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The Mechanisms of Hypoglycaemia-Induced Cell Damage in the Striatum

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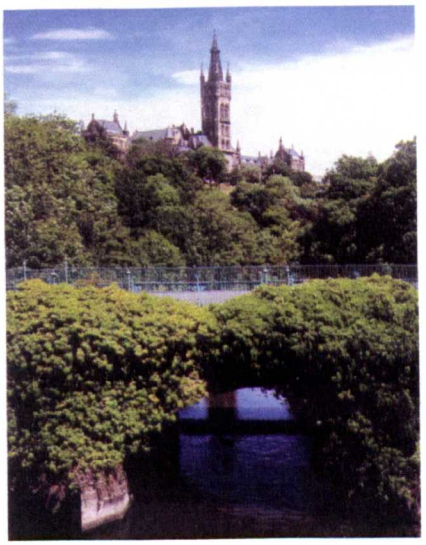

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APPENDICES

Publications.

C.J.McDermott, A.M.Palmer, B.J.Morris. 2000. Lack of neuroprotection by trifluoperazine and cyclosporin A in striatal neurones exposed to hypoglycaemic neurotoxicity. European Journal of Neuroscience; 12(11); p234 (110.15 Abstract).

C.J.McDermott, A.M. Palmer, B.J. Morris. 2000. Effect of Cyclosporin A against toxicity caused by hypoglycaemia in striatal neurones. Restorative Neurology and Neuroscience; 16 (1); p57 (Abstract).

C.J.McDermott, A.M.Palmer, B.J.Morris. 2001. Sustained recovery of striatal neurones following 24 hours of hypoglycaemia. British Neuroscience Association Abstract; 16; p126.

Declaration

I declare that all the work in this thesis was carried out by myself, except where referenced and including the Fura-2 experiments which were completed by Dr Karen Bradley, and that it has not been submitted for any previous higher degree.

Abbreviations.

Aβ	- Amyloid-beta
ABC Reagent	- Activin Biotinylate Conjugate Reagent
ACSF	- Artificial Cerebral Spinal Fluid
AD	- Alzheimer's disease
ADP	- Adenosine diphosphate
AIF	- Apoptosis-inducing factor
AMPA	- α-amino-3-hydroxy-5-methyl-isoxazole-propionate
ANOVA	- Analysis of variance
2APB	- 2-Aminoethoxydiphenylborate
APV	- D-2-amino-5-phosphonovalerate
AP7	- D-2-amino-7-phosphonoheptanoic acid
Apaf-1	- Apoptotic protease-activating factor-1
ATP	- Adenosine triphosphate
BAPTA	- 1,2 bis (o-aminophenoxy)ethane-N,N,N,N-tetraacetic acid
Bax	- Bcl-2-associated X-protein
Bcl-2	- B-cell lymphoma-2
Bcl-x_L	- B-cell lymphoma-XL
Bid	- BH3-interacting domain
BER	- Base excision repair pathway
$[Ca^{2+}]_i$	- Intracellular calcium concentration
CAD	- Caspase-activated deoxyribonuclease
cADP	- Cyclic adenosine diphosphate

cAMP	- Cyclic adenosine monophosphate
cGPx	- Classical glutathione peroxidase
CIBT	- Cold injury-induced brain trauma
CO_2	- Carbon dioxide
CNS	- Central nervous system
CsA	- Cyclosporin A
DA	- Dopamine
dATP	- Deoxyadenosine triphosphate
DDT	- 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane
DFF	- DNA fragmentation factor
dH_2O	- Deionised water
DMEM	- Dulbecco's modified essential medium
DMSO	- Dimethyl sulfoxide
DNA	- Deoxyribonuclease
DYN	- Dynorphin
EAA	- Excitatory amino acid
EDTA	- Diaminoethanetetra-acetic acid
EEG	- Electroencephalogram
EGTA	- Ethyleneglycol-bis(β-aminoethyl)-N,N,N,N-tetraacetic acid
EMSA	- Electrophoretic mobility shift assay
ENK	- Enkephalin
ER	- Endoplasmic reticulum
ERCC3	- Excision-repair cross-complementing gene 3
FAD	- Flavin-adenine dinucleotide (oxidised form)

$FADH_2$	- Flavin-adenine dinucleotide (reduced form)
FCI	- Focal cerebral ischaemia
GABA	- Gamma-aminobutyric acid
GDP	- Guanine diphosphate
GTP	- Guanine triphosphate
GPl	- Globus pallidus lateral
GPm	- Globus pallidus medial
HeLa	- Human cervix carcinoma
HD	- Huntington's disease
HEPES	- N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HIV	- Human Immunodeficiency Virus
HNE	- 4-Hydroxy-2-nonenal
H_2O_2	- Hydrogen peroxide
IAP	- Inhibitors of apoptosis
ICAM	- Inducible cellular adhesion molecule
ICE	- Interleukin-1 β-converting enzyme
IκB	- Inhibitory kappa B
IKK-1	- IκB kinase-1
IKK-2	- IκB kinase-2
IL-1β	- Interleukin 1
IP_3	- Inositol (1, 4, 5)-trisphosphate
iNOS	- Inducible nitric oxide synthase
K-ATP channels	- ATP-sensitive potassium channels
KCl	- Potassium chloride

LPS	- Lipopolysaccharide
LTP	- Long term potentiation
MAP2	- Microtubule-associated protein 2
MEM	- Minimal essential modified
MgCl	- Magnesium chloride
MK-801	-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohept-5,10-iminemaleate
Mn-SOD	- Manganese superoxide dismutase
MPP+	- 1-methyl-4-phenylpyridinium
MSH2	- Human MutS homolog
MTP	- Mitochondrial permeability transition pore
mGluR	- metabotropic glutamate receptors
mRNA	- Messenger ribonucleic acid
MTT	- 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide thiazole blue
NAD	- Nicotinamide-adenine dinucleotide (oxidised form)
NADH	- Nicotinamide-adenine dinucleotide (reduced form)
Na^+/K^+-ATPase pump	- Sodium-potassium ATPase dependent pump
NaCl	- Sodium chloride
NADPH	- Nicotinamide adenosine dinucleotide phosphate
NBQX	- 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione
NCAM	- Neuronal cell adhesion molecule
NER	- Nucleotide excision repair pathway
NFκB	- Nuclear factor kappa B
NGF	- Nerve growth factor

NIK - NFκB-inducing kinase

NLS - Nuclear localisation signal

NMDA - N-methyl-D-aspartate

NO - Nitric oxide

NOS - Nitric oxide synthase

PARP - Poly ADP-ribose polymerase

PBS - Phosphate-buffered saline

PBN - α-phenyl-t-butyl nitrone

PC12 - Rat adrenal phenochromocytoma

PD - Parkinson's disease

PDTC - Pyrrolidinedithiocarbamate

PET - Positron emission tomography

PFA - Paraformaldehyde

PHGPx - Phospholipid hydroperoxide glutathione peroxide

PKA - Protein kinase A

PKC - Protein kinase C

PMA - Phorbo-myra-acetate

PMS - Phenazine methosulphate

RHD - Rel homology domain

ROD - Relative optical density

ROS - Reactive oxygen species

SERCA - Sarco(endo)plasmic reticulum ATPase

SNc - Substantia nigra compacta

SNr - Substantia nigra reticular

STN	- Subthalamic nucleus
TCA	- Tricarboxylic acid cycle
TdT	- Terminal deoxynucleotidyl transferase
TE	- Tris-EDTA
TBE	- Tris Boric acid EDTA
TFP	- Trifluoperazine
TMB-8	- 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate
TNF	- Tumor necrosis factor
TUNEL	- Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling
VGCC	- Voltage-gated calcium channels
VIP	- Vector peroxide substrate
XRCC1	- X-ray repair cross-complementing group 1
XTT	-Sodium(2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2*H*–tetrazolium -5-carbxanilide
Z-DEVD-FMK	- Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-FMK

Abstract

Glucose deprivation involved in hypoglycaemia has been associated with neurotoxicity and cell death. It is hypothesised that this neurotoxic process is initiated by a fall in cellular ATP concentration and a dysfunction of the Na^+/K^+ ATPase pump. Consequently the plasma membrane depolarises, opening the VGCC and allowing an excessive influx of calcium, which initiates glutamate release, ROS generation and the opening of the MTP. This intracellular activity subsequently triggers the apoptotic machinery necessary to promote irreversible cell death.

In this study, primary cultures of embryonic rat striatal neurones were exposed to hypoglycaemia for periods between 1 hour and three days. Mitochondrial respiratory function and cytoskeletal integrity were affected. However several observations were found that conflicted with the general consensus of the mechanisms involved in hypoglycaemia-induced cell death. Evidence was obtained that there was :-

1. No calcium influx upon hypoglycaemia, indicating that the cell membrane does not depolarise

2. No glutamate toxicity

3. No ROS toxicity

4. No MTP involvement

5. DNA fragmentation independent of caspase activity

6. Reversal of cell damage upon the replacement of glucose

7. A decrease in intracellular calcium concentration upon glucose replacement

These data suggest that the removal of glucose from striatal cultures does not cause cell death but triggers the cell to enter a quiescent state with sufficient energy to maintain resting membrane potential but also with morphological, mitochondrial and DNA modifications. In conclusion striatal cells possess a neuroprotective mechanism against prolonged glucose deprivation and remarkably can recover metabolically with repaired DNA.

Chapter One. Introduction

1. Cell Death

In contrast to the rapid turnover of cells in proliferative tissues, neurones commonly survive for the entire lifetime of the organism. This enduring nature of neurones is necessary for maintaining the function of cells and as a consequence cell death must be regulated and controlled. However, acute insults associated with ischaemia and traumatic brain injury and neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease (HD) and Parkinson's disease (PD) are connected with substantial loss of neurones. Indeed, understanding the mechanisms which lead to neurodegeneration is vital in preventing unwanted cell death. Ultimately, this understanding will provide targets for pharmacological intervention to improve the clinical outcome of patients suffering from neurodegenerative disorders and brain injury.

The type of cell death depends on many factors, namely, cell type, the triggering stimuli, the severity of the stimulus and the age of the tissue (Martin *et al.*, 2000). Numerous signal transduction pathways which are engaged in cell death are highly influenced by these factors, however, it has not yet been illustrated whether these signal pathways converge onto a specific target, universal for all cell types. In neuronal cells excitotoxicity is a well established and documented mechanism and refers to the cytotoxic action, mediated by excessive stimulation of excitatory amino acid (EAA) receptors (Olney and de Gubareff, 1978; Choi and Rothman, 1990; for review, Meldrum and Garthwaite, 1990). Evidence strongly suggests that excitotoxicity is the predominant mechanism of cell death in numerous acute and chronic neurodegenerative diseases (Choi, 1988). The precise pathway remains to be clarified, although it is apparent that the presence of a high concentration of EAAs and an increased sustained level of intracellular calcium are essential components (Choi, 1988).

Two pathways leading to cell death were characterised and termed apoptosis and necrosis. They were originally thought of as distinct but recent evidence suggests that these pathways can interact (Colborne *et al.*, 1999- see section 1.4). Both apoptosis and necrosis can be induced by the same agents, and have been observed in excitotoxicity-mediated cell death (Bonfoco *et al.*, 1995; Qin *et al.*, 1998). Necrotic cell death occurs in the early period of acute intense insults, whereas cell death with apoptotic characteristics

occurs with less intense insults and at much later stages after the toxic event (Bonfoco *et al.*, 1995; Kerr *et al.*, 1972; Wyllie *et al.*, 1980).

1.1 Necrosis

Necrosis initiated by toxic insults causes internal energy failure within the cell with passive cell swelling and as a result membrane lysis occurs. Typical changes include swelling of the cytoplasm and organelles, especially the mitochondria, defects in membrane permeability and ion transport proteins as well as impairments in oxidative phosphorylation. Only minor changes in the nucleus are evident with chromatin clumped in irregular configurations (Kerr *et al.*, 1995). The cell's contents are released, such as reactive oxygen species and this is accompanied by an inflammatory response, which includes leukocytic infiltration and tissue oedema. Consequently surrounding cells die (Grooten *et al.*, 1993).

1.2 Apoptosis

Apoptosis is derived from the Greek word describing the process of leaves falling from trees, or petals falling from flowers. It is a fundamental requirement for embryogenesis, organ metamorphosis and tissue homeostasis (Imai *et al.*, 1999), and can be described as a programmed process as it is regulated at protein and gene level.

The concept of apoptosis was introduced 29 years ago (Kerr *et al.*, 1972). It occurs in the nervous system under both physiological and pathological conditions. During development, for example, a significant proportion of neurones die by apoptosis to permit matching cell numbers with their targets (Oppenheim, 1991). Apoptotic cell death has also been described after acute insults to the brain, such as traumatic brain injury (Rink *et al.*, 1995) and ischaemia (Li *et al.*, 1995b; Krajewski *et al.*, 1995; Chen *et al.*, 1996), as well as in chronic degenerative conditions, such as PD, AD and HD (Hartley *et al.*, 1994; Portera-Cailliau *et al.*, 1995, Mattson *et al.*, 2000a).

The morphological and molecular characteristics of apoptosis are distinguishably different from necrosis. The main features are cell shrinkage, membrane blebbing, chromatin condensation and genomic fragmentation. Unlike necrosis, the external and internal membranes of cells undergoing apoptosis are preserved, therefore safely sealing the cell contents and protecting the surrounding region until phagocytosis occurs. In tissue where apoptosis has occurred, intermingling healthy and dying cells are observed.

Apoptotic signalling includes three stages: signal induction, propagation via a protease cascade, and execution. The signal induction stage varies with the stimulus, but converges on common propagation and execution stages. The end result of apoptosis is degradation of various cellular proteins, such as nuclear lamins and cytoskeletal components, and the breakdown of the DNA into internucleosomal fragments. Ultimately, the cell and its contents are broken down to membrane-bound fragments that are phagocytosed by adjacent cells (Hale *et al.*, 1996; Rosen and Casciola-Rosen, 1997).

The propagation stage of apoptosis is complex and requires specific mediators depending on the death-inducing stimulus and cell type (Woo *et al.*, 1998). The cytoplasmic compartment of cells contains the core apoptosis machinery in a latent or sequestered form (Takahashi and Earnshaw, 1997), and it has become apparent that the activation of this machinery depends on a multiple post-translational mechanism (For review, Porter, 1999).

1.2.1 Caspases

Caspases are synthesised as inactive proenzymes that require proteolytic activation. Activation of one caspase can lead to the cleavage and activation of additional molecules of the same or other proteases, leading to an amplified protease cascade (Tewari *et al.*, 1995). The caspase family are thought to be the fundamental regulators of apoptotic cell death. Many of their genes were originally identified in the nematode *Caenorhabditis elegans*, in which three genes, *ced-3* (ced, cell-death abnormal), *ced-4*, *ced-9* are thought to play critical roles (Ellis and Horvitz, 1986). The genetic and environmental factors that trigger neuronal apoptosis may be different in various physiological and pathological settings, but the core of this execution program is highly conserved from worm to human.

The caspase family has been divided into three major components: the B-cell lymphoma-2 (Bcl-2) family proteins, the cysteine aspartic proteases (caspases), and the apoptotic protease-activating factor-1 (Apaf-1) protein that relays the signals integrated by Bcl-2 family proteins to the caspases (Adams and Cory, 1998).

The first caspase, initially discovered as a cytokine-processing enzyme, was designated interleukin-1 β-converting enzyme (ICE). Since then over 14 new caspases have been cloned (Thornberry and Lazebnik, 1998). Caspase-1 and caspase-11 have been shown to function mainly in cytokine production (Li *et al.*, 1995a), whereas caspase-2, -3, -6, -7, -8, -9, -10 and -12 are involved in the regulation and execution of apoptosis (Kuida *et al.*, 1996; Hakem *et al.*, 1998).

When cells undergo apoptosis, these caspases become activated through one or two sequential proteolytic events that cleave the single peptide precursor into large and small fragments. This forms the active enzyme which is subsequently highly regulated (Thornberry and Lazebnik, 1998). There are currently two well characterised caspase-activating cascades that regulate apoptosis: one is initiated from the cell surface death receptor activated by ligands such as tumour necrosis factor-α (TNFα) and the other is triggered by changes in mitochondrial integrity (for review, Konopleva and Andreef, 1999; Depraetere and Golstein, 1998; Song and Steller, 1999).

Some caspases are activated during the early phase of apoptosis. These upstream caspases, such as caspase-6 and 12, can then activate "effector" caspases such as caspase-3 either directly or indirectly. These effector caspases can then activate endonucleases that cleave DNA into oligonucleosome-sized fragments (Arends *et al.*, 1990; Nagata, 2000), and can also cleave various substrate proteins that may co-ordinate the cell death process (Lazebnik *et al.*, 1994; Lazebnik *et al.*, 1995).

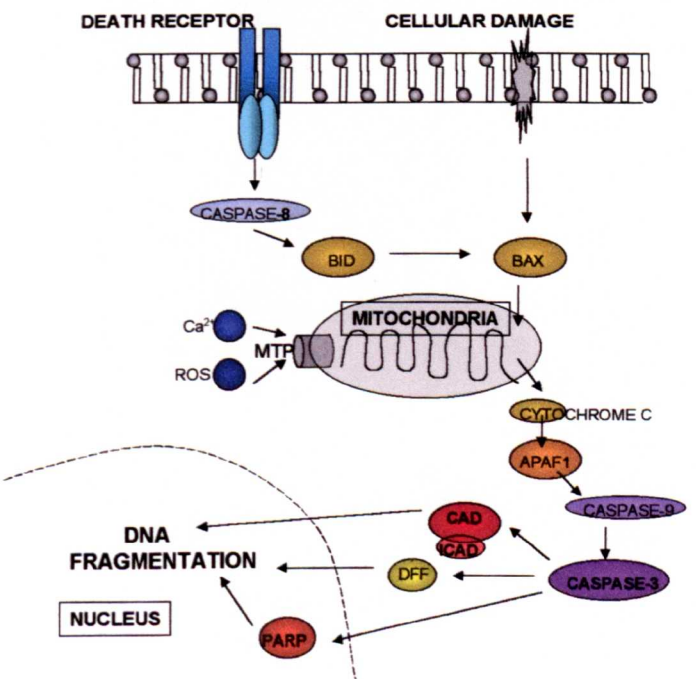

Figure 1.1

Pathways involved in apoptosis. Following signals via either activation of death receptors or cellular damage, upstream regulators caspase-8, Bid and Bax are triggered. Bax translocates to the mitochondria initiating the release of cytochrome c which binds to APAF1 and triggers caspase-9. Calcium ions and ROS can also cause the release of cytochrome c by activating the MTP. Caspase-9 triggers the activation of caspase-3. Caspase-3 has various substrate which ultimately cleaves the nuclear DNA, such as PARP, DFF and CAD.

The caspase cascade triggered by alteration of the mitochondria has been intensely investigated. This cascade begins with Bax activation and is followed by cytochrome c release, Apaf-1 activation, the cleavage of caspase-9 and the activation of caspase-3. This cascade has been confirmed *in vivo*, as shown by the recent results from the gene knockout experiments. In Apaf-1 knockout mice, caspase-9 and caspase-3 cannot be activated in response to various apoptotic stimuli, even though cytochrome c release still occurs. Likewise, caspase-3 activation is abolished in caspase-9 knockout mice (Hakem *et al.*, 1998; Kuida *et al.*, 1998; Yoshida *et al.*, 1998; Cecconi *et al.*, 1998).

1.2.1.1 Bax

The Bcl-2 family of proteins includes both pro-apoptotic and anti-apoptotic members (Pellegrini and Strasser, 1999). The best-studied anti-apoptotic member is Bcl-2. An overexpression of Bcl-2 in cell cultures and in transgenic mice increases resistance of neurones to death induced by excitotoxic, metabolic and oxidative stress (Martinou *et al.*, 1994; Guo *et al.*, 1998). The mechanism by which Bcl-2 proteins control cell death is not clear, but it may involve interactions among other family members or associations with mitochondria, resulting in altered ion movements across mitochondrial membranes (Green and Reed, 1998).

Bcl-2-associated X-protein (Bax) is the best characterised pro-apoptotic Bcl-2 family member. Bax translocates to the mitochondria, inducing mitochondrial dysfunction and caspase activation, hence neurones lacking Bax are protected against apoptosis (White *et al.*, 1998). Various apoptotic stimuli can induce the translocation of monomeric Bax from the cytosol to mitochondrial membrane (Hsu *et al.*, 1997), where it inserts as a homodimer (Gross *et al.*, 1998). It is thought that this stimuli can trigger a conformational change, exposing the N- and C- termini of Bax, that appears to be required for its insertion into mitochondria (Nechushtan *et al.*, 1999). It is unclear whether Bax homodimerisation is a cause of its translocation or a consequence of its insertion nevertheless, dimerisation is strongly associated with cell death. Bax has been shown to directly induce the release of cytochrome c from isolated mitochondria in a cell-free system and in K^+- or serum-deprived cerebellar granular cells (Desagher *et al.*, 1999; Rosse *et al.*, 1998). The mechanism underlying the function of Bax is not clear, although there is evidence that Bax possesses ion channel-forming activity which is inhibited by Bcl-2 (Schlesinger *et al.*, 1997).

1.2.1.2 Cytochrome c

Cytochrome c is a 12kDa protein that functions in the mitochondrial electron transport chain. It is a soluble protein that is localised in the mitochondrial intermembrane space and is loosely attached to the surface of the inner mitochondrial membrane (Gonzales and Neupert, 1990). At physiological ionic strength, cytochrome c diffuses in the aqueous phase between the inner and outer membrane of the mitochondria, between complex III and complex IV (Gupte and Hackenbrock, 1988).

Cytochrome c has been described as a necessary component for apoptosis (Kuida *et al.*, 1996). The mechanism by which cytochrome c is released from mitochondria remains to be determined, although it is thought that the mitochondrial permeability transition pore is involved (see section 1.3). In addition, it has been reported that cytochrome c can be released from the mitochondria early in apoptosis before mitochondrial depolarisation and the activation of caspases (Kluck *et al.*, 1997; Kroemer *et al.*, 1998).

1.2.1.3 Caspase-9

Once released cytochrome c binds to its two cofactors, Apaf-1 and procaspase-9, which are both cytosolic proteins (Srinivasula *et al.*, 1998). Apaf-1 binds to ATP and hydrolyses it to ADP. This hydrolysis, however, does not have any function consequently if cytochrome c is absent (del Peso *et al.*, 1997). Likewise, cytochrome c will bind Apaf-1 in the absence of ATP. However, this complex is unstable and inactive. In contrast, in the presence of cytochrome c, the binding and hydrolysis of ATP/dATP promote the formation of a multimeric Apaf-1/cytochrome c complex. This multimeric complex is fully functional in recruiting and activating procaspase-9 (Zou *et al.*, 1999; Srinivasula *et al.*, 1998; Li *et al.*, 1997). Activated caspase-9 is subsequently released from this complex to cleave and directly activate downstream caspases such as caspase-3, and –7. In addition, caspase-9 is capable of indirectly activating caspase -2, -6, -8 and 10 (Green, 1998; Zou *et al.*, 1997; Slee *et al.*, 1999).

The activation of caspase-9 can be regulated by two possible mechanisms. Firstly, anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L that are tethered to the outer mitochondrial membrane, could prevent cytosolic Apaf-1/procaspase-9 activation indirectly by blocking the translocation of cytochrome c from the intermembrane space (Green, 1998). Alternatively, it has been reported that Bcl-x_L might inhibit caspase-9 activation directly by binding to Apaf-1 (Adams and Cory, 1998).

1.2.1.4 Caspase-3

Caspase-3 proenzyme is proteolytically processed by caspase-9 (Li *et al.*, 1997). It has been proposed to be the main effector of apoptosis in neurones and astrocytes (Keane *et al.*, 1997). Significantly, apoptosis is completely absent in certain tissues of caspase-3 knock-out mice; during development within the CNS, massive hyperplasia with distortion of cytoarchitecture was evident by embryonic day 12 (Kuida *et al.*, 1996). This phenotype was attributed to a lack of cell death.

Caspase-3 can cleave specific cellular proteins. These include protein kinase C (Hugunin *et al.*, 1996), DNA-dependent protein kinase (Han *et al.*, 1996), and sterol regulatory element binding protein (Wang *et al.*, 1996b), as well as the cytoskeleton component actin (Mashima *et al.*, 1997). The DNA binding enzyme poly ADP-ribose polymerase (PARP) is certainly the best characterised substrate for caspase-3. PARP is a nuclear enzyme that modifies cellular proteins in response to DNA damage (Kaufmann *et al.*, 1993; Casciola-Rosen *et al.*, 1995). Its cleavage by caspase-3 may interfere with DNA repair mechanisms that would otherwise counteract apoptotic DNA degradation (Martin and Green, 1995). Likewise, cleavage of cytoskeletal proteins such as actin, may cause membrane blebbing and cellular fragmentation, leading to the appearance of apoptotic bodies (Martin and Green, 1995). Caspase-3 may promote cell death via the activation of caspase-activated deoxyribonuclease (CAD), a key DNA-cleavage enzyme responsible for DNA fragmentation during apoptosis. It is suggested that caspase-3 activates CAD by cleaving and releasing the CAD inhibitory protein that normally binds CAD (Enari *et al.*, 1998).

1.3 Mitochondria and the Mitochondrial Permeability Transition Pore (MTP)

Mitochondrial changes are pivotal in the cell death decision in many cases (Kroemer *et al.*, 1998). Increasing evidence suggests that the mitochondrion is a point of convergence for death stimuli, and is a necessary step in apoptosis. Signals triggered from extracellular stimuli cause mitochondrial membrane depolarisation and trigger the release of apoptotic-inducing proteins from the mitochondria (Green and Reed; 1998; Susin *et al.*, 1996; Zamzami *et al.*, 1996a).

Mitochondria are the main source of ATP and play a central role in cellular homeostasis. A membrane potential maintained across their inner membrane by the transport of hydrogen ions from the internal (matrix) space to the intermembrane space, is important in maintaining a pore, termed the membrane permeability transition pore (MTP), in a closed

position. The MTP may be of crucial importance in the development of cell damage and has been characterised in isolated mitochondria (Hunter and Haworth, 1979), *in vitro* (Kristal and Dubinsky, 1997) and *in vivo* (Fennel et al., 2001).

The pore is voltage dependent and acts as a tightly regulated inner membrane channel to permit the passage of molecules up to 1500 Da in size. Its composition is not yet defined although it is thought to be associated with adenine nucleotide translocase of the inner membrane, the matrix specific cyclophilin D (Halestrap *et al.*, 1998) and porin of the outer mitochondrial membrane.

Mitochondria maintain, and precisely regulate, intracellular calcium homeostasis by sequestering and releasing calcium. If cytosolic calcium concentrations become too high, as following excessive neuronal firing or severe energy shortage, the subsequent mitochondrial calcium overload will initiate the opening of the MTP pore (Zoratti and Szabo, 1995). This calcium-dependent formation of the MTP pore represents an abrupt increase of permeability to solutes normally impermeable to the inner membrane, causing osmotic swelling and rupture of the outer mitochondrial membrane, with concomitant loss of Ca^{2+}, K^{+}, Mg^{2+} and mitochondrial proteins (Green and Reed, 1998; Kroemer *et al.*, 1998). In addition, oxidant chemicals have been shown to promote the onset of the MTP. Thus, the importance of the MTP in the propagation stage of apoptotic signalling is substantial, given that MTP opening could potentially be the principal mechanism involved in the release of cytochrome c (Zamzami *et al.*, 1997; Kroemer, 1997).

1.4 The Apoptosis-Necrosis Continuum

It has recently been argued that there are more than two forms of cell death and that apoptosis and necrosis are not mutually exclusive but rather they exist as a continuum of cell death phenotypes (Portera-Cailliau *et al.*, 1995; Portera-Cailliau *et al.*, 1997; Martin *et al.*, 1998: Roy and Sapolsky, 1999). This hypothesis arose from observations that features of each type of cell death coexisted in the excitotoxically injured adult striatum (Portera-Cailliau *et al.*, 1995).

Morphologically distinct types of apoptosis have been described which differ in their molecular determination. Unlike "classical apoptosis", where DNA fragmentation and chromatin condensation are principal outcomes, a form of apoptosis where only slight chromatin condensation occurs and vacuolisation is the primary event has been reported

(Zakeri *et al.*, 1995). It is suggested that these morphologies are two extreme situations and the intermediate apoptotic-like situations could exist.

Other evidence suggests that neuronal death can still occur despite the fact that DNA fragmentation is prevented and chromatin condensation is partially blocked by caspase-inhibitors (Miller *et al.*, 1997). In another model, overexpression of Bax initiated potentially damaging processes such as membrane blebbing, decreased protein synthesis, and even a slight condensation of the chromatin, but DNA fragmentation, cleavage of caspase substrates such as PARP and late stages of chromatin condensation were blocked (Xiang *et al.*, 1996).

It is also interesting to note that apoptotic activity occurs outside cell death. For example, caspase-1 cleaves the precursor of IL-1β to its mature form (Thornberry *et al.*, 1992) as well as having a role in apoptosis (Miura *et al.*, 1993). Also, caspase-3 activation has been observed upon activation of peripheral T cells in the absence of cell death (Miossec *et al.*, 1997) and in lens cells, the activation of caspases is apparently required for nuclear disintegration and does not produce death (Ishizaki *et al.*, 1998).

It is therefore evident that there are multiple pathways leading to cell death, and some cells may have the required components whilst others may not, hence, care should be taken when classifying mechanisms of cell death (Sloviter, 2002).

1.5 The Dependence of Cell Death on ATP

The existence of apoptotic cells adjacent to necrotic cells suggests that common microenvironmental signals may be involved in both forms of cell death (Martin *et al.*, 1998). It is thought that availability of ATP is the primary factor within the tissue that determines whether apoptosis or necrosis takes place, i.e. cell death within the tissue is determined by the energy supply (Richter *et al.*, 1996; Eguchi *et al.*, 1997; Leist *et al.*, 1997). Indeed, in cells where both necrosis and apoptosis can occur, apoptosis fails when ATP levels drop below 15-25% of control cells (Lieberthal et al. 1998). In addition, a number of reports have demonstrated that necrosis is dominant in severe ischaemic cases where ATP levels are completely abolished, whereas apoptosis dominates in milder insults, where ATP is still present (Stroemer and Rothwell, 1998; Nakajima *et al.*, 2000). Therefore the concentration of ATP within the tissue is the determining factor in the nature of the cell death mechanism.

In a study to determine at which stage of apoptosis ATP was required, oligomycin, a blocker of mitochondrial ATP synthesis, was added at different times after triggering cell death with staurosporin. It was deduced that ATP was required immediately before the characteristic nuclear changes, perhaps to ensure that nuclear collapse, the packaging of chromatin, and DNA degradation occur before cell lysis (Leist *et al.*, 1997). Alternatively, it is possible that the reduction of ATP inhibits the Apaf-1-mediated activation of procaspase-9. However, it is not known whether the necrotic pathway observed under low ATP conditions is caspase-independent (Kawahara *et al.*, 1998).

2 Hypoglycaemic Neurotoxicity

The effects of hypoglycaemia on the brain have been studied since the early 1930s, almost a decade after the discovery of insulin by Frederick Banting (Pillai and Panchagnula, 2001). The brain is uniquely dependent on the availability of glucose as essentially all of it's metabolism is derived from plasma glucose rather than alternative substrates (Mobbs *et al.*, 2001; Sokoloff, 1992). Glycolysis is the main source of ATP hence when glucose is removed a decrease in ATP is observed (Kahlert and Reiser, 2000). ATP is the cell's source of energy, whereby all energy-requiring reactions utilise ATP. ATP levels have shown to decrease in hypoglycaemia in *in vitro* and *in vivo* models (Mattson *et al.*, 1993; Madl and Royer, 1999; Santos *et al.*, 1996; McGowan *et al.*, 1995). The brain utilises mainly glucose as a substrate for the generation of the ATP required for normal function. Since the brain cannot store significant carbohydrates, a steady glucose supply is required from the blood. Glucose transport into the brain is mediated by a facilitative-diffusion-type transport system. A constant availability of blood glucose must be within a specific concentration range (~5mM) (Oster and Singer, 1999). If blood glucose levels in the brain fall to the range of 2.0-2.5mM, a slowing of brain activity occurs indicated by a slowing of the electroencephalogram (EEG), but if levels fall below 1μM, brain ATP levels decrease to an extent where metabolic changes occur leading to permanent damage (Agardh *et al.*, 1981; Wieloch *et al.*, 1984), including the degradation of phospholipids, nucleic acids and nucleotides (Agardh *et al.*, 1981; Chapman *et al.*, 1981) and an elevation in free fatty acids.

Acute hypoglycaemia in diabetes patients is associated with neurological manifestations such as dizziness, behavioural changes, seizures, stupor, coma, or hemiparesis (Malouf and Brust, 1985). These may be transient, but irreversible neuronal damage develops when hypoglycaemia is severe and prolonged (Agardh *et al.*, 1982; Kalimo *et al.*, 1985). Hypoglycaemia is also particularly common in the first few hours after birth (Land *et al.*, 1994).

The phenomenon of hypoglycaemia-induced toxicity is of clinical importance, as it not only accounts for the neurological outcome of insulin overdose in diabetic patients, but is likely to contribute profoundly to the brain damage associated with ischaemia and neurodegenerative diseases. Moreover, it has been reported that the distribution of hypoglycaemia-induced damage to the caudoputamen is only marginally different from the distribution of damage via an ischaemic insult (Kalimo *et al.*, 1985). It is therefore

beneficial to consider data from all energy-deficiency models, such as ischaemia and brain trauma, when studying hypoglycaemia, in order to gain a wider regard for events occurring in neuronal damage and protective mechanisms which may be employed. However, one must be cautious when comparing data from different energy failure models as particular differences are sometimes quite profound, such as ATP levels (Norberg and Siesjo, 1976; Agardh *et al.*, 1981; Wieloch *et al.*, 1984), pH of the tissue environment (Pelligrino, 1981; Siesjo, 1981) and differences in amino acid release (Auer and Siesjo, 1988).

It is known that particular brain regions are more vulnerable to hypoglycaemic damage than others. The cerebral cortex, the hippocampus and the striatum have all shown to be predominately susceptible to metabolic failure (Kiessling *et al.*, 1984; Agardh *et al.*, 1981).

2.1 The Theory of Hypoglycaemia-induced Cell Death

A hypothesis of the mechanisms involved in hypoglycaemia-induced cell death has been proposed, (Figure 1.2), in order to devise a model for the basis of designing drugs to prevent distinct cellular events occurring which initiate irreversible cell damage (Siesjö, 1992; Siesjö, 1993).

This hypothesis proposes that: the depletion of glucose availability from neurones causes a decrease in ATP levels, which results in an insufficient amount of energy required for the function of the sodium-potassium ATP-dependent pump (Na^+/K^+-ATPase pump). The Na^+/K^+-ATPase pump maintains the membrane at resting potential, allowing an equilibrium between internal and external concentrations of both Na^+ and K^+. The maintenance of this equilibrium consumes ~40% of energy production in the brain, a surprisingly large percentage part of the total (Astrup *et al.*, 1981; Whittam, 1962). A depletion in ATP, therefore, causes a collapse of ion gradients and as a consequence the cell gains sodium and simultaneously loses potassium, causing the membrane to depolarise and the voltage-dependent ion channels to open (Harris *et al.*, 1984; Szatkowski and Attwell, 1994; Martin *et al.*, 1994) (see Figure 1.2). An opening of the voltage-gated calcium channels causes an influx of calcium which increases intracellular calcium levels that then can trigger a multitude of events, such as the release of excitatory neurotransmitters (Garthwaite and Garthwaite, 1986), activation of calcium-dependent kinases (Favaron *et al.*, 1990) and phospholipases (Farooqui *et al.*, 1991), nitric oxide production (Alagarsamy *et al.*, 1994), formation of free radical compounds (Sun *et al.*, 1979), activation of calcium-dependent endonucleases (Arends *et al.*, 1990) and lethal

alterations in cytoskeletal organisation (Schlaepfer and Zimmerman, 1985). As a result various cascades are triggered which can cause permanent dysfunction of neurones leading eventually to their death. How soon this depolarisation occurs depends upon factors such as the type of insult, the severity of insult (Jiang and Haddad, 1992) and temperature (Taylor and Weber, 1993).

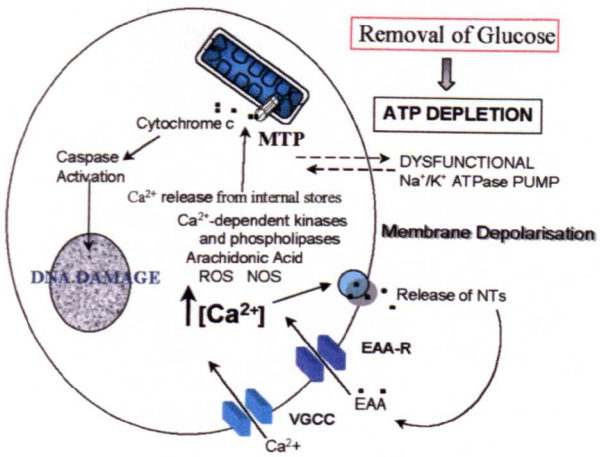

Figure 1.2

A schematic representation of events occurring in neurones subjected to a hypoglycaemic insult. Upon the removal of glucose the ATP concentration decreases, the Na^+/K^+ ATPase pump becomes dysfunctional and the plasma membrane depolarises. Calcium enters via the VGCCs initiating numerous cascades, including the release of EAA, activation of calcium-dependent kinases and phospholipases, release of calcium from internal stores, the formation of arachidonic acid, nitric oxide and free radicals and triggering apoptosis via MTP activation and caspase activity.

2.2 Calcium

Transient changes in intracellular calcium levels are very important in intracellular signalling. Fluctuations in cytoplasmic calcium levels in neurones have been shown to regulate neuronal excitability, neurotransmitter release, metabolic reactions and gene expression (For review: Verkhratsky and Peterson, 1998; Mattson *et al.*, 2000b). However, it is understood that the excessive increase in intracellular calcium concentration ($[Ca^{2+}]_i$), either as a result of influx of extracellular calcium through plasma membrane voltage and receptor operated calcium channels, or by the release from calcium stored in

intracellular pools, contribute significantly to cell death (Hass, 1983; Siesjo, 1981; Tymianski and Tator, 1996).

An extensive foundation of research has proved that toxic levels of intracellular calcium play a major role in neuronal cell death. Both *in vivo* and *in vitro* studies of energy-deficiency in models, such as cerebral ischaemia, support the association between calcium influx and damage to neural tissue (Choi, 1985; Deshpande *et al.*, 1987; Silver and Erecinska, 1990; Simon *et al.*, 1984; Uematsu *et al.*, 1989; Uematsu *et al.*, 1988). Glucose deprivation leads to a marked and rapid increase in $[Ca^{2+}]_i$ in cultured hippocampal neurones (Mattson *et al.*, 1993; Knopfel *et al.*, 1990; Tekkök *et al.*, 1999) and cortical neurones (Cheng and Mattson, 1991). An increase in intracellular calcium levels was observed in brains of acutely hypoglycaemic rats (Harris *et al.*, 1984).

Elevated $[Ca^{2+}]_i$ is thought to initiate a trigger for both necrosis (Choi, 1987) and apoptosis (Ojicius *et al.*, 1991) in neurones. The effect of the calcium ionophore, A23187, was investigated using primary cultured cortical cells. Cell death in cells incubated with <1μM of A23187, appeared to show characteristic features of apoptosis (Takei and Endo, 1994; Gwag and Canzoniero, 1999), whereas cell death occurring in cells incubated with higher concentrations showed necrotic characteristics (Emery and Lucas, 1995; Gwag and Canzoniero, 1999). This indicates that the severity of insult and the secondary phenomena precipitated by calcium excess is critical in determining the mechanism of cell death. The most studied secondary phenomena triggered by calcium excess include formation of reactive oxygen species (Dumuis *et al.*, 1988), the formation of nitric oxide (Lipton *et al.*, 1993) and the activation of calcium-dependent enzymes that degrade DNA (Ojicius *et al.*, 1991).

Whether calcium influx leads to irreversible cell damage may depend on the capacity and speed of intracellular calcium buffering systems, the attainment of critical concentrations of calcium in intracellular compartments and the mode of entry. The mechanisms of cell calcium buffering systems are dependable on cell type (Mattson *et al.*, 1989) and species. For example, human cortical neurones are more capable of buffering a calcium load compared to rat neurones (Mattson *et al.*, 1991b).

2.2.2 Intracellular Calcium Stores

Calcium entry from the extracellular space delivers most of the calcium ions for signalling in neurones, however, intracellular sources provide an important source of calcium ions.

The established calcium stores within neurones are the endoplasmic reticulum (ER) and the mitochondria.

The most characterised calcium stores are represented by specialised portions of the ER which are capable of accumulating and storing calcium ions (Pozzan *et al.*, 1994; Kostyuk and Verkhratsky, 1994). ER stores have calcium pumps (SERCA -sarco(endo)plasmic reticulum ATPase) and intraluminal calcium-binding proteins, such as calbindin and calreticulin. An electrochemical gradient towards the cytoplasm can develop as the concentration of calcium in the stores exceeds the concentration outside the store, thus releasing calcium ions into the cytoplasm through calcium release channels.

There are two major groups of calcium release channels that have been characterised (Henzi and MacDermott, 1992). Both are ligand-operated with high calcium permeability and their actions are controlled by specific binding of either inositol (1, 4, 5)-triphosphate (IP_3 receptors) or calcium ions (ryanodine receptors). The IP_3 receptor releases calcium in response to IP_3 resulting from the breakdown of membrane phospholipids initiated by numerous hormones, neurotransmitters and growth factors (Berridge, 1993). The ryanodine receptor is activated in various tissues by caffeine, calcium and cADP ribose (McPherson and Campbell, 1993). Both these receptors are found in overlapping populations of neurones throughout the nervous system, but also exhibit clear differences in relative and in subcellular localisation (Martone *et al.*, 1997).

Alterations of ER-mediated calcium homeostasis are sufficient to induce apoptosis (Lin *et al.*, 1997). Indeed, calcium released from intracellular stores in response to pro-apoptotic stimuli can exacerbate neuronal damage. For example, it has been reported that calcium is released from ryanodine stores in PC12 cells in hypoglycaemic conditions (Chung and Hong, 1998) and the depletion of ER calcium stores has been observed in hippocampal neurones following transient cerebral ischaemia in gerbils (Kohno *et al.*, 1997).

2.3 Glutamate and Glutamate Receptor Activation

Glutamate is a dicarboxylic amino acid and acts as an excitatory neurotransmitter in the CNS (Nakanishi, 1992). It is widely and fairly uniformly distributed within the CNS where it is stored in vesicles within the presynaptic terminal, to be released under normal physiological conditions by calcium-dependent exocytosis when the cell becomes depolarised.

Glutamate binds to membrane receptors, of which there are four distinct classes characterised by different pharmacological profiles and mechanism of action (Dingledine *et al.*, 1988). N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-propionate (AMPA) and kainate glutamate receptors are termed ionotropic, i.e. contain intrinsic membrane cation channels and are named according to the exogenous selective agonists that specifically bind to them. The other class of glutamate receptors is termed metabotropic, as they are coupled to G-proteins which mediate their response through intracellular second messenger systems.

It is widely accepted that excessive elevations in $[Ca^{2+}]_i$ mediate the neuronal degeneration caused by excitotoxic glutamate receptor stimulation (Choi, 1985; Schinder *et al.*, 1996). Early experiments demonstrated that local injections of glutamic acid resulted in cell death in various different brain regions, including the striatum, and this toxicity was a direct effect of EAA ionotropic receptor stimulation (Olney and de Gubareff, 1978). In addition, a low concentration of glutamate was found to kill a large proportion of neurones in culture and calcium entry was shown to be necessary in the initiation of excitotoxic mechanisms (Choi, 1988). Since then numerous studies have confirmed that excessive glutamate receptor stimulation has a deleterious neurotoxic effect (Uematsu *et al.*, 1991; Goldberg & Choi, 1993; Tymianski *et al.*, 1993; Randall and Thayer, 1992; Hansen and Zeuthen, 1981).

In addition to excessive EAA receptor stimulation, during neurotoxic conditions, such as ischaemia, there is an activation of sodium channels which induces the release of glutamate via the reverse transport of glutamate by the glutamate transporter (Taylor *et al.*, 1995). Therefore a higher concentration of glutamate is present within the extracellular space. Recent evidence has also suggested that glutamate release is induced by chloride channel activation induced by cell swelling during hypoxia (Phillis *et al.*, 1997) and that nitric oxide also has the ability to enhance vesicular glutamate release during hypoxic condition (Katchamn and Hershkowitz, 1997).

The importance of metabotropic glutamate receptors (mGluR) in neurotoxicity has recently been exposed. There are currently eight subtypes of mGlu receptors, mGlu1-mGlu8, which are divided into three groups. The activation of Group I mGluRs is likely to have significant effects on the intracellular signalling in neurones exposed to ischaemia or hypoglycaemia. For example, the activation of mGluRs mobilises IP_3, which acts on the IP_3 receptors to release Ca^{2+} from the ER, hence, increasing the $[Ca^{2+}]_i$ concentration (Nakamura *et al*, 2000). However, the activation of mGluRs has been reported to either

contribute to, or protect against, cell death in various systems (Copani *et al.*, 1995; Bond *et al.*, 1998).

Further evidence arises from the demonstration that toxic effects of EAAs were reduced by NMDA receptor antagonists (Goldberg and Choi, 1993). It is apparent that NMDA receptors play a predominant role in excitotoxicity because they have a high permeability to calcium and release calcium from internal stores. However, recent evidence suggests that non-NMDA receptors are also involved in this type of toxicity, especially in cases of chronic neurodegeneration (Nellgard and Wieloch, 1992).

Severe insulin-induced hypoglycaemia causes a marked increase in the release of glutamate and aspartate (Sandberg *et al.*, 1986; Wieloch, 1985b; Butcher *et al.*, 1987; Engelsen *et al.*, 1986). Glutamate and aspartate release has also been shown in *in vitro* models of hypoglycaemia (Williams *et al.*, 1995).

2.4 Mitochondria

Mitochondria act as fast calcium buffers and have recently been thought of as dynamic calcium stores which are directly involved in determining calcium signals. Calcium enters downhill of the electrochemical gradient through a mitochondrial calcium uniporter, which is driven by a huge negative membrane potential (-160mV) created across the inner mitochondrial membrane. The Na^+/Ca^{2+} exchanger as well as the permeability transition pore (MTP), control calcium efflux. The increase in intramitochondrial calcium levels is transient and calcium begins to leak out when $[Ca^{2+}]_i$ recovers down to the resting level after the end of the stimulus.

Calcium is sequestered into the mitochondria matrix, depolarising the mitochondrial membrane potential. As a result ATP synthesis, which is driven by a proton electrochemical gradient generated by the electron transport chain, significantly decreases and may be completely inhibited (Jacobus *et al.*, 1975). It is known that mitochondria sequester calcium from the cytoplasm after glutamate stimulation, and that this mechanism has been reported to fail due to a reduction in ATP synthesis during excessive glutamate stimulation (Budd and Nicholls, 1996). Calcium loading of the mitochondria has also been reported to indicate damage to the plasma membrane calcium extrusion pathways (Ward *et al.*, 2000). If mitochondria continue to sequester intracellular calcium and become eventually overloaded, the opening of the MTP would occur, resulting in the extrusion of mitochondrial calcium (Taylor *et al.*, 1999). The pore opening also uncouples the electron

transport system, resulting in increased free radical production. A drop in cellular ATP levels, coupled with increased generation of free oxygen radicals leads to cell death.

The involvement of the MTP is thought to be of great importance in ischaemia and hypoglycaemic-induced death, because not only does the opening of the pore disrupt normal functioning, but it also causes a release of proteins involved in the initiation of cell death cascades (Kroemer *et al.*, 1998; Lemasters *et al.*, 1998; Budd and Nicholls; 1996).

2.5 Reactive Oxygen Species (ROS)

Mitochondria are a major physiological source of reactive oxygen species (ROS), which are generated during mitochondrial respiration. ROS, e.g. superoxide, hydrogen peroxide and hydroxyl radicals, are formed when dioxygen is not completely reduced to water during respiration, and can give rise to secondary ROS, such as peroxynitrite and lipid peroxides. However, a number of enzymes exist within cells that can induce the production of ROS, e.g. cyclooxygenases, lipooxygenases and nitric oxide synthase (NOS), which generates nitric oxide (NO). NO toxicity may arise from inhibition of mitochondrial respiration and/or reaction with superoxide to form peroxynitrite (Gunasekar *et al.*, 1995). Various forms of stress such as hypoxia and UV radiation are also sources of ROS.

