glutamate was measured in NB medium of astrocytes alone (Astro). Data ± SEM, n=4 for neurone and co-culture data, n=3 for astrocytes alone, '*' marks significant difference, $p<0.05$.

We have determined the level of glutamate in the medium of neurones recovering in conditioned medium (con med) to ascertain whether or not glutamate decline is a function of astrocyte presence. Fig 6.3 shows that there is a significant decrease in the level of glutamate in medium from OGD-subjected neurones recovering in con med when compared with OGD-subjected neurones recovering in NB medium only, at all time points measured. There was a significant difference in the control culture values at 24 hours only. There was no difference between control and OGD cultures recovering in con med at each time point. Again, the data for the neurones recovering in NB medium only has been presented in fig 6.1 and so statistical significance is omitted within these values for fig 6.3.

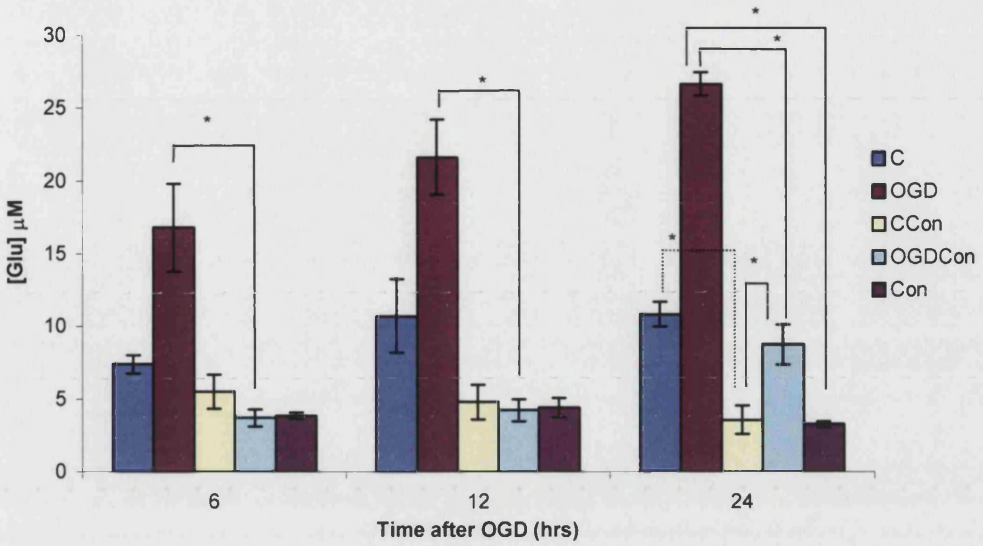


Fig 6.3 Glutamate in con med incubated with neurones. Neurones were subjected to OGD of 15min and allowed to recover in con med. The graph illustrates OGD-subjected neurones recovering in con med (OGDCon) and in NB medium only (OGD). Control cultures were also recovered in con med (CCon) and in NB medium only (C). Data ± SEM, n=4, '*' marks significant difference, $p<0.05$.

Fig 6.3 also shows that there was a basal level of glutamate present in con med that did not change significantly over time. There was no significant difference in glutamate concentration between con med without neurones and con med incubated with control and OGD-subjected neurones, at 6 and 12 hours of recovery. At 24 hours of recovery, there was significantly more glutamate in the OGD-subjected con med than in the con med incubated without neurones ($p<0.05$).

Table 6.1 summarises the combined data for all neuronal conditions and timepoints. This table shows all points of significant difference between OGD-subjected neurones at each time point and their sister control cultures.

	[Glutamate] μM in medium					
Time (hrs)	Control			OGD		
	Solo	Astro	Con	Solo	Astro	Con
6	7.39±0.63*	3.20±1.69	5.51±1.69	16.8±3.0*	7.27±2.90	3.71±0.60
12	10.73±2.55*	5.21±1.54	4.80±1.19	21.65±2.59*	4.93±1.04	4.23±0.76
24	10.84±0.85*	5.22±1.45	3.57±0.98*	26.68±0.81*	4.13±2.06	8.75±1.40*

Table 6.1 Glutamate Concentration in neurone medium following OGD. The figure shows glutamate concentration in the medium of cortical neurones subjected to 15mins OGD and allowed to recover for 24hours. Control and OGD-subjected neurones were recovered on their own (Solo), in the presence of astrocytes (Astro) or in con med (Con). Data, mean±SEM, n=4, '*' marks significant difference between C and OGD within a single condition $p<0.05$.

6.4.2 Lactate

Lactate concentration is expressed relative to volume only. We could not accurately determine the amount of protein in the NB medium due to interference with the assay. Neither could we express lactate against protein in the cell pellets as it became difficult to compare results using co-culture experiments. We did however seed exactly the same number of cells per well and coverslip, and have determined cell loss to be minimal before time of experimentation. Therefore, it is acceptable to express lactate as concentration in the medium. Concentration is expressed as total lactate minus lactate in the NB medium only.

6.4.2.1 Lactate concentration increases over time in astrocyte medium

Lactate concentration was measured in the medium of neurones subjected to OGD for 15mins and allowed to recover in NB medium alone, in astrocyte co-culture or in conditioned medium (con med). Lactate was also measured in NB medium incubated with astrocytes alone for a 24hour period and in con-med incubated alone for 24hours.

Fig 6.4 shows that lactate concentration increased in a linear manner over time in medium of astrocytes alone, from 1.67±0.13μM at 6hours to 5.62±0.25μM at 24hours (Mean±SEM, n=4). The linear accumulation was also evident in the presence of neurones. There was no significant difference between OGD-subjected and control co-cultures at 6 and 12 hours of recovery. There was also no significant difference between either control or OGD-subjected co-cultures and astrocytes cultured alone at 6 and 12 hours of recovery. However, there was significantly less lactate in the medium of OGD-subjected neurones than either control neurones or astrocytes alone at 24 hours.

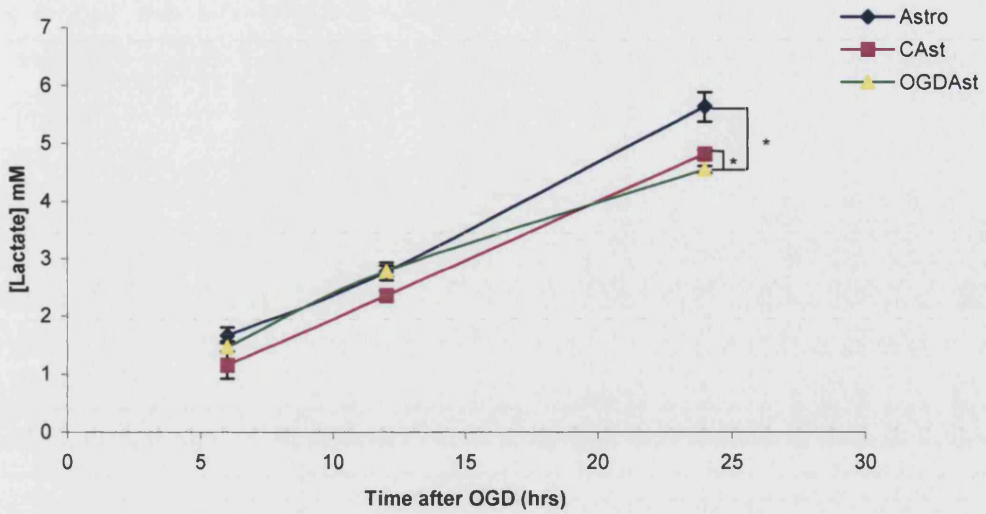


Fig 6.4 Lactate in co-culture medium. Neurones were subjected to 15mins OGD and allowed to recover in NB medium co-cultured with astrocytes. The graph shows lactate detected in culture medium of astrocytes in NB medium alone (Astro), control (CAst) and OGD-subjected co-cultures (OGDAst) at 6, 12 and 24 hours of recovery. Data ± SEM, n=4, '*' marks significant difference, $p<0.05$.

6.4.2.2 Lactate is stable in astrocyte-conditioned medium

Fig 6.5 shows that the concentration of lactate in con-med did not change significantly over time from a value of 5.60±0.30μM at 6hours (Mean ± SEM, n=3). There is no significant difference between this value and that measured in astrocyte medium alone at 24hours. There was also no significant difference in medium lactate concentration between control and OGD-subjected neurones at any time point. Furthermore, there was similarly no difference between either control or OGD-subjected neurones and con med alone ($p<0.05$).