A state of moderately increased levels of intracellular ROS is referred to as oxidative stress. ROS are capable of causing oxidative damage to macromolecules leading to lipid peroxidation, oxidation of amino acid side chains, formation of protein-protein cross links, oxidation of polypeptide backbones resulting in protein fragmentation, DNA damage, and DNA strand breaks. Cells respond to these adverse conditions by modulation of their antioxidant levels, induction of new gene expression, and protein modification. The production of ROS in mitochondria is regulated by a number of antioxidant enzymes within the mitochondria. These enzymes include phospholipid hydroperoxide glutathione peroxide (PHGPx), classical glutathione peroxidase (cGPx), and Manganese-superoxide dismutase (Mn-SOD) (Ursini *et al.*, 1985).

In hypoglycaemia, ROS production is an important component mediating cell damage. Elevated levels of intracellular calcium can activate phospholipase A_2 leading to a generation of the free fatty acid, arachidonic acid, (Kukreja *et al.*, 1986) and the metabolism of arachidonic acid consequently produces ROS and lipid peroxidation (Verity, 1994). An increase in free fatty acids has been demonstrated in the cortex of rats which were subjected to severe hypoglycaemia (Wieloch *et al.*, 1984). In addition, in

mouse striatal cells, exposed to 30 minutes of glucose deprivation, glutamate induced the release of arachidonic acid by the activation of NMDA receptors, AMPA and metabotropic receptors (Williams et al. 1995).

ROS, that are generated in excess in mitochondria via a number of stimuli, act as mediators of the apoptotic signalling pathway (Zamzami *et al.*, 1996a; Garcia-Ruiz *et al.*, 1997; Nomura *et al.*, 1999). For example, the peroxidation of a protein termed cardiolipin, via ROS activation, in the mitochondria results in the dissociation of cytochrome c from mitochondrial inner membranes, providing the initial step in the release of cytochrome c from the mitochondria (Nomura *et al.*, 2000).

2.6 ATP Sensitive Potassium Channels

ATP-sensitive K^+ channels (K-ATP channels) form a unique class of potassium channels which are thought to couple the metabolic state of the cell to its electrical activity (Noma, 1983; Ashcroft and Gribble, 1998). K-ATP channels are negatively regulated by intracellular ATP, hence a decrease in the nucleotide concentration leads to channel activation. They are found in a wide variety of tissues and regulate functions such as hormone secretion (Bernardi *et al.*, 1993), excitability of neurones (Ashford *et al.*, 1990) and cytoprotection during ischaemia (Amoroso et al., 1990).

The K-ATP channels consist of two types of subunit, an inwardly rectifying subunit (Kir6.1 or Kir6.2) and a sulphonylurea receptor subunit (Inagaki *et al.*, 1995; Sakura *et al.*, 1995). In neurones, K-ATP channels exist in many brain regions, including the striatum (Dunn-Meynell *et al.*, 1998; Zhu *et al.*, 1999), and have been reported to exist in the mitochondrial membrane (Ashford *et al.*, 1990; Debska *et al.*, 2001).

The existence of K-ATP channels in neurones which are in control of energy homeostasis, such as hypothalamic neurones (Ashford *et al.*, 1990), is apparent but it is unclear why such channels should be present in neurones not associated with energy homeostasis. One possible explanation is that K-ATP channels may play a role in neuroprotection, whereby low ATP concentrations lead to channel opening, efflux of intracellular K^+ and cellular hyperpolarisation (Fellows *et al.*, 1993). Indeed, it was reported that in hippocampal neurones, K-ATP channels are activated during hypoglycaemia with a reversible increase in activity (Zawar and Neumcke, 2000).

2.7 Classification of Hypoglycaemic Cell Death

The classification of hypoglycaemic cell death is debatable and it is probable that there is no specific form of cell death limited to hypoglycaemic neurotoxicity. Ferrand-Drake *et al.*, observed ultrastructural changes in cells comprising of both necrotic and apoptotic features after *in vivo* hypoglycaemia (Ferrand-Drake *et al.*, 1999).

It is hypothesised that depending on cell type and duration of glucose deprivation, different pathways will be switched on, eventually deciding the phenotype of cell demise. The following are examples of cellular events occurring in response to glucose deprivation, which provide evidence for this hypothesis.

In cultured striatal neurones, 10 minutes of glucose deprivation induced a progressive, slow membrane depolarisation, and the amplitude of this depolarisation was dependent on the duration of hypoglycaemia. Periods of hypoglycaemia under 30 minutes caused reversible membrane potential changes, whereas periods over 30 minutes caused irreversible membrane depolarisation (Calabresi *et al.*, 1997a). In slice cultures, hypoglycaemia caused a depression in synaptic transmission in the striatum after five minutes (Calabresi *et al.*, 1997c).

In cultured cortical cells, after a 6-8 hour period of glucose deprivation, ultrastructural investigation showed neuronal swelling and some disintegration. These morphological changes increased over subsequent hours and this slow development of injury is similar to that seen following the exposure of cortical neurones to exogenously administered glutamate (Monyer *et al.*, 1989). Similar observations were also seen in dentate gyrus granule cells. In addition, morphological examination revealed a dendro-somatic, axon-sparing damage, characteristics also observed in excitotoxin-induced neuronal damage (Schwarcz *et al.*, 1983).

It has also been reported that immediately after the hypoglycaemia insult, the most significant change is the dispersion of polyribosomes. These changes correlate with the autoradiographically demonstrated absence of protein synthesis observed in most brain structures after insulin-induced hypoglycaemia (Kiessling *et al.*, 1984; Kiessling *et al.*, 1986).

2.7.1 Apoptotic Characteristics

Apoptotic-like characteristics and machinery have been reported in every energy-depleted model described to date. In comparison to necrotic characteristics these features are more focused upon as it is thought that cells showing apoptotic features have a higher survival potential. For example, DNA fragmentation following 24 hours of insulin-induced hypoglycaemia was observed in the hippocampus (Ferrand-Drake *et al*, 1999). Bax was found to be upregulated in neurones destined to die after global ischaemia (Krajewski *et al.*, 1995; Chen *et al.*, 1996) and in the transient forebrain ischaemia model in the gerbil, it was observed that Bax immunoreactivity peaked at 72 hours, prior to DNA fragmentation, in the CA1 region of the hippocampus. (Hara *et al.*, 1996; Iwai *et al.*, 1995). Caspase-3 activation has been found in a number of neuronal toxicity paradigms, including traumatic brain injury (Yakovlev *et al.*, 1997), neurotrophin withdrawal (Keane *et al.*, 1997; Taylor *et al.*, 1997; Yakovlev *et al.*, 1997), serum and potassium deprivation (Miller and Johnson, 1996), glutamate excitotoxicity (Du *et al.*, 1997) and chemical ischaemia (Allen *et al.*, 1999). Colocalisation of DNA fragmentation and elevated caspase-3 immunoreactivity was detected in a majority of neurones in the CA1 region and striatum at 72 hours after transient global ischaemia (Chen *et al.*, 1998).

In most *in vivo* models, morphological and molecular changes consistent with apoptosis, including caspase activation, expression of pro-apoptotic genes and release of cytochrome c, are detected in the penumbra region surrounding the acute insult. This suggests that apoptosis is associated more with cells exposed to less abrupt energy deficiency, and also that these cells can be rescued by therapeutic intervention (McManus *et al.*, 1993; Goto *et al.*, 1990; Dirnagl *et al.*, 1999). It was observed that in the striatum there was a relatively high density of apoptotic cells from 6 through to 7 days after hypoxic-ischaemic insult (Nakajima *et al.*, 2000), suggesting that apoptosis is also predominantly associated with reperfused cells. In another study, evidence of apoptosis was found only 1 to 4 days after cerebral hypoxia-ischaemia in the granule cells of the inner layers of the dentate gyrus (Pulera *et al.*, 1998). However, it has been acknowledged that cells possessing apoptotic morphology even exist in the ischaemic core (Nakajima *et al.*, 2000).

2.8 Reperfusion Damage

Reperfusion damage refers to the further damage caused to existing cells when ATP is reintroducted into the system via a reperfusion of either oxygen or glucose or both. Therefore, despite restoration of normal neuronal oxygen tension or/and glucose content,

the reperfusion period can be associated with additional neuronal injury (Siesjo, 1992; Kalimo *et al.*, 1985).

It is suggested that after a period of severe cellular energy shortage, the MTP may be activated at reperfusion. The subsequent uncoupling of the electron transport chain and the loss of free radical scavengers may lead to a surge of calcium-activated cellular degeneration and free radical damage, causing necrotic cell death as well as mitochondrial dysfunction, that promote additional sodium and calcium entry (Kristian and Siesjo, 1996). For example, during the reperfusion phase after cerebral ischaemia, calcium accumulates in mitochondria, and a burst of free radical formation occurs, conditions that favour the activation of the MTP (Almeida *et al.*, 1995). Indeed, calcium concentrations were shown to be significantly higher in reperfused cells when compared with calcium levels of neurones subjected to oxygen-glucose deprivation (Taylor *et al.*, 1999). It is not known whether the calcium overload starts before or after the transition to irreversible cell damage. This provides further evidence that factors other than energy failure are operative during the post-insult period, such as excitatory transmitters. Indeed, NMDA receptor antagonists have been protective after the insult period (Wieloch *et al.*, 1985b; Tasker *et al.*, 1992) and the deafferentation of glutamatergic cortico-striatal input prevents the development of irreversible nerve cell injury within the striatum during the recovery period after 30 minutes of hypoglycaemia (Linden *et al.*, 1987).

An increase in the activity of pro-apoptotic components is apparent after reperfusion. It was reported that 24 hours after reperfusion following 1 hour of middle cerebral artery occlusion, the population of caspase immunoreactive cells was significantly increased. In addition, Bcl-2 immunoreactivity was localised in the cytoplasm of the penumbra where the cells were still viable. Bcl-2 positive neurones were few after 5 hours of reperfusion, but at 24 hours Bcl-2 expression was weak and thinly scattered in few neurones of the penumbra, whereas the expression of Bax and cytochrome c staining was strong, and was evident in the core where many neurones were already dead. TUNEL-positive cells were clearly observed at 8 hours after reperfusion and progressively increased at 24 hours (Prakasa Babu *et al.*, 2000).

Reported morphological observations after reperfusion include dendritic blebbing and pronounced somatic and mitochondrial swelling in CA1 slices subjected to oxygen and glucose deprivation and then reperfused (Taylor *et al.*, 1999). Swollen neuronal membranes are seen in cortical cells after a resupply of normal oxygen and glucose following a 6-8 hour ischaemia insult (Goldberg & Choi, 1993). After 24 hours of recovery

following transient focal ischaemia, neuronal shrinkage, nuclear condensation and fragmentation apoptotic bodies were observed in affected areas (Prakasa Babu *et al.*, 2000).

3 Protection from Hypoglycaemia-induced Cell Damage

3.1 Calcium Modulation

The idea that calcium entry is the final common pathway of cell death has provoked an enormous amount of research on the prevention of high $[Ca^{2+}]_i$ and consequent prevention of cell damage. In addition, it has been reported that neuronal survival is not determined primarily by the degree of neuronal calcium loading. Instead, it has been proposed that the main determinant of calcium toxicity is the route through which calcium ions gain access to the intracellular space, and the signalling cascades initiated along the specific route (Sattler *et al.*, 1998). Hence, blockade of calcium entry to the intracellular space has involved both VGCCs, intracellular calcium stores and glutamate receptors.

3.1.1 Calcium Channel Blockade

Voltage-gated calcium channels (VGCC) can be at present classified into at least six types named L, N, P, Q, R, T which are distinguished by their particular electrophysiological and pharmacological properties (Randall, 1998).

The blockade of VGCC has been shown to have neuroprotective effects against damage caused by focal ischaemia (Germano *et al.*, 1987; Jacewicz *et al.*, 1990; Mohamed *et al.*, 1985). In particular, there is a family of L-type calcium channel antagonists which have been widely used in numerous toxicity models. Nicardipine has shown protective effects in cerebral ischaemia models (Alps *et al.*, 1988; Alps, 1992; Rami and Krieglstein, 1994) as well as *in vitro* models of hypoglycaemia (Hayashi *et al.*, 1993). It has also been shown to inhibit K^+-induced increase in $[Ca^{2+}]_i$ in synaptosomes and hippocampal cells (Kobayashi *et al.*, 1992). Nimodipine was reported to prevent toxicity in hippocampal cells, against glutamate (Hara *et al.*, 1990) and cerebral ischaemia (Steen *et al.*, 1983; Kawaguchi *et al.*, 1999) and nifedipine has been shown to significantly reduce neuronal apoptosis induced by mitochondrial inhibitors (Keller *et al.*, 1998).

3.1.1.1 Extracellular/Intracellular Calcium Chelation

Omitting calcium from the bathing solution or adding calcium chelators provides information on whether neuroprotection is a result of removing calcium before, during or after a neurotoxic insult.

In hippocampal and cerebellar brain slices, toxicity induced by glutamate was abolished when calcium was omitted from the bathing solution (Tymianski, 1994). BAPTA-AM is an exogenous chelator which is cell permeable. Its buffering activity has been shown to be neuroprotective in depolarised CA1 pyramidal neurones (Tsubokawa *et al.*, 1994; Tsubokawa *et al.*, 1993). It has also been shown to prevent mitochondria ROS accumulation, membrane depolarisation and apoptosis (Keller *et al.*, 1998). EGTA, a cellular impermeable calcium chelator, prevented toxicity induced by glutamate in rat hippocampal cells (Hara *et al.*, 1993).

Calcium-binding proteins, such as calbindin-D_{28K} and calmodulin, have been proposed to function as neuroprotective agents due to their ability to buffer $[Ca^{2+}]_i$. For example, a systemic administration of kainate was shown to cause an induction of calbindin- D_{28K} in cortical and hippocampal astrocytes (Mattson *et al.*, 1995). In addition, cultured hippocampal astrocytes and PC12 cells expressing the calcium binding protein, calbindin-D_{28K}, were less vulnerable to excitotoxic insults (Mattson *et al.*, 1991a; McMahon *et al.*, 1998).

3.1.1.2 Blockade of Intracellular Stores

The ER can contribute to the calcium overload by releasing calcium (Wei and Perry, 1996). Treatment of neurones with agents that suppress ER-mediated calcium release has been reported to protect cells against excitotoxicity (Frandsen and Schousboe, 1991).

Blockers of the IP_3 receptors have provided neuroprotection against ischaemia. The IP_3 antagonist, TMB-8, is reported to be neuroprotective in cerebellar cultures from calcium-dependent glutamate-induced excitotoxicity (Malcolm *et al.*, 1996). TMB-8 has also been used *in vivo* to reduce neuronal damage in ischaemic stroke. The size of necrotic tissue was reduced by about 90% in TMB-8 treated animals, even if the administration is delayed for 6 hours (Chiou and Hong, 1995).

Blockade of the ryanodine receptor-regulated intracellular calcium store in PC12 cells provided protection against glucose withdrawal, indicating that calcium release from ryanodine stores, rather than calcium influx, may be the major source of $[Ca^{2+}]_i$ induced by glucose deprivation (Chung and Hong, 1998).

The blockade of ryanodine receptors with dantrolene significantly decreased the rise of $[Ca^{2+}]_i$ in neuronal cultures induced by NMDA (Frandsen and Schousboe, 1991; Lei *et al.*,

1992; Mody and MacDonald, 1995), depolarisation (Simpson *et al.*, 1995), mitochondria inhibitors (Keller *et al.*, 1998), and trauma (Yoon *et al.*, 1996). Dantrolene has also been shown to be neuroprotective *in vivo* (Zhang *et al.*, 1993; Wei and Perry, 1996).

3.1.2 Glutamate Receptor Antagonism

Compounding evidence supporting the involvement of glutamate neurotoxicity in neurodegeneration stems from both *in vitro* and *in vivo* studies. Neuronal damage in the striatum of animals, which have been subjected to severe insulin-induced hypoglycaemia, can be reduced by decortication (Wieloch *et al.*, 1985a). The glutamatergic input to the caudatoputamen was abolished by unilateral ablation of the overlying cortex, prior to the hypoglycaemic insult, and a drastic decrease in the number of injured neurones was observed after one week (Linden *et al.*, 1987). This neuroprotection is thought to arise because of the degeneration of the excitatory amino acids (EAA) innervation to the caudate neurones, resulting in a dramatic decrease of EAA receptor activity (Wieloch *et al.*, 1985a; Westerberg and Wieloch, 1986).

Studies with NMDA-receptor antagonists have also illustrated potent neuroprotection against excitotoxicity in energy failure injury. Goldberg & Choi demonstrated that the administration of a wide selection of NMDA antagonists, e.g. D-APV, CGS 19755, MK-801, during the exposure period of oxygen-glucose deprivation, blocked both acute neuronal swelling and late neuronal degeneration in cultured cortical cells (Goldberg & Choi, 1993). Local injections of an NMDA receptor antagonist, AP7, in the caudate nucleus after 30 minutes of insulin-induced hypoglycaemia, reduced 90% cell death, following one week of recovery, compared to that in saline-injected animals (Wieloch, 1985b).

Reports have shown that the administration of non-NMDA receptor antagonists did not protect cortical cultures from glucose-deprivation, even at maximum concentrations (100μM - 1mM) (Monyer *et al.*, 1989). This is in agreement with the excitotoxicity theory, which proposes that neurotoxicity is predominantly mediated by NMDA glutamate receptors. However, in an investigation into the effects of post-hypoglycaemic glutamate receptor blockade on neuronal necrosis, the NMDA receptor antagonist, MK801, and AMPA-receptor antagonist, NBQX, administered separately or in combination, decreased neuronal damage in the striatum when given to rats after a severe insulin-induced hypoglycaemic insult (Nellgard and Wieloch, 1992). This implies that AMPA receptors are involved in late secondary degeneration.

Tetanus toxin, a compound which blocks calcium-dependent vesicular transmitter release, was found to possess neuroprotective properties comparable to that of NMDA antagonists, i.e. attenuated glutamate efflux and neuronal injury following glucose deprivation and combined oxygen-glucose deprivation (Monyer *et al.*, 1992). This provides further evidence that the prevention of glutamate release increases cell survival. During the recovery period tetanus toxin must be immediately present in the glucose-containing wash to provide substantial protection from membrane injury in organotypic hippocampal culture, whereas MK801 protects even if the administration is delayed for 30 minutes into the recovery period (Tasker *et al.*, 1992).

3.1.3 MTP Blockade

The MTP may be manipulated by a variety of drugs. The most common drug used is cyclosporin A (CsA). CsA possesses two properties, it inhibits the opening of the MTP by binding to cyclophilin D within the inner mitochondrial pore (Connern and Halestrap, 1994) and it inhibits a phosphatase, calcineurin. However, CsA, has been shown to have both an anti-apoptotic (Zamzami *et al.*, 1996b) and a pro-apoptotic action (McDonald *et al.*, 1996; Mosieniak *et al.*, 1997). The mechanisms by which CsA exerts pro-apoptotic effects are yet undefined. It is known that it causes TUNEL staining with plasma membrane blebbing in neuronal cultures (McDonald *et al.*, 1996) and has direct effects on neuronal excitability and synaptic transmission in hippocampal slices (Wong *et al.*, 2000).

CsA has shown to be effective in many models of neuronal degeneration induced by a variety of stimuli, such as transient ischaemia (Yoshimoto and Siesjo, 1999; Matsumoto *et al.*, 1999) and mitochondria complex I inhibitor induced cell death (Seaton *et al.*, 1998). It has been demonstrated that CsA can exert its neuroprotective effect before, during and hours after the insult is given. This property of CsA is especially important when considering the therapeutic window of treatment of ischaemia.

The mechanism by which CsA exerts neuroprotective properties is not completely understood at present. It may be a combination of inhibiting both the MTP and calcineurin. By inhibiting calcineurin, CsA can alter the phosphorylation and function of many protein substrates including cytosketetal proteins, synapsin 1, protein phosphatase inhibitors, protein kinase A, ion channels, receptors, transcription factors and NOS (Steiner *et al.*, 1992). However, the calcineurin inhibitor, FK506, which does not interact with the MTP, has failed to produce neuroprotective effects in certain energy deprived systems (Scheff and Sullivan, 1999; Ferrand-Drake and Wieloch, 1999).

Rats treated with CsA 5 minutes before or immediately after a traumatic cortical brain injury showed a significant reduction in the amount of cortical damage 7 days following the insult. Results showed a 50% reduction in the size of the injury with a low dose pre-treatment and a 40% reduction with a post injury treatment (Scheff and Sullivan, 1999). The same experiments were repeated using FK506. The results showed that FK506 failed to protect animals against cortical damage (Scheff and Sullivan, 1999). CsA also significantly reduced the number of swollen mitochondria in the dentate gyrus and prevented DNA fragmentation from severe hypoglycaemic-induced insults. Again, FK506 did not prevent mitochondrial damage or DNA fragmentation (Ferrand-Drake and Wieloch, 1999). These experiments suggest that neuroprotection by cyclosporin A, in energy depleted conditions, is likely to be caused by blockade of the MTP and not calcineurin.

Trifluoperazine (TFP) has also been proposed to be an inhibitor of the MTP but blocks MTP via a mechanism distinct from CsA, possibly by the inhibition of phospholipase A_2 (Hoyt *et al.*, 1997). Trifluoperazine is an inhibitor of calmodulin and may exert more general inhibitory effects by competing for calcium ions binding to specific calcium recognition sites as well as inhibiting the activation of calcineurin (Levin and Weiss, 1979). In a study where glutamate-induced mitochondrial depolarisation in cultures of forebrain neurones was investigated, results showed that TFP inhibited or delayed the onset of glutamate-induced mitochondrial MTP in intact neurones (Hoyt *et al.*, 1997).

3.1.4 ROS Scavenging

It was first reported that free radicals had a role in excitotoxicity when Dykens and colleagues demonstrated that kainate-induced damage in cerebellar neurones was reduced following the administration of the anti-oxidant, superoxide dismutase (Dykens *et al.*, 1987). Since then, anti-oxidants have been shown to protect against apoptosis induced by a variety of agents in different cell types.

The administration of superoxide dismutase, was shown to improve the survival of superior cervical ganglion neurones subjected to glucose deprivation (Saez *et al.*, 1987) and following cerebral ischaemia, transgenic mice carrying the human superoxide dismutase gene, showed a reduced level of ischaemic damage compared to wild-type mice (Kinouchi *et al.*, 1991). Also, the over-expression of superoxide dismutase in mice was shown to protect against quinolinic acid and kainate acid neurotoxicity in the striatum (Schwartz, 1998).

Free radical spin traps, such as PBN, are compounds which react with free radicals to form more stable products. Administration of these compounds to excitotoxicity models have shown to be effective in attenuating damage, such as ischaemic lesions (Phillis and Clough-Helfman., 1990), and striatal NMDA-induced lesions (Schulz *et al.*, 1995). In addition, PBN and the inhibitor of lipid peroxidation, U-83836E, were shown to protect cultured striatal neurones against damage induced by glucose deprivation (Nakao *et al.*, 1996).

Pyrrolidinedithiocarbamate (PDTC) as a free radical scavenger has been used in many systems and proved to be effective in protecting tissue from ROS toxicity. Apoptosis induced by the mitochondrial complex I inhibitor, MPP+ in PC12 and SK-N-MC dopaminergic cells was decreased in cells that had been pretreated with PDTC. In this study it was suggested PDTC's neuroprotective mechanism of action was inhibition of the production of hydroxyl radicals (Seaton *et al.*, 1997).

As well as preventing the production of hydroxyl radicals, PDTC has also been associated with the production of inducible nitric oxide synthase (iNOS). The overproduction of nitric oxide has been proposed to be an important factor in neurotoxicity. In rat forebrain slices deprived of oxygen and glucose, PDTC inhibited the induction of NOS (Moro *et al.*, 1998). The inhibition of NOS has also been shown to decrease ischaemic damage both *in vitro* (Cazevielle *et al.*, 1993) and *in vivo* (Moncada *et al.*, 1992).

It has been reported recently that apoptosis can be blocked by inhibition of the generation of ROS in mitochondria by the overexpression of the enzyme phosholipid hydroperoxide glutathione peroxide (PHGPx) (Nomura *et al.*, 1999). The enzyme PHGPx is a unique intracellular antioxidant enzyme that directly reduces peroxidised lipids that have been produced in cell membranes (Ursini *et al.*, 1985). Mitochondrial PHGPx suppresses apoptotic cell death activated by a deprivation of glucose (Nomura *et al.*, 1999).

3.1.5 K^+-ATP Channel Activation

K^+ channel openers, such as diazoxide and (-)-cromakalim, have been shown to protect cultured hippocampal neurones against excitotoxicity (Abele *et al.*, 1990), amyloid-β (Aβ) toxicity (Goodman and Mattson, 1996) and, reduced the depolarisation of neocortical pyramidal cells induced by short-term hypoxia (Pissarek *et al.*, 1998).

The proposed mechanism of diazoxide-mediated neuroprotection effects is activation of K-ATP channel which would attenuate membrane depolarisation by hyperpolarising it, and suppressing Ca^{2+} influx through voltage-dependent channels. Indeed, (-)-cromokalin was shown to prevent cell death induced by either oxygen-glucose deprivation or glutamate toxicity in hippocampal cultures, and this prevention was associated with regulating calcium homeostasis (Lauritzen *et al.*, 1997).

It has also been suggested that K^+ channel openers have an antioxidant neuroprotective action independent of their effect on K^+ channel (Goodman and Mattson, 1996).

3.1.6 Modulation of the Apoptotic Execution Pathway

The intervention of components of the apoptotic execution pathway has resulted in the prevention of cell death in most models described. The administration of Z-DEVD-FMK, an inhibitor of casapse-3 activity, significantly decreased DNA fragmentation and neuronal death in the CA1 region of the hippocampus up to 7 days after ischaemia. When administered intracerebroventricularly, after transient brain injury in rats, Z-DEVD-FMK showed marked neuroprotective actions, as indicated by both improvement of neurological recovery and reduction of DNA fragmentation (Yakovlev *et al.*, 1997). It has also been shown that caspase-3 inhibitors reduce infarct volume and neurological deficits in mice subjected to focal cerebral ischaemia (Nomura, 1998) or direct excitotoxic insults (Hara *et al.*, 1997).

In an *in vitro* traumatic injury model, it was reported that the simultaneous inhibition of NMDA receptors, with MK-801, and caspase-3 activity, using Z-DEVD-FMK resulted in significantly greater protection than either treatment alone (Allen *et al.*, 1999). This provides further evidence that excitotoxicity plays an important role in the initiation period of apoptosis.

The protective effects of inhibiting caspase-3 are observed even after the toxic event. In a serum/K^+ deprivation model of toxicity in cerebellar granule cells, Z-DEVD-FMK provided protection after 12 hours of serum/K^+ deprivation and retained these protective effects after 48 hours (Eldadah *et al.*, 1997). This indicates during the recovery period caspase-3 is still active, and cell death via apoptotic mechanisms still occurs.

The overexpression of the anti-apoptosis protein, Bcl-2, has been shown to prevent apoptosis in response to various stimuli (Yang *et al.*, 1997; Kluck *et al.*, 1997, Vander

Heiden *et al.*, 1997; Liu *et al.*, 1997). This effect is thought to be due to prevention of the mitochondrial membrane depolarisation during apoptosis (Zamzami *et al.*, 1996b) perhaps by inhibiting the MTP or suppressing oxidative stress (Bruce-Keller *et al.*, 1997). It has also been suggested that Bcl-2 and its family member, Bcl-$_{XL}$, inhibit cytochrome c release (Yang *et al.*, 1997; Kluck *et al.*, 1997; Zamzami *et al.*, 1996a; Susin *et al.*, 1996; Vander Heiden *et al.*, 1997), either directly or indirectly, by regulating the flow of ions including calcium, across the mitochondrial and ER membranes (Kluck *et al.*, 1997). This indicates that Bcl-2 is an important stabiliser of calcium homeostasis (He *et al.*, 1997). Indeed, it has been shown that an overexpression of Bcl-2 in an *in vivo* model of hypoglycaemia protects hippocampal cells, even after 24 hours of hypoglycaemic insult (Lawrence *et al.*, 1996).

3.2 Alternative Substrates

Neurones can utilise alternative substrates to glucose for the synthesis of ATP during hypoglycaemia. The brain contains the enzyme system required for the metabolism of a range of substrates, including ketone bodies (Owen *et al.*, 1967; Fourest-Fontecave *et al.*, 1987), lactate (Maran *et al.*, 1994), fatty acids (Evans *et al.*, 1995), glycerol and amino acids (Weithop and Cryer, 1992). It is apparent that different brain regions may vary in which non-glucose substrate they use during hypoglycaemia, but regardless of type, endogenous substrates must be rapidly mobilised in sufficient amount to supply the cells with enough substrate to uphold the ATP necessary for ionic pumping (Wieloch *et al.*, 1984).

In the absence of glucose, exogenous pyruvate can be effectively utilised as a substitute substrate (Kauppinen and Nicholls, 1986). The final product of glycolysis in aerobic conditions is pyruvate, which enters the Krebs cycle (Figure 1.3). In anaerobic conditions, pyruvate is converted to lactate. Both pyruvate and lactate can be used as fuel molecules, however, during hypoglycaemia oxygen is present, therefore only pyruvate is produced.

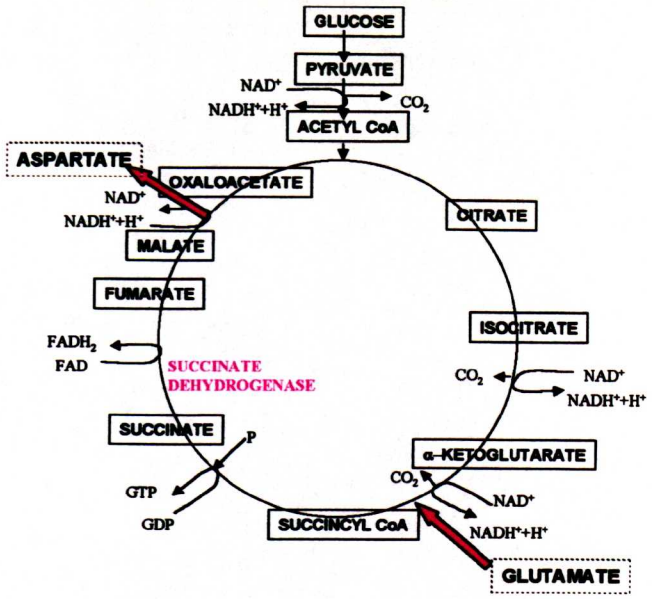

FIGURE 1.3

The tricarboxylic acid cycle (TCA). This simple view of a component of aerobic respiration shows the metabolites involved in the TCA cycle and pyruvate and glutamate as energy substrates entering the TCA cycle. Glutamate is reversibly converted to α-ketoglutarate by aspartate transaminase by which aspartate is produced. It also shows the enzyme succinate dehydrogenase which is reduced by XTT in cell viability assays (Chapter Two).

Energy failure is thought to be less severe during hypoglycaemia, compared to ischaemia, because of the capacity of the brain to oxidise endogenous non-glucose fuels (Koretz *et al.*, 1994). In addition, there is a predominant release of aspartate into the extracellular fluid in hypoglycaemia, which differs from the rise in extracellular glutamate found in ischaemia (Auer and Siesjo, 1988). In hypoglycaemia, it has been shown that glutamate catabolism is favoured over glutamate synthesis in the TCA, and therefore glutamate is used as an alternative energy source.

Astrocytes may contribute markedly to local cerebral fuel homeostatic mechanisms at times of metabolic stress in the brain (Magistretti, 1982), probably by releasing pyruvate for local neuronal oxidation. It has also been thought that astrocytes alone are capable of partially oxidising free fatty acids and producing ketone bodies (Edmond *et al.*, 1987). Hence, it is hypothesised that at times of high circulating fatty acids levels, such as are seen during hypoglycaemia, astrocytes metabolise fatty acids to form ketone bodies which are easily taken up by neurones via the monocarboxylate carrier and substantially oxidised.

It is thought that only in mild cases of hypoglycaemia can alternative substrates to glucose provide the metabolic demands of the brain. In acute hypoglycaemia, it may be that no other substrate can ameliorate the detrimental effects of hypoglycaemia, and because profound hypoglycaemia causes tissue alkalosis resulting from the ammonia formed from the deamination of amino acids, the consumption of metabolic acids, and the absence of lactic acid formation, this may be a significant cause of irreversible cell damage (Auer, 1986).

4.Cellular Recovery from Energy-Depletion

Cells employ specific protective mechanisms during energy failure, which ameliorate irreversible damage and prolong the survival of the cell. The understanding of these mechanisms is critical in the event of therapeutic pharmacological intervention and improving neurological outcome following an energy-deficient insult.

4.1 Recovery of Protein Synthesis

Protein synthesis is controlled by calcium homeostasis within the ER (Brostrom and Brostrom, 1990; Paschen, 1996). It is an energy consuming process and becomes impaired with the reduction of ATP. The suppression of global protein synthesis is a common response to metabolic stress, for example, protein synthesis impairment was observed in the brain after insulin-induced hypoglycaemic damage. After 30 minutes of severe hypoglycaemia there was a complete inhibition of amino acid incorporation into proteins of the cerebral cortex, hippocampus, and the striatum (Kiessling *et al.*, 1984).

However, inhibition of protein synthesis appears not to be indicative of irreversible damage. In the same experiment, all animals which had 30 minutes of insulin-induced hypoglycaemia were allowed to recover for 75 minutes and partial restoration of protein synthesis was reported (Kiessling *et al.*, 1984). The absence of protein synthesis was autoradiographically observed in cortical cells after hypoglycaemia, and again this did not appear to signify an irreversible change, since the neurones were shown to recover both structurally and metabolically (Auer *et al.*, 1985b; Kiessling *et al.*, 1986).

It is interesting to observe studies where cells have survived when treated with a protein synthesis inhibitor, e.g. cycloheximide, and subjected to a toxic insult. It has been suggested that protein synthesis inhibitors prevent apoptotic cell death by inhibiting the machinery to activate the apoptotic process (Goto *et al.*, 1990).

Inhibition and recovery of protein synthesis show a remarkable regional difference. Indeed, it has been shown that after transient global ischaemia, protein synthesis was completely suppressed but recovered in non-vulnerable areas, such as the cerebellum, but not in vulnerable ones, such as the hippocampus (Bodsch *et al.*, 1985; Thilmann *et al.*, 1986). This contradicts data stating that if protein synthesis was suppressed, apoptosis will be suppressed and all cells would recover. It has been suggested that the ability to recover

depends on whether regions have a more efficient glucose supply during hypoglycaemic conditions, e.g. transport sites with a higher affinity for glucose (Ratcheson *et al.*, 1981).

4.2 DNA Repair Proteins

In adult neurones, DNA damage can be generated either as a result of endogenous deamination, depurination, oxidation, polymerase misincorporation hydrolysis, alkylation or x-ray irradiation (Lindahl, 1993; Gobbel *et al.*, 1998; Wallace, 1988). Such damage occurs at a frequency too high to be compatible with life and must be corrected efficiently by DNA repair mechanisms. Repair of non-replicating DNA is expected to be of particular importance in neurones because these cells are among the longest-living cells in the body and show high rates of spontaneous DNA mutation (Andrew *et al.*, 1997).

It is generally accepted that regardless of the specific type of DNA injury generated, the cell's response follows the common principles: the damaged region is recognised, it is removed, a DNA polymerase synthesises a new strand, and a ligase replaces the lost information to restore the DNA to the original state.

Recognition of damaged DNA necessitates a very wide range of repair enzymes specific to the individual lesion. There are two established predominant pathways involved in repairing DNA damage which exist in the brain (Coudoré *et al.*, 1997); the nucleotide excision repair pathway (NER) (Setlow and Carrier, 1967), and the base excision repair pathway (BER) (Lindahl, 1971). NER handles rather infrequent lesions, whereas BER is essential for recovery from spontaneous DNA damage (Seeberg *et al.*, 1995). Both are ATP-dependent processes.

NER is the most versatile DNA repair system in mammalian cells, since it recognises and processes all the lesions tested, at least *in vitro*. It is a multistep process that has been dissected into two broad stages: first recognition, incision and excision of the lesion and the DNA polymerisation through the gap, followed by ligation to restore the strand continuity (Wood, 1996). The BER pathway is the most important cellular protection mechanism responding to oxidative DNA damage, whether it occurs from reactive oxygen species formed during normal metabolism, or from exposure to exogenous agents. The key enzymes in the BER are DNA glycosylases, which remove different types of modified or damaged bases by cleavage of the N-glycosylic bond between the base and the deoxyribose moieties of the nucleotide residues. Different DNA glycosylases remove different kinds of damage, and the specificity of the repair pathway is determined by the

type of glycosylase involved. Once the base is removed, a DNA polymerase fills the resulting gap, and the strand is sealed by DNA ligase (Seeberg *et al.*, 1995).

The mismatch of bases is another form of damage that occurs to DNA. DNA mismatch repair is the process by which incorrectly paired nucleotides in DNA are recognised and repaired. Mismatches in DNA may arise as replication errors, and as a result of base damage. The expression of DNA mismatch repair proteins in neurones may therefore represent a part of an intracellular genetic program activated by DNA damage and directed either to recognise and repair it, or to signal the presence of the damage to the apoptotic machinery.

A four-fold increase of DNA repair synthesis after 30 minutes of cerebral ischaemia suggests that nuclear DNA damage is repaired (Tobita *et al.*, 1991), and that the damage in DNA is repaired with time. The following sections describe the role of four particular genes involved in DNA repair.

4.2.1 XRCC1

The DNA protein x-ray repair cross-complementing group 1 (XRCC1), plays a central role in the DNA BER pathway (Thompson *et al.*, 1990), which is responsible for repairing damage by interacting with DNA ligase III and with DNA polymerase β (Caldecott *et al.*, 1996; Kubota *et al.*, 1996).

The XRCC1 protein has multiple roles in repairing base damage. These functions include forming a stable complex with DNA ligase IIIα, (Caldecott *et al.*, 1994; Nash *et al.*, 1997), bridging the steps in BER through protein interaction (Kubota *et al.*, 1996), directly detecting breaks, co-ordinating the binding and polymerase activity of DNA polymerase β (and perhaps its efficiency) (Kubota *et al.*, 1996), and possibly regulating PARP (Caldecott *et al.*, 1996; Masson *et al.*, 1998).

XRCC1 gene expression has been examined in rat, mouse and baboon tissue (Yoo *et al.*, 1992., Walter *et al.*, 1994., Zhou and Walter, 1995). The XRCC1 protein is constitutively expressed in the entire normal mouse brain, but has a regional predominance in the hippocampus and the caudate-putamen (Fujimura *et al.*, 1999). Its activity has been studied in models of neuronal energy-depletion producing ambiguous results.

A reduction of XRCC1 was observed in the lateral caudate putamen but not in the cortex of the rat after ten minutes of reperfusion following 1 hour of focal cerebral ischaemia (FCI). This reduction was sustained at 4 and 24 hours. (Fujimura *et al.*, 1999). Double staining with XRCC1 and TUNEL, 24 hours after FCI, showed a significant amount of TUNEL-positive cells with the characteristic features of apoptosis, however, none of these cells showed XRCC1 immunoreactivity. On the other hand, some cells had a faint expression of XRCC1, but none were TUNEL positive (Fujimura *et al.*, 1999). This indicates that early reduction of XRCC1 precedes the occurrence of DNA fragmentation in the ischaemic area and that the reduction of XRCC1 may contribute to DNA-damaged cell death after FCI.

A significant reduction of the XRCC1 expression was observed in a model of cold injury-induced brain trauma (CIBT) after fifteen minutes. The reduced expression remained until 24 hours after the insult, and again the reduction of XRCC1 preceded the occurrence of DNA fragmentation in the traumatised tissue (Fujimura *et al.*, 2000). In hypoxic rat brains, mRNA levels for XRCC1 decreased significantly after five minutes of hypoxia, but increased significantly after 15 minutes (Chiappe-Gutierrez *et al.*, 1998). This could suggest that the expression of XRCC1 is affected by the type of DNA damage the cell receives. Also, a decrease in XRCC1 mRNA in response to agents that damage DNA was found *in vitro* models (Yoo *et al.*, 1992).

It is conceivable that a selective decrease of XRCC1 protein synthesis, or a difference in post-translational regulation causes XRCC1 reduction in energy-deprived neurones. Alternatively, it could be suggested that a reduction in XRCC1 does not contribute to DNA-damage, but that reperfusion initiates the production of ROS, opening the MTP and releasing cytochrome c and thereby exacerbating DNA fragmentation after FCI (Fujimura *et al.*, 1999).

4.2.2 ERCC3

An increase in the protein levels for ERCC3 (excision-repair cross-complementing gene 3) was observed in the brains of patients with Down's Syndrome and AD (Hermon *et al.*, 1998). ERCC3 may not only reflect increased repair, but may be representing increased DNA modifying/processing. If increased ERCC3 expression is reflecting increased repair, this may be due to the permanently ongoing DNA damage, for example, by active oxygen species, or other forms of DNA stress. However, it is not known whether ongoing DNA damage is the cause of AD and Down's Syndrome.

However in hypoxic rat brain tissue, mRNA levels encoding ERCC3 never exceeded levels at normoxaemic conditions during 5 to 20 minutes observation period (Chiappe-Gutierrez *et al.*, 1998). This suggests that DNA repair genes are selected depending on the stimuli.

4.2.3 MSH2

One of the mismatch recognition components is the MSH2 protein. MSH2 recognises both single base mispairs and, with a higher affinity, multiple base insertion/deletion mispairs (Fishel *et al.*, 1994; Drummond *et al.*, 1995).

The expression of MSH2 was investigated in the human neuroblastoma SH-SY5Y cell line when exposed to doxorubicin, a toxic antibiotic that induces DNA damage and apoptosis (Tewey *et al.*, 1984). It was shown that this cell line constitutively expressed MSH2 protein, and the level of this protein increased following the exposure to neurotoxic concentrations of doxorubicin (Belloni *et al.*, 1999a).

In addition, kainate administration resulted in a marked increase of MSH2 immunoreactivity in selective, vulnerable, neurones of the hippocampus (Belloni *et al.*, 1999b). Also observed was a sparse distribution of MSH2 immunoreactivity in the striatum. In cerebellar granule cells exposed to toxic concentrations of glutamate, a dramatic increase in MSH2 expression was observed (Uberti *et al.*, 2000; Grilli and Memo, 1999). Whether MSH2 induction is a part of a protective response, or is a sign of suffering cells, remains to be investigated.

However, it is interesting to note that mouse embryonic stem cells containing defective MSH2 genes fail to efficiently execute apoptosis in response to low-level radiation treatment (DeWeese *et al.*, 1998). This suggests a possible role of mismatch repair systems contributing to neuronal cell death via apoptosis.

4.2.4 DNA Polymerase β

DNA polymerase β is the most predominant polymerase active in the nuclei of adult neurones (Kuenzle, 1985). It preferentially fills short DNA gaps, and because of this attribute, it had generally been assumed to be involved in BER. In addition to the DNA synthesis activity to fill the short gaps generated during repair, DNA polymerase β initiates the activity of necessary components for the DNA ligase to seal the nicked DNA strand (Matsumoto and Kim, 1995; see review, Budd and Campbell., 1997).

DNA polymerase β knockout cells show high sensitivity to (m)ethylating agents (Sobol *et al.*, 1996), and were not protected against methylmethanesulfonate (Chen *et al.*, 1998b).

In an ischaemia-reperfusion model, a decrease in DNA polymerase β gene was observed. The results indicate that there was an increase in DNA lesions in ischaemic samples compared with those in normal and sham-operated controls (Liu *et al.*, 1996a).

5. Nuclear Factor Kappa B

Transcription factors provide a crucial link between external stimuli and changes in gene expression, thus influencing biological responses. Nuclear factor kappa B (NFκB) is an unusual transcription factor as it resides within the cytoplasm, enabling it to receive upstream signals more directly than other nuclear proteins, and actively participate in the communication of signals from the cytoplasm to the nucleus. NFκB is activated upon the arrival of external cellular stimuli and translocates into the nucleus to modulate gene expression (Figure 1.4). It is now known that numerous stimuli activate NFκB and abundant genes are induced due to its activation, characterising the importance of this protein.

NFκB was first identified as a nuclear factor in 1986, controlling the expression of the kappa light chain of B cells of the immune system, hence the origin of its name. It is generally thought of as a mediator of stress and pathogen response due to the nature of stimuli that activate NFκB and of the target genes NFκB induces (for review, Siebenlist *et al.*, 1995; May and Ghosh,1998).

In 1993, NFκB was discovered in cultured cerebellar neurones (Rattner *et al.*, 1993) and since then the importance of elucidating NFκB's function within the CNS is extremely evident from the amount of current research. Studies have shown the existence of NFκB DNA binding activity in many brain cells, including hippocampal (Meberg *et al.*, 1996), striatal (Qin *et al.*, 1998), cerebellar (Guerrini *et al.*, 1995), and cortical cells (Kaltschmidt *et al.*, 1995). NFκB is not homogeneously expressed in the brain. The cellular distribution of NFκB varies depending on brain region and cell type. It can exist in an inducible form, i.e. bound within the cytoplasm in soma, dendrites and synapses, rendered inactive (Kaltschmidt *et al.*, 1993a) or in a constitutive form i.e. present within the nucleus and capable of DNA binding (Kaltschmidt *et al.*, 1994).

The functional roles of NFκB in neurones and glia function are associated with processes such as neuronal development, neuronal plasticity and neurodegeneration, signifying the significance of this transcription factor.

5.1 Inhibitory kappa B Regulation

Nuclear factor kappa-B is retained in most cells in an inactive form in the cytoplasm by the inhibitory protein IκB. This is an extremely important molecule as IκB provides a method of regulating the activity of NFκB DNA binding and hence regulating the transcription of target genes (for review, Matthews and Hay, 1995). A tightly controlled negative feedback loop exists, involving the degradation of IκB in the cytoplasm, re-synthesis of IκB and the subsequent inactivation of NFκB (Figure 1.4). Activation of NFκB can occur within minutes of receptor binding.

IκB family members contain multiple ankyrin domains which are essential for the interaction of IκB with NFκB proteins (Jaffray *et al.*, 1995). The ankyrin domain masks the nuclear localisation signals (NLS) of the NFκB protein, which consequently inhibits DNA binding (Liou and Baltimore, 1993). Mutations of the NLS decreases the affinity of NFκB for IκB and thus the inhibition function of IκB is diminished (Beg *et al.*, 1992).

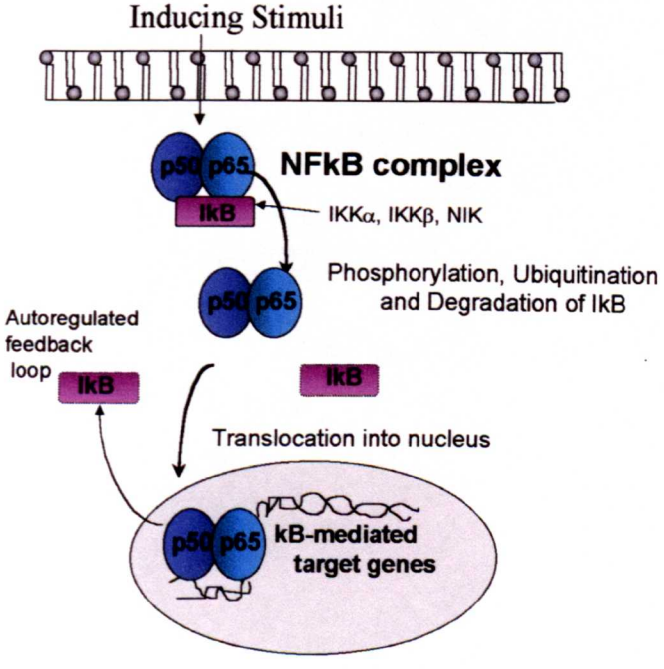

Figure 1.4

Schematic representation of the activation of NFκB upon various extracellular and intracellular stimuli, degradation of IκB and the translocation of NFκB into the nucleus inducing genes encoding κB. These include the gene encoding IκB which initiates the autoregulated feedback loop.