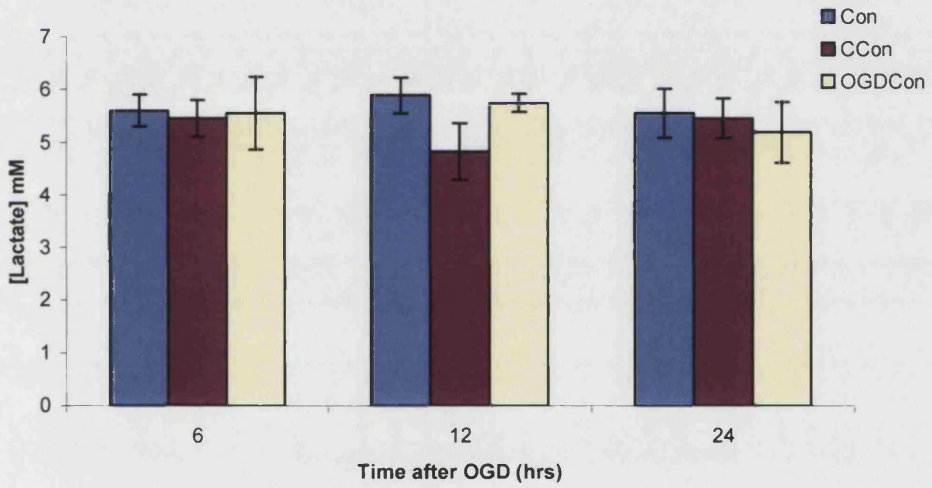


Fig 6.5 Lactate in conditioned medium. Neurones were subjected to 15mins OGD and allowed to recover in astrocyte-conditioned NB medium. The graph shows lactate detected in culture medium of con med alone (Con), control (CCon) and OGD-subjected cultures (OGDCon) in con med at 6, 12 and 24 hours of recovery. Data ± SEM, n=4, '*' marks significant difference, $p<0.05$.

Table 6.2 summarises the data obtained from the measurement of lactate and includes lactate measured in the medium of control and OGD-subjected neurones recovering in NB medium only. There was no significant difference between control and OGD cultures at any time point. There was significantly less lactate in the cultures of neurones in NB medium only than there was in cultures of neurones

in con med or in co-culture with astrocytes, at all time points ($p<0.05$).

	[Lactate] mM in medium					
Time (hrs)	Control			OGD		
	Solo	Astro	Con	Solo	Astro	Con
6	0.05±0.02	1.16±0.23*	5.46±0.35**	0.03±0.00	1.47±0.09*	5.56±0.69**
12	0.05±0.02	2.35±0.09*	4.83±0.53**	0.05±0.01	2.79±0.10*	5.74±0.17**
24	0.11±0.04	4.80±0.08*	5.46±0.38**	0.05±0.01	4.54±0.06*	5.19±1.57**

Table 6.2 Lactate Concentration in neurone medium following OGD. The figure shows [lactate] in the medium of cortical neurones subjected to 15mins OGD and allowed to recover for 24hours. There was 5.56±0.69mM lactate in conditioned medium at 0hrs. Control and OGD-subjected neurones were recovered in NB medium alone (Solo), in the presence of astrocytes (Astro) or in con med (Con). Data, mean±SEM, n=4, '*' marks significant difference between Solo and Astro groups, '**' marks significant difference between Solo and Con groups, $p<0.05$.

6.4.3 GSH

6.4.3.1 GSH in astrocyte medium

We first of all wanted to establish if astrocytes are capable of constitutively releasing GSH in to NB medium. Astrocytes were incubated in NB medium for 6, 12 and 24 hours as described in section 6.2.3.1. NB medium alone was also incubated for the same period of time. GSH was measured in the supernatant as detailed in section 6.2.3. Fig 6.6 shows that GSH concentration increased in the astrocyte medium to a value of 6.25±0.91μM (mean ± SEM, n=4) by 24hrs. There was a basal level of GSH in NB medium that did not change significantly with time and which was significantly less than the level observed in astrocyte medium at all time points.

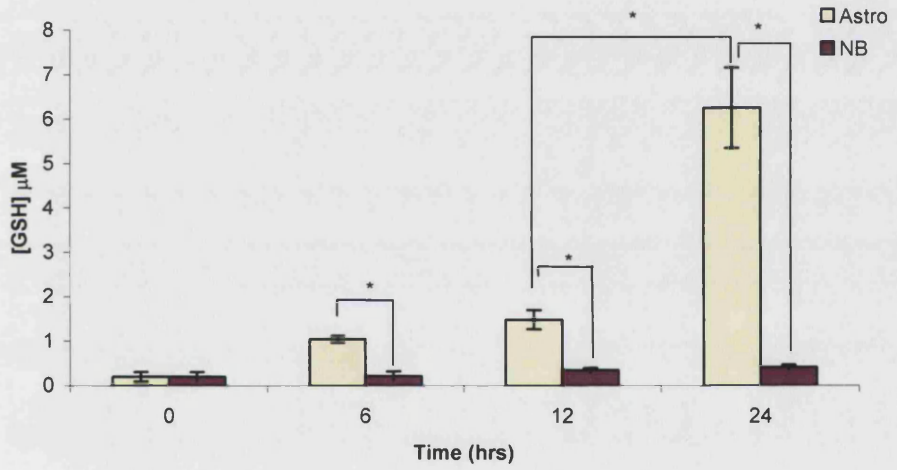


Fig 6.6 GSH in astrocyte medium. GSH in NB medium alone (NB) and NB medium in the presence of astrocytes (Astro) was measured at 6, 12 and 24 hours. The 0hrs point repeats the value for GSH concentration observed in NB at 6 hours. Data ± SEM, n=4 for Astro, n=3 for NB, '*' marks significant difference, $p<0.05$.

The first point is the GSH observed in NB medium, before being incubated and with no astrocytes present and so represents the GSH level from which both sets of data in the graph would start. The figure shows that there is a significant increase in GSH between 0 and 6 hours but this lost between 6 and 12 hours. The increase between 12 and 24 hours is significant again.

We next wanted to determine the level of GSH in astrocyte-conditioned medium (con med) as a means with which to test the stability of astrocyte-released GSH. To reiterate, con med is NB medium that has been incubated with astrocytes for 24 hours. Con med was incubated on its own for a further 24hours and GSH was measured at 6, 12 and 24 hours as detailed in section 6.2.3. Fig 6.7 compares the data obtained for con med to the data obtained for NB medium on its own that we have already seen in fig 6.6. This figure shows that GSH concentration was significantly greater in conditioned medium than in NB medium at all time points and further that GSH levels in con med did not change over the 24hour period, from the initial value of 7.57± 1.32μM (Mean ± SEM, n=4).

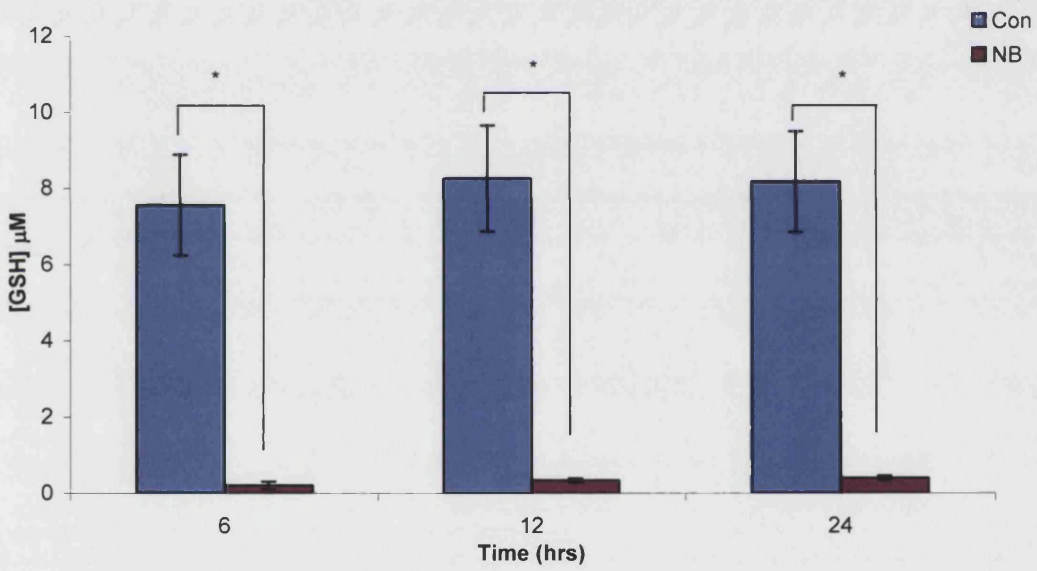


Fig 6.7 GSH concentration in conditioned medium. GSH in NB medium on its own (NB) and in con med (Con) was measured at 6, 12 and 24 hours. Data ± SEM, n=4 for Con, n=3 for NB, '*' marks significant difference, $p<0.05$.

Because of findings in our lab that GSH stability depends on the medium, and that astrocytes release a form of superoxide dismutase (SOD), we wanted to determine if GSH preservation in our conditions was a function of NB medium or if it was a function of astrocyte release. This was done by spiking the NB medium with 10μM GSH

and allowing it to incubate for 24 hours. This value was chosen, as it was slightly higher than the maximum values observed by us in the medium and would therefore stand out clearly if it were preserved. The GSH level was determined at 6, 12 and 24 hours. Fig 6.8 shows that there was no significant difference between spiked and unspiked samples at all time points measured. Therefore, NB medium is not capable of preserving GSH on its own.