5.1.1 Phosphorylation of IκB

Following cell stimulation, phosphorylation of the IκB occurs (Figure 1.4). It was shown that the IκB molecule was phosphorylated by a serine specific kinase at its regulatory N-terminus on serines 32 and 36. Since this finding much research has been involved with identifying a protein kinase responsible for the regulation of the phosphorylation process. One main problem is the belief that the kinase may exist as a part of a large complex, thus, providing difficulty in identifying it (for review, May and Ghosh, 1998; Verma and Stevenson, 1997; Israel, 1997). Two kinases referred to as IKK-1 (IκB kinase-1) and IKK-2 (IκB kinase-2) were identified. It was demonstrated that the expression of both IKK-1 and IKK-2 in tranfected HeLa cells led to the phosphorylation of serines 32 and 36 of IκB, and were activated by specific inducer of NFκB.

However, another kinase has been described, called NFκB-inducing kinase (NIK). It was found that NIK was required for IL-1 or TNFα- induced NFκB activation and that NIK associated with IKKα and IKKβ. Mutant forms of NIK have been shown to block signalling from both IL-1 and TNF receptors, suggesting that a convergence of signalling pathways, induced by these pro-inflammatory cytokines leading to NFκB activation, might be at the level of this kinase. In addition, protein kinase A (PKA) has also been reported to be an alternative candidate (Zhong *et al.*, 1997).

It is possible that the different subunits of the IκB-kinase complex are targets for separate signalling cascades or that the IκB-kinase complex responds to incoming stimuli by generating an internal signalling cascade involving kinase and substrate subunits which leads eventually to the activation of the IκB-phosphorylating kinase.

5.1.2 Ubiquitination

The phosphorylation of IκB induces ubiquitination of the molecule on N-terminal lysine residues, 21 and 22, which targets the protein for rapid proteolytic degradation i.e. 10-15 minutes, allowing NFκB to translocate into the nucleus. It is thought that the phosphorylation of the N-terminal serine residues of IκB in some way alters the conformation of the protein and exposes the lysine-containing sequences that are targeted by the ubiquitin molecules. Neither phosphorylation or ubiquitination alone is sufficient to dissociate the NFκB-IκB complex and hence free NFκB is only released after degradation of IκB.

5.1.3 IkB Autoregulation

After the degradation of IκB, the IκB pool is replenished by a production of the inhibitory protein transcriptionally regulated by NFκB p65 subunit induced nuclear NFκB, which causes an up-regulation of IκB mRNA levels due to the presence of NFκB sites in the IκB promoter (Sun *et al.*, 1993). Newly synthesised IκB can then bind to NFκB in the cytoplasm, inhibiting further NFκB activation. This ensures target genes are activated only transiently. It is thought that some newly synthesised IκB remains localised in the nucleus where it can inhibit DNA binding and the transcriptional activity of NFκB. The presence of IκB within the nucleus may trigger the transport of the IκB-NFκB complex back to the cytoplasm where it can respond to new stimuli (Arenzana-Seisdedos *et al.*, 1997).

5.2 Molecular Biology of NFκB and IκB

NFκB dimers are composed of proteins from the NFκB (Rel) family (Table 1). All family members contain a Rel homology domain (RHD) of about 300 amino acids. The conserved RHD determines DNA binding, dimerisation, nuclear translocation, and the interaction with the inhibitory protein IκB. It is thought that a short stretch at the beginning of the N-terminal domain appears critical for the binding to DNA and the extreme end of the C-terminal for the nuclear localisation signal (NLS) (for review, Verma *et al.*, 1995; Baeuerle and Henkel, 1994).

	Protein
NFκB	p50
	p65 (Rel A)
	p52
	c-Rel (Rel)
	v-Rel
	Rel B
IκB	IκBα
	IκBβ
	IκBγ
	IκBε
	Bcl-3

Table 1

NFκB and IκB Proteins nomenclature

Most members of the NFκB family can form homo- and heterodimers *in vitro* therefore various combinations of dimers may exist in different cell types and have distinct characteristics. The most common functional dimer found is the combination of p50 subunit-p65 subunit. This heteromer binds with a high affinity to DNA sequences, the most common is 5'-GGGACTTTCC-3'. There are five members of the IκB family to date (Table 1) and the two major characterised forms of IκB proteins in mammalian cells are IκBα and IκBβ.

5.3 Activators of NFκB

There are many diverse stimuli which activate NFκB DNA binding, which suggests that different upstream signalling pathways converge at some point to degrade IκB and allow NFκB to translocate into the nucleus. Regulation of the DNA binding activity of NFκB by different inhibitory proteins and kinases may explain how NFκB can be induced by such diverse stimuli.

The induction of the degradation of IκBα and IκBβ depends on the type of NFκB inducer. For example lipopolysaccharide (LPS) stimulation results in the degradation of IκBα and

IκBβ, whereas, phorbo-myra-acetate (PMA) stimulation results in the degradation of only IκBα, thus causing a transient activation of NFκB (Thompson *et al.*, 1995). The following table displays examples of the type of stimuli which activate NFκB.

NFκB Activation Stimuli
Glutamate
Nerve growth Factor
Amyloid-β peptide
Nitric Oxide
Calcium
Opioids
Depolarisation
Reactive Oxygen Species
Cytokines
Bacterial and Viral Products
Ceramide
Ionising Radiation
UV Rays

Table 2

An example of the type of diversity of stimuli which activate NFκB

5.3.1 Excitatory Amino Acids

It was first shown that glutamate and kainate could induce the activation of NFκB in cultured cerebellar granule cells (Guerrini *et al.*, 1995; Kaltschmidt *et al.*, 1995), and since then it has been shown repeatedly in other neuronal culture types (Simpson and Morris, 1999). In an *in vivo* study, an increase in NFκB binding activity in the striatum of rats administered with quinolinic acid, a glutamate agonist, was observed. The NMDA receptor antagonist, MK-801, was found to completely prevent any changes in NFκB activity (Qin *et al.*, 1998). In astrocytes, however, no NFκB activation could be detected with glutamate (Guerrini *et al.*, 1995), although a chronic administration of kainate markedly increased NFκB p65 immunoreactivity (Perez-Otana *et al.*, 1996).

5.3.2 Calcium

Studies using NMDA toxicity to stimulate NFκB have shown that this stimulation is dependent on calcium entry. NFκB activation was inhibited when calcium chelators were added to cell cultures, demonstrating that NFκB translocation into the nucleus depends on calcium influx via mainly NMDA receptors (Guerrini *et al.*, 1995; Ko and Kelly, 1999). NFκB was also found to be activated by large transient rises in the level of $[Ca^{2+}]_i$ in B cells (Dolmetsch *et al.*, 1997).

5.3.3 Reactive Oxygen Species

An increase in ROS levels activates NFκB (Muller *et al.*, 1997; Schreck *et al.*, 1992b). It was observed that micromolar concentrations of hydrogen peroxide (H_2O_2) increased the level of ROS and specifically increased the DNA-binding activity of NFκB in nuclear extracts of Jurkat T cells (Meyer *et al.*, 1993), PC12 cells (Tong and Perez-Polo, 1996), and oligodendrocytes (Vollgraf *et al.*, 1999). Moreover, micromolar concentrations of antioxidant substances, e.g. PDTC (Schreck *et al.*, 1992b) and thioredoxin (Schenk *et al.*, 1994) block the activation of NFκB in response to nearly all NFκB inducers, including H_2O_2 (Schreck *et al.*, 1992b). Many inducers of NFκB, such as excessive glutamate and calcium, TNF, PMA, UV, IL1, are known to cause oxidative stress, implying that the induction of oxidative stress is a common signal in the pathway to NFκB activation. Therefore, NFκB has been proposed to act primarily as an oxidative-stress response transcription factor (Schreck *et al.*, 1991). Additionally, an induction of NFκB was detected in striatal cultures exposed to nitric oxide-releasing compounds, i.e. S-nitroso-n-acetylpenicillamine (SNAP) (Simpson and Morris, 1999).

5.4 Genes Induced by NFκB DNA Binding

It is apparent that more target genes have been identified for NFκB than any other transcription factor (Baldwin, 1996). These include genes encoding cytokines, growth factor, chemokines and neuropeptides (Table 3).

The following table shows a few examples of NFκB target genes.

Inducible Genes
Manganese-Superoxide Dismutase
IκB and NFκB Proteins
Inducible Nitric Oxide Synthase
Neuropeptides-dynorphin, proenkephalin
Cytokines –IL-1, TNFα
Cycloxygenase enzyme
Adhesion molecules- ICAM
Viral Proteins- HIV

Table 3

An example of the types of genes induced by NFκB.

To add further complexities, certain proteins which activate NFκB and are themselves induced by NFkB, e.g. IL-1.

5.5 The Role of NFκB in Pathological Conditions

In view of a wide range of genes regulated by NFκB, it is not surprising that NFκB could play different roles in response to different insults in different cell types. The involvement of NFκB in the induction of apoptosis has recently been intensely investigated (for review, Foo and Nolan, 1999; Grilli and Memo, 1999; Baichwal and Baeuerle, 1997). Many studies suggest that NFκB plays a pivotal role in preventing cell death by inducing the expression of anti-apoptotic genes, such as Mn-SOD (Wang *et al.*, 1996; Van Antwerp *et al.*, 1996), whereas other studies suggest a role for NFκB in mediating neurodegeneration by inducing the expression of potential pro-apoptotic genes, such as inducible NOS (Kaltschmidt *et al.*, 1993b; Rupec and Baeuerle, 1995).

Increased activity of NF-κB has been reported in many brain structures from patients suffering from neurodegenerative diseases. A study performed on human post-mortem tissue of seven patients who had suffered idiopathic Parkinson's disease, showed a 70-fold increase (compared to controls) in the proportion of dopaminergic cells with immunoreactive nuclear NFκB, (Hunot *et al.*, 1997). To complement this study, it was demonstrated that the proportion of neurones displaying nuclear NFκB staining was

identical to that of neurones showing apoptotic characteristics (Hunot *et al.*, 1997). Brain sections from AD patients revealed that the p65 subunit was activated only in areas affected by the disease (Terai *et al.*, 1996), and in both neurones and astrocytes surrounding early stage plaques (Kaltschmidt *et al.*, 1997). Analysis of six post-mortem human brains following death from an ischaemic insult showed increased immunoreactive p65 in the core area of the ischaemic tissue, and to a lesser extent in the penumbra in astrocytes and in some macrophages (Terai *et al.*, 1996).

Several animal models of stroke have provided evidence that NFκB activity is increased in ischaemic areas of the brain (Gaybriel *et al.*, 1999; Hill *et al.*, 2001). Focal ischaemia-reperfusion in the cerebral cortex region caused increases in NFκB binding activity that was observed five days after the reperfusion in the ischaemic area and in the penumbra (Salminen *et al.*, 1995). It has been demonstrated that this increase in NFκB binding activity is not transient. In a model of traumatic brain injury in rats, a 250% increase in levels of NFκB DNA binding activity was seen after 1 day post injury, and carried on increasing to day 3 post-injury with levels returning to sham levels after 10 days (Yang *et al.*, 1995). In addition, it was seen that activated NFκB was present in the degenerated CA1 area of the hippocampus 72 hours following global forebrain ischaemia and DNA fragmentation was concomitant with this NFκB activation (Clemens *et al.*, 1997a; Clemens *et al.*, 1997b). However, it cannot be concluded yet that NFκB is the causative factor for the induction of apoptosis.

The delay in binding activity may be due to a delayed infiltration of inflammatory cells. Indeed, it is possible that injury leads to an increase in the production of IL-1 or TNF, which then activates NFκB, leading to an induction of the expression of a range of pro-inflammatory genes. Overexpression of cytokines is observed in the brains of patients suffering from Alzheimer's disease, stroke and traumatic brain injury (for review, Toulmond *et al.*, 1996). The increase of NF-κB activity is expected as many of the known NFκB inducers play a role in these pathophysiological conditions, i.e. ROS, cytokines, glutamate and calcium, but the fundamental question is whether the increase in NFκB activation in these conditions is a consequence of the cell employing neuroprotective mechanisms or a consequence of NF-κB mediating cell death. There is evidence to suggest both.

5.5.1 NFκB Mediates Neurotoxicity

Compelling evidence suggests that NF-κB is a part of the apoptotic process and promotes cell death in neurotoxic environments. DNA fragmentation, and a sevenfold increase in NFκB binding activity, was observed in striatal neurones, 12 hours after exposure to quinolinic acid. However, when rats were co-administered with a synthetic peptide, SN50, which prevents NFκB nuclear translocation, striatal cell death and DNA fragmentation were significantly decreased (Qin *et al.*, 1998).

Another convincing set of results demonstrated the ability of aspirin to inhibit NF-κB activity (Kopp and Gosh, 1994). Subsequently it was reported that aspirin is neuroprotective in primary cerebellar cells against excitotoxicity in a concentration-dependent manner (Grilli *et al.*, 1996). Neuroprotection by aspirin was also seen in hippocampal slices treated with NMDA, and this effect correlated with the inhibition of EAA release and NF-κB translocation to the nucleus (Moro *et al.*, 2000). It was determined that this result was independent of events associated with anti-inflammatory properties (Moro *et al.*, 2000). It was therefore suggested that this prevention of excitotoxic damage was due to the blockade of NFκB activity, and that the activation of NFκB is necessary for NMDA-induced neurotoxicity in cerebellar and hippocampal neurones (Grilli *et al.*, 1996).

It has been reported that a caspase-3-like protease contributes to the degradation of IκB-α in striatal neurones exposed to quinolinic acid. It was demonstrated that quinolinic acid administration reduced IκB-α protein to about 40% of control levels after 12-24 hour of drug administration, and that IκB-α degradation was completely blocked by the NMDA receptor antagonist MK-801 (Qin *et al.*, 2000). In addition, the caspase-inhibitor, Ac-DEVD·CHO, inhibited quinolinic acid-induced IκB-α degradation and attenuated both quinolinic acid-induced NFκB activation and DNA fragmentation (Qin *et al.*, 2000). This provides further evidence for the activation of NF-κB and apoptosis via NMDA receptor stimulation in striatal neurones.

One gene that has been intensely focused upon is the tumor suppresser protein p53 which is a well established modulator of apoptosis. Much evidence has suggested that p53 is a pro-apoptotic gene contributing to neurodegeneration (Uberti *et al.*, 1998). For example, kainic acid induces p53 expression on neurones exhibiting apoptotic cell death (Sakhi *et al.*, 1994) and in mice which lack the p53 gene, a systemic injection of kainate acid does

not induce cell death (Morrison *et al.*, 1996). It was subsequently proposed that excitotoxic stimuli-induced NFκB contributes to cell death by switching on the transcription of p53 gene (Uberti *et al.*, 1998). Indeed, increases in NFκB binding activity correlated with a marked increase in the expression of the p53 gene, suggesting NFκB is promoting the apoptotic cascade through the upregulation of pro-apoptotic genes, such as p53 (Qin *et al.*, 1999).

5.5.2 NFκB Mediates Cell Survival

The increase in activation of NFκB in ischaemia and other neurodegenerative diseases, could be a neuroprotective mechanism. There are NFκB consensus sequences on anti-apoptotic genes, i.e.Mn-SOD, thus NFκB activation could act to induce transcription of these antioxidants, preventing oxidative stress.

Evidence surrounding the hypothesis of NFκB acting as a protective agent is through convincing studies with "knock-out" mice which lack the p65 subunit. These mice died before birth and showed a massive apoptotic death in liver cells (Beg et al.., 1995). It was also demonstrated that fibroblasts and macrophage cells from p65 deficient mice and cells from IκB mutant jurkat cell lines were more susceptible to TNF-α-induced apoptosis when NFκB signalling pathway were blocked (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Liu *et al.*, 1996b). However, the outcome of neurones in these p65 knockout mice was not observed, therefore NF-κB's role maybe completely different in neurones.

However, in other systems, NFκB activity has been shown to be crucial to the survival of neurones. For example, the pretreatment of fibrosarcoma cells with IL-1, a cytokine which stimulates the activation of NFκB, but is not pro-apoptotic, protects the neurones after an addition of TNF (Van Antwerp *et al.*, 1996). Conversely, TNF-α has been shown to prevent neuronal death in experimental models of natural neuronal death and neurodegenerative disorders (Hagg and Varon, 1993; Middleton *et al.*, 2000; Mattson and Camandola, 2001), and it has been hypothesised that TNFα stimulates NF-κB which protects cells by possibly switching two genes encoding Mn-SOD (Wong *et al.*, 1989) and the zinc finger protein, A20 (Opipari *et al.*, 1992).

The peptide, Aβ is deposited in neuritic plaques. Neurodegeneration occurs around the plaques and therefore it is proposed that Aβ peptide is neurotoxic. An *in vitro* study demonstrated a time and concentration-dependent decrease in NFκB activity in cultured rat

cortical neurones, following the exposure to Aβ, along with DNA fragmentation (Bales *et al.*, 1998). Anti-oxidants can block Aβ peptide toxicity, suggesting that neurotoxicity induced by β peptide involves the generation of free radicals. It could be proposed that Aβ appears to mediate its neurodenegeneration effects by down-regulating NFκB activity, and therefore NFκB is neuroprotective.

It seems apparent that NF-κB interacts with inhibitory regulators of the apoptotic process. It was demonstrated that NFκB activates the transcription of the caspase inhibitor IAP (inhibitor of apoptosis protein), which suppresses apoptosis (Chu *et al.*, 1997) by binding to executionary caspases, such as caspase-3 (Roy *et al.*, 1997), and protecting cells from pro-apoptotic Bcl-2 family members (Orth and Dixit *et al.*, 1997). In addition, NFκB activation has shown to block MTP induction, since cells not expressing a repressor of IκB degradation did not undergo mitochondrial depolarisation or MTP activation in response to TNF-α (Hatano *et al.*, 2001). Finally, caspase 3 can cleave IκBα *in vitro*, resulting in a constitutive NFκB repressor, suggesting that distal caspases may in turn inhibit NFκB to promote apoptosis (Barkett *et al.*, 1997).

The functional significance of the increase in activation of NFκB in ischaemia and other neurodegenerative diseases is uncertain, as many factors can influence the role of NFκB whether it be pro-apoptotic or anti-apoptotic. These include the subunit composition of the NFκB complex, genetic differences within different neuronal phenotypes, the presence of other transcription factors, the type and intensity of the stimuli, the existence of multiple IκB-dependent and IκB-independent pathways influencing NFκB activity, and whether there is a functional difference between constitutive and inducible NFκB activity (Grilli and Memo, 1999). It must also be remembered that protein synthesis is inhibited in areas of energy depletion, therefore genes may not be induced by NF-κB. Additionally, IκB synthesis may also be inhibited, consequently decreasing the potential inactivator of the activated NFκB complex.

6. The Striatum

Dysfunction of the striatum is associated with disorders of movement. In Parkinson's disease, a loss of striatal dopamine is observed, whereas in Huntington's disease, a degeneration of striatal neurones occurs. Striatal lesions are also thought to be involved in the pathogenesis of dystonia and Tourette's syndrome (Salloway and Cummings, 1996). Striatal dysfunction has recently been implicated in neuropsychiatric conditions such as, obsessive-compulsive disorders, psychoses and addictive behaviours (Calabresi *et al.*, 1997b). Hence, there is reason for understanding striatal neuronal circuits and striatal functioning under normal physiological conditions as well as pathological conditions.

6.1 Neural Circuit within the Basal Ganglia

The basal ganglia are a group of interconnected sub-cortical brain nuclei, which are fundamentally associated with the initiation and the control of movement. The basal ganglia comprises of the striatum, the globus pallidus, which consists of two segments; the medial segment (GPm) and the lateral segment (GPl), the subthalamic nucleus (STN) and the substantia nigra, which is also subdivided, into substantia nigra pars reticula (SNr) and pars compacta (SNc) (Figure 1.5).

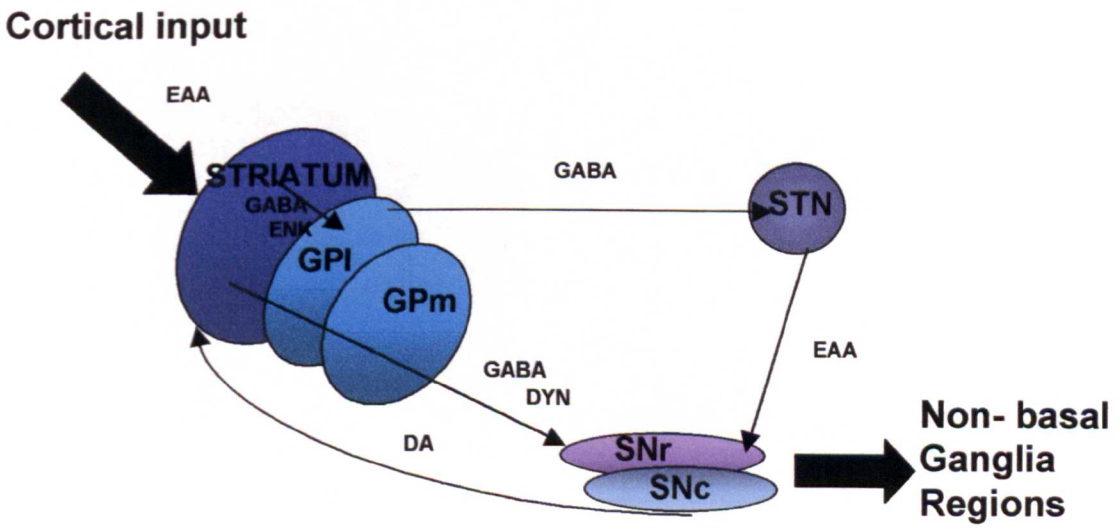

Figure 1.5

A schematic representation of the basal ganglia, indicating the nature of the interconnections between the various nuclei. Abbreviations: STN, subthalamic nuclei; GPl, globus pallidus lateral; GPm, globus pallidus medial; SNr, substantia nigra pars reticula; SNc, substantia nigra pars compacta, GABA; γ-aminobutyric acid, EAA; excitatory amino acid, DA; dopamine; DYN, dynorphin; ENK, enkephalin.

The main input to the basal ganglia arises from the cerebral cortex and terminates in the striatum, appropriately termed the cortico-striatal projection. The major output pathways are from the GPm and the SNr to the cortex via the thalamus. There are numerous neurotransmitters and neuropeptides that are involved, including dopamine, glutamate, GABA, enkephalin and opioids. Each performs a different role whether it be excitatory, inhibitory or modulatory.

6.2 Neuroanatomy and Neurochemistry of the Striatum

In the primate, the striatum is divided into the caudate nucleus and the putamen. In the rodent the striatum is undivided. The putamen is the largest area of the striatum and is located beneath the cortex lying between the external capsule and the lateral segment of the globus pallidus.

Striatal neurones receive a myriad of synaptic inputs from different sources. Afferents from all areas of the cortex and the thalamus represent the important source of excitatory amino acids, whereas the nigrostriatal pathway and intrinsic circuits provide the striatum with dopamine, acetylcholine, GABA, nitric oxide and adenosine (Hettinger *et al.*, 2001). .

The major cell type within the striatum is the medium spiny neurone which comprises over 95% of striatal neurones and is characterised by a high density of dendritic spines. They have unusual electrophysiological properties in that they show very low levels of spontaneous discharge and maintain a hyperpolarised state. They discharge phasically in response to excitatory inputs from the cortex (Kawaguchi *et al.*, 1995; Kawaguchi, 1997). Medium spiny neurones are GABAergic projecting neurones and are divided into two major types (Gerfen, 1992). One type stains positive for substance P and/or dynorphin, co-localises with GABA, expresses principally D-1 dopamine receptors and projects to the substantia nigra pars reticula. The other stains positive for enkephalin, preferentially expresses D-2 dopamine receptors and provides efferents to the globus pallidus (Bolam *et al.*, 1985; Gerfen *et al.*, 1990; Graybiel, 1990; Besson *et al.*, 1990; Simpson *et al.*, 1994).

The remaining 5% of neurones are interneurones, characterised by their morphology and their cytochemical staining. Large cholinergic aspiny interneurones have large somas, widespread dendritic trees, receive direct dopaminergic inputs and form most of their synaptic contacts with medium spiny neurones (Kawaguchi *et al.*, 1995). The postsynaptic effects of acetylcholine are mediated primarily by muscarinic receptors. Another group of

interneurones are nicotinamide adenosine dinucleotide phosphate (NADPH) diaphorase positive, this enzyme localisation being identical to NOS. These also contain somatostatin and neuropeptide Y (Bolam *et al.*, 1984; Phelps *et al.*, 1985; Graybiel, 1990; Vuillet *et al.*, 1990; Simpson *et al.*, 1994; Calabresi *et al.*, 2000).

The striatum is compartmentalised into striosomes which are embedded into a matrix, in which neurotransmitters and neuropeptides are differentially distributed and each striatal compartment has slightly different afferents and efferents to each other (Gerfen, 1985). For example, glutamate receptors are segregated, NMDA and AMPA glutamate receptors are found largely in the matrix, whereas kainate receptors are predominant in the striosome zones (Graybiel & Moratalla, 1989), and calbindin D28K is found in neurones which lie in the matrix but not in the striosome zone (Kawaguchi, 1997).

The main input to the striatum arises from the cerebral cortex, utilises glutamate as its transmitter and is excitatory (Figure 1.5). Other glutamatergic afferents to the striatum originate in the thalamus. Dopaminergic projections from the substantia nigra synapse on the same striatal neurones as excitatory afferents. Cholinergic systems in the striatum are very important and a balanced interaction between dopamine and acetylcholine is fundamental for normal striatal activity.

The main efferent pathways from the striatum project to the globus pallidus and SNr (Figure 1.5), and these projections are GABAergic. The pathway projecting to the lateral segment also utilises enkephalin as a co-transmitter, whereas the pathway leading to the medial segment/SNr uses substance P and dynorphin as co-transmitters.

7. AIMS

Although hypoglycaemia is known to be associated with severe energy failure, the exact mechanisms underlying neuronal damage after hypoglycaemic coma or ischaemia are unknown. Intracellular energy failure is also thought to be an important cofactor of the neuronal degeneration occurring in diseases such as HD, PD and AD (Rothman and Olney, 1986; Choi, 1988; Beal, 1992). Complelling evidence concerning the involvemnet of studies using positron emission tomography (PET), e.g. In both AD and HD patients a significant reduction in glucose metabolism has been reported in brain regions, such as the cortex and the basal ganglia (Beal, 1992). This reduction may be a consequence of neuronal loss. However, biochemical studies of HD postmortem tissue show a decrease in mitochondrial complex II and complex III activity in the caudate nucleus (Browne *et al.*, 1997) and ultrastructural studies of cortical biopsies from HD patients show abnormal mitochondria (Goebel *et al.*, 1978). In AD postmortem tissue, a decrease in cytochrome oxidase activity has been reported (Kish *et al.*, 1992).

Components of the excitotoxicity cascade are implicated in hypoglycaemia as well as in neuronal damage occurring in neurodegenerative conditions, including dysfunctional calcium homeostasis (German et al., 1992; Iacopino and Christakos, 1990; Mattson and Guo, 1999), EAA stimulation (Toulmond *et al.*, 1993; Klockgether and Turski, 1993), ROS production (Sun and Chen, 1998), K-ATP channel activation (Beal, 1996; Hanna and Bhatia, 1997), and DNA fragmentation (Clark *et al.*, 1999). Therefore the aim of this study is to investigate the mechanisms and components involved in hypoglycaemic-induced injury, to provide further understanding in the hope of improving therapeutic pharmacological intervention against neuronal damage induced via energy deficiency.

It is difficult to study pure hypoglycaemic toxicity *in vivo*, as hypoglycaemia *in vivo* can be accompanied by consequential alterations in metabolic parameters, including pH, cardiorespiratory status, and local cerebral blood flow. To circumvent these potential complicating factors, an *in vitro* model is used. Using an *in vitro* model most parameters can be controlled and therefore the external cellular environment can be manipulated. In addition the *in vitro* model of hypoglycaemia is relatively easy to attain and is reproducible.

The striatum is very vulnerable to energy failure, both in hypoglycaemia (Auer *et al.*, 1984; Kalimo *et al.*, 1985; Wieloch *et al.*, 1985a), and in transient reversible ischaemia (Pulsinelli *et al.*, 1982; DeGirolami *et al.*, 1984; Smith *et al.*, 1984). An *in vitro* model

was therefore designed to investigate primary events occurring in striatal cultures in conditions of glucose deprivation. It has been well established that despite the absence of extrinsic inputs, cultured neonatal striatal neurones develop a variety of cell types, synaptic connections and immunohistochemical and biochemical features of the *in vivo* striatum (Freese *et al.*, 1990, Simpson *et al.*, 1994).

Finally, much research in effective therapy for acute energy-deficient disorders and chronic neurodegeneration has concentrated on the EAA receptor itself but recently the challenge to intervene in the neurotoxic process at a stage beyond the receptor, at the level of transcription factors, has seemed more appropriate. Since NFκB was reported to be present in the brain, it is a strong candidate for this pharmacological regulation, considering the evidence for its role in excitotoxicity, the nature of genes it induced and the various stimuli which can be involved in its activation. In view of this evidence linking NF-κB to neurotoxicity in the striatum, the role of NF-κB in striatal neurodegeneration was also selected as the focus for further study.

Chapter Two. Materials and Methods

2.1 Materials

2.1.1 Primary Dissociated Cell Culture

All products used in cell culture were obtained from Life Technologies (Invitrogen, U.K) except Euthatal (Rhône Mérieux Ltd, Essex).

2.1.2 Cell Viability Assays

XTT (Molecular Probes, U.S); PMS (Sigma, U.K); MAP2 antibody (Sigma, U.K)

2.1.3 Immunocytochemistry

Phosphate Buffered Saline (PBS): 1.3M NaCl (Fisher Chemicals, U.K)

30mM Na_2HPO_4 (Sigma, U.K)

70mM NaH_2PO_4 (Sigma, U.K)

Paraformaldehyde (PFA) (Riedel-de Haën, Germany); Normal goat serum (Scottish Antibody Production Unit (SAPU), Scotland); Triton-X100 (Sigma, U.K); Activin Biotinylate Conjugate (ABC) Reagent (Vector Laboratories Ltd, U.K); Vector peroxide substrate (VIP) kit (Vector Laboratories Ltd, U.K); Ethanol (BDH Laboratory Supplies, U.K); Histoclear and Histomount (National Diagnostics, U.S).

2.1.4 TUNEL Staining

APOPDETECT™ PeroxidasePlus (The Quantum Biotechnologies Group, Q-Biogene, U.K); Acetic acid (BDH Laboratory Supplies, U.K); Methyl green (Calbiochem, U.K); n-buthanol (BDH Laboratory Supplies, U.K).

2.1.5 Preparation of Nuclear Extraction for EMSA

HEPES (Sigma, U.K); $MgCl_2$ (BDH Laboratory Supplies, U.K); EDTA (Sigma, U.K); Glycerol (BDH Laboratory Supplies, U.K); DDT (Sigma, U.K); Nonidet-p40 (NP40) (Sigma, U.K).

2.1.6 Labelling NFκB Oligonucleotide Probe

TE Buffer (pH 8.0): 1M Tris (Sigma, U.K)

0.5M EDTA (Sigma, U.K)

NFκB oligonucleotide (Promega, U.S); T4 polynucleotide kinase (Promega, U.S); T4 polynucleotide kinase 10X buffer (Promega, U.S); ^{33}P-ATP (NEN Life Sciences, U.K); Chromatography G-25 spin columns (Biorad Laboratories, U.S); Scintillation Fluid (Ecoscint, National Diagnostics, U.S).

2.1.7 Electrophoretic Mobility Shift Assay

KCl (BDH Laboratory Supplies, U.K); HeLa Cells (5mg/ml; Promega, U.S); 6% DNA Polyacrylamide gel (Novex, Invitrogen, U.K); TBE Buffer X5 (Novex, Invitrogen, U.K); Filter paper sandwich nylon membrane (Novex, Invitrogen, U.K); Biomax MR Film (Kodak, Sigma, U.K).

2.1.8 Fura-2 Calcium Measurements

Artificial Cerebral Spinal Fluid (ACSF): 25mM $NaHCO_3$ (Riedel-de Haën, Europe)

115mM NaCl (Riedel-de Haën, Europe)

10mM D-Glucose (Fluka, Sigma, U.K)

2.5mM $CaCl_2$ (BDH, U.K)

2.2mM KH_2PO_4 (BDH, U.K)

1.2mM $MgSO_4$ (BDH, U.K)

2mM KCl (BDH, U.K)

Fura-2 (Molecular Probes, U.S); Glutamate (Sigma, U.K).

2.1.9 Drugs

CsA [500μM stock], TFP [2mM stock], Ouabain [10mM stock], PDTC [10mM stock], EGTA [10mM stock], BAPTA [1mM stock], nickel [100mM stock] nicardipine [10M stock], TMB-8 [10mM stock], dantrolene [1mM stock], MK801 [10mM stock], CNQX [10mM stock], 2-APB [10mM stock], A23187 [10mM stock], aspirin [18mM stock] and parthenolide [11.8mM stock] were all obtained from Sigma (U.K).

HNE [62mM stock] (Alexis Biochemicals, U.K); Z-DEVD-FMK [1.5mM stock] (Calbiochem, U.K).

EGTA, BAPTA, A23187, nicardipine, 2-APB, CNQX, diazoxide, Z-DEVD-FMK, fura-2, parthenolide and HNE were made up in DMSO to form a stock solution and then diluted to the required concentration in PBS. The final concentration of DMSO never exceeded 0.1%. CsA was made up with ethanol and dantrolene in an alkaline aqueous solution (NaOH). All other drugs were made up with PBS.

2.1.10 Antibodies

Bax, cytochrome c, caspase-3, caspase-9, p65 and p50 were all obtained from Santa Cruz Biotechnology (U.S).

XRCC1 and DNA polymerase-β (Lab Vision Corporation – Neomarkers). ERCC3 and MSH2 (Autogen Bioclear, U.K). Secondary biotinylated antibodies (Vector Laboratories Ltd, U.K). The specificity of the antibodies used was not determined in this study. Data from other experiments using the same antibodies, at the same concentration, proved that these were specific for the proteins they were staining (Hara *et al.*, 1996; Liu *et al.*, 1996c; Srinvasula *et al.*, 1998; Kaufmann *et al.*, 1993; Caldecott *et al.*, 1996; Hermon *et al.*, 1998; Grilli and Memo, 1999; Seeger *et al.*, 2000). It can also be noted that the immunoreactivity of the antibody was measured which is not a direct measurement of the protein itself.

2.2 Primary Dissociated Cell Cultures

Pregnant Wistar rats (200-300g) gestation day 17 (E17) were killed with an overdose of sodium pentobarbitone (Euthatal), administered intraperitoneally. Embryos (10-12) were removed from the uterus, decapitated and placed in cold modified essential medium (MEM, see appendices, Table 1). Brains were removed from the crania and using the optic chiasm as the posterior marker the dorsal striata were dissected out (Misgeld and Dietzell, 1988). The subsequent procedure was carried under sterile conditions in a laminar flow hood. Striatal tissue was then minced and washed three times with ice-cold MEM. Cells were dissociated in 5ml of 2.5% trypsin solution for 45 minutes at 37°C. Trypsin was inactivated by the addition of 5ml of foetal calf serum and subsequently the tissue was washed three times again in MEM.

The tissue was triturated through a pasteur pipette in Dulbecco's modified Eagle's medium (see appendices, Table 2) with GlutaMAX I™ (1000mg/L) supplemented with 20% horse serum and 5μg/ml penicillin-streptomycin, and seeded at a density of approximately (0.15 x 10^6 cells/cm, see Simpson et al, 1994), onto either eight-well multichamber glass slides or 6-well plates successively coated with poly-D-lysine (4μg/ml) and mouse laminin (6μg/μl). The cultures were incubated at 37°C and with 5% CO_2/95% O_2 in a humidified incubator (ICN Flow).

After 72 hours the medium was changed to Neurobasal medium with penicillin-streptomycin (5μg/ml) and B27 supplement added. B27 supplement controls the proliferation of non-neuronal cells. Every three days thereafter the medium was changed. Cultures between 14-18 days old were used for experimentation. All experiments were done at 37°C, excluding fura-2 work which was at room temperature.

2.2.1 Hypoglycaemic and Recovery Conditions

Cultures subjected to hypoglycaemia were washed twice with Dulbecco's MEM (without glucose and sodium pyruvate) and Neurobasal medium was replaced with the glucose/sodium pyruvate-free medium. Normoglycaemic (control) cultures were treated the same with the equivalent Dulbecco's MEM which contained glucose (100mg/L) and

sodium pyruvate (110mg/L). The duration of hypoglycaemia-mediated toxicity was dependent on experimental procedure.

The recovery phase was determined by returning the glucose content back into the cultures, using the medium for normoglycaemic cultures. Again, normoglycaemic cultures received equivalent procedures.

Cultures were stimulated with various drugs and inhibitors for varying lengths of time to examine neuroprotective effects, as detailed in the following chapters. Initially, the original duration of the experiment was for 24 hours, i.e. cultures were incubated in glucose-free medium, with or without drugs, for 24 hours. This length of hypoglycaemia produced a 75 % decrease in XTT staining. However, it was subsequently observed that 4 hours of hypoglycaemia produced an approximately 50% decrease in XTT staining and that this percentage was sufficient to experiment with, i.e. there was a significant amount of injury that would improve if drugs were neuroprotective. Another important factor was that results were quicker to obtain.

2.3 Cell Viability Assay

2.3.1 Mitochondrial Functioning

XTT (sodium(2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-*2H*–tetrazolium-5-carbxanilide) is a tetrazolium salt which is cleaved and reduced within the active mitochondria of living cells by the enzyme succinate dehydrogenase to form soluble formazan crystals (Mosmann *et al.,* 1983). The final concentration of XTT used was 1mg/ml. In addition, phenazine methosulphate (PMS) was added to increase the rate of reduction reaction at a final concentration of 1.53µl/ml (Goodwin *et al.,* 1995). The XTT/PMS reagent was dissolved in PBS and added to the medium and incubated at 37°C and 5% CO_2/95% O_2 in a humidified incubator, for three hours. These three hours were included within the experimental period.

Subsequently, the medium was pipetted into a 96 well plate and each sample, in triplicates was read spectrophotometrically (Dynex Revelation 4.04 software) by an microplate reader at a wavelength of 450nM (Opsys MR, Dynex Technologies, U.K). Background signals, obtained from wells containing XTT/PMS in medium not exposed to cells were subtracted from the sample readings.

2.3.2 Cytoskeletal Marker

Microtubule-associated protein 2 (MAP-2) is present in dendrites and cell bodies of neurones. Toxic stimuli reduce MAP-2 immunoreactivity (Whittaker *et al.*, 1993; Arias *et al.*, 1997; Brooke *et al.*, 1999). Cells were treated with specific stimuli and immunocytochemical analyses were performed using the antibody against MAP-2 (refer to section 4).

2.4 Immunocytochemistry

Cells were fixed using ice-cold 4% paraformaldehyde in phosphate-saline buffer (PBS), for 30 minutes. After two washes with PBS, blocking serum (PBS with 15% normal goat serum) was added to the cells for one hour. The blocking serum was removed and the primary antibody solution (PBS with various specific antibodies (0.2-2μg/ml), 3% normal goat serum and 0.005% Triton-X100 was added over night at 4°C in a humidified chamber.

The cells were washed for five minutes with PBS, three times and then were incubated with the secondary antibody solution (PBS with specific species biotinylated secondary antibody (1μg/ml) and 15% normal goat serum) for 60 minutes at room temperature. Following a further three five minute washes with PBS, the cells were incubated with an ABC reagent (prepared using manufacturer's protocol) for 60 minutes at room temperature. The cells were washed again three times for five minutes per wash with PBS and then incubated with the VIP solution (prepared using manufacturer's protocol) for 10-15 minutes at room temperature, until staining was visible under the light microscope.

After the cells were rinsed once in PBS for five minutes and then in distilled water for a further five minutes, they were dehydrated gradually in increasing concentrations of alcohol; 70%, 90% and three times in 100% for 5 minutes per concentration. Cells were allowed to dry briefly (~2 minutes) and then immersed in histoclear overnight. Cells were preserved by mounting the slides with coverslips using histomount and then left to dry.

2.5 TUNEL Staining

The APOPDETECT™ kit is designed to label the free 3′ -OH DNA termini *in situ* with chemically digoxigenin labeled and unlabelled nucleotides. The nucleotides contained in the reaction buffer are enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT). TdT catalyses a template-independent addition of nucleotide

triphosphates to the 3′ -OH ends of double-stranded or single-stranded DNA. The incorporated nucleotides form an oligomer to unlabelled nucleotide, and the kit is optimised to promote anti-digoxigenin antibody binding to the oligomer. DNA fragments which have been labelled with the digoxigenin-nucleotide are then allowed to bind an anti-digoxigenin antibody that is conjugated to a peroxidase. The bound peroxidase antibody conjugate enzymatically generates a permanent, intense, localised stain from chromogenic substrates, providing sensitive detection in immunocytochemistry. This kit has been shown to be effective in neurotoxicity models (Qin *et al.*, 1998; Parsadanian *et al.*, 1998).

The APOPDETECT™ manufacturers protocol was carried out, using the reagents provided within the kit as follows. Cultures were either exposed to normoglycaemia or hypoglycaemia for a specific duration (refer to relevant chapters) and then fixed in 1% paraformaldehyde in PBS (pH 7.4) for 10 minutes at room temperature. The fixative was washed off with two changes of PBS for five minutes each wash. The slides were then placed into precooled ethanol:acetic acid solution (2:1) at –20°C for five minutes which permeabilised the cells. This solution was washed off with two changes of PBS for five minutes each wash. Excess liquid was tapped off and 13µl/cm^2 equilibration buffer was added immediately to the cells for ten seconds at room temperature. This liquid was then tapped off and 1.1µl/cm^2 TdT enzyme (70% reaction buffer and 30% TdT enzyme) added to the slides for one hour in a humidified chamber at 37°C. Following this incubation, the slides were agitated for fifteen seconds and incubated for a further ten minutes in stop/wash buffer (1ml stop/wash buffer and 34 ml deionised water (dH_2O) at room temperature. The slides were then washed three times in PBS for one minute each wash. Excess liquid was tapped off before incubating the slides with 13µl/cm^2 room temperature anti-digoxigenin peroxidase conjugate for 30 minutes in a humidified chamber at 37°C. Slides were then washed four times in PBS for two minutes per wash at room temperature and VIP substrate (refer to section 2.4) was applied for twelve minutes at room temperature. The slides were washed again three times for one minute in dH_2O and then incubated in dH_2O for five minutes at room temperature.

The cells were counterstained by leaving the slides in 0.5% (w:v) methyl green for ten minutes at room temperature and washed in three changes of dH_2O, agitating the slides for the first two washes and incubated for thirty seconds for the third. The slides were then washed with 100% n-buthanol three times agitating the slides for the first two washes and incubated for thirty seconds for the third. The slides were finally dehydrated by placing a series of alcohols and mounted the same as described in section 2.4.

2.6 Preparation of Nuclear Extraction for EMSA

The medium was removed, leaving approximately 1ml in which the cells were scraped off and spun at 500g for 10 minutes. Subsequently the pellet of cells were lysed with 50µl of extraction buffer (5mM HEPES, 1.5mM $MgCl_2$, 0.2mM EDTA and 26% glycerol, 300mM $NaCl_2$, 0.1% DDT, 0.025% nonidet-p40 (NP40) and one protease cocktail inhibitor tablet/5ml). The samples were then homogenised, frozen at -70°C and thawed out at 37°C, three times and finally centrifuged at 4°C, at 2100g for 20 minutes. Aliquots of the supernatant containing nuclear protein were frozen at –70°C, until needed.

2.7 Labelling NFκB Oligonucleotide Probe

The probe used for all EMSAs contained the double stranded NFκB oligonucleotide (0.6pmol/ml; '5-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAA GGGTCCG-5'), 1µl T4 polynucleotide kinase 10X buffer, 1µl T4 polynucleotide kinase, 2.65µl deionised water and 5µl ^{33}P-ATP (2000Ci/mmol). This was incubated at 37°C for 10 minutes. The reaction was stopped by adding 1µl of 0.5M EDTA and equilibrated with 39µl of TE (Tris-EDTA) buffer. The labelled oligonucleotide was then separated from unincorporated nucleotides by running through a gel chromatography G-25 spin column. Finally, 2µl of labelled oligonucleotide was added to 8ml of scintillation fluid and put through a liquid scintillation analyser (Packard Tri-Carb 2100TR) to determine the radioisotope concentration of the probe.

2.8 Electrophoretic Mobility Shift Assay

Approximately 4µl-6µl (5-10mg, see Simpson and Morris, 1994) of nuclear protein was incubated for 30 minutes at 4°C with 4µl labelled NF-kB oligonucleotide probe in binding buffer (10mM HEPES, 50mM KCl, 0.2mM EDTA, 2.5mM DDT, 10% glycerol, 0.05% NP40 and a tablet of protease inhibitors/5ml). The total reaction volume was 20µl. Labelled samples were loaded into a non-denaturing 6% polyacrylamide gel and run in 0.5 x TBE (Tris boric acid EDTA) buffer for 70-80 minutes at 100 volts/0.01 amps. The DNA-protein complexes were transferred from the gel to a nylon membrane in 0.5 x TBE buffer for 120 minutes at 25 volts/0.36 amps. The membrane was then irradiated with UV light for 1 minute and exposed to Biomax MR film (Kodak) for 14 days. Nuclear protein extract (2µl) from the human tumor cell line, HeLa cells, was used as a positive control.

2.9 Fura-2 Calcium Measurements

Cells were grown on poly-D-lysine and laminin coated coverslips (No. 0, Chance Propper Ltd, England) within 6-well plates, treated accordingly and loaded with fura-2AM (1µl/ml; dissolved as a 1mM stock in DMSO) for 30 minutes at 37°C and 5% CO_2/95% O_2 in a humidified incubator. This is membrane permeable such that the cells are able to convert it into a membrane-impermeable form in their cytosol, i.e. the acetoxymethyl ester (AM) linkage is cleaved by ubiquitous intracellular esterases.

Small sections of coverslip were placed on a recording bath (No. 0) within a bath filled with ACSF (approximately 1ml). Constant perfusion (1ml per minute) with ACSF occurred during the recording at room temperature (~20°C). Glucose-free ACSF was used with hypoglycaemic-treated cells. Fluorescence measurements were made using a microfluorimeter consisting of an inverted fluorescence microscope (Nikon Diaphot) and a photomultiplier tube with a bi-alkali photocathode, as described by McCarron and Muir (McCarron and Muir, 1999). Single cells were alternatively excited with wavelengths of 340nM and 380nM every 10 ms for 8.5ms at each wavelength to give a ratio at baseline using a PTI delta scan (Photon Technology International Inc, East Sheen, London, U.K). The binding of calcium to fura-2 increases the absorption at 340nm and decreases the absorption at 380nm, therefore a ratio of emission from 340nm excitation to emission from 380nm excitation is related to intracellular calcium concentration, and is independent of variation caused by differences in cell thickness, dye quantity or photobleaching. Baseline recordings were allowed to run for approximately 1 minute before measuring the ratios.

Glutamate (1mM and 10mM) was added to the bathing medium to obtain a response to depolarisation to determine the viability of the cell.

The calibration of the ratios obtained from the fura-2 imaging was performed at the end of the experiment. Ratios from two different solutions of fura-2, one containing a saturating concentration of Ca^{2+} (100mM), and the other containing zero Ca^{2+} and EGTA (10mM) were obtained.

The following equation, devised by Grynkiewicz *et al.*, was used to calculate the concentration of intracellular calcium (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = Kd \frac{(R-R_{min})}{(R_{max}-R)} * \frac{sf_2}{sb_2}$$

Where R is the experimental ratio obtained, R_{min} (0.4) the ratio obtained from a solution of fura-2 lacking calcium, R_{max} (8.0) the ratio obtained from a solution of fura-2 containing calcium, sf_2 the emission detected from 380nM excitation from the calcium-free solution, sb_2 the emission detected from 380nm excitation of the calcium-containing solution and Kd (224nM), is the dissociation constant of fura-2 for calcium (Grynkiewicz *et al.*, 1985).

Fluorescence equipment was controlled by PTI Felix software (Photon Technology International Inc, East Sheen, London, U.K), which also performed the storage and analysis of the fluorescence.

2.10 Image Analysis and Statistics

The number of individual experiments within each pooled set of data was between three and five. Individual experiments were obtained from separate animals.

For each experimental culture thirty immunoreactive cells were measured from randomly chosen fields of view (five cells per field of view). The cytoplasmic area and/or the nuclear area was isolated and the relative optical density (ROD) of immunoreactive staining was measured and a mean ROD was calculated. In order to measure MAP2 immunoreactive staining, a ROD threshold was set and the mean number of pixels per unit area within that threshold was recorded for each of the fifteen fields of view. A mean area of the specific range of ROD was calculated. Consistency was maintained by measuring cells within the same culture and within the same experiment on the same day. All recordings were made by using Image NIH version 1.52 (Macintosh) software.

Data were converted to percentages of control (except TUNEL staining data) in order to normalise data (where control was equal to 100%) and the standard error of the mean was calculated. Using the statistical package Minitab, statistical significance was assessed using two-way analysis of variance (ANOVA) with post hoc Tukey's test for comparison between groups. One-way ANOVA with post hoc Fisher's test and the non-parametric Mann-Whitney test was used for comparison within groups. The 95% confidence intervals were tested for comparison between treatment groups and control.

Chapter Three.

Components of Hypoglycaemic toxicity

3.1 Introduction

The modulation of specific components active in hypoglycaemic neurotoxicity has been reported to prevent cell death (Nellgard *et al.*, 1992; Chung and Lee, 1998; Tasker *et al.*, 1992; Ferrand-Drake and Wieloch, 1985b). In this study a number of these components were manipulated with defined pharmacological tools, in the effort to determine whether they were involved in hypoglycaemia-induced cell death in striatal cultured cells. Figure 3 is a schematic diagram showing the site of action of each compound used in the attempt to promote survival in cells exposed to hypoglycaemia.

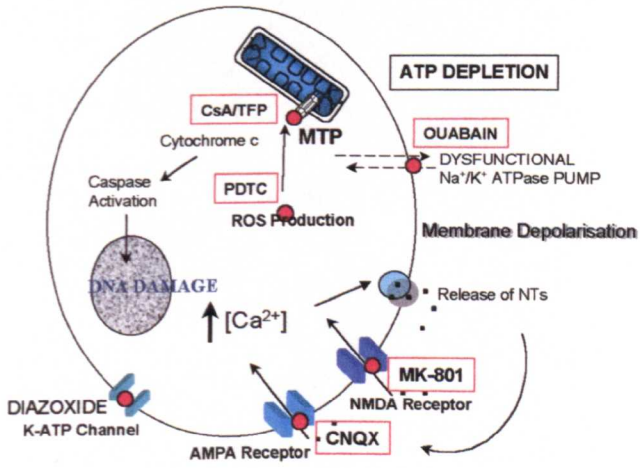

Figure 3
A schematic diagram of the mechanisms involved in hypoglycaemic-induced cell death and the site of pharmacological action, including glutamate receptors and potassium channels, free radical production and the mitochondrial permeability pore. Inhibitory factors are shown in red boxes.

Diazoxide is a channel opener for the ATP-dependent K^+ channels (K-ATP). It mediates its stimulatory effect by binding to the sulfonylurea receptor subunit of the K-ATP channel. Diazoxide is lipid soluble and can activate K-ATP channels regardless of the side of the

membrane to which it is applied (see Ashford review, 1988). The theory is that diazoxide would increase the activity of K-ATP channels, thereby hyperpolarising the membrane, reducing excitability and preventing the opening of the VGCC.

MK801 is an anti-convulsant, which shows high antagonist potency for NMDA receptors. It is a non-competitive NMDA receptor antagonist, which binds to the phencyclidine site within the channel pore. It is dependent on the presence of glutamate agonists, i.e. it inhibits NMDA receptor responses by binding when the receptor is in the agonist-activated open state (Dingledine *et al.*, 1988). Assuming that the membrane is depolarised in the absence of glucose, MK801 should prevent excitotoxic damage through the NMDA receptor.

CNQX is a competitive AMPA receptor antagonist (For review, Davies, 1990). It is thought that by blocking AMPA receptors, the prevention of a glutamate-dependent sustained depolarisation and secondary excessive influx of calcium will occur, therefore CNQX will be acting as an early neuroprotectant in the excitotoxicity cascade.

PDTC is a stable derivative of dithiocarbamate. Dithiocarbamate is widely used, from insecticide to therapeutic agents for alcoholism, metal intoxication and cancer. PDTC has at least two chemical properties. One is a chelating activity for heavy metals, the other is an antioxidative activity of its dithiocarboxy group. It is known to inhibit the production of hydroxyl radicals and inducible nitric oxide (Seaton *et al.*, 1997; Moro *et al.*, 1998). In normoglycaemic conditions PDTC may exert neuroprotective effects by scavenging the excess of ROS thus preventing irreversible damage, such as lipid peroxidation.

Cyclosporin A (CsA) is a member of a class of potent immunosuppressants. In human medicine, CsA is widely used in transplantation to prevent rejection of the transplanted organ (Keown, 1990; Liu, 1993). CsA is a lipophilic molecule, so can diffuse through the cell membrane, where it binds to a cytosolic protein termed cyclophilin. The receptor for the cyclophilin/CsA complex is found in the inner membrane of the MTP and therefore it is thought that CsA blocks the pore by binding to this receptor (Connern and Halestrap, 1994).

Trifluoperazine (TFP) is a known calmodulin antagonist but also inhibits the MTP by mechanisms not yet understood (Hoyt *et al.*, 1997). Additionally, it was also reported that trifluoperazine can inhibit mitochondrial Na^+/Ca^+ exchange in intact neurones (Hoyt *et al.*,

1997). The hypothesis is, that by blocking the MTP with either CsA or TFP, mitochondrial swelling and the release of ROS and cytochrome c will be prevented.

Finally, if the ATP-dependent Na^+/K^+ pump function is inhibited by the ATP depletion during hypoglycaemia, it can be suggested that pharmacological inhibition of the pump would produce the same effects as hypoglycaemia, with membrane depolarisation leading to a toxic influx of calcium. This would be an insight as to whether the removal of glucose does specifically prevent the normal functioning of the ATP-dependent Na^+/K^+ pump. Ouabain is an ATP-dependent Na^+/K^+ pump inhibitor and if the hypothesis is correct should have deleterious effects on normoglycaemic cells. It may produce further damage to hypoglycaemic cells.

In this series of experiments, the above compounds were tested for their effects on hypoglycaemic neurotoxicity as assessed using either XTT staining (for mitochondrial respiratory function) or MAP2 immunoreactivity (for cytoskeletal integrity).

3.2 Results

Cell viability, following the exposure to hypoglycaemia for either 4 or 24 hours was analysed using the XTT assay. Cell viability was significantly reduced by 43.6% (±16.9) compared to normoglycaemia after 4 hours and by 74.0% (±8.4) after 24 hours (Figure 3.1).

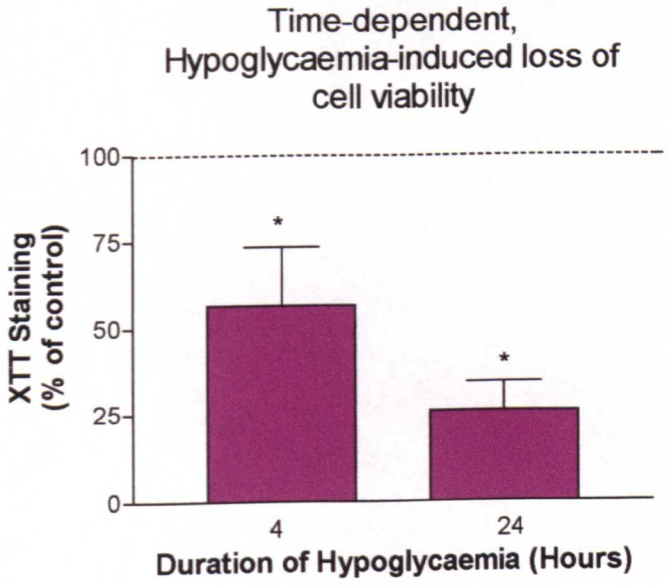

Figure 3.1
The effect of hypoglycaemia on striatal cells. The glucose content was removed from cultures for either 4 or 24 hours. Cell viability was measured by XTT staining. There is a significant reduction in the percentage of cell viability in hypoglycaemic cultures after both 4 and 24 hours compared to normoglycaemic cultures (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). The cell viability of cultures exposed to 24 hours of hypoglycaemia is significantly lower than cultures exposed to only four hours of hypoglycaemia (one-way ANOVA, $F(1,4)=7.86$, $p<0.05$). Data are expressed as percentage of XTT staining in normoglycaemic cultures (100%) (n=5).

Figure 3.2a compares the morphological changes in hypoglycaemic cells (B) compared to normoglycaemic cells (A). In hypoglycaemic cultures, the glucose was removed for 24 hours prior to staining for MAP2. Staining shows shortened, fragmented dendrites in hypoglycaemic cultures compared to normoglycaemic cultures. Clumps of cells can be observed in hypoglycaemic cultures where it appears cells have migrated towards each other. In contrast, cells in normoglycaemic cultures are evenly spread with long dendrites making contact with each other. There are also less cells in hypoglycaemic cultures, and it is assumed that profoundly sick cells have become unstuck from the adhesive coating lining the culture chambers and consequently are removed during fixation. The morphological changes in Figure 3.2a are reflected in Figure 3.2b, which is a quantitative

histogram of MAP2 immunoreactivity in normoglycaemic and hypoglycaemic cultures. MAP2 immunoreactivity decreased by 59.2% ± 23.3 in hypoglycaemic cultures compared to normoglycaemic cultures.

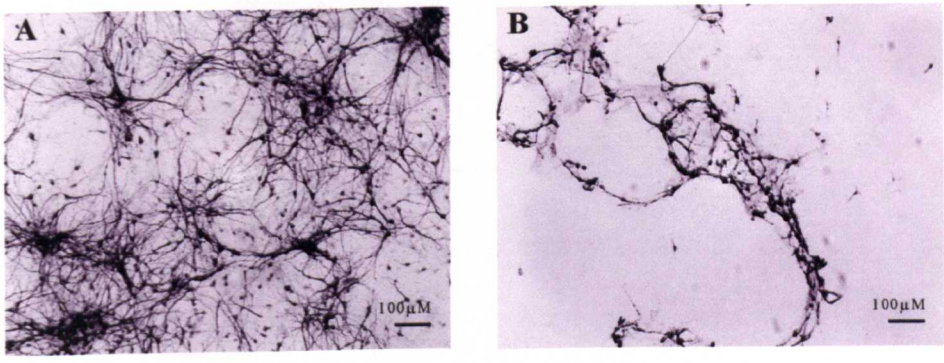

Figure 3.2a
MAP-2 immunostaining in cultures treated in normoglycaemic conditions, (A) or hypoglycaemic conditions for 24 hours (B). The dendrites in hypoglycaemic cultures are retracted or fragmented, there are fewer cells and the remaining cells appear to be in clumps.

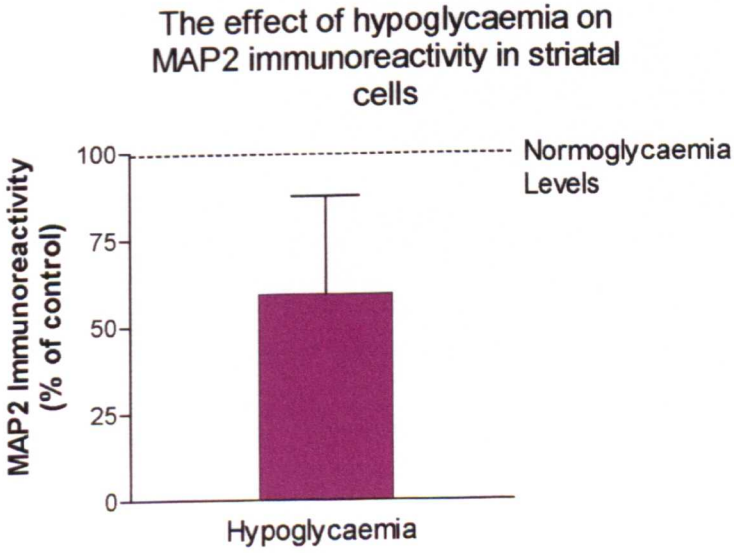

Figure 3.2b
MAP-2 immunostaining in cultures treated in normoglycaemic conditions or hypoglycaemic conditions for 24 hours. In hypoglycaemic cultures MAP2 immunoreactivity significantly decreases to 59.2% ± 23.3 when compared with normoglycaemic cultures at 100% (n=5).

In normoglycaemic cultures, all concentrations of ouabain produced a significant reduction in cell viability after 4 hours compared to non-treated normoglycaemic cultures. After 4 hours of hypoglycaemia alone, cell viability was significantly reduced from 100% but there was no further reduction with ouabain. The percentage of cell death remained the same, regardless of the concentration of ouabain (Figure 3.3).

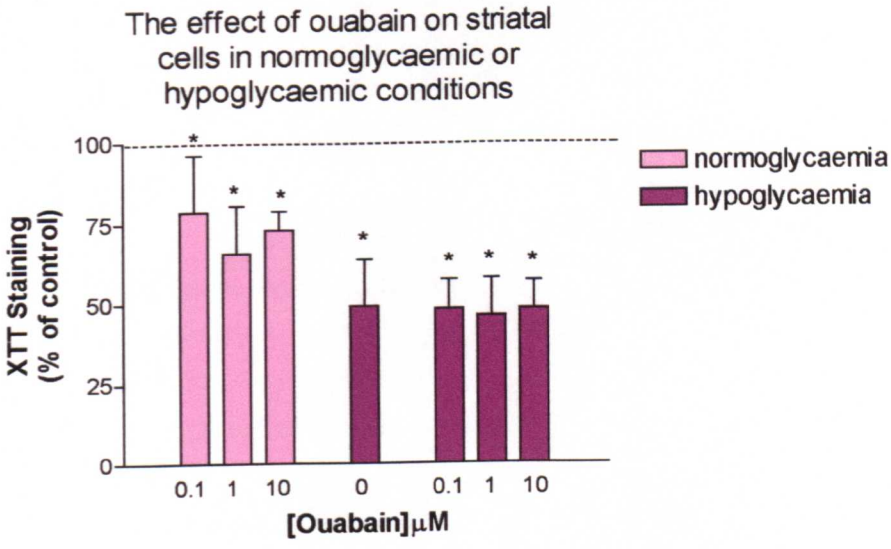

Figure 3.3
The effect of ouabain on striatal cells in normoglycaemic and hypoglycaemic cultures. Ouabain was added at various concentrations to cultures for 4 hours. In hypoglycaemic cultures the glucose was removed just prior to the addition of ouabain. Cell viability was measured by XTT staining. Removing the glucose produced a significant decrease in cell viability in the absence of ouabain. There is an effect of hypoglycaemia but no effect was seen with ouabain (two-way ANOVA, effect of hypoglycaemia, $F(1,16)=43.9$, $p<0.001$; effect of ouabain, $F(3,16)=2.8$, $p=0.08$). There is no effect of ouabain in hypoglycaemic cultures (one-way ANOVA, $F(3,8)=0.04$, $p=1.0$) but a significant reduction in cell viability is seen in normoglycaemic cultures at each concentration (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). Data are represented as a percentage of XTT staining in normoglycaemic cultures without oubain treatment (100%) (n=3).

There were no neuroprotective effects observed with MK801 in hypoglycaemic treated cells. Cell viability was significantly reduced after 4 hours of hypoglycaemia. However there was no significant change in cell survival when MK801 was added. Thus, MK801 had no effects on normoglycaemic cultures (Figure 3.4).

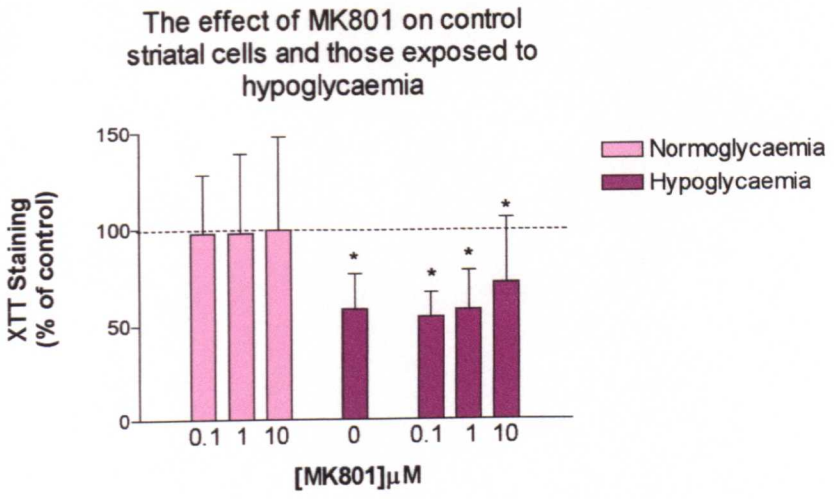

Figure 3.4
The effect of MK801 on normoglycaemic and hypoglycaemic cultures. MK-801 was added at various concentrations to cultures for 4 hours. In hypoglycaemic cultures, the glucose was removed just prior to addition of MK-801. Cell viability was measured by XTT staining. There was a significant reduction of cell viability in hypoglycaemic cultures (*$p<0.05$ versus 100%; 95% confidence intervals of the mean) but no significant effects of MK801, (two-way ANOVA, $F(3,24)=0.16$, $p=0.9$, no significant effect of MK801, $F(1,24)=13.2$, significant effect of hypoglycaemia, $p<0.001$). In particular, MK-801 did not show significant neuroprotection against hypoglycaemic cell damage at any concentration (one-way ANOVA, $p=0.7$). The percentage of cell viability did not reach normoglycaemic levels. Data are represented as a percentage of XTT staining normoglycaemic cultures without MK-801 treatment (100%) (n=4).

Similarly the blockade of AMPA receptors, by CNQX, has no neuroprotective effect against hypoglycaemic-induced injury. A significant decrease in cell viability was observed in hypoglycaemic cultures, after 4 hours, compared to normoglycaemia cultures but CNQX showed no significant ability to reduce this effect. Therefore, CNQX had no effect on normoglycaemic cells (Figure 3.5).

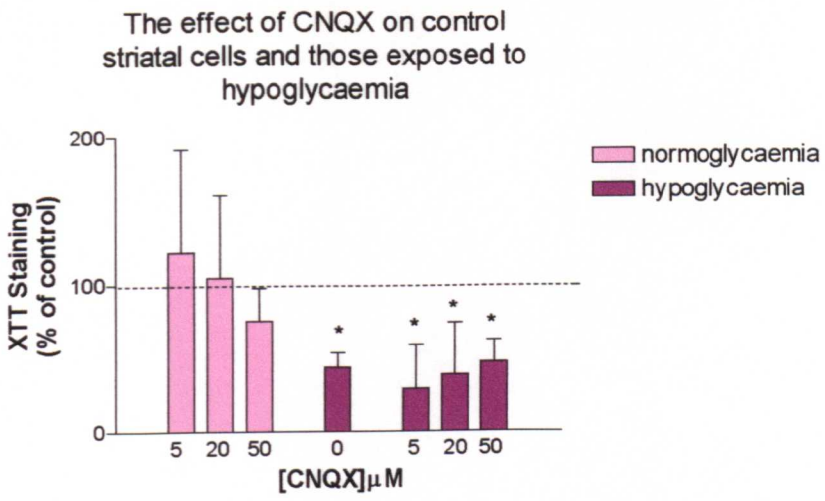

Figure 3.5
The effect of CNQX on normoglycaemic and hypoglycaemic cultures. CNQX was added to cultures at various concentrations to cultures for 4 hours. In hypoglycaemic cultures, the glucose was removed just prior to the addition of CNQX. XTT staining significantly decreased in hypoglycaemic cultures (*$p<0.05$ versus 100%; 95% confidence intervals of the mean) but there was no significant effect of CNQX (two-way ANOVA, $F(1,16)=16.1$, significant effect of hypoglycaemia, $p<0.001$. effect of CNQX dose, $F(3,16)=0.16$, $p=0.9$), in particular, there was no significant neuroprotection against hypoglycaemic cell damage when cells were treated with CNQX (one-way ANOVA, $F(1,3)=0.3$, $p=0.8$). Cell viability was measured by XTT. Data are represented as a percentage of XTT staining normoglycaemic cultures without CNQX treatment (100%) ($n=4$).

After 24 hours of hypoglycaemia, the viability of cells was significantly reduced compared to cells in normoglycaemic cultures (Figure 3.1). The addition of CsA to hypoglycaemic treated cells had no significant effect at any concentration (0.1μM - 10μM). Similarly, CsA had no significant effect on normoglycaemic cells (Figure 3.6). Figure 3.7 shows the effect of CsA on cell viability measured by MAP2 staining. A significant decrease was seen in MAP2 staining in cells treated with 24 hours of hypoglycaemia, compared to staining measured in normoglycaemic cells. There was no significant difference in MAP2 staining between hypoglycaemic cells and hypoglycaemic cells treated with CsA (Figure 3.7).

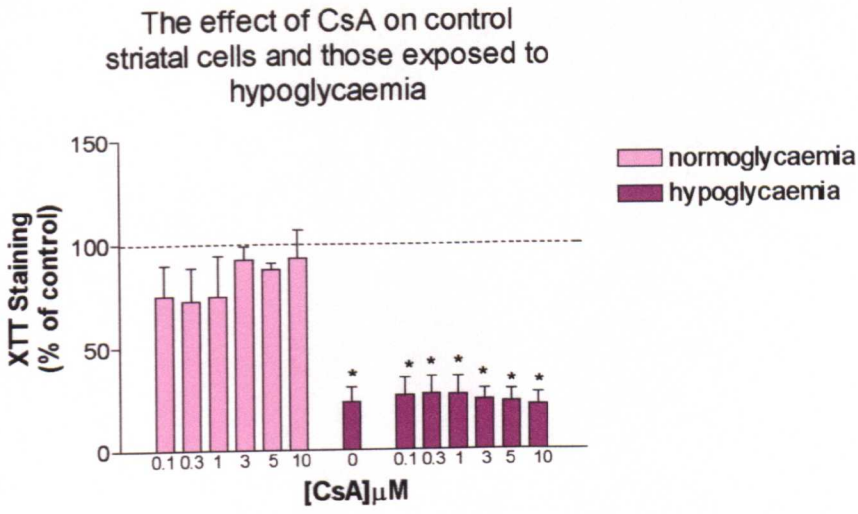

Figure 3.6

The effect of CsA on normoglycaemic and hypoglycaemic cultures. CsA was added at various concentrations to cultures for 24 hours. In hypoglycaemic cultures, the glucose was removed just prior to the addition of CsA. Cell viability was measured by XTT staining. There is a significant reduction of cell viability in hypoglycaemic cells (*$p<0.05$ versus 100%; 95% confidence intervals of the mean) but viability was not affected by CsA concentration (two-way ANOVA, $F(1,28)=365.5$, significant effect of hypoglycaemia, $p<0.001$; effect of CsA, $F(6,28)=1.26$, $p=0.3$). CsA did not show neuroprotection against hypoglycaemic cell damage at any concentration (one-way ANOVA, $F(6,14)=0.23$, $p=0.96$). Data are represented as a percentage of XTT staining in normoglycaemic cultures without CsA treatment (100%) (n=3).

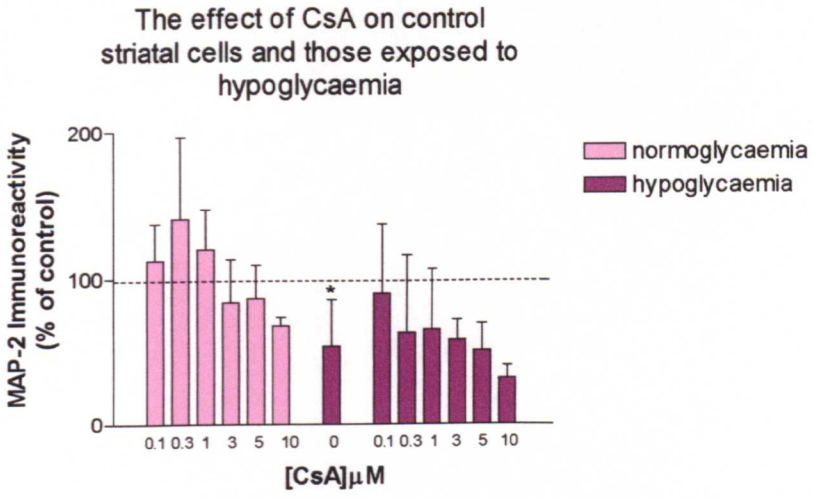

Figure 3.7

The effect of CsA on normoglycaemic and hypoglycaemic cultures. CsA was added at various concentrations to cultures for 24 hours. In hypoglycaemic cultures, the glucose was removed just prior to the addition of CsA. Cell viability was measured by MAP-2 immunoreactivity. There is a significant effect of hypoglycaemia on MAP2-immunorectivity, but no significant effect of CsA concentration (two-way ANOVA, effect of hypoglycaemia, $F(1,14)=12.6$, $p<0.01$; effect of CsA concentration, $F(6,14)=1.46$, $p=0.26$). There was no significant difference in MAP2 staining between hypoglycaemic cells and hypoglycaemic cells treated with CsA at any concentration (one-way ANOVA, $F(6,7)=0.5$, $p=0.8$). Data are represented as a percentage of normoclycaemic cultures without CsA treatment (100%) (n=3).

The cell viability of hypoglycaemic treated cultures was significantly reduced after 24 hours compared to normoglycaemic cultures, however TFP had no significant effect, at any concentration, on hypoglycaemic cultures. In normoglycaemic cultures there was no significant difference when TFP was added apart from at 50μM, where a dramatic reduction in cell viability was observed (Figure 3.8). Cell viability was also measured using MAP2 staining. There was no significant difference in MAP2 staining between hypoglycaemic cells treated with or without TFP. As expected, at 50μM, MAP2 staining significantly decreased in normoglycaemic cells compared to normoglycaemic cells without TFP treatment (Figure 3.9).

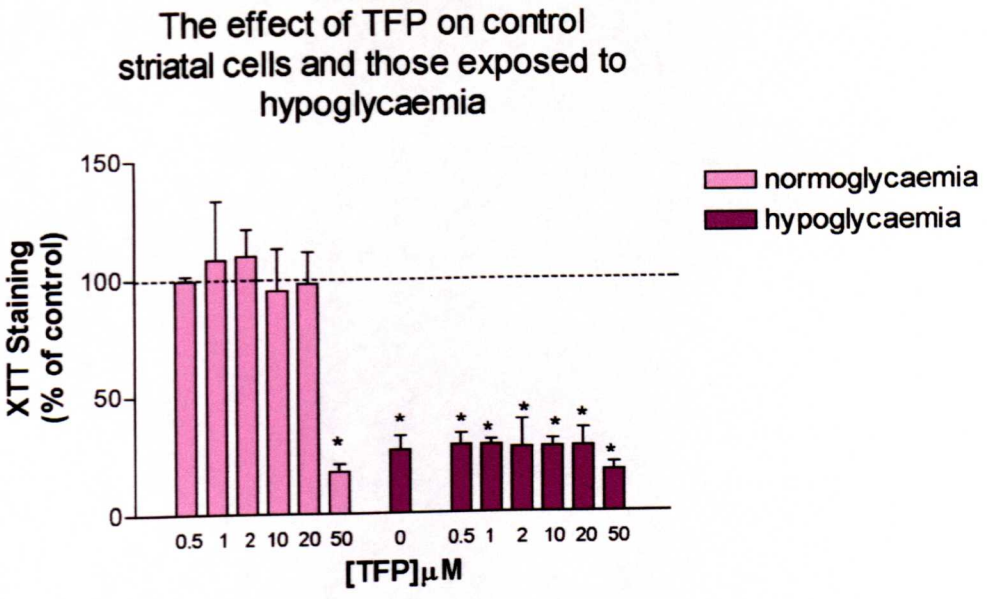

Figure 3.8

The effect of TFP on normoglycaemic and hypoglycaemic cultures. TFP was added at various concentrations to cultures for 24 hours. In hypoglycaemic cultures, the glucose was removed just prior to the addition of TFP. Cell viability was measured by XTT staining. There was a significant reduction of cell viability in hypoglycaemic cells and in normoglycaemic cells exposed to 50μM TFP (*$p<0.05$ versus 100%; 95% confidence intervals of the mean) and a significant effect of TFP (two-way ANOVA, effect of hypoglycaemia, $F(1,28)=384.6$, $p<0.001$; effect of TFP, $F(6,28)=17.9$, $p<0.001$). TFP did not show neuroprotection against hypoglycaemic cell damage at any concentration (one-way ANOVA, $F(6,14)=0.4$, $p=0.4$). Data are represented as a percentage of XTT staining in normoglycaemic cultures without TFP treatment (100%) (n=3).

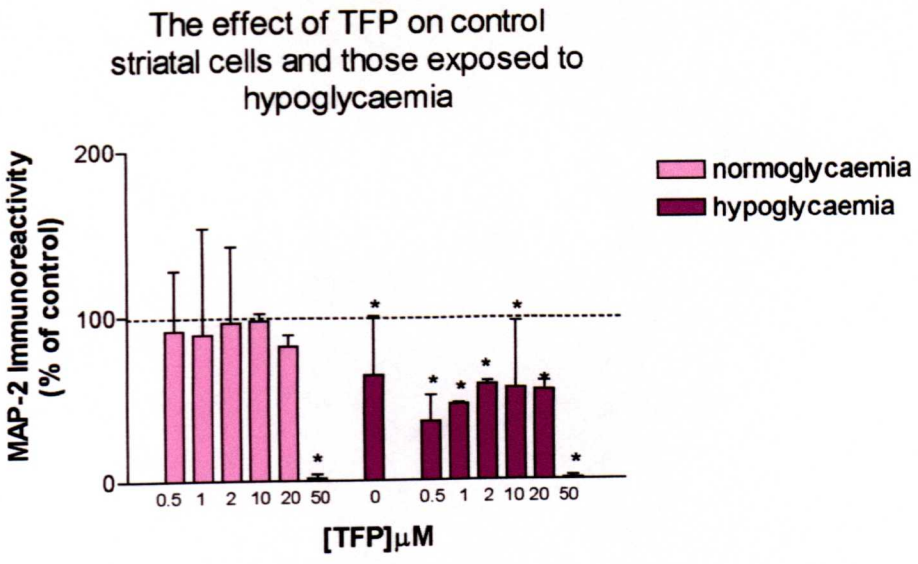

Figure 3.9
The effect of TFP on normoglycaemic and hypoglycaemic cultures measured by MAP2-immunoreactivity. TFP was added at various concentrations to cultures for 24 hours. In hypoglycaemic cultures, the glucose was removed just prior to the addition of the TFP. Cell viability was measured by MAP2 staining. There was a significant reduction in MAP-2 staining in hypoglycaemic cells and in normoglycaemic cells exposed to 50μM (*$p<0.05$ versus 100%; 95% confidence intervals of the mean) and a significant effect of TFP (two-way ANOVA, effect of hypoglycaemia, $F(1,14)=10.7$, $p<0.01$; effect of TFP, $F(6,14)=4.1$, $p<0.05$). TFP did not show any neuroprotection against hypoglycaemic cell damage (one-way ANOVA, $F(6,7)=2.03$, $p=0.2$). Data are represented as a percentage of MAP2 staining in normoglycaemic cultures without TFP treatment (100%) (n=3).

PDTC showed no significant effect on cell viability in cultures treated with 4 hours hypoglycaemia. However, in normoglycaemic cultures treated with 50μM PDTC there was a significant increase in cell viability compared to normoglycaemic cells incubated in the absence of PDTC (Figure 3.10).

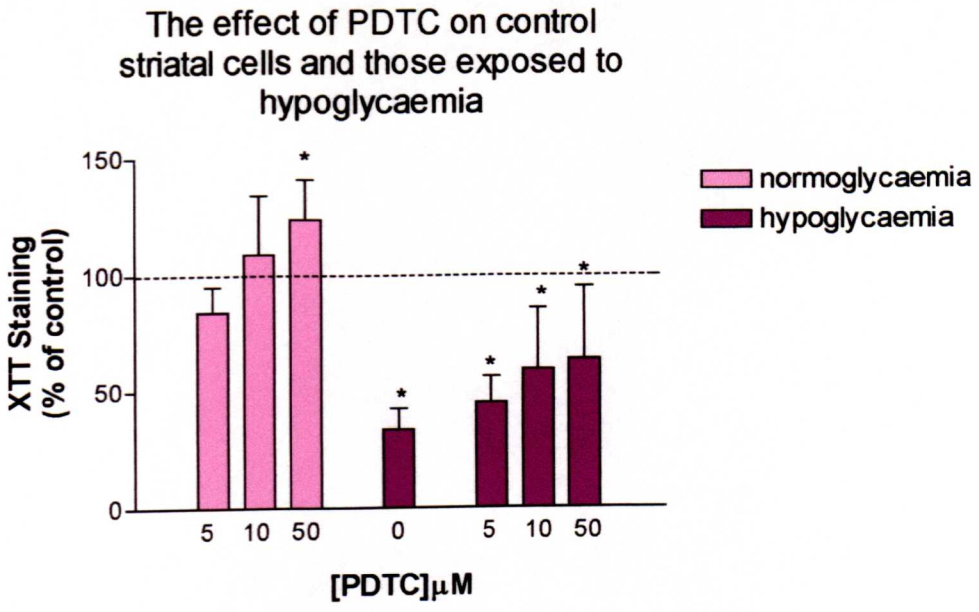

Figure 3.10
The effect of PDTC on normoglycaemic and hypoglycaemic cultures. PDTC was added at various concentrations to cultures for 4 hours. In hypoglycaemia cultures, the glucose was removed just prior to the addition of PDTC. Cell viability was measured by XTT staining. There is a significant reduction of cell viability in hypoglycaemic cultures (*$p<0.5$ versus 100%; 95% confidence intervals of the mean) along with a significant effect of PDTC concentration (two-way ANOVA, $F(1,24)=64.8$, effect of hypoglycaemia, $p<0.001$; effect of PDTC, $F(3,24)=4.4$, $p<0.01$), however, PDTC did not show neuroprotection against hypoglycaemic cell damage at any concentrations (one-way ANOVA, $F(3,12)=0.2$, $p=0.2$). In normoglycaemic cultures, 50μM PDTC significantly increased cell viability (*$p<0.05$ versus 100%; 95% confidence intervals of the mean); significant effect of PDTC concentration, $F(2,9)=4.7$, $p<0.5$ one way ANOVA). Data are represented as a percentage of XTT staining in normoglycaemic cultures without PDTC treatment (100%) (n=5).

Figure 3.11 shows that opening K-ATP channels with diazoxide at various concentrations had no neuroprotective effect on hypoglycaemic treated cells. Four hours of hypoglycaemia alone produced a significant reduction in cell viability but no significant change was observed when hypoglycaemic cells were incubated with diazoxide. Thus, diazoxide had no significant effect on normoglycaemic cells (Figure 3.11).

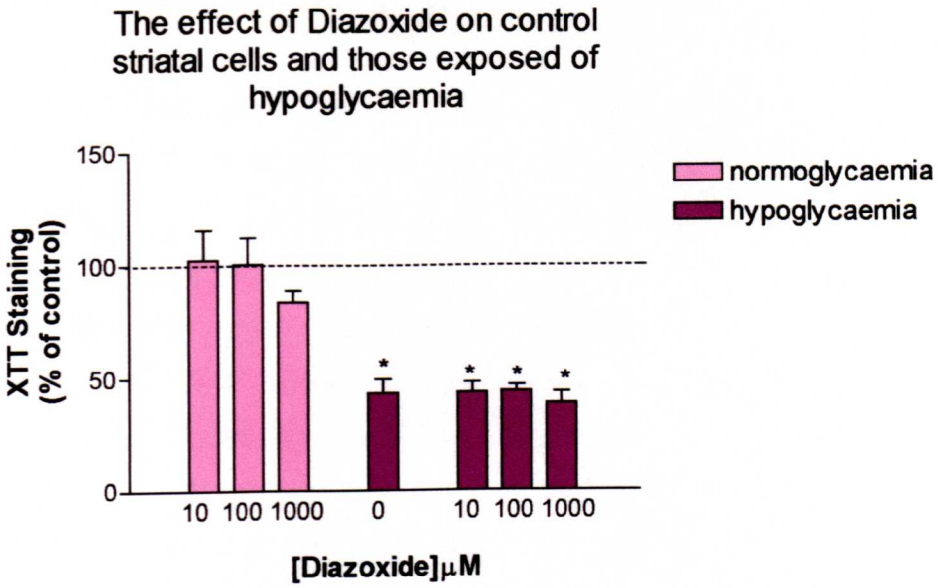

Figure 3.11

The effect of diazoxide on normoglycaemic and hypoglycaemic cultures. Diazoxide was added at various concentrations to cultures for 4 hours. In hypoglycaemic cultures, the glucose was removed just prior to the addition of diazoxide. Cell viability was measured by XTT staining. There was a significant reduction of cell viability in hypoglycaemic treated cells (*$p<0.5$ versus 100%; 95% confidence intervals of the mean) and a significant effect of diazoxide (two-way ANOVA, effect of hypoglycaemia, $F(1,16)=326.9$, $p<0.001$; effect of diazoxide, $F(3,16)=3.4$, $p<0.5$) but the addition of diazoxide did not show any increase in cell viability (one-way ANOVA, $F(3,8)=0.8$, $p=0.53$). Data are represented as a percentage of XTT staining in normoglycaemic cultures without diazoxide treatment (100%) (n=3).

3.3 Discussion

Hypoglycaemia-induced toxicity caused a significant reduction in cell viability in striatal cultures after 4 and 24 hours (Figure 3.1). This is consistent with previous studies (Williams *et al.*, 1995; Nakao *et al.*, 1995; Calabresi *et al.*, 1997a) and suggests that an alteration in mitochondrial functioning has occurred by 4 hours. Indeed, it has been reported that twelve minutes after oxygen and glucose deprivation, dysfunctional mitochondria are observed in hippocampal slice cultures (Taylor *et al.*, 1999). It is interesting that the difference in the percentage of cell viability between 4 and 24 hours of hypoglycaemia, although significantly different, is relatively small and that the percentage does not reach zero (Figure 3.1). It could be assumed that either there is a specific type of neurone or neurones within the culture which are relatively resistant to hypoglycaemic cell damage. For example, it is known that cholinergic interneurones are much less vulnerable to hypoglycaemia compared with medium spiny neurones (Calabresi *et al.*, 1997a). This could have been tested via neurochemical markers such as choline acetyltransferase which would have specifically stained cholinergic interneurones. Thus, following 4 hours of hypoglycaemia, the majority of medium spiny neurones may have ceased to function but after 24 hours, all of the medium spiny neurones may have stopped functioning, leaving the resistant interneurones, which can still reduce XTT. However, 95% of the cell population within the cultures are the same so it may be postulated that even after 24 hours of hypoglycaemia, ATP production is not totally depleted due to the surviving mitochondria within all cells. It could be that within these functional mitochondria the energy production is low, possibly due to TCA cycle alterations from utilising alternative substrates, such as glutamate.

The hypothesised initial event in hypoglycaemia is a dysfunction of the Na^+ /K^+ ATPase pump, a result of the progressive decline in ATP concentration. In normoglycaemic cultures the addition of ouabain produced a significant reduction in cell viability (Figure 3.3). This is expected as inhibiting the Na^+ /K^+ ATPase pump, disrupts the ionic gradients and causes the membrane to depolarise. These results are similar to other studies, although, it has been shown that at low concentrations of ouabain (1-3μM), the membrane potential of medium spiny neurones in slice cultures did not alter (Calabresi *et al.*, 1995). This discrepancy could be due to differences in the microenvironment between slice cultures as used by Calabresi et al. and primary cell cultures as used here. The fact that the cell viability of normoglycaemic cultures incubated with ouabain was not reduced to levels observed in hypoglycaemic cultures incubated with ouabain, suggests either that the cell

viability reduction in hypoglycaemic cultures is not solely as consequence of Na^+ /K^+ ATPase pump inhibition, or that membrane depolarisation in normoglycaemic cultures is not as detrimental to cellular functioning as in hypoglycaemic cultures. This may be due to other factors associated with glucose deprivation influencing the outcome of survival.

It is also interesting to see that ouabain did not produce any additive deleterious effects in hypoglycaemic cultures (Figure 3.3). Ouabain has been shown to produce a dose-dependent increase in NMDA- and AMPA-induced membrane depolarisation (Calabresi *et al.*, 1995; Westerink *et al.*, 1989), an absence of a graded response with ouabain suggests that in hypoglycaemia the Na^+ /K^+ ATPase pump is inhibited independent of ouabain's actions.

It has been previously suggested that because the Na^+ /K^+ ATPase pump requires most of the cell's energy, if the pump was partially inhibited without damaging consequences in times of energy depletion, energy could be conserved (Whittam, 1962). It is possible that the concentrations of ouabain used in this experiment were too high and provided full inhibition, whereas a lower concentration may have provided a neuroprotective effect by partially inhibiting the pump and distributing energy to other functions, such as protein synthesis.

Most studies have found that MK801 has a neuroprotective effect on hypoglycaemic-induced cell damage in the striatum (Williams *et al.*, 1995; Wieloch *et al.*, 1986). It is possible that a higher dose of MK801 in this system may have shown neuroprotective results as in Figure 3.4 XTT staining begins to increase, although there is no significant differences between each dose. In addition, it is possible that the concentration of magnesium present in the culture medium effected the NMDA receptor activity. However, other studies show the presence of magnesium at concentrations comparable with concentrations used in this study to have no overall effect (Williams *et al.*, 1995). It should also be noted here that all potential neuroprotective compounds in Chapter One and Chapter Two (and in Chapter Seven) gave the same results regardless of whether cultures were pre-incubated with them or incubated with them on the onset of glucose-deprivation. This proves that the addition of the drugs was not too late.

Studies have reported MK801 being ineffective in other systems such as, in transient ischaemia (Nellgärd *et al.*, 1991) and global ischaemia (Block and Pusinelli, 1987; Lanier *et al.*, 1990). Discrepancies between results have been attributed to temperature (Minamisawa *et al.*, 1990), the density and duration of ischaemia (Siesjo, 1988) and pH

(NMDA receptors are inhibited at low pH) (Giffard *et al.*, 1990). It is therefore possible that MK801 was ineffective in protecting the cells even though EAA-induced damage is important during the hypoglycaemic insult.

Alternatively, it could be proposed that EAA are not released in this hypoglycaemia model and cell death is attributable to other glutamate receptor-independent mechanisms. It has been shown that hypoglycaemia causes neuronal damage when calcium is excluded from the extracellular medium (Nedergaard, 1991), suggesting that cell death was not via a direct calcium-mediated excitotoxic mechanism.

Studies using slice cultures or *in vivo* models maintain excitatory extrinsic inputs into the striatum, whereas *in vitro* striatal cultures do not possess extrinsic inputs, have few or no intrinsic glutamatergic neurones and possess predominantly inhibitory neurones (Freese *et al.*, 1990). It could be assumed that under hypoglycaemic conditions, membrane depolarisation does not release toxic amounts of glutamate or aspartate because GABA is the predominant neurotransmitter within the presynaptic terminals. Indeed it has been shown that GABA can provide protection from the effects of ischaemia (Shuaib *et al.*, 1993).

Many studies, investigating the effects of hypoglycaemia use cortical, hippocampal or cerebellar cultures which possess glutamatergic neurones. In addition, it has been proposed that the excitation input from the cortex and /or thalamus to the striatum appears to promote the maturation of glutamate receptors on striatal neurones (Chen *et al.*, 1999). It is possible that striatal cells in cultures do not possess fully mature and functional glutamate receptors due to the absence of the excitatory input (Chen *et al.*, 1999). However, other groups would suggest this unlikely as excitotoxicity has been reported in striatal cultures (Williams *et al.*, 1995; Peng *et al.*, 1998) and excessive toxic levels of glutamate and aspartate have been reported to be released in hypoglycaemia-induced injury within striatal cultures (Williams *et al.*, 1995).

CNQX also proved to be ineffective in protecting cells against hypoglycaemia. This is in line with other studies showing the ineffectiveness of CNQX (Hara *et al.*, 1993). Again, this suggests that either CNQX did not provide complete antagonism of AMPA receptors. However, it has been reported that CNQX is a glycine antagonist between concentrations 10-30 μM (Lester *et al.*, 1989) which would provide further EAA receptor blockade. Therefore it is more likely that AMPA receptors are not involved in the mechanism of cell damage or that cell damage is independent of glutamate and aspartate. The concentration

of CNQX used in these studies is sufficient to block all AMPA receptor-mediated transmission (Garthwaite and Garthwaite, 1989). CsA has potent neuroprotective effects in many energy deprived systems (Yoshimoto and Siesjo, 1999; Matsumoto *et al.*, 1999; Seaton *et al.*, 1997), but it showed no protective effects in this system, detected either by XTT or MAP2 staining (Figures 3.6 and 3.7). This suggests that either CsA is not effective in inhibiting the opening of the pore or that the pore does not initially open and cell damage is independent of MTP activity. In addition, it has been reported that CsA possesses toxic properties in rats (Tanaka *et al.*, 1993; Famiglio *et al.*, 1989), neuronal cultures (Stoltenburg-Didinger *et al.*, 1992; Martin *et al.*, 1988; Seaton *et al.*, 1998; Ryu *et al.*, 1999) and humans (Walker and Brochstein, 1988; Berden *et al.*, 1985), highlighting the magnitude of properties associated with CsA.

It is known that the MTP plays a role in cell death in most neurones (Kroemer *et al.*, 1998; Lemasters *et al.*, 1998; Taylor *et al.*, 1999), with the notable exception of cerebellar granular cells (Castilho *et al.*, 1998), verifying that the same inducer of cell death can induce different mechanisms depending on cell type. The CsA data in this study suggests that the cell death pathway involving the MTP is not the predominant pathway in striatal cells exposed to hypoglycaemia. Indeed, it has been shown that in conditions of restricted oxidative activity, calcium-induced mitochondrial depolarisation occurred without concomitant swelling and MTP opening (Brustovetsky and Dublinsky, 2000). Therefore, a disruption of mitochondrial functioning could occur in the absence of MTP activity.

If the MTP was not open, it is possible that toxic levels of calcium could efflux from the mitochondria via the Na^+/Ca^{2+} exchanger and not the MTP. The importance of Na^+/K^+ exchange varies considerably between tissues (Duchen *et al.*, 1990). This has already been reported in ischaemic rat hippocampal dendrites (Zhang *et al.*, 1999), in cultured rat dorsal root ganglion neurones (Baron and Thayer, 1997) and in glutamate stimulated cultured cortical neurones (White and Reynolds, 1996). However TFP, is an apparent Na^+/Ca^{2+} exchanger inhibitor and MTP inhibitor (Hoyt *et al.*, 1997). Hence, neuroprotection would be seen with TFP according to this theory. Figures 3.8 and 3.9 show no evidence of TFP preventing cell damage.

In addition, CsA is an inhibitor of calcineurin and TFP inhibits calmodulin. In contrast to other studies (Ruiz *et al.*, 2000), these results suggest that calcineurin and calmodulin play no role in hypoglycaemic-induced toxicity in striatal cells. It could also be assumed that the reduction of cell viability in striatal cultures is not due to lipid peroxidation of the

membrane as TFP has been shown to protect cells by inhibiting lipid peroxidation (Kuroda *et al.*, 1997).

PDTC has been shown to have anti-oxidant properties in many different types of cells (Seaton *et al.*, 1997; Seaton *et al.*, 1998; Moro *et al.*, 1998). However, it was shown to have no significant effect on reducing cell viability of hypoglycaemic-induced cell death (Figure 3.10). This suggests that PDTC is not an effective ROS scavenger, or that the production of ROS was too extensive for PDTC to cope with. It should be noted that the doses of PDTC used here are sufficient to produce profound neuroprotective effects in other culture systems (Moro *et al.*, 1998; Seaton *et al.*, 1997). It has been reported that antioxidants are only effective in cultures with low cell densities (Banker, 1980). However, striatal cultures used in these experiments were 14-18 days old and therefore the cell density is quite high, providing a possible explanation why PDTC is ineffective.