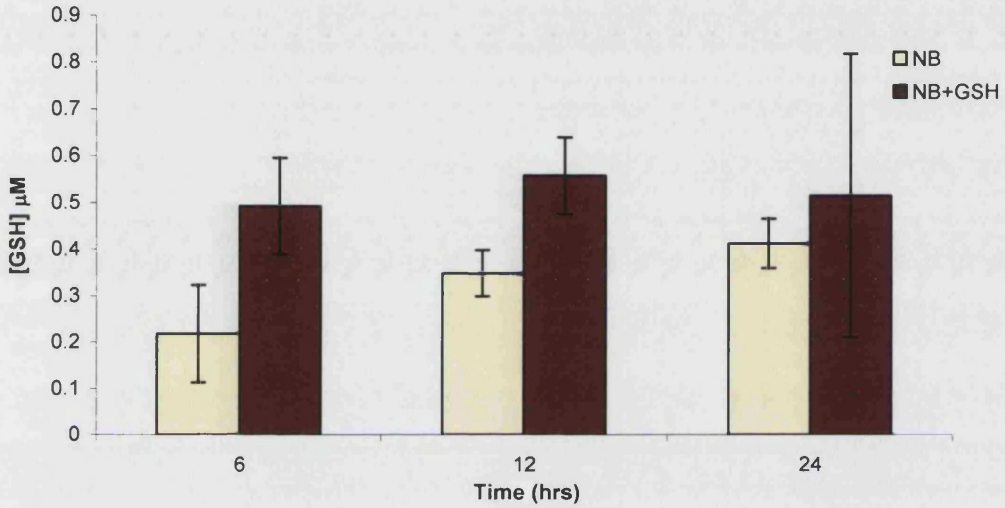


Fig 6.8 GSH-spiked NB medium. NB medium was spiked with GSH to a concentration of 10µM and was allowed to incubate for up to 24 hours. GSH level was determined at 6, 12 and 24 hours. Graph shows spiked (NB+GSH) and unspiked NB medium (NB). Data ± SEM, n=3, $p<0.05$.

We finally wished to determine if either con med or NB medium in the presence of astrocytes, were capable of preserving exogenously applied GSH. NB medium was spiked with 10µM GSH and allowed to incubate with astrocytes for up to 24 hours. Likewise, con med was spiked with 10µM GSH and allowed to incubate on its own for up to 24 hours. Fig 6.9 compares the GSH levels in spiked samples at 6, 12 and 24 hours with unspiked samples. All data from unspiked samples were seen previously in either fig 6.6 or 6.7. There was no significant difference between spiked and unspiked samples of the same condition at any time point ($p<0.05$). The figure also shows

that by 24hours [GSH] in NB incubated with astrocytes reaches a similar level to con med (6.25±0.91 and 8.18±1.33μM respectively, mean ± SEM, $p<0.05$). This confirms that exogenously applied GSH is not preserved and furthermore reiterates the point that astrocyte-derived GSH is preserved in con med.

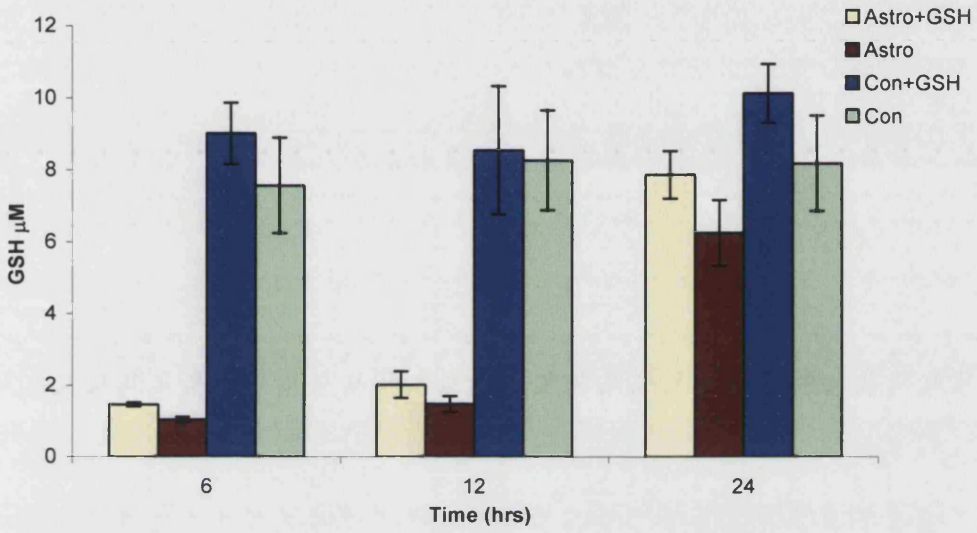


Fig 6.9 Astrocyte-mediated stability of GSH. Con med and astrocyte-exposed NB medium were spiked with GSH to a concentration of 10μM and were allowed to incubate for up to 24 hours. GSH level was determined at 6, 12 and 24 hours. Con med was spiked (Con+GSH) or unspiked (Con). NB medium in the presence of astrocytes was spiked (Astro+GSH) or unspiked (Astro). Data ± SEM, n=4, $p<0.05$.

6.4.3.2 GSH in recovery medium of OGD-subjected neurones

Having established that astrocytes release GSH in this system and furthermore that this GSH is preserved in con med, we now wish to investigate the possible role of released GSH in neuroprotection. We have subjected cortical neurone cultures to O_2 glucose deprivation (OGD) for 15mins and allowed them to recover for up to 24hours in NB medium, in con med or in NB medium whilst in co-culture with astrocytes.

First of all we want to determine if there is a correlation between GSH concentration in the extracellular environment and exposure to OGD. GSH concentration in the culture medium was measured at 6, 12 and 24hours of recovery. Fig 6.10A shows that there is no significant difference between control and OGD levels at any time point measured. We have also used this figure to compare GSH levels in neuronal medium to the levels of GSH in NB on its own (first presented in fig 6.6). There is significantly greater GSH in the neuronal medium from both control and OGD groups at 6 hours of recovery.

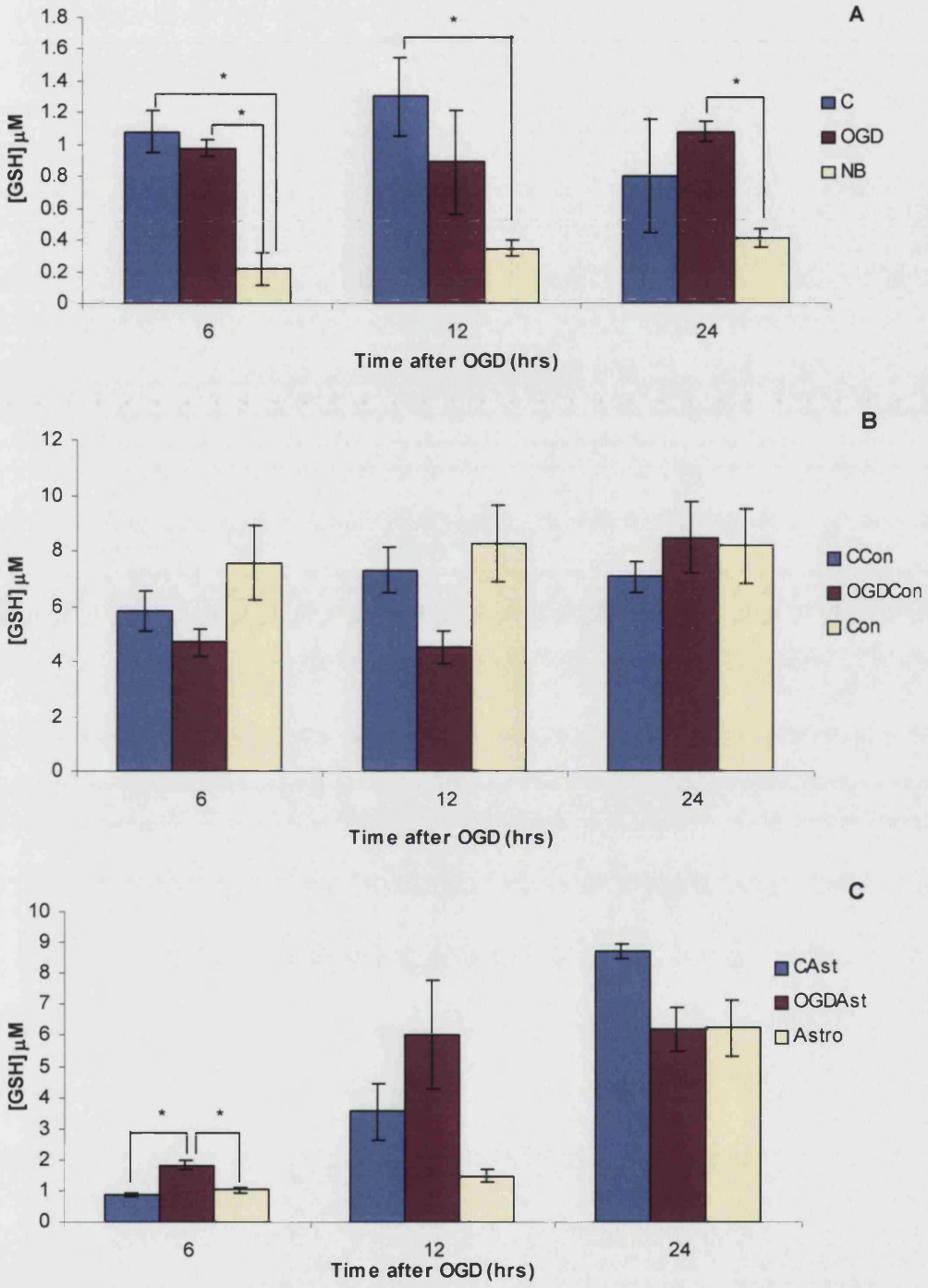


Fig 6.10 Extracellular GSH concentration following OGD. Cortical neurones were subjected to an OGD of 15mins and allowed to recover for 24hrs in NB medium only (A), in con med (B) or in astrocyte coculture (C). Control cultures are marked as (C) and OGD subjected cultures are marked as (OGD) in each panel. **A)** includes GSH in NB medium without neurones, **B)** includes GSH in con med without neurones **C)** includes GSH in NB medium of astrocytes without neurones. Data ± SEM, n=4, '*' marks significant difference, $p<0.05$.