Another possibility is that ROS do not play a part in the mechanisms of cell damage in this system. If the MTP is not open then there would be no release of ROS from the mitochondria, however, MTP opening has been reported to be downstream from ROS production in cortical cells treated with toxic levels of glutamate (Ruiz *et al.*, 2000). Therefore, it can be concluded that ROS production independent of MTP also does not occur or does not play a part in hypoglycaemic-induced cell death in cultured striatal cells. This is in contrast to studies where ROS production was inhibited using free radical spin traps and lipid peroxidation inhibitor which protected striatal cells from glucose deprivation (Nakao *et al.*, 1996).

It is interesting to note an increase of cell viability in normoglycaemic cultures with the highest concentration of PDTC (Figure 3.10). One explanation for this observation is that under normal physiological conditions, ROS are produced as a by product of respiration but are buffered to prevent damage, however, if an extra anti-oxidant such as PDTC, is added to the culture, it is possible that a more efficient buffering system could be expected. An alternative explanation is that PDTC increases basal respiration in normoglycaemic cultures.

Medium spiny neurones possess K-ATP channels (Schwanstecher and Bassen, 1997) but diazoxide did not show any effects on hypoglycaemia toxicity in striatal cells although the concentrations of diazoxide used in this study have been shown to be neuroprotective in other culture systems (Zawar and Neumcke, 2000). However, ambiguous results show that diazoxide can be ineffective (Calabresi *et al.*, 1997), protective (Koretz *et al.*, 1994; Liss

et al., 1999) or toxic in cells under glucose deprived conditions, suggesting that K-ATP channels are not implicated in the neuroprotection of hypoglycaemia.

One reason for the ambiguous actions of diazoxide is that there are five different putative K-ATP channels, which have different pharmacological properties and display varying sensitivities to K-ATP channels openers. It is also apparent that a single neurone can express different types of K-ATP channels and that these channels can exhibit significant differences in metabolic sensitivities (Gribble and Ashcroft, 2000).

In addition, diazoxide has been shown to be 2000 times more active for the mitochondrial K-ATP channels (Garlid *et al.*, 1996a) compared to the plasma membrane K-ATP channels and has been reported to cause mitochondrial depolarisation, increasing the matrix volume and inducing a release of cytochrome c (Debska *et al.*, 2001). One study demonstrated that moderate opening of the mitochondrial K-ATP channel by diazoxide contributes to a more efficient formation of ATP, by accelerating the electron transport through the inner mitochondrial membrane, whereas full opening results in the uncoupling of oxidative phosphorylation (Garlid, 1996b).

It appears that the mechanisms and components theorised to be essential in other models of hypoglycaemic cell death are not relevant in this *in vitro* model of hypoglycaemia. These results suggest that the lack of involvement of NMDA and AMPA glutamate receptors indicate that glutamate toxicity is not implicated. The MTP, ROS toxicity and K-ATP channel activity are also not involved. Further investigation into how cell functioning is perturbed is essential as well as a new revised hypothesis explaining what are the mechanisms of cell death in hypoglycaemia. It is therefore proposed that the removal of glucose from the medium depletes the ATP concentration available to cells and causes the plasma membrane to depolarise via Na^+ /K^+ ATPase pump dysfunction. Calcium enters the cell through the VGCC which consequentially causes a release of calcium from intracellular stores. This overload of calcium exceeds the rate of intracellular sequestration and cell death is via calcium-dependent necrosis. Therefore, an examination of the behaviour of calcium ions during hypoglycaemia is the next logical step in this study.

Chapter Four.

Calcium Modulation in Hypoglycaemic toxicity

4.1 Introduction

Calcium plays an important pathogenic role via mechanisms such as activating calcium-dependent enzymes including phospholipases, proteases, and endonucleases and by changing the phosphorylation state of proteins (Farooqui *et al.*, 1991; Arends *et al.*, 1990). Therefore, it seems likely that if the levels of intracellular calcium were reduced during hypoglycaemic toxicity via a blockade of either extracellular or intracellular calcium stores, cells would have a better chance of survival. Figure 4 is a schematic diagram showing the site of action of each calcium modulating compound used in the attempt to promote survival in cells exposed to hypoglycaemia.

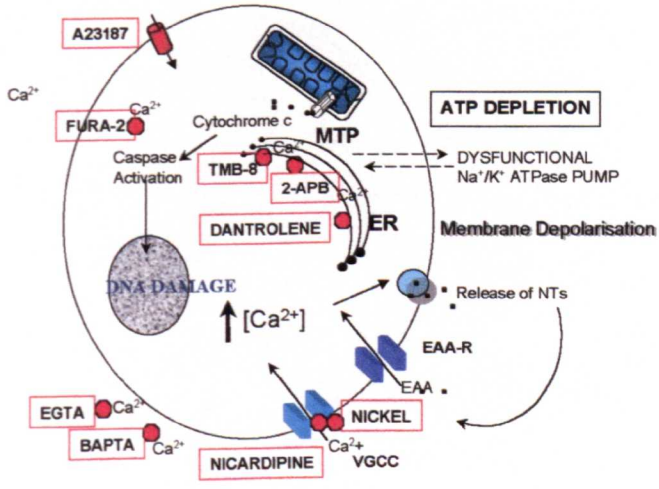

Figure 4
A schematic diagram of the site of pharmacological action of the various calcium-modulating compounds, including extracellular chelators, VGCC blockers and antagonists of intracellular stores. It shows the insertion of the ionophore, A23187 and the Fura-2 calcium indicator dye within the cytoplasm.

Extracellular calcium chelators, EGTA and BAPTA have a binding cavity within their structure specific for calcium and other divalents ions. It must be noted that the magnesium

concentration within the culture medium was not compensated for when EGTA and BAPTA were added to the cultures.

BAPTA has two advantages over EGTA. It is insensitive to pH and is a faster buffer, important in chelating transient calcium responses (Tsien *et al.*, 1980). The cell membrane impermeable form of BAPTA was used to see if reducing the extracellular calcium levels in hypoglycaemic conditions would be protective for the cells.

It is thought that L-type channels are the dominant pathways for calcium influx (Ikeda *et al.*, 1997) and are believed to play unique roles in enduring changes in neuronal structure and function (Tsien *et al.*, 1989). Therefore nicardipine, a L-type VGCC blocker, was used (Figure 4). However, T-type VGCC have been implicated in neurotoxicity, therefore nickel, a competitive antagonist of the T-type VGCC (Mlinar and Enyeart, 1993), was also assessed (Figure 4). It was hypothesised that blocking VGCCs would produce a neuroprotective effect by preventing excessive calcium influx if the membrane is depolarised during hypoglycaemia.

Ryanodine and IP_3 receptors are expressed by medium spiny striatal neurones (Sharp *et al.*, 1993), whereas only ryanodine receptors are present extensively within aspiny neurones (Martone *et al.*, 1997). TMB-8 was one of the first compounds to be described as an exclusive intracellular calcium antagonist (Malagodi and Chiou, 1974). It is thought to block the release of intracellular bound calcium ions at the IP_3 store (Figure 4). However, it also acts as a general calcium antagonist on the calcium channels located on the plasma membrane (Doutheil and Paschen, 1999). A more recent IP_3 receptor antagonist, 2-APB, was found to be more specific as it does not affect the calcium influx from outside the cell (Maruyama *et al.*, 1997). 2-APB does not inhibit the binding of IP_3 to its receptor and presumably binds to a recognition site different from the IP_3 recognition site (Maruyama *et al.*, 1997) (Figure 4).

Dantrolene has been used clinically since 1974 to treat malignant hyperthermia and muscle spasticity. It acts by inhibiting calcium release from stores via the ryanodine receptor (Ward *et al.*, 1986; Simpson *et al.*, 1995; Mody and McDonald, 1995) (Figure 4). By antagonising intracellular calcium store receptors during hypoglycaemia, intracellular calcium released in response to calcium influx would be inhibited and this could potentially prevent a perturbed calcium homeostasis and possibly cell death.

The calcium ionophore, A23187, forms a lipid soluble complex with calcium ions, thereby permitting their transport across the membrane and increasing intracellular calcium concentrations (Schaffer *et al.*, 1973) (Figure 4). Adding A23187 to normoglycaemic cells provides information to determine whether calcium alone is toxic to striatal cells independent of the hypoglycaemic insult.

In this series of experiments, all these compounds were assessed via XTT measurement, to investigate their effect on hypoglycaemic cells. The final experiment using Fura-2 examines changes in the intracellular calcium levels during hypoglycaemia.

4.2 Results

Cell viability was measured, using XTT, in normoglycaemic cultures incubated with a range of concentrations of A23187 for 24 hours. A dose-dependent response to A23187 was observed. Concentrations above 1μM significantly decreased the XTT staining of the cultured cells (Figure 4.1).

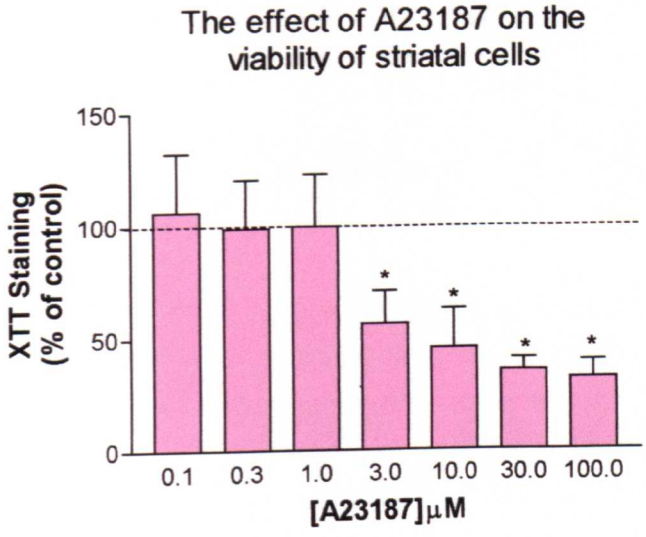

Figure 4.1
The effect of A23187 on the cell viability of striatal cells. Cultures were incubated with different concentrations of A23187 (0.1 -100μM) for 24 hours. Cell viability was measured by XTT staining. A dose-response curve was obtained. Concentrations of A23187 >1μM cause a significant decrease in cell viability compared to cultures incubated in the absence of A23187 viability (one-way ANOVA for the effect of dose, $F(6,14)=19.18$, $p<0.001$; *$p<0.05$ versus 100%; 95% confidence intervals of the mean). Data are represented as a percentage of XTT staining in cultures without A23187 treatment (n=5).

EGTA in the extracellular medium did not have any significant effect on hypoglycaemic-mediated damaged cells. Moreover, in normoglycaemic cultures the presence of EGTA produced a significant decrease in cell survival at 1mM but not at 5mM(Figure 4.2).

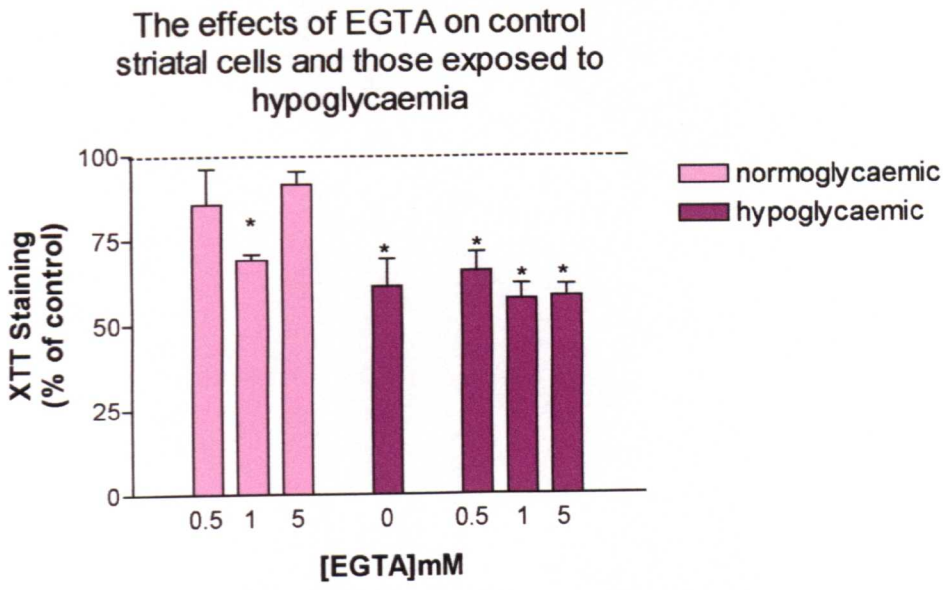

Figure 4.2
The effect of EGTA on striatal cells in normoglycaemic and hypoglycaemic cultures. EGTA was added to cultures at varying concentrations for 4 hours. In hypoglycaemic cultures the glucose was removed just prior to the addition of EGTA. Cell viability was measured by XTT staining. There is a significant effect of both EGTA and hypoglycaemia (two-way ANOVA; effect of hypoglycaemia, $F(1,12)=62.7$, $p<0.001$; effect of EGTA, $F(2,12)=8.68$, $p<0.01$). There is a significant decrease in cell viability in cells exposed to hypoglycaemia (*$p<0.05$ versus 100%; 95% confidence intervals of the mean) but no neuroprotection is observed with EGTA (one-way ANOVA, $p=0.35$). Moreover, a significant reduction in cell survival was observed when 1mM EGTA was added to normoglycaemic cultures. Data are represented as a percentage of XTT staining in normoglycaemia cultures without EGTA treatment (100%) (n=3).

There was no neuroprotective effect using BAPTA as an extracellular calcium chelator. Cultures treated with hypoglycaemia for 4 hours showed a significant reduction in cell viability and there was no change when hypoglycaemic cells were treated with varying concentrations of BAPTA. In contrast, the presence of BAPTA in normoglycaemic cultures, appeared to increase XTT staining compared to staining in normoglycaemic cultures (shown as 100%) (Figure 4.3).

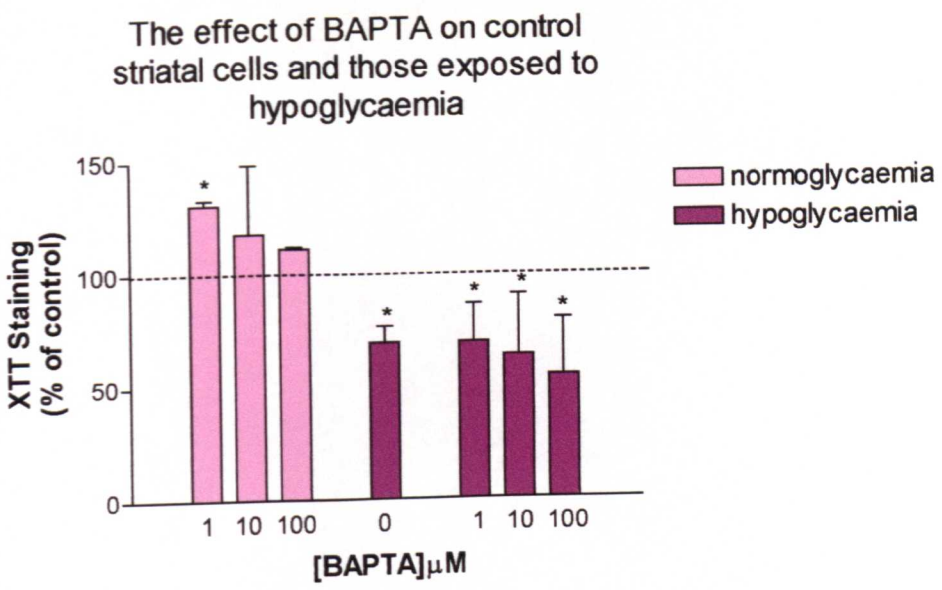

Figure 4.3
The effect of BAPTA on striatal cells in normoglycaemic and hypoglycaemic cultures. BAPTA was added to cultures at varying concentrations for 4 hours. In hypoglycaemic cultures the glucose was removed just prior to the addition of BAPTA. Cell viability was measured by XTT staining. The cell vialibity in hypoglycaemic cultures is significantly decreased compared to normoglycaemic cultures (*$p<0.05$ versus 100%; 95% confidence intervals of the mean) (two-way ANOVA, effect of hypoglycaemia, $F(1,16)=46.1$, $p<0.001$; effect of BAPTA, $F(3,16)=1.1$, $p=0.4$) and no significant effect is seen with BAPTA in hypoglycaemic cultures (one-way ANOVA, $p=0.8$). An increase in cell viability is observed in normoglycaemic cultures with 1μM BAPTA (*$p<0.5$ versus 100%; 95% confidence intervals of the mean). Data are represented as a percentage of XTT staining in normoglycaemic cultures without BAPTA treatment (100%) ($n=4$).

There was a significant reduction in XTT staining in hypoglycaemic cultures but in the presence of $NiCl_2$, no significant changes were measured. $NiCl_2$ had deleterious effects on normoglycaemic cultures. At 5mM, XTT staining significantly decreased compared to normoglycaemic cultures without $NiCl_2$ treatment (Figure 4.4).

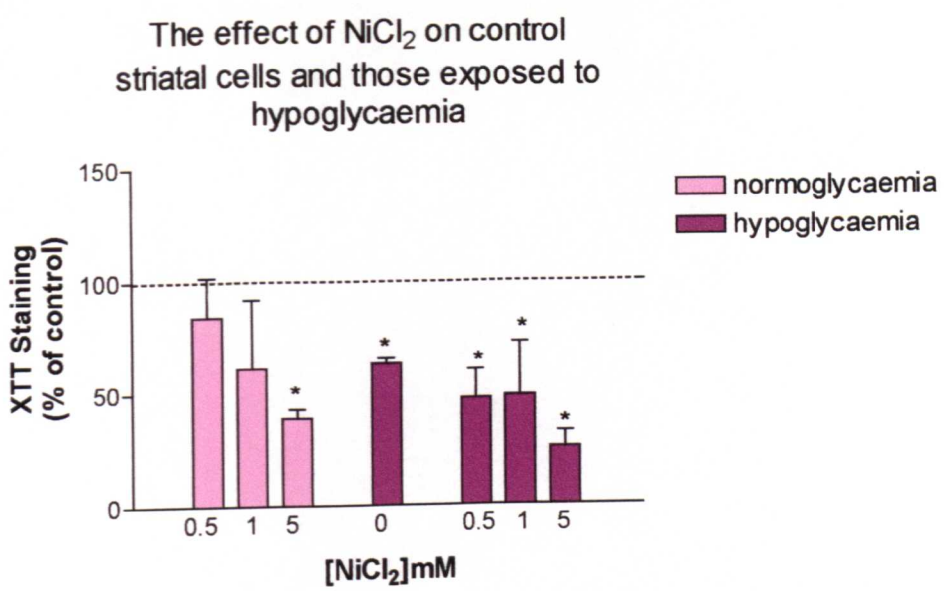

Figure 4.4

The effect of nickel on striatal cells in normoglycaemic and hypoglycaemic cultures. Nickel chloride ($NiCl_2$) was added to cultures at varying concentrations for 4 hours. In hypoglycaemic cultures the glucose was removed just prior to the addition of $NiCl_2$. Cell viability was measured by XTT staining. There is a significant effect of both $NiCl_2$ and hypoglycaemia (two-way ANOVA, effect of $NiCl_2$, $F(3,8)=6.7$, $p<0.05$; effect of hypoglycaemia, $F(1,8)=9.6$, $p<0.05$). Hypoglycaemia produced a significant decrease in cell viability (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). $NiCl_2$ did not show a significant difference in cell survival (one-way ANOVA, $p=0.2$), moreover, in normoglycaemic cultures at 5mM, $NiCl_2$ causes a significant decrease in cell viability (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). Data are represented as a percentage of XTT staining in normoglycaemia cultures without $NiCl_2$ treatment (100%) (n=3).

The blockade of L-type VGCCs with nicardipine showed no neuroprotective effects. In fact at higher concentrations nicardipine was toxic itself. There was a significant reduction in XTT staining in hypoglycaemic treated cultures after 4 hours but no significant increase was observed with the presence of nicardipine. Similar to $NiCl_2$, nicardipine significantly decreased XTT staining in normoglycaemic cultures at 10 and 100μM (Figure 4.5).

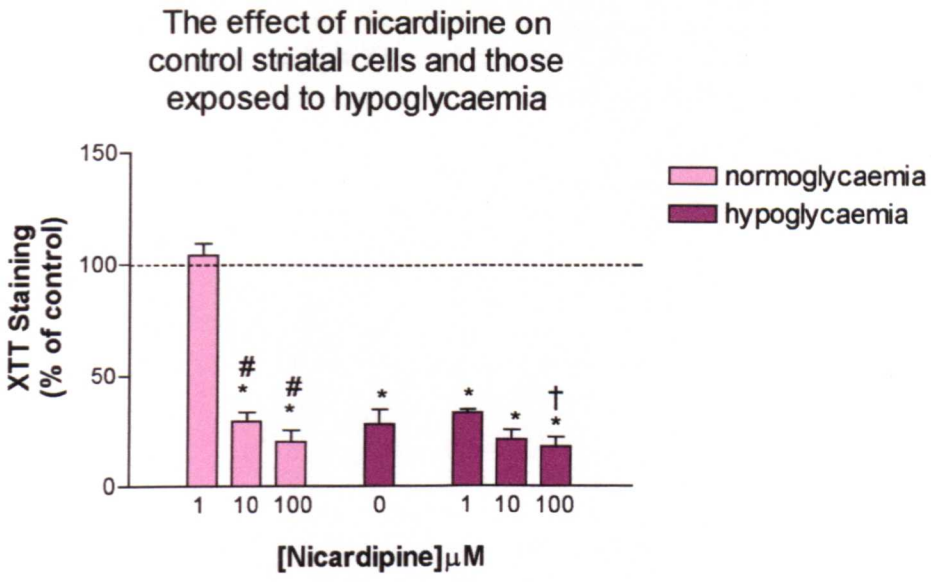

Figure 4.5

The effect of nicardipine on striatal cells in normoglycaemic or hypoglycaemic cultures. Nicardipine was added to cultures at different concentrations for 4 hours. In hypoglycaemic cultures the glucose was removed just prior to the addition of nicardipine. Cell viability was measured by XTT staining. Both nicardipine and hypoglycaemia produce an effect in cultures (two-way ANOVA; effect of nicardipine, $F(3,16)=210.6$, $p<0.001$; effect of hypoglycaemia, $F(1,16)=462.7$, $p<0.001$). There is a significant decrease in cell viability in hypoglycaemic-treated cultures (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). Nicardipine produces a significant effect on both hypoglycaemic (one-way ANOVA, $F(3,8)=6.9$, $p<0.01$) and normoglycaemic cultures (one-way ANOVA, $F(2,6)=283$, $p<0.01$). The cell viability of hypoglycaemic cultures treated with 100μM nicardipine is significantly reduced compared to hypoglycaemic cultures treated with no nicardipine or 1μM nicardipine (†$p<0.05$, Fishers post-hoc test). The cell viability of normoglycaemic cultures treated with 10μM and 100μM nicardipine is significantly decreased from normoglycaemic cultures treated with 1μM nicardipine (#$p<0.05$, Fishers post-hoc test). Data are represented as a percentage of XTT staining in normoglycaemia cultures without nicardipine treatment (100%) (n=4).

TMB-8 had no significant effect on hypoglycaemic or normoglycaemic cultures. There was a significant decrease in cell viability from cultures treated with hypoglycaemia but no significant change when incubated with TMB-8 (Figure 4.6).

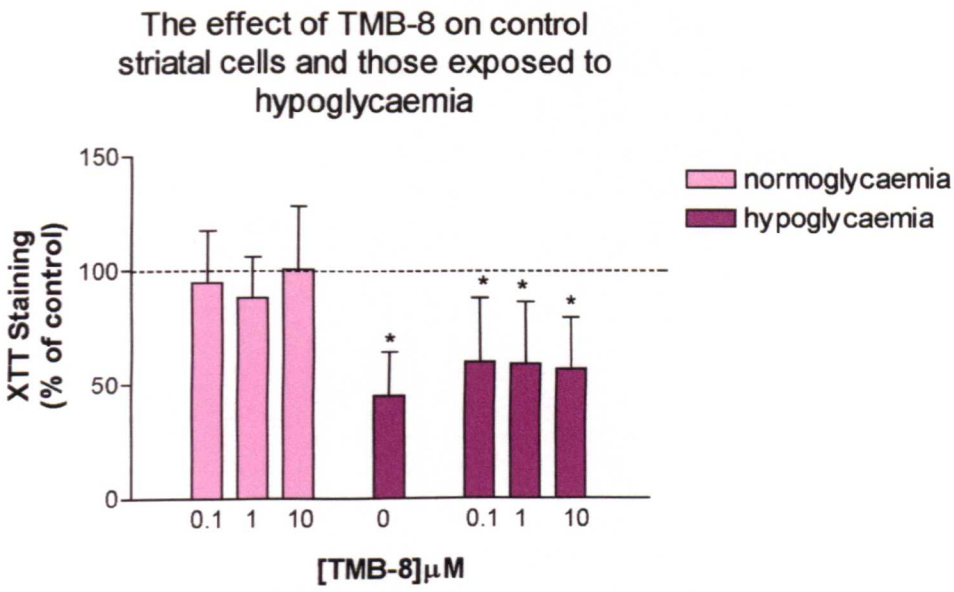

Figure 4.6

The effect of TMB-8 on striatal cells in normoglycaemic and hypoglycaemic cultures. TMB-8 was added to cultures at different concentrations for 4 hours. In hypoglycaemic cultures the glucose was removed just prior to the addition of TMB-8. Cell viability was measured by XTT staining. Hypoglycaemia produces a significant decreases in cell viability compared to normoglycamia (*$p<0.05$ versus 100%; 95% confidence intervals of the mean), however, TMB-8 showed no effect (two-way ANOVA, effect of hypoglycaemia, $F(1,24)=26.7$, $p<0.001$; effect of TMB-8, $F(3,24)=0.15$, $p=0.9$). TMB-8 did not show significant neuroprotection in hypoglycaemic cultures (one-way ANOVA, $p=0.8$). Data are represented as a percentage of XTT staining in normoglycaemia cultures without TMB-8 treatment (100%) (n=5).

In agreement with TMB-8 data, 2-APB did not significantly increase cell survival in hypoglycaemic-treated cells. Moreover at 100μM, 2-APB significantly decreased cell viability in normoglycaemic cultures (Figure 4.7).

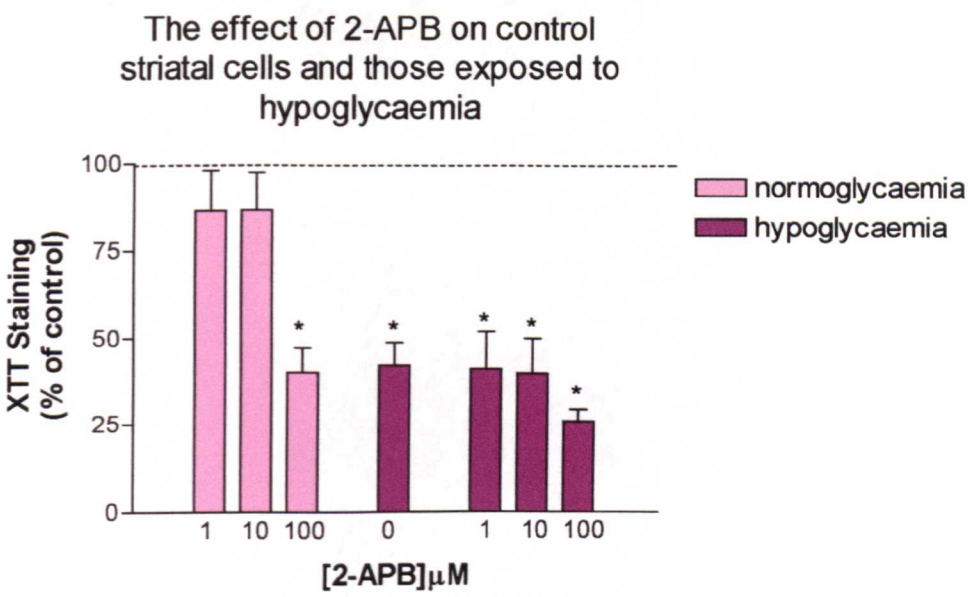

Figure 4.7

The effect of 2-APB on striatal cells in normoglycaemic and hypoglycaemic cultures. 2-APB was added to cultures at varying concentrations for 4 hours. In hypoglycaemic cultures the glucose was removed just prior to the addition of 2-APB. Cell viability was measured by XTT staining. There is a significant effect of 2-APB and hypoglycaemia (two-way ANOVA; effect of 2-APB, $F(3,8)=16$, $p<0.001$; effect of hypoglycaemia, $F(1,8)=94.4$, $p<0.001$). There was a significant decrease in XTT staining in cultures treated with hypoglycaemia (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). Hypoglycaemic cultures treated with 2-APB show no difference in XTT staining (one-way ANOVA, $p=0.3$). In normoglycaemic cultures, 100μM 2-APB significantly decreased cell viability (one-way, $F(2,3)=14.4$, $p<0.05$). Data are represented as a percentage of XTT staining normoglycaemia cultures without 2-APB treatment (100%) ($n=3$).

Dantrolene showed no neuroprotective effects in hypoglycaemic-damaged cells. There was a significant decrease in cell viability in hypoglycaemic-treated cells compared to normoglycaemic cells but the addition of dantrolene, had no significant effect on cell viability. Moreover, dantrolene significantly decreased the cell viability of normoglycaemic cells (Figure 4.8).

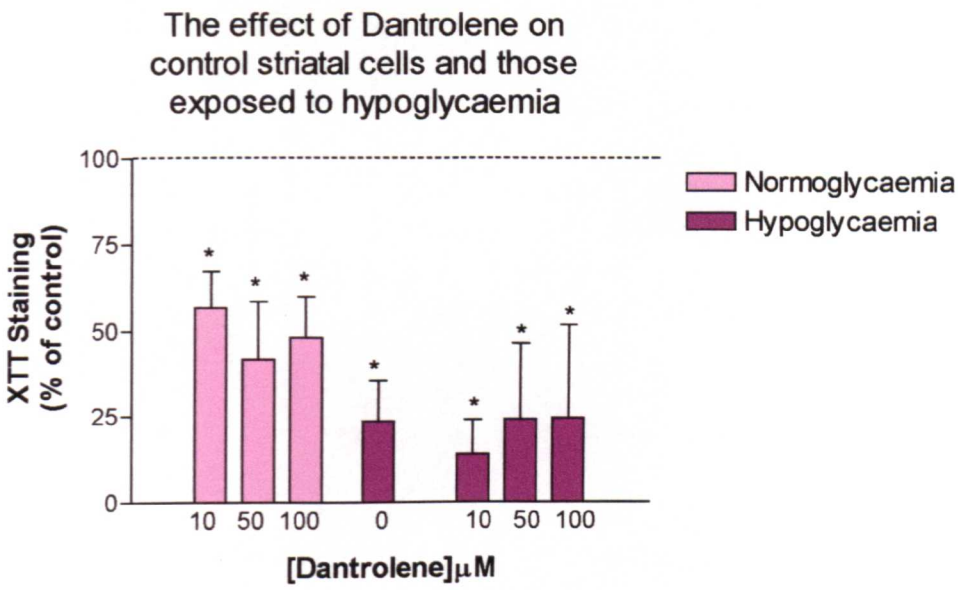

Figure 4.8

The effect of dantrolene on striatal cells in normoglycaemic and hypoglycaemic cultures. Dantrolene was added to cultures at different concentrations for 4 hours. In hypoglycaemic cultures the glucose was removed just prior to the addition of dantrolene. Cell viability was measured by XTT staining. Both hypoglycaemia and dantrolene produce a significant effect (two-way ANOVA, effect of hypoglycaemia, $F(1,16)=38$, $p<0.001$; effect of dantrolene, $F(3,16)=4.3$, $p<0.05$), however, dantrolene has no effect on cell viability in hypoglycaemic cultures (one-way ANOVA, $p=0.9$). A significant decrease in cell viability is seen normoglycaemic cultures with each concentration of dantrolene (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). Data are represented as a percentage of XTT staining in normoglycaemia cultures without dantrolene treatment (100%) ($n=3$).

It was proposed that a neuroprotective effect might be observed when several components, thought to be involved in hypoglycaemia were manipulated. A combination of drugs were used in normoglycaemic and hypoglycaemic cultures. Firstly, the XTT staining of cultures exposed to hypoglycaemia for 4 hours was significantly decreased compared to normoglyaemic cultures. The antioxidant, PDTC and BAPTA provided no neuroprotective effects neither did the combination of the MTP inhibitor, CsA, 2-APB and BAPTA. Finally, all four compounds, PDTC, BAPTA, CsA and 2-APB in combination also had no significant effect on hypoglycaemic-mediated damage in striatal cultures. Interestingly, each combination significantly decreased XTT staining in normoglycaemic cultures (Figure 4.9).

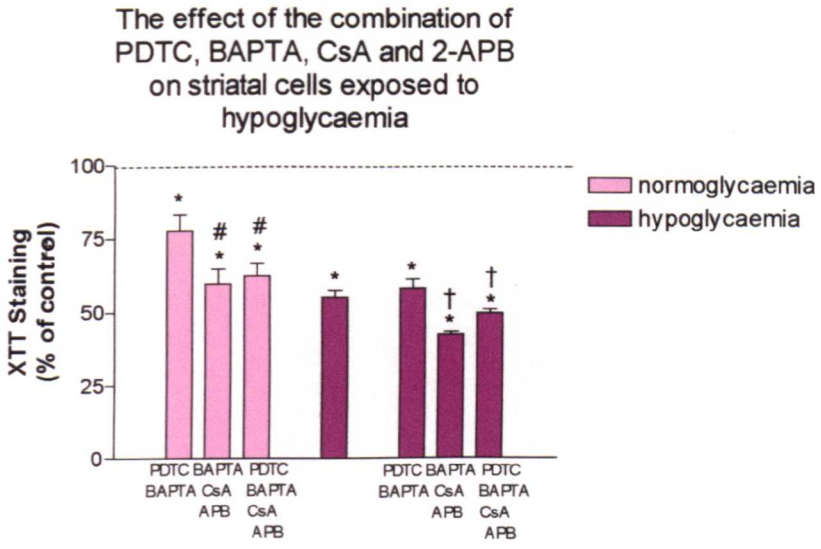

Figure 4.9

The effect of the combination of PDTC (5μM), BAPTA (1μM), CsA (1μM) and 2-APB (1μM) on striatal cells in normoglycaemic and hypoglycaemic cultures. Various combinations of the compounds were added to cultures at varying concentrations for 4 hours. In hypoglycaemic cultures the glucose was removed just prior to the addition of drugs. Cell viability was measured by XTT staining. Both the addition of the cocktail of drugs and hypoglycaemia produces a significant effect (two-way ANOVA; effect of cocktail, $F(3,16)=74.3$, $p<0.001$; effect of hypoglycaemia, $F(1,16)=285.7$, $p<0.001$). There is a significant decrease of XTT staining in hypoglycaemic-treated cells. The combination of BAPTA, CsA and 2-APB and the combination of PDTC, BAPTA, CsA and 2-APB produce significantly greater effects on XTT staining than hypoglycaemic alone (one-way ANOVA, $p<0.001$; †$p<0.05$, Fishers post-hoc test). In normoglycaemic cultures, all combinations of compounds causes a significant decrease in XTT staining compared to normoglycaemic cultures without any treatment (*$p<0.05$ versus 100%; 95% confidence intervals of the mean) and the combination of BAPTA, CsA and 2APB and PDTC, BAPTA, CsA and 2-APB again produced the most damaging effects (one-way ANOVA, $p<0.01$; #$p<0.05$, Fishers post-hoc test). Data are represented as a percentage of XTT staining in normoglycaemia cultures without drug treatment (100%) (n=3).

Free intracellular calcium was measured in cells treated with 24 and 48 hours of normoglycaemia or hypoglycaemia by the change in the fura-2 signal. After 24 hours, the mean calcium concentration for cells incubated in normoglycaemic conditions was 162nM ± 47, whereas the mean concentration for cells incubated in hypoglycaemic conditions was 158nM ± 47. Therefore there was no significant difference in the levels of cytosolic calcium between normoglycaemic and hypoglycaemic cells. After 48 hours, the mean calcium concentration in hypoglycaemic cells decreases by 31nM compared to normoglycaemic cells. However this change in the levels of cytosolic calcium was not significantly different (Figure 4.10).

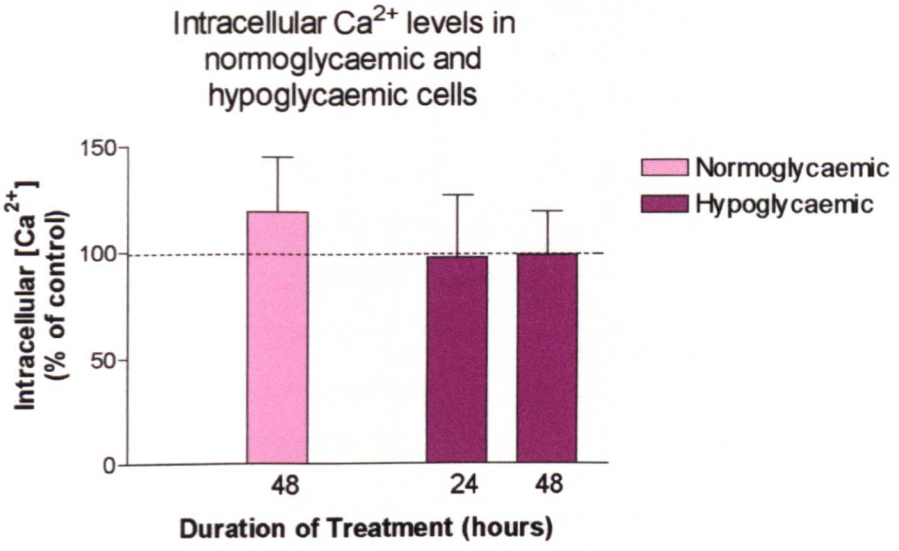

Figure 4.10

The percentage difference in $[Ca^{2+}]_i$ from striatal cells exposed to either 24 or 48 hours of normoglycaemia or hypoglycaemia. Intracellular calcium levels were measured by the change in the fura-2 signal. There was no significant change in levels of calcium in hypoglycaemic cells relative to levels of calcium measured in normoglycaemic cells after 24 hours and 48 hours (one-way ANOVA, $p=1.0$). Data are represented as a percentage of cultures in normoglycaemic conditions for 24 hours (100%).

4.3 Discussion

An excessive influx of calcium into cultured striatal cells causes a significant decrease in XTT staining, which is regarded as a precise index of cell viability (Figure 4.1). Low concentrations (0.1μM - 1μM) of A23187 produced no effects on cultures, whereas higher concentrations did significantly affect cell viability which is consistent with previous studies (Emery and Lucas, 1995). This result suggests that striatal neurones can cope with a specific calcium concentration range but when exceeded beyond a critical point, buffer systems are overloaded and $[Ca^{2+}]_i$ levels rise to toxic levels. The binding of calcium ions to buffering molecules is an energy-independent process, whereas a slower process, requiring energy, then transports calcium ions up their concentration gradients into organelles or out of the cells. It is possible that at high concentrations of A23187 $[Ca^{2+}]_i$ levels simply exceed the intracellular extrusion capacity of the cell. It is also possible that energy failure occurs either due to excessive activation of calcium-dependent proteases. These act on neural membrane phospholipids resulting in the accumulation of free fatty acids which can disrupt ATP synthesis within mitochondria and damage the extrusion system to transport calcium ions out of the cell (Ward *et al.*, 2000).

It has been repeatedly demonstrated that an attenuation of calcium influx protects neurones from various toxic insults. Both extracellular calcium chelators, EGTA and BAPTA had no effect in this system. EGTA provided no significant increase in cell viability in hypoglycaemic neurones and in fact, at 1mM EGTA became toxic in normoglycaemic cultures (Figure 4.2). The data show that the lowest concentration of EGTA provides maximal effect and a lower contration in micromole may have provided more information pharmacologically. This calcium chelator has been known to dramatically change the pH of the culture medium, depending on the concentration (Tsien, 1980). It is possible that the pH change reduced the cell viability in normoglycaemic cells but was masked by the effects of hypoglycaemia, hence no further decrease in cell viability was seen in hypoglycaemic cultures.

BAPTA is reportedly a superior calcium chelator to EGTA, which does not affect the pH of the culture medium (Tsien, 1980). However, no neuroprotection was seen with any of the concentrations used (Figure 4.3), a result that has previously been reported in hippocampal cultures during excitotoxic exposure (Dubinsky, 1993). The results of both EGTA and BAPTA suggest that either these compounds are not effective in chelating toxic amounts of calcium or that the concentrations used were not high enough to provide

complete extracellular calcium chelation. However, the doses of these compounds used here are sufficient to produce profound neuroprotection in other culture systems (Hara *et al.*, 1993; Tsubokawa *et al.*, 1994; Tekkok *et al.*, 1999). Another reason could be that cell damage is dependent on increased $[Ca^{2+}]_i$ and therefore extracellular calcium concentrations are irrelevant. In this case it would have been interesting to observe any differences between BAPTA and BAPTA-AM as BAPTA-AM passes into the cell cytosol where it chelates $[Ca^{2+}]_i$.

Nickel has previously been described as a "dirty" pharmacological tool as it affects other systems and does not only block T-type VGCC but R-types as well (Kobayashi *et al.*, 1992). It is clear, that at high concentrations (5mM), nickel shows toxic effects in normoglycaemic cultures and has an additive toxic effect in hypoglycaemic cultures (Figure 4.4). This is probably due to its non-specific behaviour as opposed to toxic effects caused by blocking T-type VGCC. On the other hand, nicardipine belongs to a family of compounds which specifically block L-type channels and has shown to be neuroprotective in many models, including *in vitro* hypoglycaemia (Hayashi *et al.*, 1993). Again, lower concentrations of both nickel and nicardipine should have been used to observe a dose-response profile of both drugs (this also refers to TMB-8 and dantrolene data). However, in this model no neuroprotection was observed (Figure 4.5). In agreement with this data, previous reports have described a lack of protection with nicardipine and other L-type channels blockers, against glutamate (Godfraind *et al.*, 1986; Hochstrate *et al.*, 1995), combined oxygen and glucose-deprivation (Goldberg *et al.*, 1993) and *in vivo* hypoglycaemic brain damage (Ohta *et al.*, 1991). At high concentrations (10μM and 100μM) nicardipine was markedly toxic in normoglycaemic cultures. It has been reported that low cytosolic calcium levels can be detrimental to neuronal survival (Dubinsky, 1993) and in this study it is evident that striatal cells in normoglycaemic conditions are very sensitive to alterations in calcium concentrations. Figures 4.2, 4.3, 4.4, 4.5, 4.7 and 4.8 show XTT measurements that have decreased from control levels when calcium-modulating compounds have been added to the cultures.

The explanation for nickel and nicardipine being ineffective in hypoglycaemia at concentrations not toxic in normoglycaemic cells, could be that other VGCC, such as P-, Q- or N-type channels play a more predominant role hypoglycaemic-induced damage in striatal cultures. Their ineffectiveness may indicate either that the VGCCs do not open because the membrane does not depolarise sufficiently or that the rise in $[Ca^{2+}]_i$ is caused by the release of calcium from intracellular stores and the calcium influx from the VGCC

is insignificant in comparison (Himmel and Ravens, 1990). This has previously been shown in other *in vitro* models (Murchison and Griffith, 2000).

However, there was no prevention of hypoglycaemic-induced cell death by intracellular calcium store antagonists. The IP_3 antagonist, TMB-8, possesses diverse effects in different systems, for example, it has shown to be an antagonist at nicotinic acetylcholine receptor subtypes (Bencherif *et al.*, 1995), to inhibit the ATP-sensitive K^+ channel (Szewcyk *et al.*, 1992) and to inhibit mitochondrial ATP production and global protein synthesis (Brand and Felber, 1984; Doutheil *et al.*, 1997). In addition TMB-8 has been shown to exhibit an intrinsic toxicity in neuronal cultures (Malcolm *et al.*, 1996) and it has been demonstrated that the blocking effect of TMB-8 on the depletion of IP_3 stores was only transient and thereafter TMB-8 produced an even more pronounced increase in $[Ca^{2+}]_i$ (Doutheil and Paschen, 1999). The ineffectiveness of TMB-8 to protect cells could be due to the compound acting on other sites, as described above. However, 2-APB has been shown to be much more selective than TMB-8 for IP_3 stores but again it shows no effect apart from an undesired toxic effect on normoglycaemic cells at a high concentration (Figure 4.7). Similar results were seen with dantrolene (Figure 4.8).

It has been shown that medium spiny neurones contain both IP_3 and ryanodine receptors (Sharp *et al.*, 1993). This suggests that both IP_3 and ryanodine stores are not involved in the toxicity produced by hypoglycaemia, assuming that these drugs are antagonising the receptors efficiently. Alternative methods to determine whether the release of calcium from intracellular calcium stores is predominant in hypoglycaemic-induced toxicity would have been to inhibit the ER SERCA-pump using thapsigargin (Nguyen *et al.*, 2002) or by the reversal of SERCA pump mechanism with cyclopiazonic acid (Kahlert and Reiser, 2000).

Data from this study is in contrast to studies reporting that calcium release from intracellular stores is the major source of calcium during glucose deprivation in PC12 cells (Chung and Lee, 1998). Another possibility as to why no protection was seen with dantrolene is that there was an absence of an intracellular calcium influx to trigger the release of calcium from the ryanodine stores. This would be consistent with the results obtained using EGTA, BAPTA, nicardipine and $NiCl_2$.

The idea that only one pathway is active in hypoglycaemic cell damage is implausible, considering the complexity and the numerous components involved. It is possible that blocking a combination of these components would potentiate the likelihood of obtaining protection. Figure 4.9, shows a combination of drugs used to block ROS generation

(PDTC), extracellular calcium (BAPTA), MTP activity (CsA) and intracellular calcium levels (2-ABP) had no effect on hypoglycaemic-induced cell death. Although the combination of these components did prove to reduce cell viability in normoglycaemic cultures (Figure 4.9), these results consolidate previous data suggesting that these components might not be involved in hypoglycaemic-induced cell death in striatal cultures.

One of the most interesting and informative experiments showed data measuring the levels of intracellular calcium. After 24 and 48 hours of hypoglycaemia no change in $[Ca^{2+}]_i$ was seen in surviving cells when compared to normoglycaemic cells (Figure 4.10) contrary to most other studies (Mattson *et al.,* 1993; Knopfel *et al.,* 1990; Tekkök *et al.,* 1999; Cheng *et al.,* 1991; Harris *et al.,* 1984). This provides a crucial insight to the effect on cells exposed to glucose deprivation and explains why none of the drugs used were effective in preventing the attenuation of cell viability. The fact that there is no calcium influx into the cells after 24 hours of hypoglycaemia is evidence that the cell membrane is not depolarised as the VGCC have not opened, explaining why nickel and nicardipine having no effect. This does not rule out the possibility that the membrane may have been depolarised sometime during the hypoglycaemic insult. If there were no depolarisation, no VGCC opening and no calcium influx, there would be no trigger to release calcium from internal stores. This provides reasons why EGTA, BAPTA and ER store antagonists provided no change to hypoglycaemic cells. In addition, there would be no calcium-dependent release of EAA, no sustained depolarisation via AMPA receptors and no calcium influx via NMDA receptors, therefore CNQX and MK801 showed to be ineffective. However, the lack of EAA release in hypoglycaemic striatal cultures is more likely because 95% of cells in striatal cultures are GABAergic and not glutamatergic but PDTC, CsA and TFP were also ineffective in preventing cell death. This is possibly because if there was no calcium influx, the MTP would not open and there would be no release of ROS.

This lack of change in $[Ca^{2+}]_i$ during hypoglycaemia has been reported previously. In mouse striatal neurones, the $[Ca^{2+}]_i$ level of cells exposed to 30 minutes of hypoglycaemia was not significantly different from normoglycaemic cells but changes occurred after 30 minutes (Williams *et al.,* 1995). Williams *et al.,* did not measure $[Ca^{2+}]_i$ at 24 hours but it may be assumed that at 24 hours changes in $[Ca^{2+}]_i$ would be observed as cells death was measured after 4 hours. In myocytes, exposed to severe ATP depletion, little or no increase in $[Ca^{2+}]_i$ was also observed (Cobbold and Bourne, 1984; Haworth *et al.,* 1987).