At 12 hours there is significantly more GSH in the control group only and at 24hours there is significantly more GSH in the OGD group only.

Fig 6.10B illustrates the data obtained when neurones were recovered in con med. It also includes GSH levels measured in con med without neurones, which was first presented in fig 6.7. It can be seen that there was no statistically significant difference in GSH levels between control and OGD groups at any time point. Furthermore there was no difference between con-med only and neuronal medium, from either control or OGD groups, at any time point.

Fig 6.10C illustrates the data obtained when neurones were recovered in co-culture with astrocytes. It also includes GSH levels measured in NB medium incubated with astrocytes only, which was first presented in fig 6.7. We have measured the GSH concentration in the medium at 6, 12 and 24 hours and compared them with the GSH levels found for astrocytes incubated on their own. OGD-exposed medium contained significantly more GSH at 6hours of recovery than either the control culture medium or the medium from astrocytes on their own. There was no statistically significant difference between any other groups at either 12 or 24 hours.

6.4.3.3 Intraneuronal GSH

The concentration of intraneuronal GSH was measured following recovery in the presence of astrocytes in order to determine if astrocytes influenced GSH level in neuronal cells that had been subjected to OGD-reoxygenation. Fig 6.11 shows the intraneuronal GSH concentration of neurones subjected to OGD and allowed to recover in NB medium only or in NB medium co-cultured with astrocytes, measured at 6, 12 and 24 hours of recovery. GSH concentration was also measured immediately at the end of the OGD period for control and OGD-subjected cultures. Therefore the 0hours

GSH level of both the astrocyte and con med lines start in exactly the same place for control and OGD groups respectively.

There is significantly less GSH in neurones subjected to OGD than in control neurones, marked by '**' on the graph, at 0 hours (12.47±1.43 and 7.02±0.63 nmol/mg respectively, mean ± SEM, n=4). There was no significant difference between control and OGD groups of each condition (ie with and without astrocytes) at each time point. There was however significantly less GSH in OGD-subjected neurones recovering in NB medium only, than in OGD-subjected neurones recovering in the presence of astrocytes at 6, 12 and 24 hours of recovery. There was also significantly more GSH in control neurones recovering with astrocytes than without astrocytes at 24hours of recovery only.

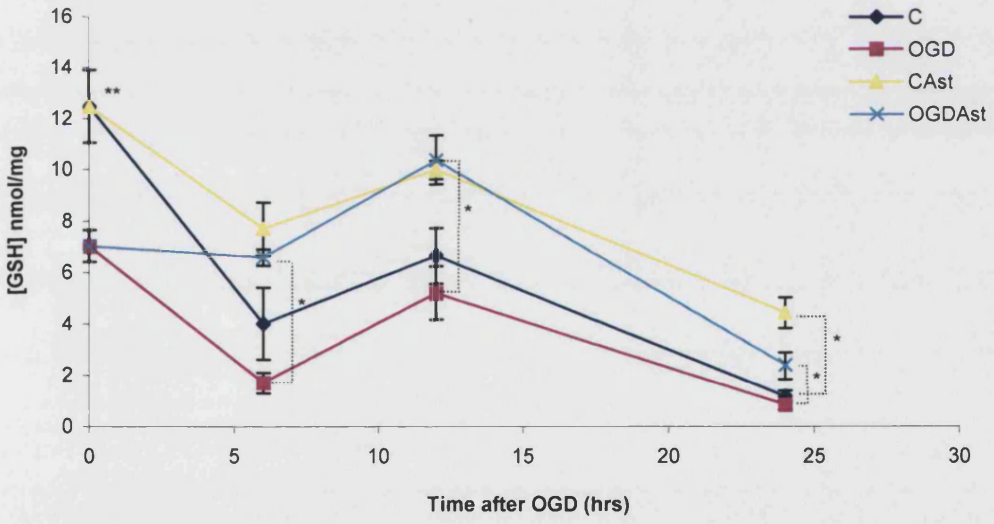


Fig 6.11 Intracellular neuronal GSH . Neurones were subjected to 15mins OGD and allowed to recover in the presence or absence of astrocytes. Neuronal GSH was measured and expressed as nmolGSH/mg protein. OGD-subjected neurones were recovered with astrocytes (OGDAst) or without astrocytes (OGD); control neurones were also recovered with astrocytes (CAst) or without astrocytes (C). Data ± SEM, n=4, '*' marks significance between conditions, '**' marks significance within conditions, $p<0.05$.

GSH levels dropped considerably and significantly over time in all conditions measured from the outset of the recovery period to 24hours of recovery. There was a trend to an increase in GSH in all groups between 6 and 12hours of recovery however, this rise was only significant in OGD cultures recovering in NB only.

6.4.3.4 Intracellular astrocyte GSH levels

The intracellular GSH level of astrocytes incubating on their own in NB medium and incubating in the presence of OGD-exposed and control neurones was determined in order to see if the presence of injured neurones influences astrocyte GSH turnover. Fig 6.12 shows that there is no significant difference in GSH levels of astrocytes incubated with control and OGD exposed neurones at 6 and 24hours of recovery but there is at 12hours of recovery. There is also a significant drop in GSH level of astrocytes incubated with neurones (C and OGD) at 24hours of recovery (Not indicated as significant on figure due to clutter). The GSH level of astrocytes incubating on their own did not change significantly over time from a level of 34.5±0.9nmol/mg (mean ± SEM, n=4) at 6hours. This was significantly greater than the level in astrocytes incubated with neurones at 6 and 24 hours. The difference at 12hours was not statistically significant.

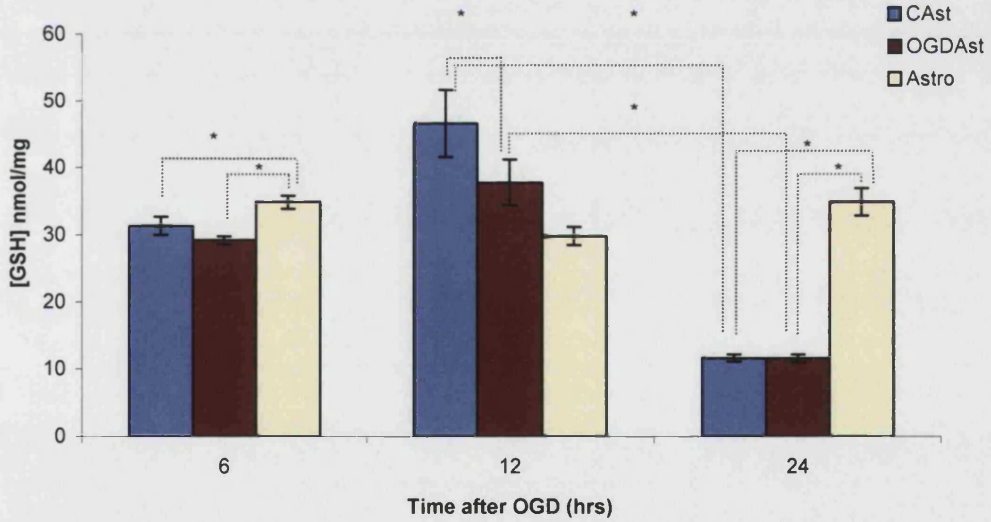


Fig 6.12 Intracellular astrocyte GSH. Neurones were subjected to 15mins OGD and allowed to recover in the presence of astrocytes. Intracellular GSH of astrocytes co-cultured with control neurones (CAst) and OGD neurones (OGDAst) was measured at 6, 12 and 24hours of recovery. The intracellular GSH level of astrocytes incubating on their own for 24hours in NB medium was also determined (Astro). Data±SEM, n=4, '*' marks significant difference, $p<0.05$.

6.5 Discussion

In meeting the aims of this chapter we have begun to investigate the neuroprotective properties of astrocytes within the confines of the current model by looking at the behaviour of three separate molecules. Each is representative of one of the three lines of investigation stated in the opening paragraph of this chapter; glutamate, the neurotoxin to be removed; lactate, the metabolic substrate; glutathione, the protective antioxidant. To be absolutely clear, the aim of this chapter is not to conclusively determine if the use of these molecules can elicit neuroprotection per se but rather to determine if they elicit neuroprotection in our model. We want to know if the presence of astrocytes makes them available to, or in the case of glutamate, removes them from, neurones in this model specifically.

6.5.1 Removing a neurotoxin?

We have detailed quite clearly in chapter one, how glutamate acts as a neurotoxin. The sequence of events in ischaemia has so far been established to follow this order. Energy deprivation causes a drop in ATP, which stops the plasma membrane K^+/Na^+ pump from working. The increase in extracellular K^+ causes the cells to depolarise, and the Na^+ gradient is diminished. There is then no driving force for released glutamate to be pumped back in to the cell. The extracellular glutamate concentration increases and glutamate activates NMDA receptors (primed by the depolarisation), which causes further depolarisation and release of intracellular glutamate.