What is causing a reduction in cell viability in these striatal cultures exposed to hypoglycaemia, if it is not calcium elevations and calcium-dependent processes? Why is the cell not depolarised after 24 and 48 hours of hypoglycaemia and how is glucose exerting its regulatory effect if not by increases in calcium? It has been previously suggested that ATP depletion can directly lead to the collapse of mitochondrial membrane potential independently of an elevation of $[Ca^{2+}]_i$ (Lemasters *et al.*, 1987). However, it is interesting to note that after 48 hours of hypoglycaemia, the levels of $[Ca^{2+}]_i$ have not decreased below normoglycaemic levels (Figure 4.10), suggesting that the membrane is still intact as there is no calcium leakage due to disrupted membrane.

In conclusion, it is apparent that altered calcium homeostasis is not a key factor in hypoglycaemia-induced cell death in striatal cells, therefore the hypothesis must be once again amended. The depletion of glucose from the medium causes a decrease in ATP concentration, which triggers a calcium-independent dysfunction of the mitochondria. Consequently, the mitochondria releases apoptotic inducing factors which initiate the cascade for apoptosis. It has been reported that some cells can undergo apoptosis under essentially calcium-free conditions (Lennon *et al.*, 1992). Therefore the next step in this study is to investigate whether apoptosis is the mechanism of cell death in this model of hypoglycaemia.

Chapter Five.

Apoptosis in Hypoglycaemic toxicity

5.1 Introduction

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) examines DNA fragmentation (Gavrieli *et al.*, 1992). The DNA strand breaks are detected by enzymatically labelling the free 3′ -OH termini with modified nucleotides. It was hypothesised that hypoglycaemic-induced insults might induce apoptotic cell death, characterised by DNA damage, hence an increase in TUNEL staining would be observed.

The mature caspase-3 enzyme is formed from 17kDa (p17) and 12kDa (p12) subunits, which are produced from a 32-kDa precursor. In the 32-kDa caspase-3 precursor protein, there are two cleavage sites for the production of the p17 and p12 subunits (Han *et al.*, 1997). Z-DEVD-FMK, is a caspase-3 activity inhibitor which appears to prevent the p17 subunit being cleaved (Han *et al.*, 1997). It is proposed that if downstream caspase activity is inhibited by prevention of caspase-3 activation, this will prevent the onset of the execution stage of apoptosis and, therefore promote cell survival during hypoglycaemia.

Immunostaining for Bax, cytochrome c, caspase-9 and caspase-3 in cells exposed to hypoglycaemia, is important in the understanding of whether cells are dying via an apoptotic mechanism and can be use to confirm TUNEL staining data. It is hypothesised that the expression of one or more of these components will be increased from basal levels in hypoglycaemic cells if apoptotic cell death is occurring. Nuclear activity of the two downstream caspases, caspase-9 and caspase-3 should also be measured as several lines of evidence suggest that some caspases are translocated to the nucleus and trigger specific substrates that contributes to nuclear apoptosis (Nakagawara *et al.*, 1997).

In this series of experiments, the assessment of these various apoptotic components and characteristics were conducted to shed some light upon whether hypoglycaemia produces apoptotic cell death.

5.2 Results

TUNEL staining was detected in cultures exposed to 24 hours of hypoglycaemia and compared to normoglycaemic cultures. There was a significant difference in TUNEL staining between the two conditions. In hypoglycaemic cultures there were significantly more TUNEL positive cells (40.8% ± 16.6) compared to normoglycaemic cultures (7.4% ± 0.6). (Figure 5.1a). Figure 5.1b is an example of TUNEL positive staining in a culture exposed to 24 hours of hypoglycaemia.

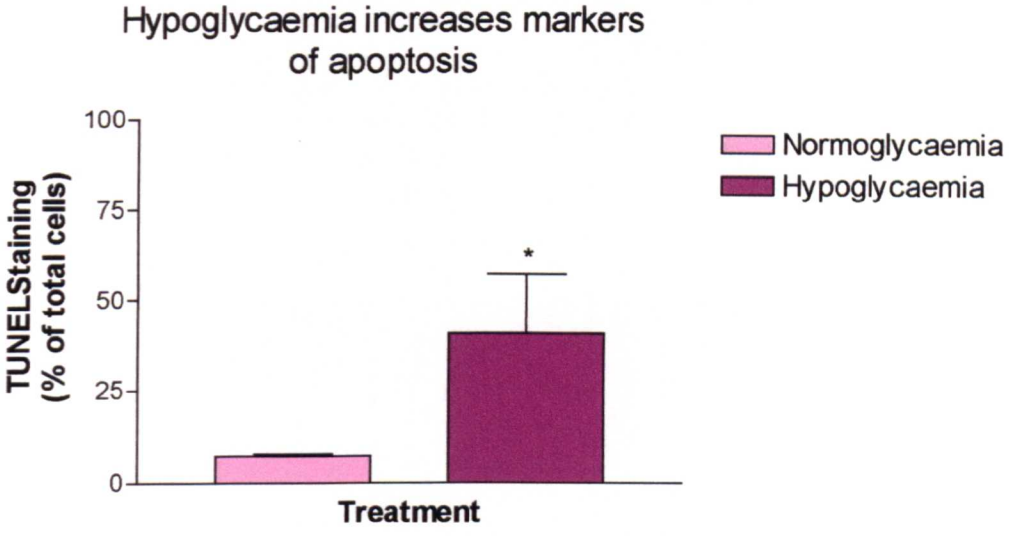

Figure 5.1a

The percentage of cells showing TUNEL staining in cultures exposed to either 24 hours of normoglycaemia or hypoglycaemia. In hypoglycaemic cultures the glucose was removed for 24 hours before fixing for TUNEL staining. There is a significant increase between the percentage of the cells expressing TUNEL staining in hypoglycaemic cultures compared to normoglycaemic cultures (one-way ANOVA, $F(1,4)=12.5$, $*p<0.05$). Data are presented as a percentage of TUNEL positive cells from the total number of cells in culture.

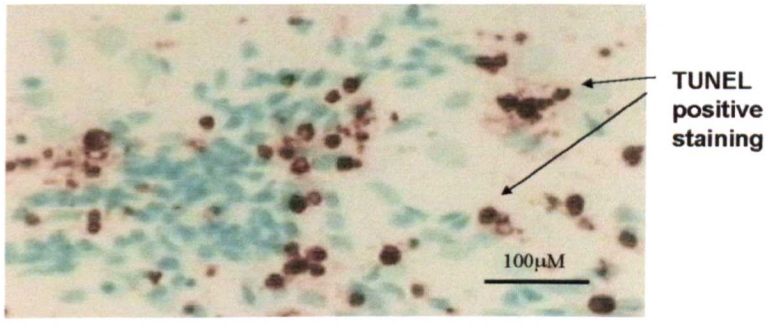

Figure 5.1b
TUNEL positive staining in a culture exposed to 24 hours of hypoglycaemia. Cells with nicked DNA contain dark nuclei and are surrounded by cells containing no nicked DNA that are counterstained light green.

The caspase-3 inhibitor, Z-DEVD-FMK, did not significantly increase the cell viability of the hypoglycaemic-treated cultures. In fact, at concentrations used, it appeared to be toxic itself as the cell viability in normoglycaemic cultures was significantly reduced.

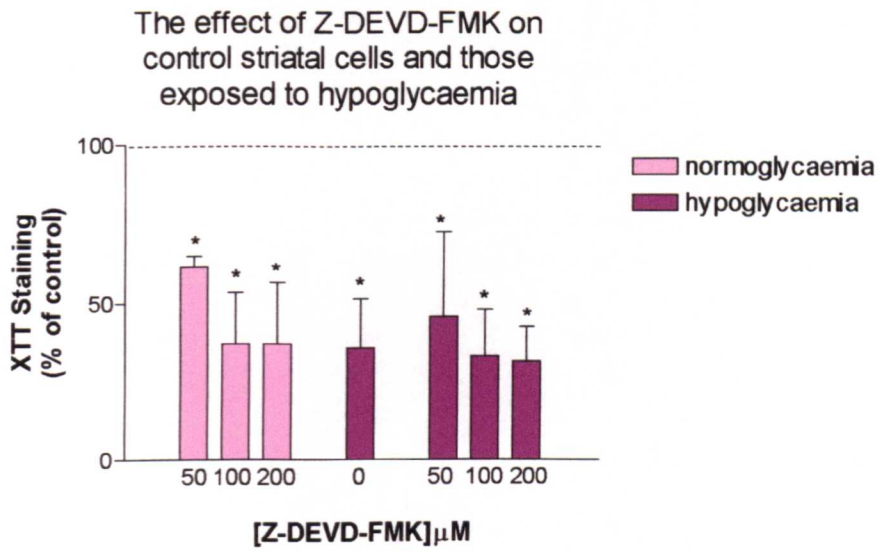

Figure 5.2

The effect of the caspase-3 inhibitor, Z-DEVD-FMK, on striatal cells in normoglycaemic and hypoglycaemic cultures. The glucose was removed from hypoglycaemic cultures prior to the addition of the caspase inhibitor. Different concentrations of Z-DEVD-FMK were added to cultures for 4 hours before cell viability was measured. Cell viability was measured by XTT staining. Both Z-DEVD-FMK and hypoglycaemia produce no significant effect (two-way ANOVA; effect of Z-DEVD-FMK, $F(2,12)=2.9$, $p=0.1$; effect of hypoglycaemia, $F(1,12)=1.35$, $p=0.3$). There is a significant decrease of XTT staining in hypoglycaemic-treated cultures compared to normoglycaemic cultures without Z-DEVD-FMK. In normoglycaemic cultures the addition of Z-DEVD-FMK also produces a significant decrease in cell viability compared to normoglycaemic cultures without Z-DEVD-FMK (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). Z-DEVD-FMK showed no neuroprotection against hypoglycaemic-induced toxicity (one-way ANOVA, $p=0.8$). Data are represented as a percentage of XTT staining in normoglycaemic cultures without Z-DEVD-FMK treatment (100%) (n=5).

Hypoglycaemia had no effect on the expression of cytoplasmic Bax at any of the time points measured (Figure 5.3).

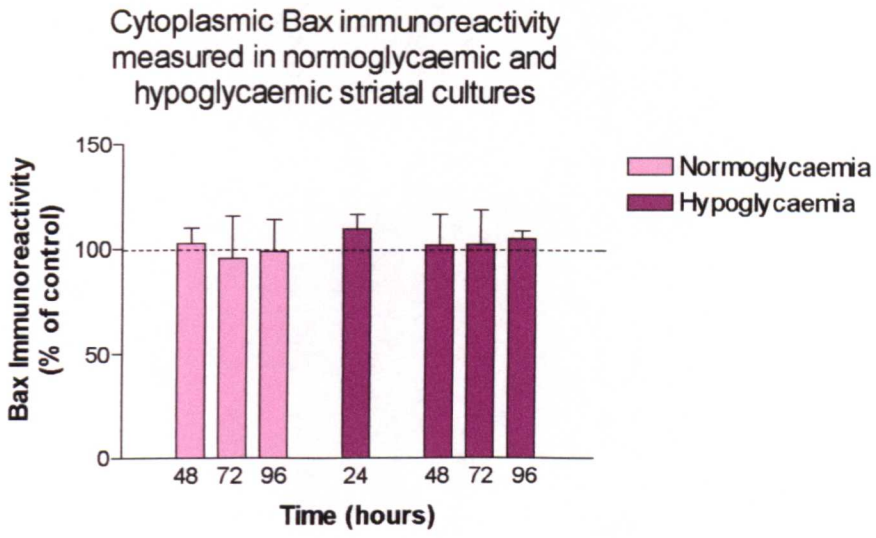

Figure 5.3

Cytoplasmic Bax immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. Cells were either treated with 24, 48, 72 or 96 hours of normoglycaemia or hypoglycaemia. There was no change in Bax cytoplasmic expression in hypoglycaemic cultures relative to normoglycaemic cultures and there was no change in expression of cytoplasmic Bax over time (one-way ANOVA, $p=0.8$). Data are expressed as a percentage of Bax immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%) ($n=3$).

Cytosolic cytochrome c immunoreactivity appears significantly higher in hypoglycaemic cells after 24 hours compared to normoglycaemic cells. This expression seems to decrease to non-significant levels (normoglycaemic levels) after 48 hours but significantly rises again after 72 and 96 hours. There were no changes in expression in normoglycaemic cultures (Figure 5.4).

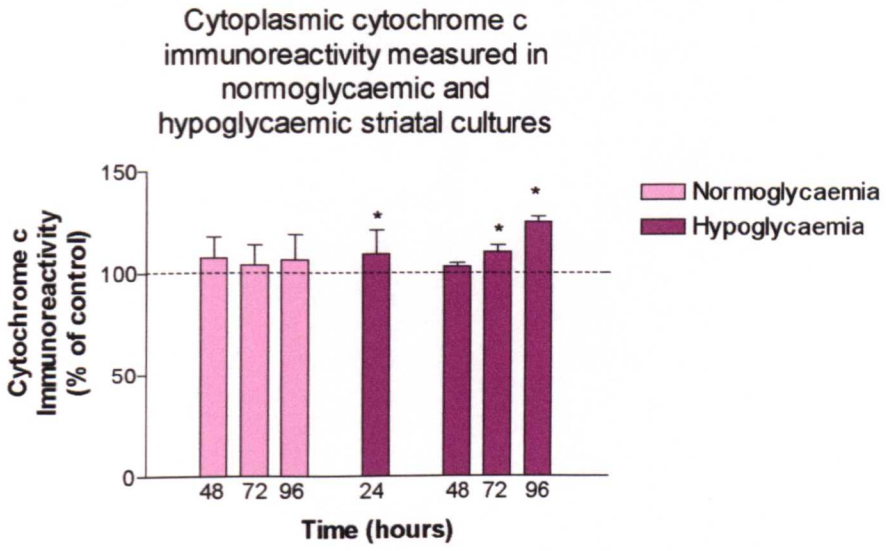

Figure 5.4

Cytoplasmic cytochrome c immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. Cells were either treated with 24, 48, 72 or 96 hours of normoglycaemia or hypoglycaemia. There is a significant increase in cytoplasmic cytochrome c expression with time in cultures treated with 24, 72 and 96 hours of hypoglycaemia (one-way ANOVA, $F(3,8)=24.4$, $p<0.001$; $^*p<0.05$ versus 100%; 95% confidence intervals of the mean)). Data are expressed as a percentage of cytoplasmic cytochrome c immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%) (n=3).

Hypoglycaemia had no effect on cytoplasmic caspase-9 expression at any of the time points observed. However, after 96 hours, there was a significant decrease in caspase-9 expression in normoglycaemic cultures (Figure 5.5).

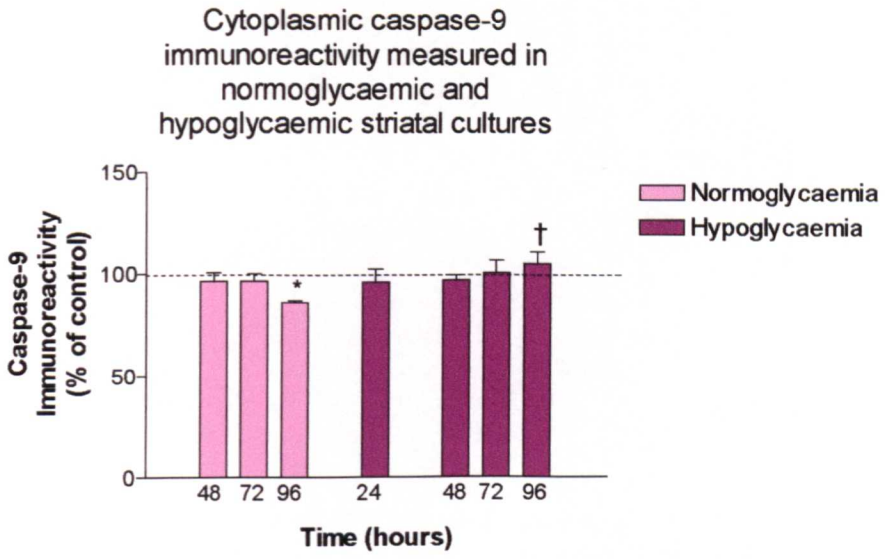

Figure 5.5

Cytoplasmic caspase-9 immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. Cells were either treated with 24, 48, 72 or 96 hours of normoglycaemia or hypoglycaemia. There is no change in caspase-9 cytoplasmic expression in hypoglycaemic cultures relative to normoglycaemic cultures, except at 96 hours. At 96 hours caspase-9 cytoplasmic expression increased in hypoglycaemic cultures (†$p<0.01$,Tukey pairwise comparison). However, in normoglycaemic cultures after 96 hours an significant decrease in cytoplasmic caspase-9 expression is observed compared to all other time points (one-way ANOVA, $F(2,6)=11.8$, $p<0.01$; *$p<0.05$ versus 100%; 95% confidence intervals of the mean). Data are expressed as a percentage of cytoplasmic caspase-9 immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%) (n=3).

Similar results were seen when nuclear caspase-9 was measured. Hypoglycaemia had no effect on nuclear caspase-9 expression but in normoglycaemic cells after 96 hours, a decrease in nuclear caspase-9 expression was observed (Figure 5.6).

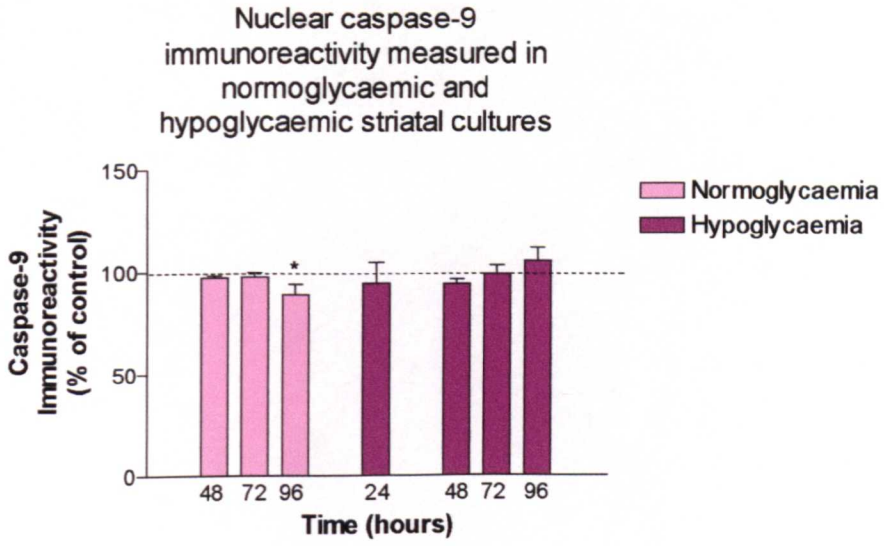

Figure 5.6

Nuclear caspase-9 immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. Cells were either treated with 24, 48, 72 or 96 hours of normoglycaemia or hypoglycaemia. There is no change in caspase-9 nuclear expression in hypoglycaemic cultures relative to normoglycaemic cultures (one-way ANOVA, $p=0.4$). In normoglycaemic cultures after 96 hours, a significant decrease in nuclear caspase-9 expression is observed compared to all other time points (one-way ANOVA, $F(2,6)=12.3$, $p<0.01$; $^*p<0.05$ versus 100%; 95% confidence intervals of the mean). Data are expressed as a percentage of nuclear caspase-9 immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%) (n=3).

There was no change in either cytoplasmic (Figure 5.7) or nuclear (Figure 5.8) caspase-3 expression after 24, 48 or 72 hours of hypoglycaemic treatment.

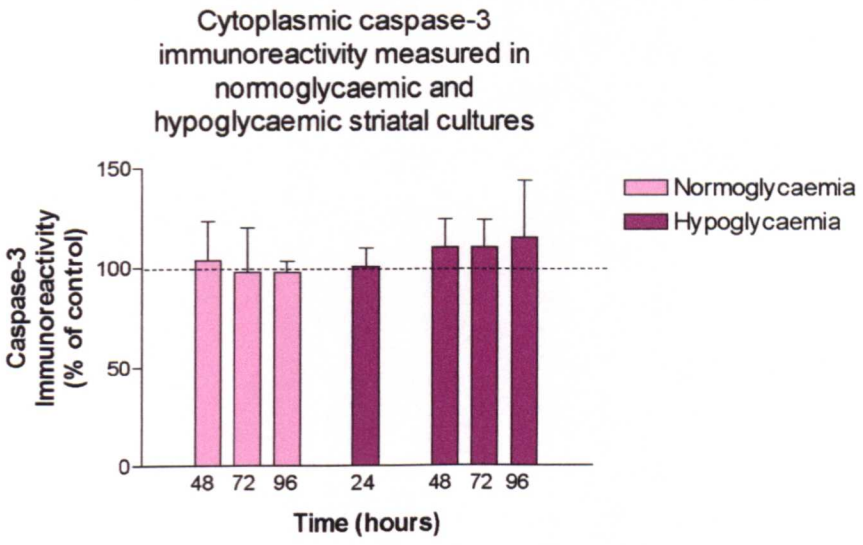

Figure 5.7

Cytoplasmic caspase-3 immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. Cells were either treated with 24, 48, 72 or 96 hours of normoglycaemia or hypoglycaemia. There is no change in caspase-3 cytoplasmic expression in hypoglycaemic cultures relative to normoglycaemic cultures and there is no change in expression of cytoplasmic caspase-3 over time (one-way ANOVA, p=1.0). Data is expressed as a percentage of cytoplasmic caspase-3 immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%) (n=3).

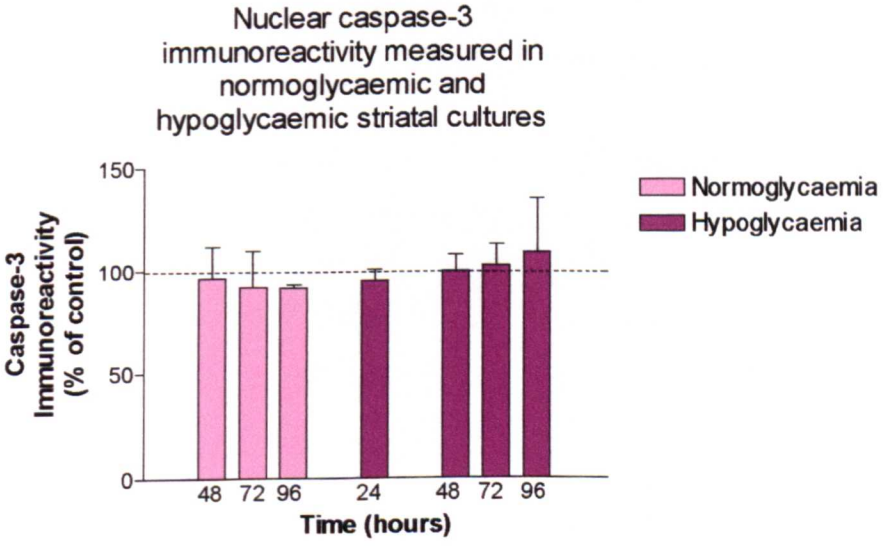

Figure 5.8

Nuclear caspase-3 immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. Cells were either treated with 24, 48, 72 or 96 hours of normoglycaemia or hypoglycaemia. There is no change in caspase-3 nuclear expression in hypoglycaemic cultures relative to normoglycaemic cultures and there is no change in expression of nuclear caspase-3 over time (one-way ANOVA, p=0.9). Data is expressed as a percentage of nuclear caspase-3 immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%) (n=3).

5.3 Discussion

TUNEL staining was markedly increased in cells subjected to 24 hours of hypoglycaemia (Figure 5.1) suggesting that glucose deprivation produces DNA fragmentation, a distinct marker of apoptosis. This has been repeatedly reported in many models, including *in vitro* and *in vivo* models of hypoglycaemia (Lawrence *et al.*, 1996; Ferrand-Drake *et al.*, 1999 REF). The presence of TUNEL staining implies that the DNA has been nicked by specific endonucleases. It has been reported that calcium-independent endonucleases exist, for example, Dnase II (Barry and Eastman, 1993), so that it is plausible that in this model DNA nicking enzymes are active. In addition it has also been reported that ATP is required for caspase activation but not for DNA fragmentation (Leist *et al.*, 1997). Therefore, it is possible that even if ATP levels in hypoglycaemia have diminshed DNA fragmentation can still occur.

The caspase-3 inhibitor, Z-DEVD-FMK, produced no neuroprotective effect in hypoglycaemic cultures (Figure 5.2). This suggests that either Z-DEVD-FMK is not specific and inadequately inhibits caspase-3 or that caspase-3 is not involved in the apoptotic pathway initiated by the removal of glucose. However, it is becoming increasingly evident that apoptotic-features are found when cells are treated with caspase-inhibitors (Nicotera *et al.*, 2000) and it has been described that some caspase inhibitors can prevent the appearance of certain, but not all features of apoptosis in certain model systems (McCarthy *et al.*, 1997). Z-DEVD-FMK did not increase survival in striatal neurones undergoing apoptosis in a Huntington disease model (Kim *et al.*, 1999a) and DNA fragmentation caused by traumatised brain injury was also not affected by the administration of Z-DEVD-FMK (Yakovlev *et al.*, 1997; Allen *et al.*, 1999). Z-DEVD-FMK was shown to be toxic to normoglycaemic cells, suggesting that either this compound inhibits other enzymes or that caspase-3 is involved in physiological as well as pathological processes. It has been reported, in other cell types, that caspase-3 is activated in the absence of cell death (Miossec *et al.*, 1997), indicating that caspase-3 has functions distinct from executing cell death. However, it has also been reported that concentrations of Z-DEVD-FMK used in this experiment can inhibit all caspases. It is therefore possible that inhibiting all caspases, even members of the family which are not involved in apoptosis, could be detrimental to healthy cells.

The non-specific background staining of the Bax, cytochrome c, caspase-9 and caspase-3 antibodies used, were determined by designing a control experiment where

immunocytochemistry procedure is identical but the primary antibodies were are omitted. In these experiments no staining was observed suggesting that the antibodies were specific for the particular protein. In addition, these antibodies are used in most experimental studies investigating apoptotic machinery (Fernandes-Alnenari, 1996; Liu *et al.*, 1997; Hockenbery *et al.*, 1991).

After 24 hours of hypoglycaemia, the expression of immunoreactive Bax remained at baseline levels (Figure 5.3). This suggests that either Bax is not activated by the removal of glucose or that the optimum time for the expression of Bax was prior to 24 hours, hence a change was not detected. However, it was shown that in transient forebrain ischaemia, the expression of Bax protein peaked at 72 hours following the insult (Hara *et al.*, 1996). Cytochrome c expression increased in cultures treated with hypoglycaemia for 24 hours, compared with normoglycaemic cultures (Figure 5.4). It is not necessary for Bax to translocate to the mitochondria, in order to release cytochrome c suggesting that although no increase in Bax expression was observed in hypoglycaemic cells, apoptotic-related changes in cytoplasmic c expression can still occur. The gradual increase in cytochrome c expression maybe a result of mitochondrial membrane depolarisation or the interaction of other Bcl-2 pro-apoptotic family proteins, such as Bid (Li *et al.*, 1998) which translocate to the mitochondria upon the onset of the hypoglycaemic insult.

After 96 hours immunoreactive caspase-9 expression in both the cytoplasm and the nucleus is decreased in normoglycaemic cells (Figures 5.5 and 5.6). However no change in either the cytoplasmic or nuclear expression of caspase-3 in hypoglycaemic cells compared with normoglycaemic cells was observed (Figures 5.7 and 5.8). This is in contrast to the majority of literature describing caspase-3 as the major apoptotic executing factor, and a report where caspase-9 has been shown to peak at 24-72 hours in the striatum after ischaemia (Chen *et al.*, 1998). It is possible that apoptosis independent of these specific caspases is initiated. It is difficult to interpret why a decrease in caspase-9 expression was observed after 96 hours of normoglycaemia. It would be postulated that if there were any change in caspase expression then an increase would be expected due to apoptosis occurring triggered by a lack of neurotrophic support after 96 hours. It is not certain that this decrease has any functional significance.

Depending on the cell type, the stimuli and the severity of the stimuli, different caspases may be employed. For example, the overexpression of caspase-12 induces apoptosis and is associated with the stresses induced to the ER in neurones, for example by A23187 or amyloid-β protein (Nakagawa *et al.*, 2000). It was observed that although other caspases

may be activated downstream from caspase-12, caspase-12 appears to be necessary for apoptosis (Nakagawa *et al.*, 2000). Caspase-9 also cleaves and activates caspase-6 and –7 as well as caspase-3. It is apparent that caspase-8 can also activate caspase-3, -6, -7 and Bid (Figure 1.1) (Muzio *et al.*, 1996; Luo *et al.*, 1998; Li *et al.*, 1998). Caspase-dependent pathways, which are independent of mitochondria, have also been described. For example, caspase-8 translocates from the receptor within the plasma membrane to the cytoplasm where it initiates the death cascade by proteolytic activation of downstream caspases (Cryns and Yuan, 1998). It is therefore very plausible that a different, not well established, cascade might be active in hypoglycaemic-induced cells. Therefore, the lack of evidence for caspase-3 and capase-9 activation in hypoglycaemia does not rule out the possibility that apoptosis is occurring.

There has been much debate over whether caspase-independent apoptosis exists (Nicotera *et al.*, 2000). The discovery of other proteases, unrelated to caspases, involved in the programmed cell death pathways, have provided evidence for the reality of multiple converging pathways (Hirsch *et al.*, 1997; Sarin *et al.*, 1997; Xiang *et al.*, 1996). It has been suggested that serine proteases, cathepsin and calpains may be active in apoptosis (Nicotera *et al.*, 2000). In addition, gene knockout mice studies have shown that mice lacking cytochrome c, apaf1 and caspase-9 exhibit apoptosis (Kuida *et al.*, 1998; Li *et al.*, 2000).

Recently, another pathway linking the mitochondria to apoptosis has been reported, which is independent of caspases but dependent on a molecule termed apoptosis-inducing factor (AIF) (Josa *et al.*, 2001). AIF is localised in the mitochondria, similar to cytochrome c, and is released in response to death stimuli, but is not associated with Apaf1 or caspase-9 (Josa *et al.*, 2001). It has been cloned in embryonic stem cells and provides strong evidence that components other than those previously described exist in neurones.

The endonuclease that is essential for the internucleosomal fragmentation of DNA is DNA fragmentation factor 45 (DFF 45). It is apparent that DFF 45 is cleaved by caspase-3 but once cleaved it can induce DNA fragmentation in the absence of caspase activity (Liu *et al.*, 1997). Transgenic mice lacking DFF 45 or its caspase cleavage site have significantly reduced DNA fragmentation. However, these mice still show DNA fragmentation and are phenotypically normal (Li *et al.*, 2001). Another nuclease has been characterised, termed endonuclease G. Once released from the mitochondria triggered by apoptotic stimuli, it translocates to the nucleus and cleaves DNA independently of caspases (Li *et al.*, 2001).

It has been repeatedly disputed that TUNEL staining is not an accurate indicator of classical apoptosis and that DNA fragmentation does not necessarily imply cell death will be apoptotic (Takei and Endo, 1994). Studies have shown TUNEL staining labelling and DNA fragmentation in necrotic cells (de Torres *et al.*, 1997; Collins *et al.*, 1992).

Indeed, apoptosis is an energy requiring process. Therefore if ATP is depleted during hypoglycaemic injury it would be assumed that the mode of cell death would switch to an ATP-independent mechanism, namely necrosis (Leist *et al.*, 1999). For example, it has been suggested that glucose-deprivation would affect apoptotic pathways by preventing the binding of Apaf-1 to procaspase-9 protein (Zou *et al.*, 1999) which is dependent on ATP, so what the subsequent activation of downstream degradative processes including caspase-3 activation and PARP cleavage would also be precluded.

Apoptosis and necrosis have been reported to coexist in the same damaged tissue and more interestingly, that cells dying from a type of cell death comprise of both apoptotic- and necrotic-like characteristics, such as mitochondrial swelling and cellular shrinkage (Ferrand-Drake *et al.*, 1999). This provides further complications when assessing whether cultured striatal cells exposed to hypoglycaemia die due to apoptosis or necrosis or both.

In conclusion, it seems that the integral apoptosis process, observed in most neurotoxic models, is not involved in hypoglycaemia measured by XTT staining and MAP2 staining. It is possible that the process of apoptosis may be halted in the absence of glucose as ATP is required to drive apoptotic components. However, DNA nicking has occurred introducing the possibility that cell demise occurring in this model is one that cannot be classified into apoptosis or necrosis. Therefore if classic apoptosis is not the mode of cell death in this model and calcium-dependent necrosis is not involved, what is the primary mechanism triggering the reduction in cell viability and DNA fragmentation? It is plausible to suggest that the cells are metabolically and structurally damaged but not actually dead. The next section of this study focuses on whether the cells can recover from the hypoglycaemic-insult, which indicates that the cells are not dead. It also examines the possible repair mechanisms involved.

Chapter Six.

The Recovery of Hypoglycaemic cells

6.1 Introduction

It has been demonstrated that the re-introduction of glucose into a previously energy-deficient system is less beneficial to the survival of neurones than would be expected (Siesjo, 1992; Tasker *et al.*, 1992; Almeida *et al.*, 1995). It has also been demonstrated that after a period of hypoglycaemia in both *in vitro* and *in vivo* models that EAA-receptor activation and EAA release occurs when the glucose content is replaced (Tasker *et al.*, 1992; Linden *et al.*, 1987). It has also been suggested that a re-introduction of glucose initiates opening of the MTP and triggers the formation of ROS (Almeida *et al.*, 1995; Kristian and Siesjo, 1996). It is therefore hypothesised that after 24 hours, neuronal survival will not increase upon the replacement of the glucose content. The duration of the return to normoglycaemia (recovery) is an apparent consequential factor, hence the effect of different lengths of recovery on the outcome of neuronal viability are observed.

It is also unclear what effect re-introduction of glucose would have on the process of apoptosis. Hence it is interesting to see whether any apoptotic machinery is employed during the recovery stage, since when glucose is added, the system has energy to switch on the ATP-dependent death-promoting components which can subsequently influence the fate of the cell.

DNA damage can occur as a result of many exogenous stimuli, such as oxidative stress. It is hypothesised that during hypoglycaemia, DNA damage occurs, detected by TUNEL staining, but it is of interest to observe whether DNA damage is exacerbated by the re-introduction of glucose. In this study, it is postulated that an increase in the expression of DNA repair proteins will be observed in association with decreased TUNEL staining, suggesting that the repair proteins are attempting to preserve the DNA structure.

In this series of experiments, the consequences of re-introduction of glucose on the viability of cells in hypoglycaemic cultures was assessed. In addition, experiments were designed to assess the activity of components of the apoptotic cascade, along with DNA fragmentation and DNA repair proteins during this recovery phase, in an attempt to determine the mechanisms involved in the recovery phase post-hypoglycaemia.

6.2 Results

There was a significant decrease in XTT staining in cells treated with hypoglycaemia for 24, 48, 72 and 96 hours. There was no significant difference observed in the percentage of cell viability between the difference lengths of hypoglycaemia (Figure 6.1).

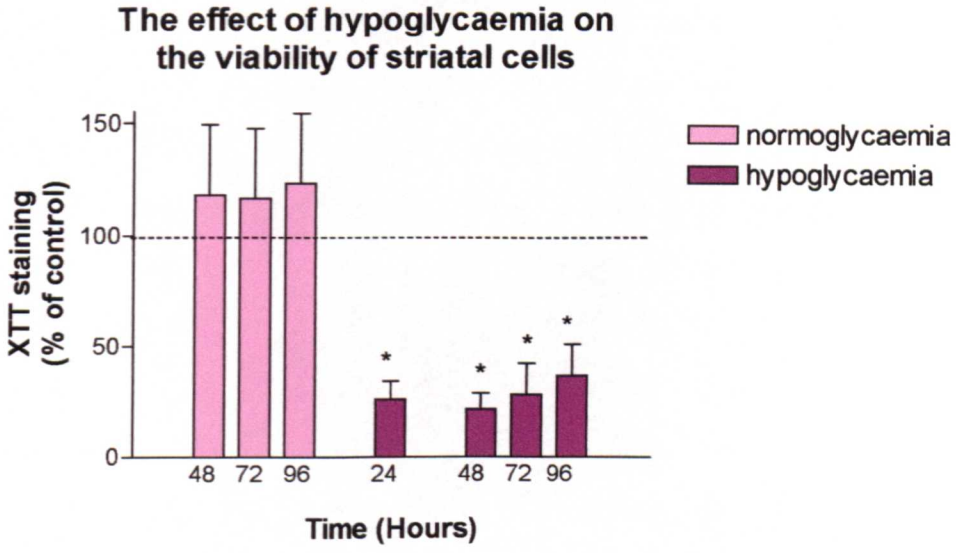

Figure 6.1
The effect of removing the glucose for 24, 48, 72 and 96 hours compared to normoglycaemic cultures. All durations of hypoglycaemia studied produce a significant decrease in XTT staining (two-way ANOVA, $F(1,16)=105.1$ significant effect of hypoglycaemia, $p<0.001$, effect of time $F(3,16)=0.7$, $p=0.6$; *$p<0.05$ versus 100%; 95% confidence intervals of the mean) but there was no difference with time (one-way ANOVA, $p=0.5$). Data are presented as a percentage of XTT staining in normoglycaemic cultures incubated for 24 hours after the onset of treatment (100%) (n=3).

Cultures treated with 24 hours of hypoglycaemia showed a significant decrease in cell viability compared to normoglycaemic cultures. However, when the glucose was replaced for 24 hours, XTT staining dramatically increased to normoglycaemic levels. Glucose replacement for a further 24 and 48 hours sustained the increased levels of cell viability (Figure 6.2a). The cell viability in normoglycaemic cultures had significantly decreased after 24 and 48 hours of recovery but not after 72 hours.

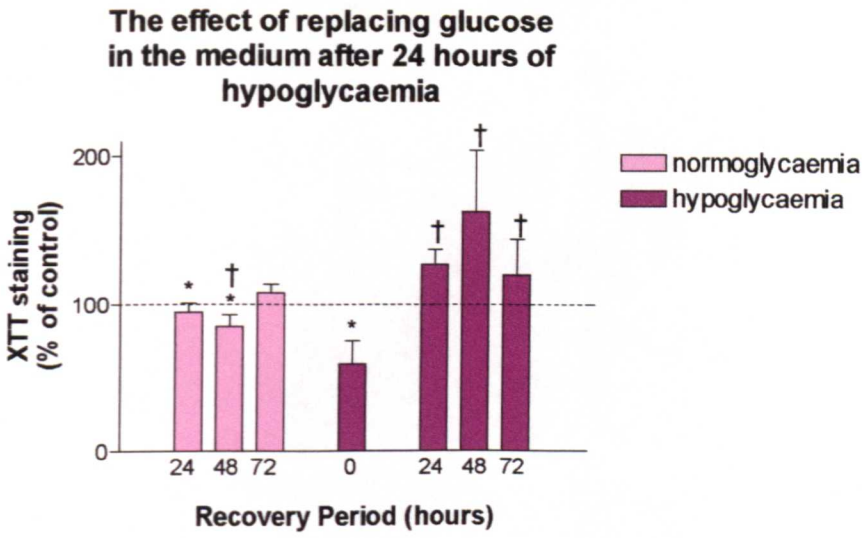

Figure 6.2a

The effect of replacing the glucose in cultures exposed to 24 hours of normoglycaemia or hypoglycaemia. In hypoglycaemic cultures the glucose was removed for 24 hours and then replaced for either 24, 48 or 72 hours. Cell viability was measured by XTT staining. Both glucose replacement and duration of glucose replacement produce significant effects (two-way ANOVA, effect of glucose replacement, $F(1,16)=7.1$, $p<0.05$; effect of recovery time, $F(3,16)=6.2$, $p<0.01$). The XTT staining of hypoglycaemic cultures after 24 hours (0 recovery period) is significantly decreased compared to normoglycaemic cultures (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). After glucose replacement for 24, 48 and 72 hours in hypoglycaemic cultures, cell viability significantly increases (one-way ANOVA, $F(3,8)=8.2$, †$p<0.01$). However, a significant decrease in XTT staining in normoglycaemic cultures is observed after 24 and 48 hours of replacing the glucose. At 48 hours XTT staining in normoglycaemic cultures is significantly decreased compared to hypoglycaemic cultures (†$p<0.01$, Tukeys pairwise comparison). Data are presented as a percentage of XTT staining in normoglycaemic cultures incubated for 24 hours (100%) (n=5).

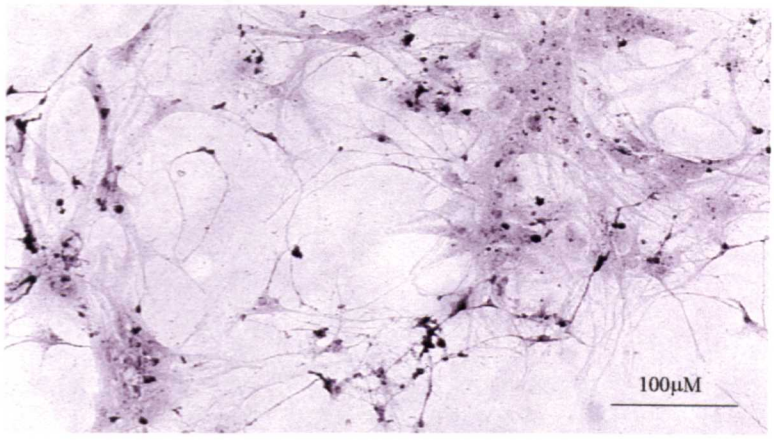

Figure 6.2b
A morphological observation of striatal cells after 24 hours of hypoglycaemia followed by 24 hours of recovery. The dendrites have spread out connecting the remaining cells and there are more glia present which may provide crucial metabolic support. Cells are stained with cytochrome c antibody.

A significant increase in TUNEL staining in cultures treated with hypoglycaemia for 24 hours was observed when compared to normoglycaemic cultures. Nevertheless, when glucose was replaced in these cultures, TUNEL staining significantly decreased to normoglycaemic levels. After 48 and 72 hours of recovery, TUNEL staining was still reduced in comparison to staining in hypoglycaemic cultures (Figure 6.3).

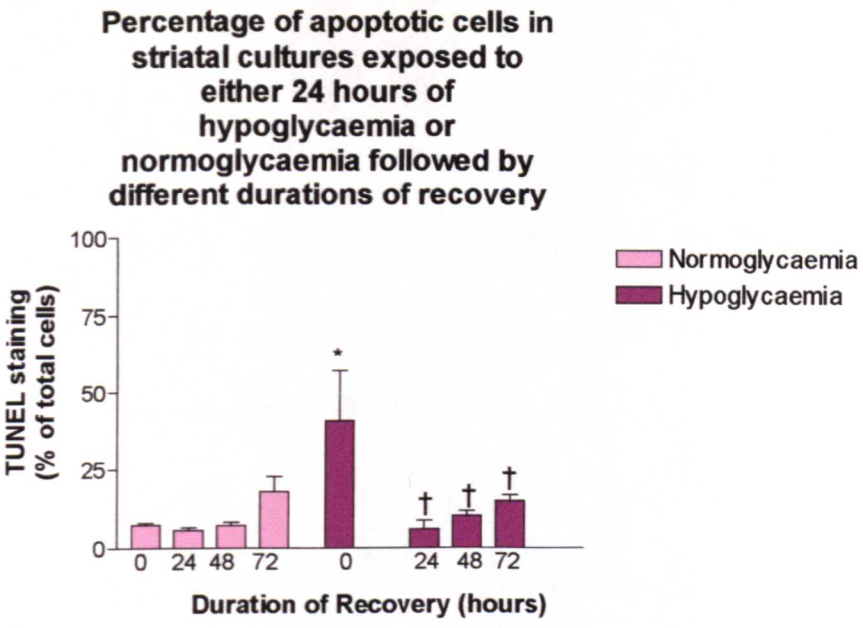

Figure 6.3

The percentage of cells showing TUNEL staining in cultures exposed to 24 hours of normoglycaemia or hypoglycaemia and subsequently either 24, 48 or 72 hours of glucose replacement. In hypoglycaemic cultures the glucose was removed for 24 hours and then replaced. Both treatment and time produce a significant effect (two-way ANOVA, effect of glucose replacement, $F(1,16)=10.28$, $p<0.01$; effect of recovery time, $F(3,16)=10.81$, $p<0.001$). Cultures exposed to 24 hours of hypoglycaemia show a significant increase in TUNEL staining compared to normoglycaemic cultures (*$p<0.001$, Tukey pairwise comparison). This staining is significantly reduced when glucose is re-introduced for 24 hours ($p<0.001$, Tukey pairwise comparison), 48 hours ($p<0.001$, Tukey pairwise comparison) and 72 hours ($p<0.01$, Tukey pairwise comparison). Data are presented as a percentage of the total number of cells in culture ($n=4$).

Figures 6.4 to Figure 6.9 show cytoplasmic or nuclear immunostaining of either bax, cytochrome c, caspase-9 or caspase-3 in cells recovered from hypoglycaemia. At all the time points studied, there was no significant changes between the conditions.

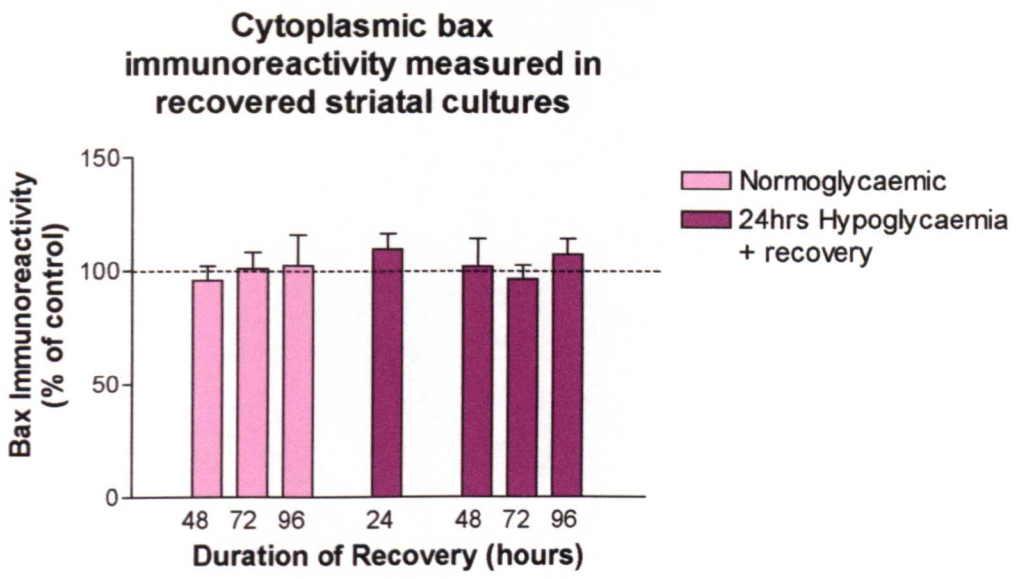

Figure 6.4
Cytoplasmic bax immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. In hypoglycaemic cultures the glucose was removed for 24 hours and then replaced for either 24, 48 or 72 hours There is no change in the expression of cytopasmic bax in recovered cells relative to hypoglycaemic cultures (one-way ANOVA, p=0.4). Data are expressed as a percentage of cytoplasmic bax immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%) (n=3).