The accepted theory on glutamate release is that reversal of glutamate transporters, predominantly present on glia, cause the increase in extracellular glutamate (Rossi et al 2000, Phillis et al 2000). This increase is responsible for excitotoxic neuronal death during the ischaemic insult (Choi et al 1990). Glutamate release during reperfusion is less well characterised but it has been documented following brain ischaemia *in vivo* (Taguchi et al 1996, Yang et al 2001, Mori et al 2004).

In agreement with these observations, we have shown that there is an increase in the concentration of glutamate with time in the NB medium of neurones recovering from OGD of 15mins compared to the concentration in NB medium of their sister control cultures. It can be assumed therefore that the increase in glutamate reflects a release event from the neurones themselves, as there are no other cell types present. All glutamate detected was released during the recovery period only as medium was changed immediately at the end of the 15min injury time.

It is fair to say that we were initially surprised to find that there was no difference in extracellular glutamate concentration between control and OGD-subjected cultures immediately following 15mins of OGD. But on reflection, it is probably too short a period for a significant difference to be observed in a system such as ours. Goldberg et al (1990) report that OGD of less than 30mins had no impact on extracellular glutamate concentration in cortical cultures. Marcoli et al (2004) show in their human cortical slice model that significant glutamate efflux occurs at 18mins of OGD, returning to normal quickly upon reperfusion. Toner et al (2001) show that glutamate efflux during OGD is significant only after 15mins in their corticostriatal slice model.

An important point to note is that we culture our cells in a very large volume (2ml), which may dilute and hence make it difficult to measure the glutamate accumulated. It also means that glutamate could be lost to the bulk medium rather than staying at the level of the cell so there would be less glutamate in the immediate vicinity of the cell than there would be *in vivo*. Furthermore our measurements and indeed those of the groups mentioned, only give an average concentration in the whole solution, however it may be possible that glutamate at the level of the cell surface is in fact far more concentrated, and a gradient exists that slopes away from the cell. Jabaudon et al (2000) show that glutamate concentration does actually rise in their hippocampal slice model (chemical ischaemia) from around 650nM to 1μM in the vicinity of the crucial NMDA receptors, within 10mins.

As we have shown, glutamate levels do rise significantly in the medium of OGD-subjected neurones throughout the recovery period and in chapter one we have discussed at length the abilities of glutamate to cause neurodegeneration in its capacity as an excitotoxin. Could the increase during recovery possibly perpetuate neuronal death or more specifically, is glutamate acting as a neurotoxin in our model?

It is common practice to model neuronal injury by an excitotoxic mechanism, through the addition of high concentrations of glutamate. The concentrations, which are normally used to induce cell death, are between 100 and 500μM (Machetti et al 2004, Nagy et al 2004, Sinor et al 2000). The figures we have reported for glutamate are underestimating the actual concentration that the cells are exposed to by 25μM, as there is 25μM glutamate in the B27 serum supplement. The way in which we have carried out the glutamate assay is responsible for this because we measure samples against their respective media and hence subtract the amount of glutamate added to the medium by the manufacturers. The maximum concentration of 25μM that we have observed at 24hours is far less than that necessary to induce significant death (at the level we observe at least) but so too is 50μM. Neither is there sufficient glutamate released during the OGD insult itself or at 6 or 12hours. However, as mentioned previously, the concentration of glutamate at the cell surface may be a lot higher. Also, the OGD insult itself may sensitise the neurones to much lower concentrations of glutamate. For the moment we can only link glutamate with cell death in a circumstantial manner, in a sense we are assuming its guilt before proving its innocence!

This brings us on to the principal aim of this section, to find out if the neuroprotection we observed is elicited by the removal of glutamate, a proven neurotoxin at least in other systems. We have shown that coculture with astrocytes significantly reduces the amount of glutamate in the recovery medium of not only OGD-subjected neurones but their sister controls as well. In fact, there is no difference between OGD-subjected and control cultures in the presence of astrocytes at any time point.

There does appear to be a basal amount of glutamate release from astrocytes alone, however we did not factor this in to the co-culture data as we could not be certain that this release would stay the same in the presence of neurones. Directly comparing the data between co-culture and astrocytes alone, it would seem that glutamate from the astrocytes could account for some of that measured and so there may be even less attributable to the neurones.

At first glance it is tempting to suggest that astrocytes are mopping up the accumulated glutamate through a transporter activity, essentially removing the neurotoxin. Astrocyte cultures have been shown to express functional glutamate transporters on numerous occasions so this option is at least possible (Hansson 1985, Waniewski et al 1986, Gegelashvili et al 1996, Perego et al 2000). However the presence of conditioned medium also reduces the amount of glutamate detected so removal seems less likely (we are not ruling this option out completely and will discuss it further in chapter 7). What is most striking about the data is that for the first 12 hours of recovery, there is no difference between the glutamate level in con med alone and in either the OGD-subjected or the control neuronal cultures recovering in con med. The glutamate levels of the OGD-subjected con med cultures become significantly greater than control values only at 24 hours of recovery, which is most likely a reflection of cell death. Therefore, we must conclude that nearly all the glutamate present has come from astrocytes. Have the astrocytes merely prevented neuronal glutamate accumulation in the first place?

It is interesting to note that there is always significantly more cell death in the OGD-subjected cultures than in the sister control cultures of every condition at all time points (see chapter 5). But this is not the case with glutamate accumulation as more often than not control and OGD values are the same when neurones are recovered in con med or co-culture. Shouldn't more neuronal death in OGD-subjected cultures equate to more glutamate release, particularly as our method of cell death detection is largely based on membrane rupture as a marker of cell death? Perhaps

it should in which case the following chain of logic emerges. Because the control cultures from each of the three conditions have the same amount of cell death it would appear that the glutamate detected in the cultures recovered in NB medium alone was released in a controlled manner and not released due to membrane lysis. So, because the neurone membranes of the cultures recovered in con med and co-culture are of a similar permeability to those recovered in NB medium alone it would appear that glutamate transport has stopped. Furthermore it has stopped due to the influence of astrocyte-derived factors.

The function of astrocyte-derived elements therefore may be to prevent the glutamate accumulation, not directly remove it. It would appear that in the absence of these elements early events in recovery cause the spiralling release of more glutamate, resulting in the death we observe for neurones recovering in NB alone.

So in answer to our question, yes, astrocytes remove a neurotoxin, but by preventing its accumulation in the first place. Precisely how glutamate accumulates and therefore where the astrocytes fit in, is a matter that we have begun to look in to. It is unclear if the rise in glutamate is the direct result of increased vesicular release or membrane lysis or if it is the result of transporter reversal or malfunction. Similarly, we have begun to address the role of glutamate in eliciting cell death in the absence of soluble astrocyte derived factors. These are issues we will address in the final chapter. For now, we will look further in to the putative neuroprotective factors.

6.5.2 Metabolic support

Our results suggest that lactate is produced by astrocytes at a steady rate and is stable for at least 24 hours as part of conditioned medium. So, there is an abundant supply of lactate ready for neuronal use. However, neither our control nor OGD-subjected neurones appear to use this lactate during the recovery period. This is most clearly illustrated by the fact that the lactate concentration in con med incubated with neurones

does not differ from con med incubated alone over the entire 24 hour period. Clearly, usage by neurones would result in a reduction in lactate in con med as there are no astrocytes present to replace it. More pertinent to our aims is the fact that the OGD insult has placed no extra demands on lactate usage by neurones at least as far as we can tell from the unwavering con med lactate concentration.

For much of the recovery period in co-culture there are exactly the same trends in lactate concentration as there are for con med i.e. the concentrations at each time point are similar. Neurones are releasing a small amount of lactate but not enough to significantly alter the data when added on to the lactate values for co-culture. It is interesting however that at 24 hours of recovery there is less lactate in the medium of OGD-subjected neurones recovering in co-culture than there is in either its sister control or in the medium of astrocytes alone. This does suggest the possibility of neuronal lactate uptake and poses an intriguing question. Will the neurones only use lactate if it comes directly from astrocytes?

If they do, a number of scenarios are possible to resolve our co-culture data. First, neurones could have used lactate when it was most needed at the start of the recovery period but astrocytes have since continued to pump it out so that levels are even later on. Essentially by 6 hours the system has become balanced again. The reduction in OGD culture lactate at 24 hours may reflect a second period of intense use. Second, the astrocytes may actually be pumping out lactate more quickly when in co-culture with neurones, the neurones may be taking it up and using it leaving the concentration in the medium appearing the same as astrocyte medium alone. Waltz et al (1990) suggest that astrocytes do not accumulate lactate under increased production, they actually release it. This option certainly fits in with the evidence of a neuronal stimulation of astrocyte glucose uptake and glycolysis (Magistretti et al 1999).

This discussion raises an issue we have hinted at in chapter 5 and which we will continue discussing in chapter 7. The co-culture of astrocytes and neurones allows for a two-way communication and therefore for neurones to stimulate astrocytes so that the mechanism of neuroprotection may actually be different between con med and co-culture.

Returning to the issue of preferential lactate use for just a moment, there is one small argument to follow through. There is a considerable amount of glucose in the recovery medium (as part of the NB medium formula) in the presence and absence of astrocytes, which would immediately prompt one to suggest that they do in fact have an abundant source of metabolic fuel so why use or need lactate? It has been shown that in the presence of glucose and lactate, cultured neurones appear to use lactate more (Tabernero et al 1996, Bouzier-Sore et al 2003). A possible consequence of the presence of lactate during recovery is that neurones could use their glucose through, for example, the pentose-phosphate pathway for the synthesis of NADPH. The consequence of this will be discussed later. For now, we have succeeded only in showing that lactate is available to be used but the question of whether it is used as a metabolic fuel remains open.

6.5.3 Antioxidant support

6.5.3.1 Preservation of released GSH

Our first step in elucidating a possible role of astrocyte-derived GSH in neuroprotection was to characterise any GSH release in this system with respect to quantity and stability. We have shown that our rat astrocyte cultures are indeed capable of releasing GSH in this system, which is in agreement with the published literature (Yudkoff et al 1990, Sagara et al 1996, Dringen et al 1997, Stewart et al 2002, Hirrlinger et al 2002). Such release has been shown to be carrier mediated and is suggested to occur via the multidrug resistance protein 1 (Sagara et al 1996, Hirrlinger et al 2002). It should be noted that it was not our intention to study GSH release per se, and so we did not use the 'GSH-preservation friendly' minimal medium preferred by Dringen et al (1997). We wanted to see if

GSH could be released into, and be preserved by, NB medium, the medium used for all neuronal re-oxygenation experiments, as this more accurately reflects what would be available to neurones.

We have noted a rise in extracellular GSH concentration in the medium of astrocytes cultured alone to a level of 6.25±0.91μM by 24 hours. There was a very small amount of GSH detected in NB medium that remained constant over time. We can assume therefore that all GSH observed in the extracellular medium of astrocytes comes directly from the astrocytes themselves.

Our observations suggest that the rate of GSH accumulation is not constant over the entire 24hour period as there was no significant rise over the 6 hours period between 6 and 12 hours of incubation, the levels remaining at a plateau of around 1.2μM (median value). This suggests the possibility of a biphasic increase in GSH concentration. Although two distinct periods of increase are evidenced by our data, there is not sufficient data before 6 hours to establish where the first increase stops or whether the increases are linear or not. So how does our system of GSH release compare with others?

There is some discrepancy in the literature as to the rate and level of GSH release from astrocyte cultures. Yudkoff et al (1990) recorded a linear increase to a plateau value of around 3μM after 1 hour of incubation. Like Yudkoff et al, Sagara et al (1996) report a linear accumulation of GSH within the first hour of incubation to 1.9μM, however they do not report any later values and the existence of a plateau is not implied. Similarly, Stone et al (1999) also report a linear increase in GSH over an 8-hour period but to a level of 1.13μM. However this group later reports a linear increase to a maximum of 2.6μM at 4hours (Stewart et al 2002). Dringen et al (1997) also report GSH increase to be entirely linear and in later work show an accumulation profile similar to ours over 24 hours in co-culture medium (Dringen et al 1999). It would appear that the medium chosen might be behind this discrepancy. Yudkoff et al (1990)

use a complicated mixture, Ham's F-12 medium, composed of amino-acids, metal based salts and a host of potential GSH scavenging molecules (http://www.cambrex.com/Content/Documents/Bioscience/Hams.pdf for formulation). In contrast Sagara et al use a simple phosphate buffer whilst the Stone, Stewart and Dringen systems use a simplified minimal medium, which is essentially a balanced salt solution with glucose added.

We have looked at GSH release on a much longer timescale than the works mentioned (with the exception of Dringen et al 1999) and have no data within the first 6 hours to compare directly. However, other elements remain the same and so comparison of release data is justified in trying to understand our system. It is important to realise however that GSH accumulation, such as we have documented, is not the same as GSH release. Our results show clearly that the accumulation of GSH is not entirely linear but that does not mean that release isn't. There may well be factors in the medium that oxidise, scavenge or breakdown released GSH. In fact we have shown that exogenously applied GSH is lost from NB medium by 6 hours of incubation which proves the existence of medium-related GSH removal. With respect to medium complexity, our system would therefore appear to closely resemble that of Yudkoff et al. We report a value of around 1.2μM by the time of the plateau which is approximately half that observed by Yudkoff. This difference could be accounted for by the greater density of astrocytes ($2x10^6$ cells) in the Yudkoff preparation, as it is double the density we use.

But what about the increase in GSH after 12 hours? The most obvious explanation for this would be that there isn't actually a plateau in GSH accumulation, there is just a very very slow increase up to 12 hours. By constantly pumping out GSH, and indeed other elements, the astrocytes are managing to overcome whatever factor it is that may be removing GSH in the period up to 6 and 12 hours. It is likely then that the more GSH is released at this point, the further the redox balance is swung in favour of an antioxidant environment and so there may actually be an

exponential accumulation of GSH. The inclusion of extra data points would test this idea and would allow for a rate to be calculated, however it is not entirely relevant to the main questions being asked by this chapter.

We did however attempt to measure total GSH in the medium (GSSG plus GSH) by converting GSSG to GSH by the method of Anderson et al (1985) in an effort to tackle the question of GSH disappearance. However we could not get the assay to work with NB medium, which is probably further proof of its GSH scavenging abilities! Hirrlinger et al (2002) and Stone et al (1999) both show that only GSH is released from astrocytes, not GSSG. This point is particularly relevant to neuronal survival because it means there may be more GSH immediately available to neurones than we can see.

Of particular relevance at this juncture is the fact that only GSH that is released by astrocytes is preserved, at least partly, in NB medium. We have shown that astrocyte-conditioned medium retains its GSH concentration when incubating alone for 24 hours. More importantly, we have further shown that exogenous GSH is lost from the system by 6 hours of incubation, not only from NB medium but also from con med and from NB medium incubating with astrocytes.

Stewart et al (2002) have shown that astrocytes release a factor, which they identify as superoxide dismutase, concomitant with GSH release that slows down its oxidation. Such a process may be happening here. The released factor may only be able to cope with the protection of just the amount of GSH released by the astrocyte. Any excess GSH is not protected and immediately lost. Stewart et al do show some protection of exogenous GSH. The oxidation of the applied GSH was merely slowed in con med compared to native medium so that over a 5 hour period there was a significant drop in GSH. Extracting this data and comparing it to ours, the results observed by us at 6hours of incubation are quite comparable to the results obtained by the Stewart model at 5hours. Nevertheless, we were surprised to see how well GSH was preserved in

con med over the 24 hour incubation (following the 24 hour conditioning period) and can only conclude that all potential GSH sinks had been completely overwhelmed over the course of the 24 hour conditioning period.

We have shown that the intracellular GSH concentration of astrocytes in this system is around 35nmol.mg protein^{-1}. This level does not change appreciably over the 24hour incubation period when extracellular GSH is increasing in NB medium. Our astrocytes are incubating in 'normal' medium, replete with sufficient GSH precursors to replace lost GSH over the 24 hour period so the result is not surprising. Sagara et al (1996) report a higher initial level of intra-astrocyte GSH but also show no reduction in level over the course of their 1-hour release experiment.

Juurlink et al (1996) claim that there is no GSH release from astrocytes under normal conditions and in fact state that any release in their system is due to cell death and subsequent membrane rupture. This is unlikely in our system as a) morphological analysis shows the astrocytes to be healthy b) there is no significant membrane rupture (deduced from results in chapter 4) and c) release of GSH does not result in intracellular decline.

6.5.3.2 GSH loss may perpetuate neurone death

We have measured GSH in the recovery medium of control and OGD subjected neurones and compared this with the basal levels found in NB medium. We did find a greater amount in the neuronal medium of both control and OGD-subjected cultures particularly at 6 hours of recovery and so must conclude that neurones are also releasing some GSH. Hirrlinger et al (2002) detected GSH release from their neuronal cultures but conclude that it was due to glial contamination and damage to neuronal membranes. Although we do not have a significant glial presence in our neuronal cultures (see chapter 3), we have shown that there is damage to both control and OGD-subjected cultures (see chapter 3). Compounding this is the fact that we did not see the steady rise in

extracellular GSH evident in astrocyte cultures and a significant difference from NB medium alone was not consistent over time. The evidence so far suggests therefore that release is passive and due to slight membrane rupture. The most important part of these data however is that there was no difference in release between control and OGD-exposed cells, despite significantly more death occurring in the OGD cultures. This is reflected also in the values obtained for intracellular GSH.

There was significantly more intracellular GSH in control neurones than there was in OGD-subjected neurones immediately following the15mins OGD period. However both conditions resulted in severe GSH loss as the reoxygenation period continued, so much so that there was no difference in GSH level between the two conditions throughout recovery, again, despite greater cell death in the OGD cultures. Looking at the 0-hours time point only, we have shown in chapter 4 that there is no significant difference in cell death between control and test cultures. As our cell death method is based mostly on membrane permeability, the reduction in GSH is unlikely to be due to leakage as a result of membrane rupture. It is more probable that OGD-subjected cells have used their endogenous GSH in an intracellular defensive effort.