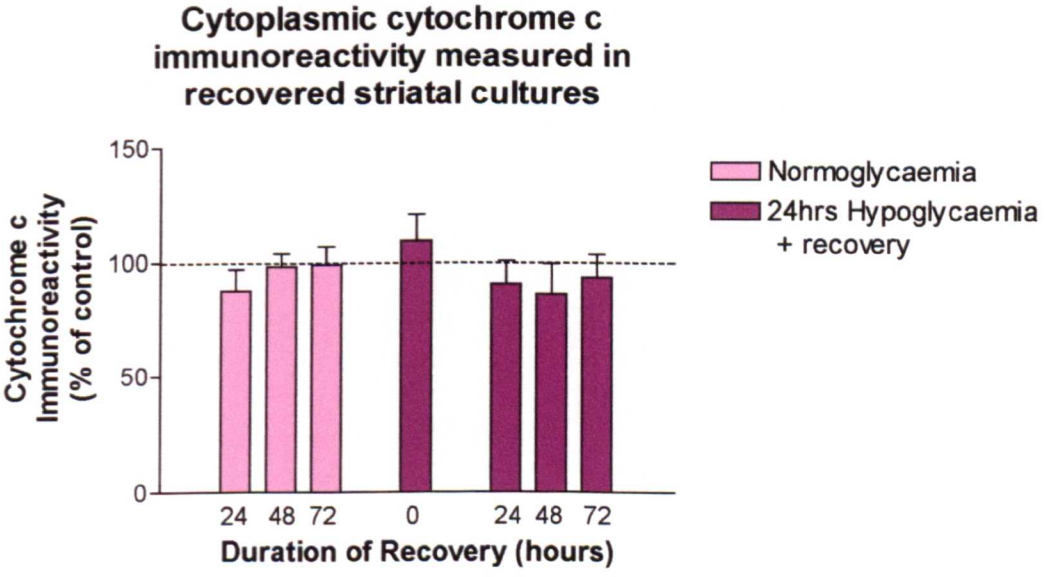

Figure 6.5
Cytoplasmic cytochrome c immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. In hypoglycaemic cultures the glucose was removed for 24 hours and then replaced for either 24, 48 or 72 hours There is no change in the expression of cytopasmic cytochrome c in recovered cells relative to hypoglycaemic cultures (one-way ANOVA, p=0.7). Data are expressed as a percentage of cytoplasmic cytochrome c immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%) (n=3).

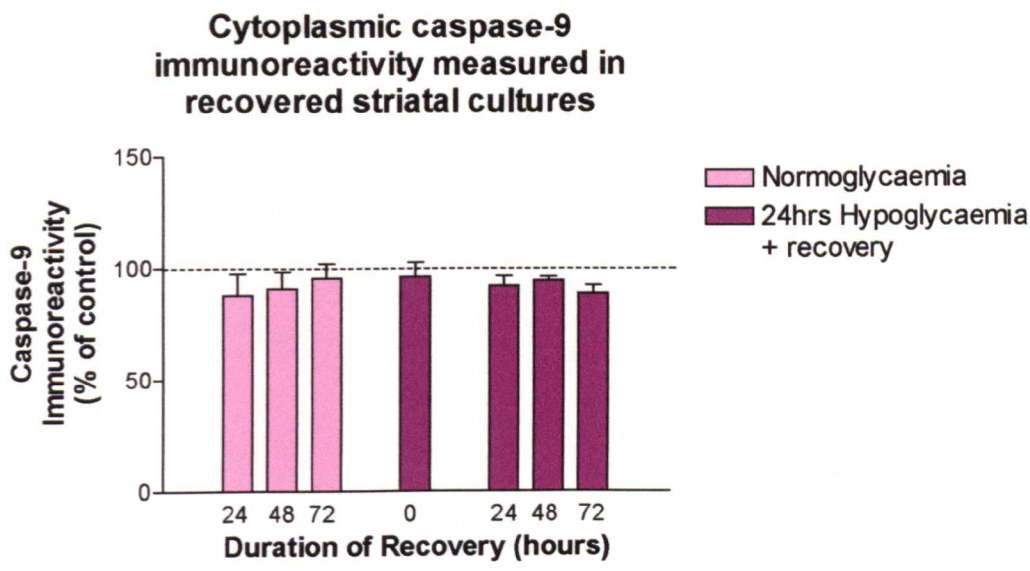

Figure 6.6
Cytoplasmic caspase-9 immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. In hypoglycaemic cultures the glucose was removed for 24 hours and then replaced for either 24, 48 or 72 hours. There was no change in the expression of cytopasmic caspase-9 in recovered cells relative to hypoglycaemic cultures (one-way ANOVA, p=0.7). Data are expressed as a percentage of cytoplasmic caspase-9 immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%)(n=3).

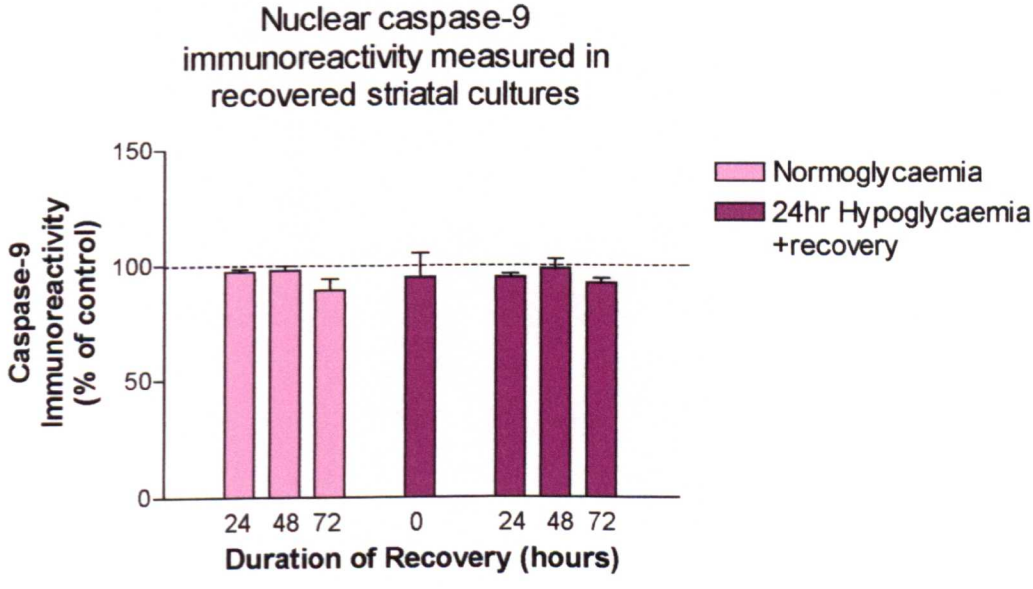

Figure 6.7
Nuclear caspase-9 immunoreactivity in normoglycaemic and hypoglycaemic striatal cultures. In hypoglycaemic cultures the glucose was removed for 24 hours and then replaced for either 24, 48 or 72 hours. There was no change in the expression of nuclear caspase-9 in recovered cells relative to hypoglycaemic cultures (one-way ANOVA, p=0.8). Data are expressed as a percentage of nuclear caspase-9 immunoreactivity in normoglycaemic cultures incubated for 24 hours (100%) (n=3).

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6.3 Discussion

Replacing the glucose in the culture medium after 24 hours of hypoglycaemia, markedly increases the XTT staining of the cells (Figure 6.2a), compared to hypoglycaemic cultures without glucose replacement (Figure 6.1). This strongly suggests an increase in cell survival that is prolonged and not a transient effect, as after 72 hours of recovery, the cell viability of these cultures is still at normoglycaemic levels (Figure 6.2a). It is also possible to suggest the dramatic increase in hypoglycaemic cultures when glucose is replaced is partly associated with the removal of toxic elements, such as ROS, as well as the re-introduction of glucose. In Figure 6.2b, recovered cells have regained long processes and are more spread out compared to Figure 3.2b. Although the staining is cytochrome c immunoreactivity (no MAP2 staining was done on these cells) and therefore is not uniform throughout the cell's structure it does provide a morphological picture of recovery in surviving cells. It also shows an increase in glia proliferation indicating that the prevention of glia cells is more difficult in older cultures and suggests that glia may have a pivotal role in neuronal recovery. Figure 6.2b complements the XTT results shown in Figure 6.2a.

This is in contrast to many studies which have reported biochemical, morphological and genetic alterations to cells upon the re-introduction of energy that are detrimental to cell survival (Taylor *et al.*, 1999; Goldberg and Choi, 1993; Prakasa Babu *et al.*, 2000). For example, during the recovery period following insulin-induced hypoglycaemia, membranes of hippocampal cells ruptured, and mitochondrial densities and condensation of the nucleus was observed (Auer *et al.*, 1985b). It is also well documented that during the recovery phase there is an increase in mitochondrial production of ROS, as a consequence of injured mitochondria (White and Reynolds, 1996), and also a release of cytochome c causing DNA fragmentation (Fujimura *et al.*, 1999). However, it has been demonstrated that during apoptosis the mitochondria remain morphologically intact (Portera-Caillau *et al.*, 1997; Martin *et al.*, 1994; MacManus *et al.*, 1993) and that mitochondrial function can be restored and cell survival maintained if glucose is replaced before the late elevation of $[Ca^{2+}]_i$ (Mattson *et al.*, 1993).

Indeed, one study showed that upon the replacement of glucose, a transient increase in viability, after 24 hours of hypoglycaemia in hippocampal cultures was observed (Mattson *et al.*, 1993). After 8 hours of recovery, the percentage of neurones surviving had increased by roughly 70% and $[Ca^{2+}]_i$ had decreased from 500nM to 100nM but after a

further four hours neuronal survival had decreased concomitantly with a progressive increase in $[Ca^{2+}]_i$ (Mattson *et al.*, 1993). In this model, there is no elevation of $[Ca^{2+}]_i$, suggesting that mitochondrial functioning will not be irreversibly damaged. In addition, in response to glutamate, a dramatic elevation in $[Ca^{2+}]_i$, was observed in recovered cells, suggesting membrane integrity and normal receptor functioning within these cells (Figure 6.15).

It is also interesting to observe the decrease in cell viability in normoglycaemic cultures after 24 and 48 hours of recovery. This suggests that changing the medium, i.e. removing elements, such as tropic factors, appears to have an adverse effect in normoglycaemic cells, but cells which are initially hypoglycaemic, are resistant to this effect. Results also show that this effect is only transient as cell viability in normoglycaemic cells return to baseline levels after 72 hours of recovery (Figure 6.2a).

Remarkably, TUNEL staining reduces concominantly with glucose replacement, suggesting that as ATP levels rise in the culture, this repairs the DNA fragmentation via energy-dependent repairing mechanisms (Figure 6.3). A decrease in TUNEL staining was observed after up to 72 hours of recovery. This is in disagreement with other studies (Prakasa Babu *et al.*, 2000; Ferrand-Drake *et al.*, 1999). For example, DNA fragmentation induced by severe insulin-hypoglycaemia was not observed after 3 hours of recovery in hippocampal dentate granule cells. However TUNEL staining was seen after 24 hours and was markedly enhanced after 48 hours of recovery (Ferrand-Drake *et al.*, 1999)

The reintroduction of glucose initiates the production of energy-dependent mechanisms, such as apoptosis. For example, the apoptotic pathway may have already begun during early stages of ATP depletion but halted when energy levels were too low. This process may re-initiated itself when energy was put back into the system. It is also possible that apoptosis is initiated upon the re-introduction of glucose. However, there are three sets of data suggesting that this does not occur. Firstly, that mitochondrial functioning has increased to normal levels (Figure 6.2a), secondly, that TUNEL staining decreases to normal levels (Figure 6.3) and finally, that caspase activity is not observed, i.e Bax, cytochrome c, caspase-9 and caspase-3 did not change from normoglycaemic levels in recovered cells (Figures 6.4 - Figures 6.9).

Levels of expression of four different DNA repair proteins, implicated in preventing cellular death, were monitored during up to 72 hours of recovery. The levels of these proteins have all been shown to increase during periods of DNA repair (Uberti *et al.*,

2000; Ahlers *et al.*, 1999; Grilli and Memo, 1999). Considering that TUNEL staining had returned to normoglycaemic levels by 24 hours, it would be assumed that the DNA repair proteins had already performed their function. However, no change in the expression of XRCC1, ERCC3, MHS2 and DNA polymerase-β was observed at any of the time points before 24 hours and no delayed change in expression was seen between 24 and 72 hours of recovery (Figure 6.10 – Figure 6.13).

It is possible that other DNA repair proteins, not assessed in this study exist, which are specific for the repair of DNA fragmentation in cultured striatal neurones exposed to hypoglycaemia. Also, it is conceivable that the repair proteins have been irreversibly damaged after 24 hours of glucose deprivation but this seems unlikely due to the cell regaining function after recovery as shown by XTT measurement (Figure 6.2a).

One reason for the observed lack of change in these proteins could be that during hypoglycaemia, no $[Ca^{2+}]_i$ change occurs (Figure 6.14) as it has previously been demonstrated that a calcium-trigger, via a calcium ionophore, is sufficient for the induction of DNA polymerase β (Ahlers *et al.*, 1999), therefore there is no trigger for the upregulation of DNA polymerase β in this system.

It is interesting to observe that the $[Ca^{2+}]_i$ levels in cells treated with 24 hours of hypoglycaemia followed by 24 hours of recovery, should decrease by approximately half (Figure 6.14). Previous studies have reported a dramatic increase in $[Ca^{2+}]_i$ levels after 24 hours of glucose replacement (Taylor *et al.*, 1999). In some studies a decrease is observed but for only a few hours before the levels rise above normoglycaemic levels (Mattson *et al.*, 1993; Tymianski *et al.*, 1993). However, in this study even after 48 hours of recovery, $[Ca^{2+}]_i$ levels still had not exceeded normoglycaemic concentrations (Figure 6.14).

It is possible that the cell up-regulates its buffering systems during the initial stages of hypoglycaemia before the ATP levels have fallen to a particular threshold. For example, the cell may detect, via some glucose-responsive receptor (Ashford, 1990), that the concentration of glucose has decreased and signals for an up-regulation of calcium-binding proteins such as calbindin-D_{28K}. In this system there is no calcium influx, during hypoglycaemia (Figure 6.14) so the calcium-binding proteins have nothing to buffer and with ATP levels declining, an inhibition of protein synthesis activity occurs. Subsequently, during the recovery period, ATP levels increases, protein synthesis is initiated and the up-regulation of calcium buffering systems causes an unregulated extensive sequestration, or extrusion of calcium out of the cell and the $[Ca^{2+}]_i$ is dramatically reduced. Indeed, the

traces shown in Figure 6.15, indicates that in the recovered cell a faster extrusion mechanism is activated as the time taken for the intracellular calcium level to return to baseline after glutamate administration is markedly shorter when compared to normoglycaemic cells. However, the trace is recorded from an individual cell and can not be interpretated as a representation of all cells. This should also be kept in mind when observing the higher resting level of $[Ca^{2+}]_i$ in Figure 6.15b and recovery cells in Figure 6.14.

Another possibility is that the membrane is hyperpolarised during hypoglycaemia but upon recovery the membrane depolarises (Ashford *et al.*, 1990). This membrane depolarisation causes influx of $[Ca^{2+}]_i$, of non-toxic magnitude, but the ATP-dependent processes, such as the internal calcium stores and the Na^+/Ca^{2+} exchanger are altered by the initial ATP depletion and begin to chelate calcium, regardless of the $[Ca^{2+}]_i$ concentration. The net result is an abnormally low concentration of $[Ca^{2+}]_i$. It is also conceivable that an altered gene expression after DNA damage could be responsible for cell death at a later time (Zhang *et al.*, 1994) which perturbs the ER calcium homeostasis and causes a disarray of calcium buffering signals or an over expression in calcium binding protein synthesis.

However, it could be speculated that the near significant difference in TUNEL staining between 24 hours of recovery and 72 hours of recovery (one-way ANOVA, $p=0.05$; Figure 6.3) indicates that after 72 hours of recovery death promoting processes are being initiated, in line with observations seen in other energy replenishment paradigms. Therefore if the recovered cells were observed after 96 hours, TUNEL staining may have increased to levels comparable to hypoglycaemic cultures, signifying an extremely delayed cell death. In addition, the $[Ca^{2+}]_i$ levels after 48 hours of recovery begin to increase to normoglycaemic levels (Figure 6.14). Thus, after 96 hours of recovery $[Ca^{2+}]_i$ levels may have progressively increased to toxic concentrations. However, it is possible that like normoglycaemic cultures after 72 hours of recovery, TUNEL staining has increased in recovered cells (Figure 6.3), indicating that DNA fragmentation occurs via intrinsic processes, such as lack of neurotrophic factors.

Overall, these results suggest that a decrease in XTT staining and an increase in TUNEL staining does not signify death as affected cells can recover function and DNA integrity when glucose is replaced. Mechanisms of DNA repair remain to be elucidated. They may involve different DNA repair proteins or unidentified processes which regenerate DNA structure in cells destined not to die.

It is possible that by investigating components associated with both the promotion of neuronal survival and the mediation of neurotoxicity may provide an insight into whether a single component possesses the power to decide cell fate, i.e. death versus survival. One possible component is the transcription factor, NF-κB, which has been proposed to possess a dual role in neuronal survival. It could be possible that transcription factors are the primary candidates in deciding which pro- or anti-apoptotic genes to switch on in the event of glucose deprivation. The next section investigates the role of NF-κB in hypoglycaemic cell death.

Chapter Seven.

The Role of Nuclear Factor–kappa B

7.1 Introduction

In view of the emerging concept of NF-κB as a key mediator in neurodegenerative and neuroprotective processes, studies were designed to test the hypothesis that NF-κB modulates hypoglycaemia-toxicity. A highly specific, non-toxic NF-κB inhibitor would be an important tool in the investigation of the role of NF-κB in particular scenarios. There are NF-κB inhibitors available as well as reagents which have been reported to inhibit NF-κB as one of their properties.

The inhibition of NF-κB has been reported to be a property of aspirin and salicylic acid (Grilli *et al.*, 1996). Aspirin is usually prescribed as an anti-inflammatory agent which irreversibly inhibits cyclooxygenases, enzymes responsible for the control of the production of prostaglandins. Their mechanism of action is thought to involve the specific inhibition of IκB kinase-β (IKK-β) which catalyses the transfer of phosphate from ATP to IκB (Yin *et al.*, 1998). Therefore, if aspirin binds to IKK-β, which in turn reduces ATP binding, NF-κB would remain sequestered by IκB in the cytoplasm.

High doses of aspirin (and salicylic acid), have been shown to inhibit the translocation of NFkB into the nucleus in human Jurkat T cell lines (Kopp & Ghosh, 1994; Yin *et al.*, 1998), mouse pre-B cell lines (Kopp & Ghosh, 1994), primary rat cerebellar granule cells, hippocampal slices (Grilli *et al.*, 1996) and within an *in vivo* rat model (Yin *et al.*, 1998).

The antioxidant, PDTC, has been repeatedly reported to be an inhibitor of NFkB activation (Sherman *et al.*, 1993; Xie *et al.*, 1994; Schreck *et al.*, 1992a). PDTC provided a specific and dose-dependent inhibitory effect on the inducibility of NFkB DNA binding. It does not affect the level of the cytoplasmic NFkB-IkB complex and does not interfere with DNA binding or nuclear uptake of NFkB. Its most likely inhibitory mechanism of action is blocking the release of IkB from NFkB. This reaction seems to require metal ions and hydroxyl radicals (Schreck *et al.*, 1992a).

Parthenolide, a sesquiterpene lactone, is used as an anti-inflammatory and has been shown to prevent the degradation of IκB-α and IκB-β, thus inhibiting NF-κB activity at concentrations from 5μM to 40μM (Hehner *et al.*, 1998; Hehner *et al.*, 1999). This inhibition has been shown in HeLa cells (Hehner *et al.*, 1999), Jurkat T cells (Hehner *et al.*, 1998), aortic smooth muscle cells (Wong and Menendez, 1999) and human hepatocarcinoma HepG2 cells (Sobota *et al.*, 2000).

4-Hydroxy-2-nonenal (HNE), is analdehydic product of lipid peroxidation. It has been reported that HNE prevents NF-κB activation in a dose-dependent manner, with a marked inhibition at 25μM and complete inhibition at 50μM by preventing the degradation of IκB-α and IκB-β in human monocytic cells (Page *et al.*, 1999).

It has also been reported that the caspase-3 inhibitor, Ac-DEVD CHO, inhibited IκB-α in a dose-dependent manner, in response to quinolinic acid (Qin *et al.*, 2000). In this study, a different caspase-3 inhibitor, Z-DEVD-FMK, was used.

In this series of experiments, the activity of NFκB is observed, in order to investigate whether this transcription factor has a potential role in hypoglycaemia. In most of the experiments the NF-κB subunits, p50 and p65, were measured, via nuclear immunoreactive staining using specific antibodies to these subunits. In addition NF-κB DNA binding activity was detected via the electrophoresis of nuclear extracts containing the oligonucleotide specific for the NF-κB DNA binding site consensus.

The NF-κB inhibitors discussed are employed to test whether NF-κB plays a significant role in the reduction of cell viability associated with hypoglycaemia. The calcium ionophore, A23187, induces NF-κB activity. This is a reliable, reproducible stimulus to activate NF-κB, and it is used to investigate whether aspirin significantly inhibits NFκB protein translocation. Finally, NF-κB activity was monitored in cells recovered from hypoglycaemia, in order to investigate the possibility that NF-κB is involved in the recovery process.

7.2 Results

The expression of p65 and p50 NF-κB subunits was observed during a period of 2 hours in either normoglycaemic or hypoglycaemic conditions. The expression of both the subunits was measured at time 0 before the medium was changed to either hypoglycaemic or normoglycaemic.

Hypoglycaemia produced a significant effect on the induction of p65 (Figure 7.1). At 15 and 30 minutes, hypoglycaemia increased the expression of p65 compared to normoglycaemia. At 60, 120, 180 and 240 minutes, there was no significant difference in p65 expression between hypoglycaemia and normoglycaemia. In normoglycaemic cultures, at 15, 30, 60 and 120 minutes of normoglycaemia, p65 expression had significantly decreased from time 0. At 180 and 240 minutes, normoglycaemic levels had returned to time 0 levels (Figure 7.1).

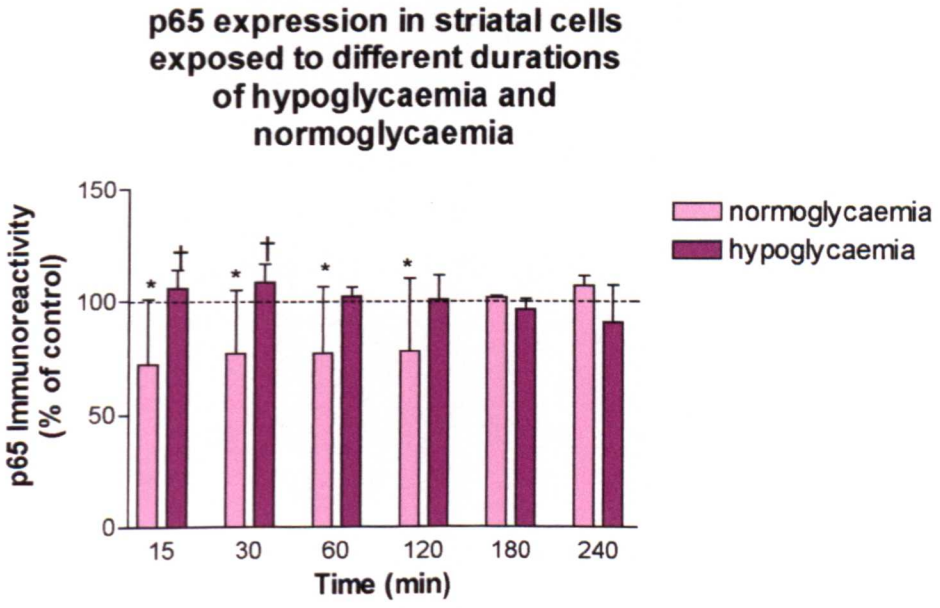

Figure 7.1

The nuclear expression of the NFκB p65 subunit immunoreactivity in striatal cultures exposed to different durations of hypoglycaemia. In hypoglycaemic cultures the glucose was removed for either, 15 minutes, 30 minutes, 1, 2, 3,or 4 hours before fixing. There is a significant effect of hypoglycaemia (two-way ANOVA, effect of hypoglycaemia, $F(1,84)=24.0$, $p<0.001$; effect of time, $F(6,84)=2.19$, $p=0.052$). Hypoglycaemia produces a significant increase in p65 expression compared to normoglycaemia after 15 (†$p<0.001$, Tukey's test) and 30 minutes (†$p<0.01$, Tukey's test). In normoglycaemic cultures, at all time points except 180 and 240 minutes, p65 expression was significantly less than time 0 (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). The data are represented as a percentage of change in p65 expression from normoglycaemic cultures at 0 time point (100%) (n=4).

Both time and normoglycaemia had a significant effect on the induction p50. After 15, 30 and 120 minutes, hypoglycaemia significantly increased the expression of p50 compared to normoglycaemia. In normoglycaemic cultures, p50 induction was significantly suppressed from the level at time 0 after 15, 30, 60 and 120 minutes but not after 180 and 240 minutes (Figure 7.2).

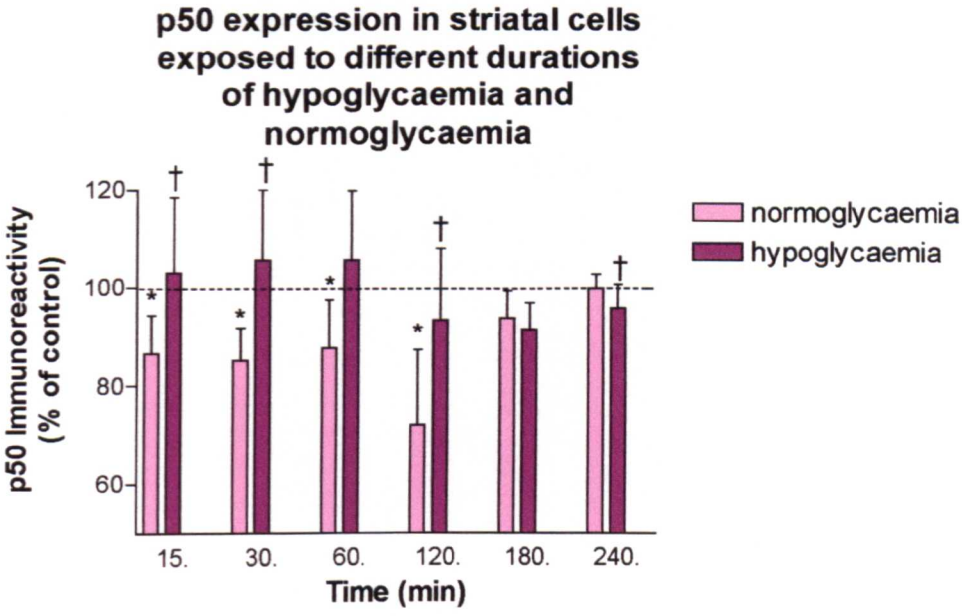

Figure 7.2

The nuclear expression of the NFκB p50 subunit immunoreactivity in striatal cultures exposed to different duration's of hypoglycaemia. In hypoglycaemic cultures the glucose was removed for either, 15 minutes, 30 minutes, 1, 2, 3,or 4 hours before fixing. There is a significant effect of both normoglycaemia and time (two-way ANOVA; effect of hypoglycaemia, $F(1,56)=56.0$, $p<0.001$; effect of time; $F(6,56)=4.49$, $p<0.001$). Hypoglycaemia produces a significant increase in p50 expression compared to normoglycaemia after 15 (†$p<0.05$, Tukey's test), 30 (†$p<0.01$, Tukey's test) and 120 minutes (†$p<0.01$, Tukey's test). After 240 minutes hypoglycaemia causes a significant decrease in p50 expression compared to normoglycaemia (one-way ANOVA, $F(1,12)=13.6$, †$p<0.01$). In normoglycaemic cultures, at all time points except 180 and 240 minutes, p50 expression was significantly less than time 0 (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). The data are represented as a percentage of change in p50 expression from normoglycaemic cultures at 0 time point (100%) (n=4).

The measurement of NF-κB DNA binding activity shows that cells incubated in hypoglycaemic conditions for 30 minutes produced an increase of 9.5% ± 66.3% in DNA binding compared to normoglycaemic cells and cells incubated for 60 minutes produced a 3.2% ± 53.5% decrease. This indicates that 30 and 60 minutes of hypoglycaemia did not significantly change NF-κB DNA binding. Figure 7.3a shows an example of an experiment

where hypoglycaemia at 30 and 60 minutes did increase NF-κB binding activity. However Figure 7.3b presents all the individual data which exhibits the variability within the data.

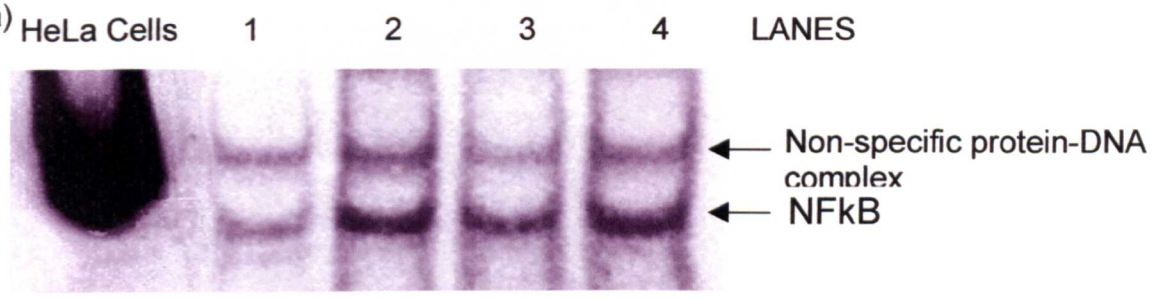

Figure 7.3a

An example of differences in the density of the NF-κB specific protein-DNA complexes from nuclear extracts from cells exposed to either 30 or 60 minutes of hypoglycaemia or normoglycaemia. The different lanes indicate samples of nuclear extracts from cells with different treatment, where 1 is 30 minutes of normoglycaemia, 2 is 30 minutes of hypoglycaemia, 3 is 60 minutes of normoglycaemia and lane 4 is 60 minutes of hypoglycaemia. The first band indicates non-specific protein-DNA complexes and the second band indicates the NF-κB DNA binding. In this experiment an increase in NF-κB DNA binding activity is observed in cells exposed to hypoglycaemia for 30 and 60 minutes (111% and 35% increase respectively).

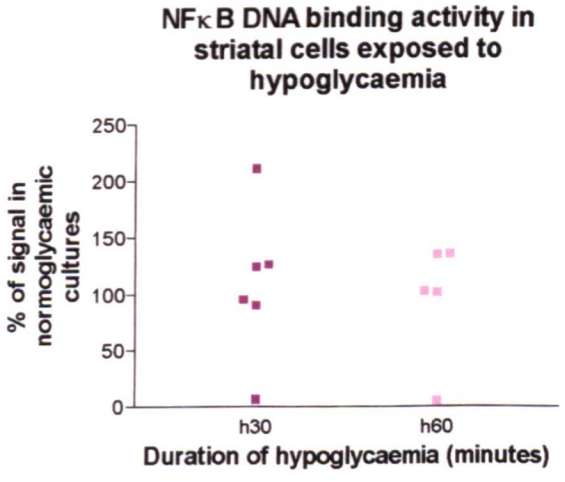

Figure 7.3b

The effect of hypoglycaemia on NF-κB DNA binding. The scatterplot shows data from individual experiments from cells either treated with normoglycaemia or hypoglycaemia for either 30 or 60 minutes. Data are expressed as a percentage of normoglycaemic cells

Aspirin (3mM) had no neuroprotective effect on A23187-induced toxicity. Cultures incubated with A23187 and aspirin and cultures incubated with A23187 and SN50 showed similar pattern in the decrease in cell viability to cultures incubated with A23187 alone (Figure 7.4).

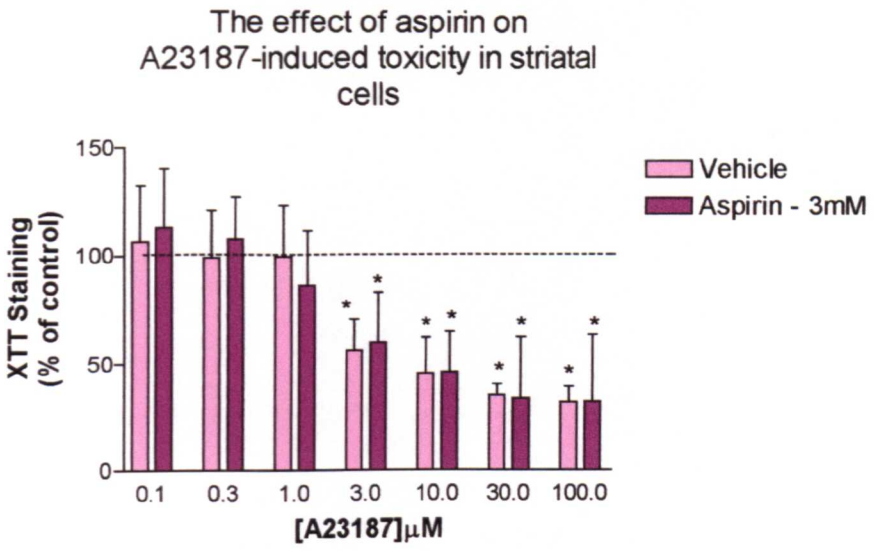

Figure 7.4
The effect of aspirin on A23187-induced toxicity in striatal cells. Cultures were preincubated with aspirin for 30 minutes prior to the addition of varying concentrations of A23187 for 24 hours. Cell viability was measured by XTT staining. A dose-response curve is observed. There is no significant effect of aspirin on cell viability. At 3μM, 10μM, 30μM and 100μM A23187, cell viability significantly decreases compared to cultures without A23187 treatment, regardless of whether aspirin is added (*$p<0.5$ versus 100%; 95% confidence intervals of the mean). Data are presented as a percentage of XTT staining in cultures incubated in the absence of A23187(100%) (n=3).

There was a significant effect of A23187 on the expression of nuclear p65. At concentrations 1μM, 10μM and 100μM, A23187 induced a significant increase in p65 induction compared to cultures incubated in the absence of A23187. If aspirin specifically inhibits NF-κB, a decrease in the expression of p65 is expected. However, in cells pretreated with aspirin and then 1μM of A23187, p65 expression increased (Figure 7.5).

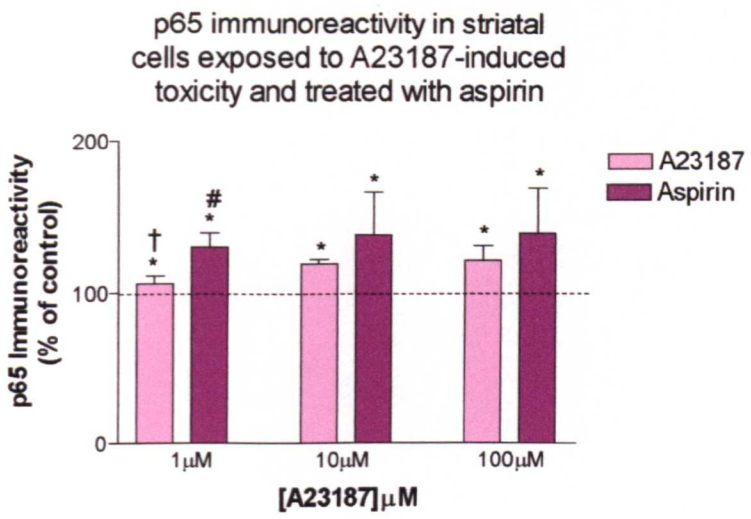

Figure 7.5

The measurement of nuclear p65 immunoreactivity in striatal cultures treated with A23187 and aspirin. Cultures were treated with different concentrations of A23187. Aspirin (3mM) was added to cultures for 30 minutes prior to the addition of A23187 for one hour. Aspirin produces a significant effect on the expression of p65 but A23187 did not (two-way ANOVA, effect of aspirin $F(1,24)=9.64$, $p<0.01$, effect of A23187,$F(2,24)=1.38$, $p=0.27$). A23187 alone causes a significant increase in the p65 expression at 10μM and 100μM concentrations and cells treated with all concentrations of A23187 and aspirin causes a significant increase relative to cultures incubated without A23187 (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). The p65 expression in cultures treated with 1μM A23187 is significantly lower than in cultures treated with 10μM and 100μM A23187 (†$p<0.05$, Fisher's post-hoc test). The administration of aspirin causes an increase in p65 expression in cells treated with 1μM A23187 (#$p<0.01$, Mann-Whittney test). Data are presented as a percentage of p65 expression in cultures with no drug treatment (100%) (n=3).

PDTC had a significant effect on p65 expression. In normoglycaemic-treated cells the expression of p65 was suppressed with 10μM and 50μM PDTC compared to normoglycaemic cells in the absence of PDTC. There were no significant changes in the expression of p65 in hypoglycaemic cells treated with PDTC, moreover, there was no changes in p65 expression in hypoglycaemic cells prior to PDTC treatment (Figure 7.6).

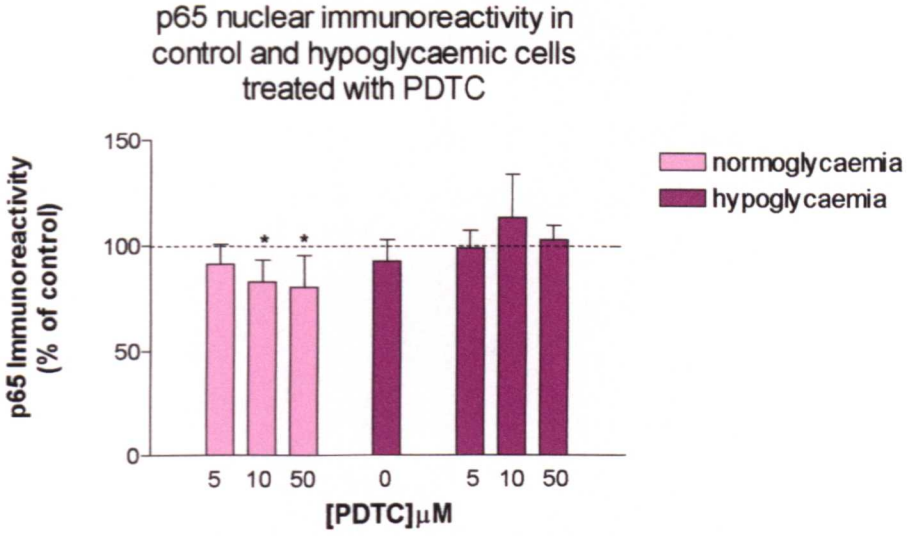

Figure 7.6

The effect of PDTC on the expression of nuclear p65 subunit immunoreactivity in striatal cultures exposed to one hour of hypoglycaemia. Glucose from hypoglycaemic cultures was removed just prior to the addition of PDTC. In normoglycaemic cultures treated with PDTC, at concentrations of 10μM and 50μM, a significant decrease in p65 expression is observed compared with normoglycaemic cultures without PDTC treatment (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). However, no significant change is observed in hypoglycaemic cultures treated with PDTC. Data are presented as a percentage of p65 expression in normoglycaemic cultures without PDTC treatment (100%) (n=3).

In hypoglycaemic conditions, 10μM PDTC significantly increased the expression of p50 compared to normoglycaemic and hypoglycaemic conditions in the absence of PDTC. PDTC had no effect on p50 expression in normoglycaemic cells (Figure 7.7).

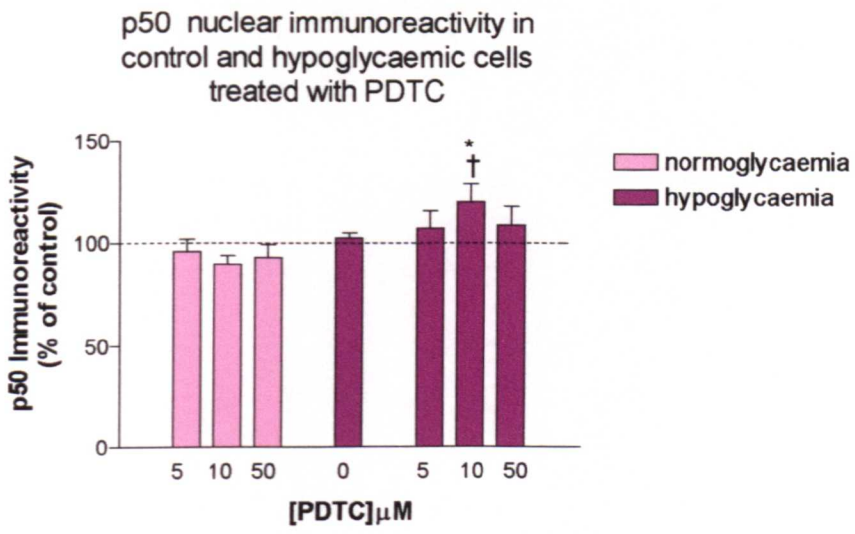

Figure 7.7

The effect of PDTC on the expression of nuclear p50 subunit in striatal cultures exposed to one hour of hypoglycaemia. Glucose from hypoglycaemic cultures was removed just prior to the addition of PDTC. In hypoglycaemic cultures treated with 10μM PDTC, a significant increase in p65 expression is observed compared to hypoglycaemic cultures (†$p<0.05$, Fisher's post-hoc test) and normoglycaemic cultures without PDTC treatment (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). PDTC had no effect on p50 expression in normoglycaemic cultures. Data are presented as a percentage of p50 expression in normoglycaemic cultures without PDTC treatment (100%) (n=3).

Parthenolide had no effect on the cell viability of hypoglycaemic cells or normoglycaemic cells (Figure 7.8).

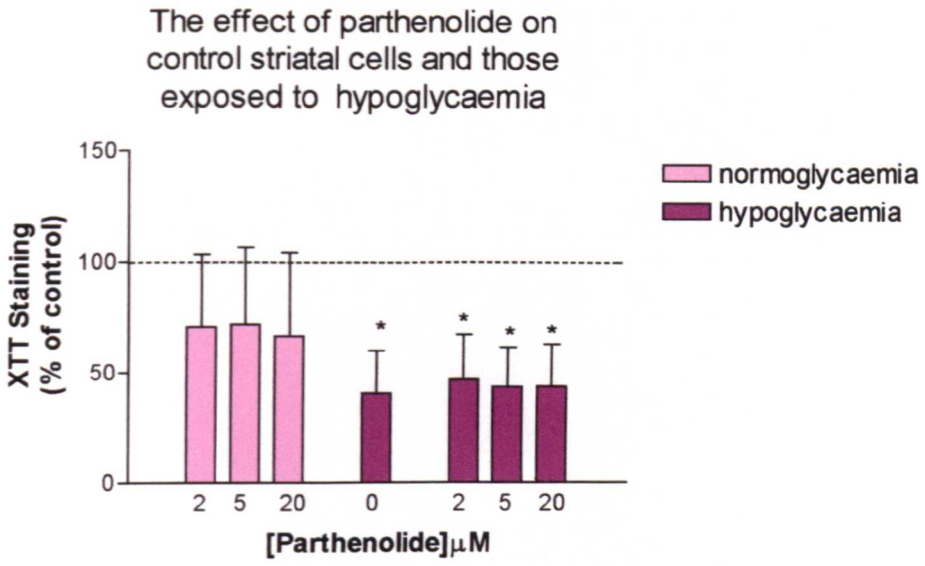

Figure 7.8

The effect of parthenolide on the cell viability of striatal cultures exposed to hypoglycaemia. The glucose was removed from hypoglycaemic cultures just prior to the addition of parthenolide and then incubated for 4 hours. Cell viability is measured by XTT staining. Hypoglycaemia causes a significant effect on XTT staining (two-way ANOVA, effect of hypoglycaemia, $F(1,24)=14.2$, $p<0.001$; effect of parthenolide, $F(3,24)=0.6$, $p=0.7$). XTT staining was significantly lower in hypoglycaemic cultures compared to normoglycaemic cultures (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). There is no significant protection by parthenolide against hypoglycaemia-induced toxicity, at any concentration (One-way ANOVA, $F(3,12)=0.07$, $p=1.0$). Data are presented as a percentage of XTT staining in normoglycaemic cultures without parthenolide treatment (100%) (n=3).

There was a suppression of p65 expression in hypoglycaemic cells treated with 2μM parthenolide compared to normoglycaemic cells treated with 2μM parthenolide. All other concentrations were ineffective. However, the expression of p65 was significantly suppressed by 2μM and 5μM of parthenolide in hypoglycaemic cells when compared to normoglycaemic levels in the absence of parthenolide (Figure 7.9).

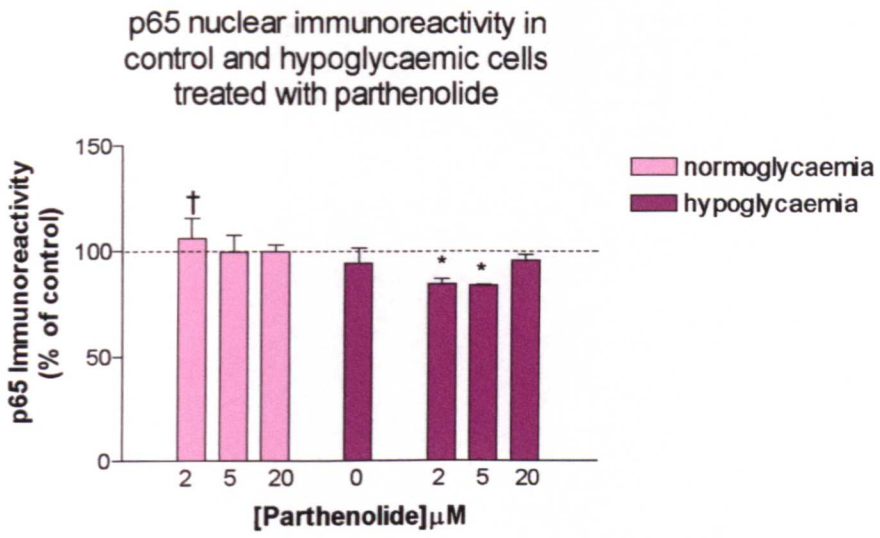

Figure 7.9

The effect of parthenolide on the expression of nuclear p65 subunit in striatal cultures exposed to one hour of hypoglycaemia. Glucose from hypoglycaemic cultures was removed just prior to the addition of parthenolide. Hypoglycaemia produces a significant effect (two-way ANOVA, effect of hypoglycaemia, $F(1,8)=19.5$, $p<0.01$, effect of parthenolide, $F(3,8)=1.05$, $p=0.4$). There is no significant difference in p65 expression between hypoglycaemic cells and hypoglycaemic cells treated with parthenolide (one-way ANOVA, $F(3,4)=4.9$, $p=0.08$). However, 2μM and 5μM parthenolide significantly decreases p65 expression in hypoglycaemic cultures relative to normoglycaemic cultures without parthenolide treatment (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). There is a significant increase in p65 expression in normoglycaemic cultures treated with 2μM parthenolide compared with hypoglycaemic cultures treated with 2μM (†$p<0.05$, Tukey pairwise comparison). Data are presented as a percentage of p65 expression in normoglycaemic cultures without parthenolide treatment (100%) (n=3).

HNE had no significant effect on the cell viability of hypoglycaemic cultures but in normoglycaemic cultures HNE had a significant neurotoxic effect at 50μM compared to the cell viability of normoglycaemic cultures incubated in the absence of HNE. (Figure 7.10).

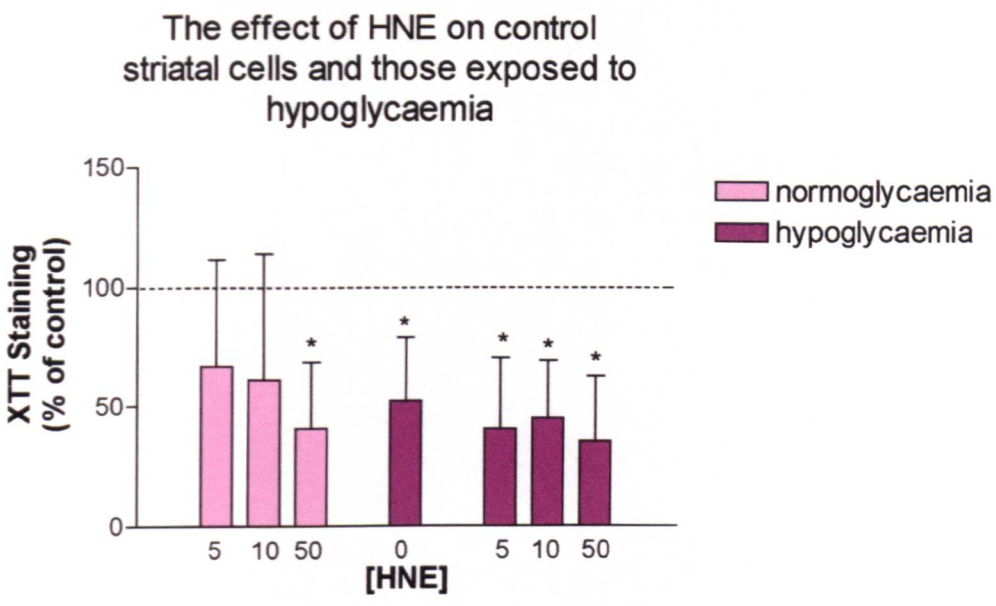

Figure 7.10

The effect of HNE on the cell viability of striatal cultures exposed to hypoglycaemia. The glucose was removed from hypoglycaemic cultures just prior to the addition of HNE and then incubated for 4 hours. Cell viability was measured by XTT staining. Hypoglycaemia causes a significant effect (two-way ANOVA, effect of hypoglycaemia, $F(1,24)=4.4$, $p<0.05$; effect of HNE, $F(3,24)=1.9$, $p=0.16$). XTT staining in hypoglycaemic cultures is significantly decreased from normoglycemic cultures (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). The addition of HNE to hypoglycaemic cultures has no significant effect (one-way ANOVA, $F(3,12)=0.3$, $p=0.84$). In normoglycaemic cultures, 50μM HNE significantly decreases cell viability compared to normoglycaemic cultures incubated without HNE (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). Data are presented as a percentage of XTT staining in normoglycaemic cultures without HNE treatment (100%) (n=3).