Looking at the recovery period, it is curious that control cultures exhibit far less cell death yet they have an equivalent amount of GSH. How do we reconcile these data? It is possible that there is a significant amount of release of both GSH and intracellular protein from OGD-subjected cultures so that they would appear to have the same specific concentration as the control cultures. We have shown that OGD-subjected neurones have a more permeable membrane than control neurones so this is quite a plausible explanation (see chapter 4, cell death data). This should however result in GSH accumulation in the medium, which we don't see. We have already shown in section 6.5.3.1 that NB medium rapidly degrades GSH so it is possible that any GSH released by neurones, due to cell death or otherwise, cannot be

registered by us. Again, we did try to determine if GSH was being oxidised but interference with the assay prevented this.

Stewart et al (2002) report that neurones release GSH and a GSH protective element, in the same manner as astrocytes, which makes it unlikely that released GSH was significantly degraded. Instead we favour the possibility that the intracellular demand on neuronal GSH in our system attenuates any release. Retained GSH is oxidised by the cell itself, as concluded previously.

We do see cell death in the control cultures and we have shown that they have a certain amount of membrane leakiness. The very fact that cell death is seen in the controls at all is a sign that just placing the neurones in the Dulbecco's modified eagle's medium (DMEM) for 15mins is stressful (see chapter 3) and so the controls are also open to 'reperfusion injury'. The origin of this stress may actually be GSH related. DMEM does not contain cysteine, which is necessary for GSH synthesis and this may affect cell physiology. This is in fact the principal reason why all experiments are carried out with careful consideration of control conditions to match all aspects of the injury except metabolic substrate withdrawal.

The higher GSH level in the controls at T0 could explain why they suffer less cell death during recovery with an equivalent amount of GSH. Although they lose GSH during the recovery period they may have had it when they most needed it, at the start of recovery. Without intending to labour the point, the higher level of GSH in the controls at the start of reoxygenation, would appear to make them better able to cope with the stress. This leads us directly on to a possibility first suggested by the glutamate data.

Is the fight against cell death fought entirely at the start of the reoxygenation period? The evidence suggests it is. We have hypothesised that glutamate release, as a consequence of stress in early

recovery, begets further glutamate release and cell death. Astrocyte-derived factors attenuate early stress and stop the cycle before it continues. At this point we can only assume that our cultures are losing their intracellular GSH as a result of fighting oxidative stress immediately after OGD so can we fit astrocytes in to this picture?

6.5.3.3 Astrocytes to the rescue

We have shown in chapters 4 and 5 that neurone cultures subjected to 15mins OGD die in a steady progressive manner over a 24-hour reoxygenation period. However the presence of astrocytes and astrocyte-conditioned medium significantly reduce the amount of death observed. We have proven that GSH is released from astrocytes and is preserved well in the form of conditioned medium. So the crucial question is could GSH release by astrocytes play a role in this neuroprotection?

Based on observations in the literature we wanted to test two hypotheses with respect to a link between astrocyte-released GSH and neuroprotection in our system of OGD-reoxygenation. First, it is used to scavenge extracellular radicals and thus reduce attack on neurones. Second it is used as a substrate for increasing neuronal GSH.

There was no difference in GSH concentration in the medium of control and OGD-subjected neurones recovering in con med. Furthermore, at no time point was there a significant reduction in GSH when compared with con med alone. Initially, much the same picture is seen when neurones are co-cultured with astrocytes. There is no difference between either the GSH levels in co-cultured control or OGD-subjected populations and the levels in astrocytes incubating alone in NB medium for most of the recovery period. However, at 6 hours of recovery there is more GSH in the OGD-subjected culture medium than either of the other two cultures.

We will start with the first hypothesis. All things being equal, one would expect there to be more GSH in the medium of control and OGD co-cultures than in the astrocyte cultures alone if passive neuronal release,

shown earlier in section 6.5.5 is factored in (indeed, the same is also true of con med). This is possibly what has happened in the OGD cultures at 6 hours. GSH concentration is 1.829±0.16μM, which is roughly equivalent to the addition of 1.04±0.08μM in the astrocyte medium, and 0.98±0.05μM in the OGD-subjected cultures recovering in NB medium only. So why is this the only point of significant difference?

We have accepted in section 6.5.5 that, to a certain extent, both control and OGD-subjected cultures are stressed. Astrocyte-derived factors may therefore be used to the same extent in both sister cultures during the recovery period explaining why GSH values are the same at each time point. We will continue with this in mind for the time being. It may very well be that 'extra' GSH has actually been used to rid the medium of radical species but as it has been used, we can't detect it. Again, this would explain why there is no difference between control and OGD values and astrocyte cultures. However, if radical scavenging were occurring, one would expect there to be less GSH in the con med over time. Indeed, as we know OGD-subjected neurones are more stressed than controls (as judged by cell death, chapter 5) even in co-culture and in con med, we would expect them to have even less GSH in their con med than controls. This is in fact the same argument we made with lactate. Our first hypothesis therefore seems unlikely to be true unless of course, as we have mentioned in the discussion on lactate, the online release of GSH from astrocytes themselves changes the way the system behaves. And our second?

The presence of astrocytes significantly increased neurone GSH levels in both control and OGD-subjected cells when compared with their counterparts recovering in NB medium only. This agrees with previous studies showing that co-culture with astrocytes results in increased neuronal GSH (Yudkoff et al 1990, Sagara et al 1993, Dringen et al 1999, Wang et al 2000). We should make the point here again that the increased GSH is not due to retention by the neurones as a consequence of reduced cell death and membrane permeability as we have already

shown that there is no difference in cell death particularly between control neurones recovering in NB medium only and control neurones recovering in either con med or co-culture (see chapter 5). The neurones appear to have actually synthesised GSH. Is the astrocyte-released GSH itself causing the increase in GSH or is there another factor involved?

Neuronal usage of astrocyte-derived GSH requires that it be broken down extracellularly before acting as a precursor for neuronal GSH synthesis. Dringen et al (1999) show that breakdown is mediated by the astrocyte ectoenzyme γ-glutamyl-transpeptidase, which cleaves GSH to produce Cys-Gly, which is then taken up and used by the neurones. Wang et al (2000) show that the astrocyte-derived GSH is actually used to reduce cystine to cysteine, which the neurones then use for GSH synthesis. Previously, because we did not see a reduction in GSH in con med of neurones compared with con med alone, we had weakened the hypothesis that GSH acts as an extracellular scavenger. The same argument also applies to the breakdown of GSH for the provision of neuronal precursors. On the other side of the coin, Iwata-Ichikawaw et al (1999) elegantly show that neurones incubated in glial conditioned medium upregulate the transcription of γ-glutamylcysteine synthase (the rate limiting enzyme in GSH synthesis) when confronted with an oxidative insult. The only problem with this option is the length of time it would take for a transcription event to translate to increased GSH within the cell. We are assuming in this model that the neuroprotective factor(s) must exert its effects at the very beginning of recovery which may not give enough time for a growth/survival factor to elicit its effects.

One of the key points that has emerged in this chapter is whether the presence of injured neurones stimulates astrocytes in to action. It would seem that neuronal injury has no effect on the GSH response but the very presence of neurones, injured or otherwise, does. We have shown that the intra-astrocyte GSH level does actually change when neurones are present. At both 6 and 24 hours of recovery, there is significantly less GSH in astrocytes co-cultured with neurones than there is in astrocytes

incubating in NB medium alone. There is in fact a massive drop in GSH level between the 12 and 24-hour period. What's going on?

One possibility is that astrocytes are becoming stressed in the presence of the neurones and so are using up their own GSH in defence. However, we showed in chapter 5 that they exhibit no sign of injury that we could detect. Another option is that the astrocytes are releasing their GSH more quickly than it can be synthesised particularly at 24 hours. Our data shows that there is no extra accumulation but this may be due to significant scavenging activities or indeed extra GSH breakdown. Alternatively, the presence of neurones has caused a reduction in GSH synthesis itself. This is a matter we will explore in chapter 7.

6.5.4 Bringing it all together

Putting all our data together the following scenario seems most likely. The OGD insult somehow causes a reduction in intraneuronal GSH. The introduction of O_2 at the start of recovery further stresses the neurone whose GSH is already too low to cope. This results in an accumulation of glutamate and the excitotoxic release of further glutamate and cell death. Astrocyte-derived factors stimulate an increase in neuronal GSH, which attenuates significant oxidative stress at the beginning of recovery and the subsequent mass release of glutamate and cell death.

We are suggesting that cell death during recovery is directly related to an inability to replace GSH during the OGD event. During the 15mins incubation the neurones do not have access to cysteine as DMEM contains cystine not cysteine. OGD-subjected cultures therefore cannot synthesise GSH they are using as a direct result of the insult, nor can they recycle GSH due to lack of glucose derived NADPH, which reduces the GSH available and accelerates its loss. No such burden is placed on the control cells, the stress they are exposed to is clearly much milder as they at least have energy substrates.

We can fit lactate in to this story by recalling that, using lactate as a fuel rather than glucose, allows glucose to be used for other pathways. In this instance, perhaps glucose can be used to generate NADPH via the pentose phosphate pathway, which is then used to regenerate GSH. This is certainly faster than the protein synthesis mentioned previously. Delgado-Esteban et al (2000) have actually shown that neurones are capable of attenuating glutamate-induced injury by using exogenously applied glucose for the regeneration of GSH. Of course lactate only has a place here if astrocytes are present themselves. We will continue this story in the next chapter.

6.6 Conclusions

We have tested the three hypotheses stated in the aim of this chapter and have arrived at a possible mechanism for neuronal death following O_2/glucose deprivation. Our data has opened up avenues of thinking, which was the main intention, rather than present any conclusive data. Furthermore we have hinted at the fact that a) other factors of neuroprotection are involved and b) the mechanism of neuroprotection may differ between conditioned medium and astrocyte co-culture.

Chapter 7

'Where do we go from here?'

7.1 Summary

Chapters 3 and 4 were entirely focussed on creating a working model of ischaemia/reperfusion injury. In chapter 3 we set out to build a robust model of ischaemia in a tissue culture environment. This chapter produced a method for the culture of almost pure cortical neurones, a novel approach to co-culturing these neurones with cortical astrocytes and finally a simple method for the simulation of ischaemia. In chapter 4 we built on this foundation to arrive at a protocol for the induction of 'reperfusion' injury in cortical neurones. Essentially, we set out to create a model that would result in neuronal death during the recovery phase that had a relatively linear rate profile.

Having finally arrived at our model in chapter 4, we set out to apply it by testing the hypothesis in chapter 5, that astrocytes can attenuate neuronal death. As expected, astrocytes proved to be neuroprotective, as did astrocyte-conditioned medium. This begged the question as to how this was possible and so in chapter 6 we proposed a number of likely mechanisms based on our observations.

In this chapter we will detail possible avenues of future research directly related to the work in chapter 6. This includes points of notice that surfaced repeatedly in chapter 6 and preliminary data on the role of glutamate in our model system.

7.2 Points to consider

7.2.1 Co-culture V conditioned medium

In chapter 6 we tended to use the data gained from the conditioned medium experiments to prove or disprove astrocyte involvement directly. However as alluded to previously, the mechanisms of neuroprotection afforded by con med and astrocyte co-culture may be quite different. This is probable because of the ability of neurones to directly influence astrocyte physiology. Although we could not prove it conclusively, neurones do stimulate astrocyte activity. This is of particular relevance to the clearance of glutamate.

The astrocyte glutamate transporters GLAST and GLT-1 are credited most heavily with maintaining extracellular glutamate levels within physiological norms. Both glutamate directly and neurone-derived factors have been shown to alter astrocyte transporter expression (Gegelashvili et al 1996 and 1997). In fact Swanson et al (1997) demonstrated that astrocytes in co-culture with neurones express both GLAST and GLT-1 whereas astrocytes in mono-culture express GLAST only. Further to this Perego et al (2000) showed that the neuronal soluble factors responsible were released by neurones in an activity dependent manner. Clearly, glutamate (and other factors) released from neurones can directly modify astrocytes to alter the neuronal environment. This is not a possibility in conditioned medium. The consequences of not having astrocytes present may be more exaggerated than they first seem.

Consider that neurones release factors to astrocytes, which release factors to neurones, which then release further factors to astrocytes. For example, we have just mentioned how astrocyte glutamate transport depends on neuronal activity. The opposite is also true, astrocytes effect neuronal glutamate transporter expression (Canolle et al 2004). It is not difficult to picture a scenario whereby the signals are volleyed back and forth, each time the cells are adjusting to the message until an acceptable level of activity is reached for both cell types to function happily.

We have given a number of reasons in chapter 6 as to why we may not have seen a difference between OGD-subjected and control neurones with respect to lactate and GSH in the presence of astrocytes. These are quite valid if conditioned medium and astrocytes do ultimately differ in their mechanisms of neuroprotection.

7.2.2 Future direction

We have made the point on a number of occasions in chapter 6 that the events leading to neuronal death are likely to occur in the early part of reperfusion. We have also said that as a consequence, at least in co-culture, the levels of lactate and GSH may be quite different to controls in the OGD-exposed cultures at this point. The most obvious way to determine the accuracy of this assumption would therefore be to focus on time-points before the 6-hour recovery point and specifically within the first hour. This may also as a consequence differentiate between conditioned medium and co-culture.

It would be of great interest to determine exactly what mechanism is behind this early dysfunction. We have postulated that oxidative stress is a likely perpetrator of early damage. So, obviously we need to determine if the cultures are indeed exposed to an oxidative stress. This would in part clarify the matter of glutamate accumulation and glutamate-related cell death. It would also place the proposal of GSH as a neuroprotectant in a more reasonable context.

We have questioned the ability of neurones to use the lactate available to them and have suggested that the actual presence of astrocytes may be a determinant. There is no doubt that neurones can and do use lactate in other culture systems and so we should consider why we do not see this in ours. It is possible that lactate usage (and indeed GSH usage for that matter) occurs within a concentration band that is hidden by the error in our experimental design. A more sensitive method of neurone metabolism, such as carbon labelling, should therefore be considered. Furthermore, we have also made the point that neurones may demand

lactate and GSH from astrocytes, encouraging a systems upregulation similar to glutamate transport. This could be tested simply by placing neurone conditioned medium ie medium that has been sitting on OGD-exposed neurones for at least one hour, on astrocytes and then measuring the release of molecules such as lactate and GSH.

The work in chapter 6 has generated a lot of ifs and maybes, most of which we have not been able to follow up. We suggest that the most immediate question to be answered is that of the function of glutamate/glutamate toxicity in this instance. To this end, the following section builds a case in support of this proposition.

7.3 Suggestion for glutamate experiments

In chapter 6 we suggested that glutamate accumulation during reoxygenation following O_2 glucose deprivation (OGD) of 15mins was responsible for neuronal death during the recovery period. In this section we will outline work being carried out whereby this hypothesis can be tested. Quite apart from the obvious pattern in our data of accumulating glutamate with increasing cell death, we speculate that glutamate is perpetuating death as a result of its track record as a potent neurotoxin.

The neurotoxic actions of glutamate are mediated through over activation of cell surface receptors (described in chapter 1) and so these have become targets for the prevention of neuronal death. The NMDA receptor has been well documented as contributing to neuronal damage following ischaemia largely due to the neuroprotection afforded by its competitive antagonist MK801 both in vivo (Gerriets et al 2003, Gorgulu et al 2000, Li et al 1999) and in vitro (Finley et al 2004, Velly et al 2003, Gerlach et al 2002). More recently, the metabotropic glutamate receptors have gained attention in the precipitation of neurodegeneration particularly the group I member mGluR1 (Bruno et al 2001). The mGlu1 subtype has been shown to be involved in post ischaemic neuronal death both in vivo and in vitro (Meli et al 2002) as judged by the neuroprotection

afforded by inhibitor, 1-aminoindan-1,5-dicarboxylic acid (AIDA). 2-Methyl-6-(phenylethynyl)-pyridine (MPEP) an inhibitor of the other group 1 member, mGluR5, has been attributed with neuroprotective activities but this is thought to be a consequence of cross reaction with NMDA receptors at high concentrations (Pellegrini-Giampietro 2003).

We have also hypothesised in chapter 6 that an early event of reoxygenation is the release of glutamate. We further speculated that the early release was carrier mediated, leading to later release as a result of combined membrane rupture and a carrier mediated event. There are a few different ways of looking at the observed glutamate accumulation; transporter malfunction leads to decreased uptake or reversal of transporter leads to increased release; release may also be vesicular. Although a rise in extracellular glutamate has been recorded during reperfusion (Erecinska et al 1984, Molchanova et al 2004,Taguchi et al 1996, Yang et al 2001, Mori et al 2004) there is sparse evidence to explain it. During the actual ischaemic event a sequential occurrence of different mechanisms has been observed. Asai et al (2000) present evidence that an early increase in extracellular glutamate is due to vesicular release, whilst a late phase is due to reversal of the Na^+ dependent transporter. Jabaudon et al (2000) show that uptake inhibition is also an early event and add to an already convincing body of data that later accumulation is due to transporter reversal.

We have suggested that an oxidative stress at the beginning of reperfusion is responsible for dysregulation of glutamate homeostasis. This is supported by Tretter et al (2002) who provide evidence that the Na^+ loading observed during ischaemia followed by the oxidative stress at the start of reperfusion determines the extent of glutamate release during reperfusion. This would suggest a role for the dysregulation of Na^+ glutamate transporters. Glutamate transporters have been shown to be vulnerable to oxidative stress and therefore allow an extracellular accumulation of glutamate (Trotti et al 1998).

It would be our intention therefore to explore the possibilities of vesicular release using botulinum toxin (an inhibitor of exocytosis) and transporter involvement using DL-*threo*-β-benzyloxyaspartate (TBOA), which inhibits all classes of glutamate transporters mentioned in section 1.2.2. We would also try to correlate the prevention of glutamate accumulation with an attenuation of cell death. This directly tests our hypothesis that early glutamate release stimulates further release and cell death. Finally as a further test of this hypothesis, we would attempt to inhibit glutamate receptor mediated damage through the inhibition of the NMDA receptor and the mGluR1 receptor.

7.4 Conclusion

The current thesis has largely been designed to establish a reproducible model of ischaemia within a tissue culture environment such that astrocyte-neuronal cross talk could be explored. We have successfully met this aim and have laid the foundations for an investigation into the specific elements important to astrocyte-communication following an ischaemic insult.

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