There was no significant difference in the expression of p65 between normoglycaemic and hypoglycaemic cultures, except for a decrease in expression in normoglycaemic cultures with 5μM HNE and the addition of HNE produced no significant effects in hypoglycaemic cultures (Figure 7.11).

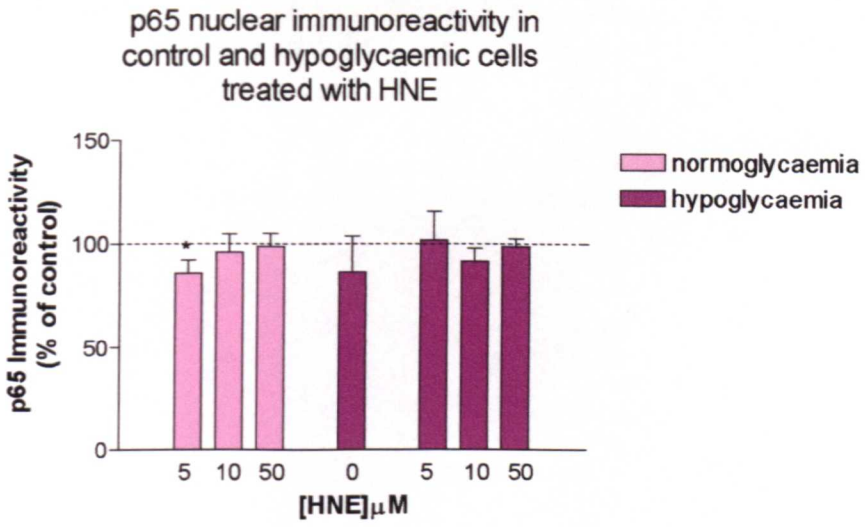

Figure 7.11

The effect of HNE on the expression of nuclear p65 subunit in striatal cultures exposed to one hour of hypoglycaemia. Glucose from hypoglycaemic cultures was removed just prior to the addition of HNE. There is no significant change in p65 expression in hypoglycaemic cultures incubated with HNE treatment (one-way ANOVA, $F(3,4)=0.86$, $p=0.5$). HNE produces a significant decrease in normoglycaemic cultures at 5μM (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). Data are presented as a percentage of p65 expression normoglycaemic cultures without HNE treatment (100%) (n=3).

In this experiment, the expression of p50 in hypoglycemic cultures has increased compared to normoglycaemic cultures. In hypoglycaemic cultures, HNE decreased p50 expression at 5μM and 50μM. HNE had no effect on p50 expression in normoglycaemic cultures (Figure 7.12).

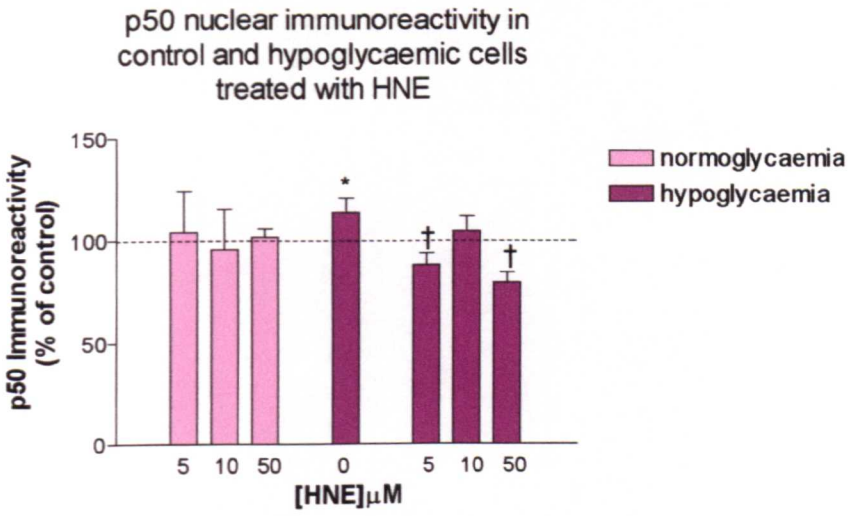

Figure 7.12
The effect of HNE on the expression of nuclear p50 subunit in striatal cultures exposed to one hour of hypoglycaemia. Glucose from hypoglycaemic cultures was removed just prior to the addition of HNE. In hypoglycaemic cultures a significant increase in p50 expression compared to normoglycaemic cultures is observed (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). In hypoglycaemic cultures, HNE, caused a significant decrease in p50 expression at concentrations of 5μM and 50μM (one way ANOVA, $F(3,4)=17.0$, †$p<0.01$, Fishers post-hoc test). HNE produces no significant changes in normoglycaemic cultures. Data are presented as a percentage of p50 expression in normoglycaemic cultures without HNE treatment (100%) (n=3).

A significant decrease was observed when hypoglycaemic cultures treated with either 50μM, 100μM or 200μM Z-DEVD-FMK were compared with normoglycaemic cultures incubated without Z-DEVD-FMK. In normoglycaemic cultures, Z-DEVD-FMK at concentrations of 50μM and 100μM produced a significant increase in p65 expression but at 200μM, a decrease was observed (Figure 7.13).

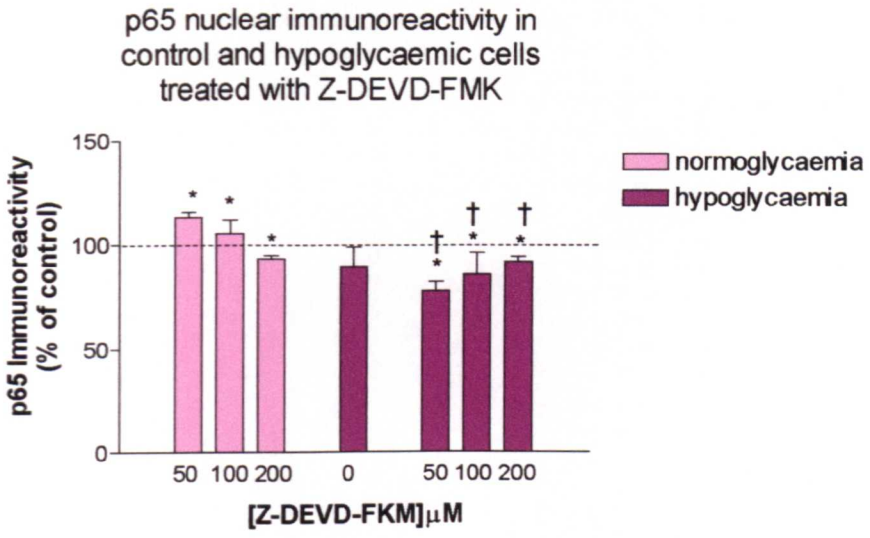

Figure 7.13

The effect of Z-DEVD-FMK on the expression of nuclear p65 subunit in striatal cultures exposed to one hour of hypoglycaemia. Glucose from hypoglycaemic cultures was removed just prior to the addition of Z-DEVD-FMK. There is a significant effect on p65-immunoreactivity produced by hypoglycaemia as opposed to normoglycaemia but not by Z-DEVD-FMK concentration (two-way ANOVA, effect of hypoglycaemia, $F(1,12)=64.3$, $p<0.001$; effect of Z-DEVD-FMK, $F(2,12)=0.76$, $p=0.5$). In hypoglycaemic cultures, the p65 expression was significantly decreased compared to normoglycaemic cultures (*$p<0.05$ versus 100%; 95% confidence intervals of the mean) and also relative to the levels of p65-immunoreactivity in normoglycaemic cultures at the same concentrations of Z-DEVD-FMK (Fisher's posthoc test †$p<0.05$). Z-DEVD-FMK did not show any dose-dependent effect on p65 expression in hypoglycaemic cultures (one-way ANOVA, $F(3,8)=2.4$, $p=0.14$). The p65 expression in normoglycaemic cultures treated with all concentrations of Z-DEVD-FMK is significantly different from normoglycaemic cultures without Z-DEVD-FMK (*$p<0.05$ versus 100%; 95% confidence intervals of the mean). Data is presented as a percentage of p65 expression in normoglycaemic cultures without Z-DEVD-FMK treatment (100%) (n=3).

There was no significant effect on the expression of p65 or p50 in cells which have been recovered from hypoglycaemia. There was no change in the expression of either subunit after 24 hours of hypoglycaemia or 24 hours of hypoglycaemia and 30 or 60 minutes of glucose replacement (Figure 7.14 and Figure 7.15).

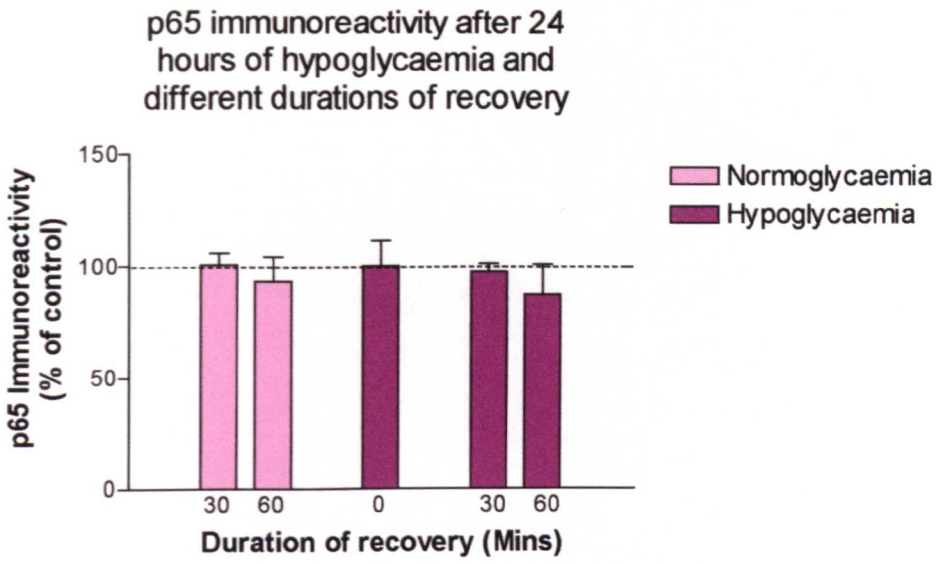

Figure 7.14

The effect of replacing the glucose on the expression of nuclear p65 subunit in striatal cultures exposed to 24 hours of hypoglycaemia. Glucose from hypoglycaemic cultures was removed for 24 hours and then replaced for either 30 or 60 minutes. There is no significant difference in p65 expression in hypoglycaemic cells compared to either cells which have been recovered for 30 or 60 minutes (one-way ANOVA, $F(2,6)=1.26$, $p=0.4$). Data are presented as a percentage of p65 expression in normoglycaemic cultures (100%) (n=3).

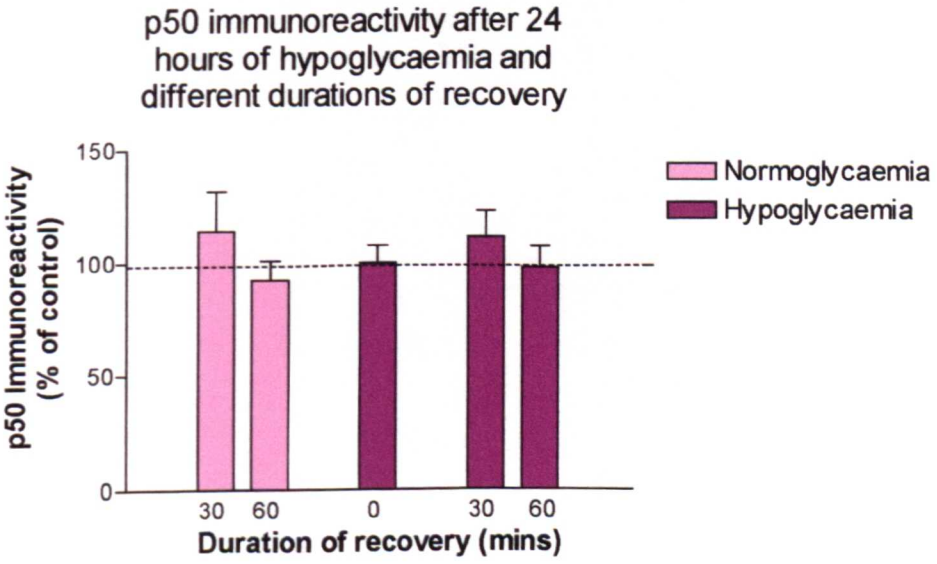

Figure 7.15

The effect of replacing the glucose on the expression of nuclear p50 subunit in striatal cultures exposed to 24 hours of hypoglycaemia. Glucose from hypoglycaemic cultures was removed for 24 hours and then replaced for either 30 or 60 minutes. There is no significant difference in p50 expression in hypoglycaemic cells compared to either cells which have been recovered for 30 or 60 minutes (one-way ANOVA, $F(2,6)=1.8$, $p=0.3$). Data are presented as a percentage of p50 expression in normoglycaemic cultures (100%) (n=3).

7.3 Discussion

The specificity of the p65 and p50 antibodies was determined by designing a control experiment where the immunocytochemistry procedure is identical but the primary antibodies are omitted. In these experiments no staining was observed suggesting that the antibodies were specific for p65 and p50. In addition, these particular antibodies are used in most experimental studies investigating NF-κB subunits (Simpson and Morris, 1994; Qin *et al.*, 2000).

The removal of glucose from cultures increases the expression of both the p65 and p50 subunits of NFκB (Figure 7.1 and Figure 7.2). This increase is only observed up to 120 minutes, thereafter, the expressions of these subunits return to normoglycaemic levels. This is in line with previous reports which have demonstrated that hypoglycaemia and ischaemia activate NF-κB (Seegers *et al.*, 2000; Moro *et al.*, 2000; Takuma *et al.*, 1999; Clemens *et al.*, 1997a; Clemens *et al.*, 1997b; Tong *et al.*, 1995).

A number of components associated with the theory of hypoglycaemia-induced damage are potent activators of NF-κB, i.e. glutamate (Sandberg *et al.*, 1986), calcium, (Glazner *et al.*, 2001), ROS (Sun *et al.*, 1979) and nitric oxide (Alagarsamy *et al.*, 1994) and therefore it is not surprising that an increase in nuclear NF-κB is detected. However, in this study no increase in $[Ca^{2+}]_i$ was measured and there is no EAA receptor activation but it is possible that ROS production via the dysfunction of mitochondrial respiration during hypoglycaemia does occur (which is unaffected by PDTC). Alternatively, other constituents, not yet identified, may activate NF-κB and be triggered by glucose deprivation.

In most studies measuring NF-κB activation, significant increases in nuclear detection are observed between 30 minutes and two hours, after the onset of the stimuli. Optimum expression is dependent on cell type and the type of stimulus, however these results are consistent with other reports. Between two and three hours, both p65 and p50 expression, in hypoglycaemic cells, no longer exceed normoglycaemic levels. There are two reasons for this, firstly, it could be suggested that after two hours of hypoglycaemia, protein synthesis is inhibited within the cell and consequently there is an inhibition of translocation of NF-κB to the nucleus and the synthesis of new NF-κB and IκB subunits. Secondly, the decrease in NF-κB expression after two hours could be due to the transient activation of NF-κB. It is translocated to the nucleus where it targets specific genes and is subsequently

inhibited via a specialised autoregulatory mechanism (Arenzana-Seisdedos *et al.*, 1997). This explanation is most probable as, in the early event of a toxic stimulus, gene transcription factors are required to switch on particular genes which will determine the final cellular outcome.

It was not possible to have the same time scale for NFκB activation and XTT staining. The XTT staining incubation was for three hours, whereas no change in NFκB subunit expression would have been observed after two hours. However, it can not be assummed that a decrease in XTT staining in hypoglycaemia cultures would have occurred within the first two hours but it is important to observe changes preceding mitochondrial failure, i.e. NFκB protein expression.

Within these first two hours, normoglycaemic levels of nuclear p65 and p50 immunoreactivity in normoglycaemic cultures, measured at 15, 30, 60 and 120 minutes, are significantly lower than levels detected in normoglycaemic cultures at time 0, i.e. in cultures where medium has not been changed. This suggests that changing the medium has a profound effect on the induction of NFκB in normoglycaemic cells and that this effect is only transient as p65 and p50 subunit expression returns to levels equivalent to normoglycaemic cells without medium change and hypoglycaemic cell with medium change, after 2 hours. How is changing the medium affecting NFκB activation? It is possible that a slight temperature and/or pH difference in the new medium causes a suppression of basal NF-κB activation. It is equally plausible that the stress associated with the medium changing procedure suppresses NFκB basal levels for a short period and then returns to normal when the homeostasis of the cell is equiliberated. Alternatively, cultured cells secrete growth factors into the medium, and changing to fresh medium may remove a growth factor receptor-mediated induction of NF-κB.

It is interesting to observe that medium change does not appear to affect NF-κB activity in hypoglycaemic cells. This suggests that events associated with hypoglycaemic neurotoxicity, mask the effect of changing the medium. It would be understandable if in hypoglycaemia, the cell is being bombarded with an overload of NF-κB stimuli, namely, calcium, EAAs and ROS, this would override the suppression of NF-κB caused by changing the medium. Although, in this study no increase in $[Ca^{2+}]_i$ is detected, the dysfunction of mitochondrial respiration during hypoglycaemia may directly or indirectly activate NF-κB and override the medium changing effect. In addition, the behaviour of the p65 and the p50 subunits are similar upon the cell's exposure to hypoglycaemia.

In the following set of experiments, there are discrepancies in the results as to whether NF-κB subunits after 60 minutes of hypoglycaemia are induced above 100% (normoglycaemic levels at time 0). In Figures 7.1, 7.2 and 7.12, hypoglycaemia increases the expression of NF-κB subunits, whereas in Figures 7.6, 7.7, 7.9, 7.11 and 7.13 a decrease in subunit expression is detected. The inconsistency is hard to interpret as all cultures and experimental conditions remain the same. There are a few possibilities, such as the age of the tissue varied occasionally. Also, inaccuracies may have occurred during the dissection to obtain the tissue, therefore different population of cells from different structures surrounding the striatum were incorporated into the cultures altering the behaviour of NF-κB subunits in response to hypoglycaemia. Alternatively, it is possible that NF-κB does not play a key role in the effects of hypoglycaemic toxicity, hence no consistent induction is observed.

Data obtained from NF-κB DNA binding studies also confirm this inconsistent activity of NF-κB induced by either 30 or 60 minutes of hypoglycaemia (Figure 7.3b). The data shown in Figure 7.3a shows an increase in DNA binding activity after both 30 and 60 minutes of hypoglycaemia. However, it is clear in results from individual experiments that the NF-κB DNA binding activity varies considerably, complementing results observed with p65 and p50 subunits induction.

There have been various reports, stating that aspirin (Grilli *et al.*, 1996), PDTC (Xie *et al.*, 1994), parthenolide (Hehner *et al.*, 1998), HNE (Page *et al.*, 1999) and caspase-3 inhibitors (Qin *et al.*, 2000) inhibit NF-κB activity. If NF-κB activity can be specifically inhibited in neurones, this would provide crucial information on the role of NF-κB, in response to neurotoxic stimuli.

A23187 was initially used to investigate whether aspirin inhibited the induction of NF-κB subunits because calcium is a critical downstream mediator in cell death and is known to activate NF-κB. A23187, at all concentrations, increased nuclear p65 immunoreactivity (Figure 7.5). There was more nuclear p65 immunoreactivity detected at higher concentrations of A23187, this suggesting that p65 induction is proportional to $[Ca^{2+}]_i$. Cell viability of cultures treated with A23187 is also proportional to $[Ca^{2+}]_i$ (Figure 7.4). However, these data do not indicate whether this increase in p65 expression is a result of NF-κB attempting to promote cell survival or that the increase is due to NF-κB mediating cell death.

Aspirin dramatically increased p65 staining, suggesting that not only was it ineffective in inhibiting p65 induction but that its other properties, such as interactions with inflammatory proteins, influence p65 activity (Figure 7.5). The high concentration used in this experiment was taken from the report by Grilli *et al.,* who had tested this concentration on cerebellular granule cells and who also stated that these high concentrations correlate with amounts of aspirin in plasma during chronic anti-inflammatory therapy (Grilli *et al.,* 1996). It is interesting to observe that although aspirin increases p65 induction, this had no effect on the cell viability measured in these cells (Figure 7.4). It is possible that NF-κB, induced by A23187, is not involved in the events leading to cell death, hence, the increase of NF-κB has no significant effect on cell viability. It must be noted that although an increase in nuclear p65 expression is seen in A23187-treated cells, it is not conclusive that NF-κB is affecting gene induction, NF-κB DNA binding activity assays are needed to provide decisive evidence.

The A23187 model of toxicity was changed to the hypoglycaemia model as it was thought that a more subtle, less abrupt form of toxicity would be more appropriate in the observation of NF-κB subunits.

PDTC did not show any inhibitory effects on the nuclear expression of the p65 or p50 subunits in hypoglycaemic cells (Figure 7.6 and Figure 7.7). This is consistent with other reports (Chung *et al.,* 2000). In both experiments, hypoglycaemia for one hour alone did not affect the expression of either subunit compared to their expression in normoglycaemia. This is in contrast to results seen in Figure 7.1 and 7.2. Thus, it could be that either PDTC is ineffective at inhibiting NF-κB at the concentrations observed or that there was no inducible activated NF-κB subunits for PDTC to inhibit because p65 and p50 subunits are at basal levels as hypoglycaemia provided no stimulus to induce NF-κB activation. Indeed, previous results have showed PDTC alone has no inhibitory effect on the activation of NFkB, even at concentrations of 10mM. However, in cells pretreated with PDTC and then stimulated with TNF-α, a dose-dependent suppression of nuclear NF-κB activity is detected (Herrmann *et al.,* 1997).

However, it does appear to have different effects on p65 and p50 expression in normoglycaemic and hypoglycaemic cells. PDTC caused a decrease in p65 expression in normoglycaemic cells. This could be attributable to PDTC's other properties, such as ROS chelation, which may affect energy competent cells but not energy depleted cells (Figure 7.6). This effect was not seen in the p50 expression of normoglycaemic cells, suggesting

the two subunits can act differently depending on the type of stimuli (Figure 7.7). Moreover, p50 expression increased in hypoglycaemic cells with 10μM PDTC. Again, this observation could be attributable to PDTC's other properties as this effect was not seen at any other concentration (Figure 7.7). A dual effect of PDTC on NF-κB DNA binding activity was recently documented (Chung *et al.*, 2000). It is interesting to note that the dose-dependent increase in XTT staining in normoglycaemic and hypoglycaemic cultures (Figure 3.10) does not correspond to any changes in p65 or p50 expression in response to hypoglycaemia.

Hypoglycaemia alone did not increase p65 expression and therefore there was no activated p65 for parthenolide to inhibit. This may have been expected, as parthenolide did not affect the viability of cells exposed to one hour of hypoglycaemia (Figure 7.8). However, parthenolide did provide inhibition in p65 expression at 2μM in hypoglycaemic cells compared with normoglycaemic cells treated equivalently (Figure 7.9). It is interesting to see that 2μM and 5μM parthenolide in hypoglycaemic cells caused a significant decrease in p65 expression compared with normoglycaemic cells incubated in the absence of pathenolide. This suggests that parthenolide alone has no effect on p65 expression (shown in normoglycaemic cells) and neither does hypoglycaemia but when parthenolide is added to hypoglycaemic cells a decrease in expression is detected. This indicates that events occurring in hypoglycaemic cells, such as the activation of IκB kinases to degrade IκB subunit, activates parthenolide to function as an inhibitor IκB degradation and consequently p65 translocation.

There was no inhibition of the p65 subunit with HNE in hypoglycaemic cells (Figure 7.11). However, it did inhibit p65 expression, at the lowest concentration, in normoglycaemic cells, suggesting that may be HNE, which has shown to have neurotoxic properties (Song *et al.*, 2001), is inhibiting p65 expression via non-specific mechanisms. Although these toxic non-specific mechanisms do not appear to effect normoglycaemic cells incubated with 10μM and 50μM HNE. HNE has been reported only to inhibit NF-κB depending on what particular stimulus induces NF-κB activation, e.g. HNE inhibits NF-κB when cells are stimulated by LPS, but not by TNF (Page *et al.*, 1999). It is therefore possible that at low concentrations of HNE that the p65 subunit is inhibited. In addition, it is possible that HNE can inhibit p50 and p65 only when there is an increase expression of these subunits in response to hypoglycaemia.

In the experiment studying the effect of HNE on p50 expression, hypoglycaemia without HNE causes an increase in p50 nuclear induction (Figure 7.12). HNE had various effects on p50 expression in hypoglycaemic cells. At 5μM and 50μM HNE inhibits p50 expression but at 10μM HNE has no effect. It could be suggested that only the lowest concentration specifically inhibits the p50 subunit, because at 50μM, HNE significantly decreases the cell viability in normoglycaemic cultures, implying at this concentration HNE is producing non-specific toxic effects (Figure 7.10). Interestingly, HNE did not have the same dose-response profile in normoglycaemic cultures. There was no significant decrease in p50 expression at 50μM, whereas Figure 7.10 shows massive reduction in cell viability. This suggests that glucose deprivation produces an additive detrimental effect on cells treated with high concentrations of HNE.

The caspase-3 inhibitor, Z-DEVD-FMK, produced different effects on the expression of the p65 subunit in normoglycaemic and hypoglycaemic cells. In hypoglycaemic cells, alone, p65 expression was reduced from normoglycaemic levels, providing further evidence to suggest that the induction of NF-κB in hypoglycaemic conditions is variable. There was no effect of Z-DEVD-FMK in hypoglycaemic cells; p65 expression remained significantly reduced from normoglycaemic levels. In normoglycaemic cells, Z-DEVD-FMK, caused an increase in p65 expression at low concentrations and then caused an inhibition at the highest concentration, suggesting that this compound can produce a multitude of concentration-dependent effects within an energy competent cell.

Finally, both p65 and p50 expression were monitored after 30 minutes and one hour of glucose replacement. The purpose of this experiment was to investigate whether NF-κB had a role in the survival mechanisms associated with recovery in striatal cultures. It could be hypothesised that NF-κB is induced upon recovery and ATP replenishment which switches on the relevant neuroprotecting genes, such as, Mn-SOD. However, the expression of both of the subunits were unchanged from hypoglycaemic cells, suggesting that NF-κB is not induced during recovery. However, there are two queries associated with these experiments, firstly, unlike the data presented in Figures 7.1 and 7.2, there was no increase in either p65 or p50 during hypoglycaemia. Secondly, the expression of p65 and p50 in normoglycaemic cells, which had had a medium change (as a control for recovered cells), did not change from normoglycaemic levels without medium change. Therefore it seems likely that p65 and p50 do not play key roles in the recovery response.

In conclusion, the removal of glucose from striatal cultures appears to produce a variation in the expression of p65 and p50 subunits and NF-κB DNA binding activity. Apart from this fundamental discrepancy creating difficulty to compare sets of experiments, this implies that NF-κB subunits are not a key mediator in hypoglycaemic cell death or recovery and suggests that this transcription factor is not significantly important in hypoglycaemia. NF-κB has been termed an oxidative-stress response transcription factor (Schreck *et al.*, 1991), therefore it is possible that the hypoglycaemic environment is not conducive for the induction of NF-κB. In hypoglycaemia there is a constant availability of oxygen and in this model ROS have shown not to be involved. It is also possible other NF-κB subunits are involved and further investigation may confirm this. In addition, none of the potential NF-κB inhibitors in this study showed adequate inhibition of p65 and p50 subunits without any undesirable effects, perhaps because all possess other properties and none are specific NF-κB inhibitors.

Chapter Eight. General Discussion

The data collected from this study suggest that hypoglycaemia decreases mitochondrial functioning (XTT staining) and damages neuronal structure (MAP2 immunoreactivity). In addition, some signs of apoptosis are present (TUNEL staining) whereas other are not (Bax, cytochrome c, caspase-9 and caspase-3 immunoreactivity), suggesting that hypoglycaemia produces a neurotoxic effect with some characteristics of apoptosis. However, this neurotoxic effect is not produced by calcium, glutamate, MTP activation or ROS although it is possible that NFκB activation is involved. In spite of this apoptotic-like neurotoxicity induced by hypoglycaemia, cells can recover following the re-introduction of glucose (XTT staining) and show signs of DNA repair (TUNEL staining).

Data from this study indicate a different type of cell dysfunction occurring in striatal cultures exposed to hypoglycaemia compared to the generally accepted hypothesis (Siesjo, 1992). Most studies have confirmed that hypoglycaemia produces neuronal cell death as a consequence of membrane depolarisation, $[Ca^{2+}]_i$ overload, excessive EAA receptor stimulation, MTP activity and an excessive generation of ROS.

There are number of reasons why differences are observed in this model. Striatal neurones possess glutamate receptors, but in striatal cultures there are no intrinsic glutamate-releasing neurones and no extrinsic glutamatergic inputs from other brain structures to stimulate these receptors. Hence a toxic release of synaptic glutamate in response to hypoglycaemia is unfeasible, thus, glutamate toxicity is not a feature in this specific cell culture.

Aspiny striatal interneurones possess a wide network of dendrites, synapsing on spiny neurones and are known to be more resistant to hypoglycaemia than spiny striatal neurones (Ferreante *et al.*, 1985; Beal *et al.*, 1992). Therefore it is possible that aspiny neurones provide energy for spiny neurones during hypoglycaemia. Data from this study shows that, after 24 hours, a reproducible 75% decrease in mitochondrial function occurs in hypoglycaemic cultures, suggesting that the remaining 25% of the culture possess functional mitochondria. This 25% could be cells more resistant to hypoglycaemia, such as interneurones which could process alternative substrates and provide energy for the whole culture to keep the culture in a suspended animated state. Alternatively, the remaining 25% could represent viable mitochondria from all cell types within the culture and that each cell

possesses a reduced number of functioning mitochondria. However, the MAP2 staining in Figure 3.2 suggests that only a certain population of cells survive.

The methods involved in dissociated primary neuronal culture are designed to prevent the proliferation of glia within the system. However, a pure neuronal population is near impossible if cultures are incubated for two weeks, and a limited glial population could influence cultured neurones. It is known that astrocytes can provide important sources of energy and they appear to have a full complement of enzymes for both glycogenesis and glycogenolysis. Therefore astrocytes may contribute markedly to fuel homeostatic mechanisms at times of metabolic stress in the brain, probably by releasing alternative substrates such as pyruvate, for local neuronal oxidation (Magistretti, 1982; Schurr *et al.*, 1988; Schurr *et al.*, 1997; Selak *et al.*, 1986; Matsumoto *et al.*, 1994; Edmond *et al.*, 1987). In addition, it has been reported that glia cells are not affected by hypoglycaemia (Golberg and Choi, 1993) possibly due to the accumulated glycogen in these cells (Hara *et al.*, 1989). This attribute of glia could perhaps counteract neuronal vulnerability by forming a functional energy supply unit and hence exert a protective profound influence on vulnerable cells exposed to hypoglycaemia. Future experiments could involve comparing the amount of glia present and the survival of cultures, perhaps by observing the antibody for glial fibrillary acidic protein.

Hypoglycaemia is known to change the glucose transport system in the brain (Pramming *et al.*, 1986), suggesting that there should be glucose-regulated mechanisms associated with the transport of glucose. A neuroprotective mechanism could involve an increase in glucose-transporters on the aspiny cells surface (LaManna and Harik, 1986; Nagamatsu *et al.*, 1992). It has been demonstrated that a 4-fold increase in the level of the glucose-transporter, GLUT3 mRNA was observed after 48 hours of hypoglycaemia indicating that glucose starvation effectively activates the transcriptional rate of the GLUT3 gene in vulnerable neurones (Nagamatsu *et al.*, 1994).

GABA, an inhibitory neurotransmitter, is the predominant neurotransmitter within the striatum, and has been reported to be both elevated (Sandberg *et al.*, 1986) and decreased (Engelsen *et al.*, 1986; Paulsen and Fonnum, 1988) during hypoglycaemia, dependent upon energy deficiency model and cell type is studied. The release of GABA and other inhibitory transmitters have been shown to protect neurones from damage caused by energy deficiency (Madl and Royer, 2000; Saeansaari and Oja, 1997; Johansen and Diemer, 1991; Shuaib *et al.*, 1993). Since the majority of neurones in the striatum are GABAergic, it is possible that the release of GABA in this model during hypoglycaemic

neurotoxicity, prevents the membrane from depolarising and is responsible for keeping the cell in a quiescent state. Alternatively, adenosine, which is also an inhibitory modulator could have a profound effect on the stability of the membrane. Extracellular levels of adenosine are elevated during hypoglycaemia and adenosine receptor agonists have been demonstrated to delay rapid depolarisation by hyperpolarising cells via the activation of K^+ channels (Martin *et al.*, 1994; Greene and Haas, 1991; Calaliere *et al.*, 2001). Hence striatal cells could be protected from irreversible toxicity either by a suppression of apoptosis (due to energy depletion) or to the presence of high levels of inhibitory neurotransmitters such as GABA or adenosine.

The intracellular effect following a metabolic insult within a neurone is multifactorial, therefore it is not surprising that variations of the response to hypoglycaemia differ substantially.

There may be a number of reasons why data in this study do not show cell death induced by hypoglycaemia and are therefore not in accordance with other studies. Most research is carried out using cortical, hippocampal and cerebellar cells. Cultures using these cell types have more of a heterogeneous population, compared to striatal cultures and this could significantly alter the influence of glucose deprivation (Freese *et al.*, 1990). Discrepancies in results are also largely due to the differences between *in vitro* and *in vivo* studies, perhaps as a result of intact neuronal networks between different structures and the presence of a glia network and blood capillaries in *in vivo* (Minamisawa *et al.*, 1990; Siesjo, 1998; Matsumoto *et al.*, 1994). Other factors include the method of producing hypoglycaemia. In this study medium containing glucose has been replaced with glucose-free medium, whereas hypoglycaemia can be induced via chemicals, such as 3-NP which inhibits succinate dehydrogenase and in *in vivo* studies administering an overdose of insulin induces hypoglycaemia is the most common method. The vulnerability of cells to toxic stimuli has been shown to be dependent on the age of the culture (Freese *et al.*, 1990), so that culture preparation could influence results. In addition, the insult caused by glucose deprivation could be unevenly distributed throughout the culture due to differences in glycogen content between culture regions and between cultures (Tasker *et al.*, 1992).

There a number of questions which need to be addressed, in attempt to clarify what cellular processes are occurring in striatal cells deprived of glucose.

What is causing mitochondrial dysfunction in striatal cells during hypoglycaemia? The detection of cell damage is by XTT measurement, and it is assumed that hypoglycaemia is

preventing the succinate dehydrogenase enzyme from functioning within the tricarboxylic acid cycle. The assumption is that the concentration of ATP is reduced during glucose deprivation because of the succinate dehydrogenase decrease in activity. However, this has not formally been demonstrated in these studies. Conclusive evidence would only arise with experiments measuring the absolute ATP concentration in control and hypoglycaemic cultures but abundant literature has demonstrated decreases in ATP levels following hypoglycaemia (Mattson *et al.*, 1993; Madl and Royer, 1999; Santos *et al.*, 1996). Combining this assumption with the present results, it could be proposed that the concentraion of ATP is reduced which initiates multiple effects that injury the cell, e.g. the actvation of a substrate to cleave the DNA.

It appears that the plasma membrane remains intact after 24 hours of hypoglycaemia and it could be proposed that the mitochondrial membrane also remains intact. Evidence arises from the lack of MTP activation in hypoglycaemic cells. However, monitoring the mitochondrial membrane potential would be the only method to test this theory. It is almost certain that calcium is not the trigger which causes disruption to mitochondrial function, although $[Ca^{2+}]_i$ was not measured until after 24 hours of hypoglycaemia. Each calcium modulating drug used had no effect when incubated with hypoglycaemic cells. In addition, it has also been reported that depolarisation of mitochondrial membranes can occur independently of calcium influx (Nowicky and Duchen, 1998). It seems apparent that ROS are not involved in the dysfunction of mitochondria since PDTC had no effect in these experiments. It has been proposed that ROS are not formed in hypoglycaemia because there is sufficient O_2 available to accept electrons passed along the mitochondrial electron transport chain.

What mechanisms are affecting mitochondrial function? It is possible that the brain has evolved mechanisms for sensing and regulating glucose homeostasis and it could be argued that proteins or secondary messengers, perhaps yet unidentified, are triggered when the cell detects a decrease in extracellular glucose content. It has been suggested that the presence of K-ATP sensitive channels may function predominantly as "gluco-sensing" receptors, whereby they open, hyperpolarising the plasma membrane (Dunn-Meynell *et al.*, 1998). This action may trigger proteins to translocate to the mitochondria and signal for either the preservation of cellular energy, or signal for the cell to undergo an apoptotic-type of cell death. In this study, the K-ATP channel opener, diazoxide, did not have any neuroprotective effects. Therefore, it may be proposed that either K-ATP channel are insensitive to diazoxide or that "gluco-sensing" receptors distinct from K-ATP channels exist within striatal neurones.

In addition, apoptotic-like cell death, resulting from glucose deprivation, causing mitochondrial dysfunction and DNA fragmentation, may be initiated from cell surface death receptors. These receptors are a family of transmembrane proteins which belong to the TNF/NGF receptor superfamily. Ligand-induced receptor activation would cause the recruitment of intracellular receptor-associated proteins, and consequently the initiation of upstream caspase activation. The activation of these upstream caspases could initiate the proteolytic cleavage of proteins from cytosolic members of the Bcl family, which translocate to the mitochondria, and may cause a disruption in oxidative phosphorylation when they insert themselves. However, it is not known whether the ligands, which activate these surface receptors, are present in this model of hypoglycaemia. Still, it is possible that other cell surface death receptors exist which cause a direct alteration of mitochondrial integrity when stimulated during conditions of low glucose concentrations.

Neurotrophins are released from cells to condition their environment. It is known that a withdrawal of growth factors, such as NGF, can cause both apoptosis and necrosis (Greenlund *et al.*, 1995; Castagne and Clarke, 1996; Mignotte and Vayssiere, 1998; Levi-Montalcini and Booker, 1960). Therefore, it is possible that the effect of the lack of neurotrophins in the medium after 24 hours of hypoglycaemia, due to decreased energy available for the production and release NGF, is a trigger for the cell to stop functioning. In addition, the increase in NF-κB observed in hypoglycaemic cells, may be indicative of subsequent attempted transcription of NGF, in the attempt to prevent cells undergoing apoptosis. NGF has been reported to protect cultured neurones from hypoglycaemia (Chend *et al.*, 1991; Cheng *et al.*, 1994; Kokaia *et al.*, 1994) but not prevent the hypoglycaemic-induced reduction in ATP levels (Mattson *et al.*, 1993). It has been reported that new protein synthesis contributes little to NFκB activation, following neurotoxicity (Qin *et al.*, 1998). Therefore if protein synthesis is inhibited during hypoglycaemia, this would not affect NF-κB's ability to induce genes which regulate apoptotic proteins but would affect the synthesis of NGF induced by NFκB.

Alternatively, a change in intracellular pH could be the predominant trigger, which causes mitochondrial dysfunction (Pelligrino and Siesjo, 1981; Norberg and Siesjo, 1976; Agardh *et al.*, 1981). It is known that intracellular pH levels start to change at a time when ATP levels are still relatively high as a result of ATP being consumed faster than it is being produced (Busa and Nuccitelli, 1984). In hypoglycaemia, alkalosis occurs due to the oxidation of alternative substrates and this change in pH could trigger conformational alterations of cell surface receptors, thereby initiating a shut down of the mitochondria.

If DNA fragmentation is a result of mitochondrial dysfunction then what signalling cascades are employed to initiate substrates which ultimately fragment DNA? It is possible that caspases other than those monitored in this study are initiated or that unidentified proteins are triggered. For example, one mechanism might involve apoptosis-inducing factor (AIF). Various triggers of cell death are known to cause the release of AIF from the mitochondrial intermembrane space which translocates to the nucleus where it activates specific endonucleases (Lorenzo *et al.*, 1999). It is known that a Ca^{2+}-independent endonuclease exists (Barry and Eastman, 1993), and it is possible that this endonuclease is activated in this model.

It has been demonstrated that glucose removal depolarises mitochondria long before the ATP/ADP ratio changes substantially (Kauppinenn and Nicholls, 1986), hence active processes such as caspase activation can occur. However, it is possible that only partial reversible DNA damage occurs in hypoglycaemic cultures because ATP is eventually reduced to a level that provides insufficient energy required to fully fragment the DNA. However, it has been reported that DNA fragmentation is an ATP-independent process (Leist *et al.*, 1997).

There is much debate concerning the nature of death in cells deprived of ATP. It is generally believed that in severe metabolic conditions the dominant pathway is necrotic whereas in more subtle metabolic conditions the dominant pathway is apoptosis (Kroemer, 1997). Both necrosis and apoptosis have mitochondrial dysfunction in common (Zamzami *et al.*, 1997) and DNA fragmentation has been reported in both apoptosis and necrosis. However, in this model of hypoglycaemia, it could be assumed that necrosis is not apparent considering the recovery of cells upon glucose replacement. It may be suggested that the neurones that did not survive, and therefore would not have recovered, could have died via a necrotic cell death. The lack of caspase activity does not necessarily confirm that cell death was necrotic as it has been shown that apoptotic cell death can occur even in the presence of caspase-inhibitors (Borner and Monnet, 1999).

An interesting theory is that protein ubiquitination and aggregation is the primary neurotoxic event involved in hypoglycaemia (Hu *et al.*, 2000). Under physiological conditions, newly synthesised, unfolded or misfolded proteins are quickly removed by the ubiquitin-proteosome system (Ciechanover *et al.*, 2000). Protein ubiquitination involves a series of ATP-dependent reactions which ligate ubiquitin to abnormal proteins, forming ubiquitinated proteins (ubi-proteins). However, under pathological conditions, when ubi-proteins in cells are produced too much to be degraded, they will aggregate through their

hydrophobic segments which threatens cellular survival and function. Indeed, ubi-protein aggregations have been consistently observed in both chronic neurodegeneration (Alves-Rodrigues *et al.*, 1998) and acute brain injuries (Hu *et al.*, 2000).

In a recent study, ubi-protein aggregates were formed in the vulnerable brain regions, under conditions of hypoglycaemic coma (Ouyang and Hu, 2001) suggesting that proteins were severely damaged after hypoglycaemia. It has been reported that hypoglycaemia provides optimum conditions to induce the overproduction of unfolded proteins in the ER and along the protein maturation pathway (Brostrom and Brostrom, 1998). Therefore, it is possible that DNA fragmentation is not a consequence of mitochondrial dysfunction but a consequence of the formation of these aggregates which initiates a signal to activate the apoptotic execution machinery.

How do striatal cells recover from hypoglycaemia? It has been suggested that there are two forms of cell injury induced by energy failure. The first is reversible and is characterised by the insufficiency of the Na^+/K^+ ATPase to meet the requirements for maintenance of membrane potential and normal ion permeability. The second is irreversible and is characterised by hyperactivity of glutamate receptors (Pissarek *et al.*, 1998). Thus, since glutamate releasing neurones are absent in striatal cultures, the form of cell damage that predominates in this model is the former one, a reversible mode of cell injury. Indeed, energy metabolism and Na^+/K^+ transport have been shown to recover fully from complete global ischaemia even following prolonged periods (Hossmann *et al.*, 1973; Kleihues *et al.*, 1975; Nordstrom *et al.*, 1978).

If cell death is reversible, the cell can not be classified officially dead. It was previously reported, that during hypoglycaemia the mitochondria are not irreversibly damaged. This suggests that mitochondria shut down temporarily whilst there is a reduction in glucose, perhaps to preserve energy levels, or perhaps there is insufficient amount of energy for the mitochondria to function effectively therefore respiration becomes suspended. This may render the cell not technically dead but electrically quiescent.

It has been reported that hyperpolarisation via K-ATP channel activation is a short-term method of conserving energy in response to hypoglycaemia (Duchen and Somjen, 1988; Duchen, 1990; Calabresi *et al.*, 1997; Spuler *et al.*, 1988). Therefore, it could be suggested that either cells in this model are hyperpolarised or they produce enough energy to remain at resting potential and remain in a suspended animation state with little mitochondria functioning. Suspended animation could resemble a hypothermic state. It is

well established that hypothermia protects against delayed neuronal death following ischaemia (Busto *et al.*, 1987). It is thought that the mechanism of this protective effect is associated with a slowing of the cells' metabolism by inhibiting oxygen and glucose consumption in the brain (Bering, 1974; Hagerdal *et al.*, 1975) and by producing a "membrane-stabilising" effect, perhaps reducing the Na^+/K^+ leakage.

It was initially thought that the cell is irreversibly damaged by glucose deprivation, which is in agreement with most other studies. This assumption was based on results with TUNEL staining. It has been proposed that DNA fragmentation does not necessarily reflect DNA damage but that the condensation of DNA that follows fragmentation, is the ultimate feature of cell death, as this is irreversible. This may explain why fragmented DNA can be repaired within minutes of glucose re-introduction. The initial apoptotic signal could have triggered DNase proteins, such as endonucleases, to cleave the DNA but under low energy conditions these proteins function less efficiently and as a result DNA fragmentation is incomplete. Therefore, is it possible that the original apoptotic death signal, initiated during early hypoglycaemia, is prevented from proceeding to completion while energy levels are low, and is redundant when glucose levels are restored? One example is the controversial role of PARP which has been reported to be involved in DNA repair as well as being a nuclear caspase substrate involved in cleaving DNA (Kaufmann *et al.*, 1993). Is it possible that the role of this protein could be altered during the period of energy deprivation and recovery? For example, during the beginning of hypoglycaemia, PARP is activated by caspases to fragment the DNA, however, as ATP content reduces and the cell enters a suspended state and PARP remains inactive. At the beginning of recovery, signals could travel to the nucleus, subsequent to the restoration of mitochondrial functioning and hence cause a change in PARP, allowing PARP to repair the DNA it had already begun to cleave.

Numerous studies have reported that an inhibition of new protein synthesis protects cells from apoptosis, possibly by preventing the functions of endonucleases, and consequently the irreversible DNA damage (Goto *et al.*, 1990) and therefore it is possible that hypoglycaemia inhibits new protein synthesis. The purpose of DNA fragmentation during apoptosis is still uncertain. It is possible that it facilitates the breakdown of the DNA upon the uptake of apoptotic cells by phagocytes or it may serve to destroy the information content of the cell. Alternatively, it may simply be a by-product of ion redistributions that occur during apoptosis (Martin *et al.*, 1994). If the latter is the case, it is possible that if ionic disturbances occur during hypoglycaemia when ionic distribution equilibrates during recovery DNA fragmentation could be repaired.

Apoptosis regulators, a family of proteins termed inhibitors of apoptosis (IAP) apparently set up an endogenous threshold level for caspase activation. It is suggested that the cellular levels of IAPs may determine the difference in sensitivities to apoptosis-inducing stimuli in various cell types. IAPs are also direct targets of transcriptional regulation by NF-κB (Chu *et al.*, 1997). Therefore, it is possible that in hypoglycaemic cells, IAPs are activated via NF-κB induction, which then inhibit downstream caspase activity. This could prevent complete DNA fragmentation and regulate inhibitory caspase activation during recovery (assuming protein synthesis is not impaired).

Intracellular calcium concentration has been referred to in toxic conditions as the decisive element within the cell. Could this be true in recovery conditions? Is a reduction in $[Ca^{2+}]_i$ during recovery a protective mechanism? It is possible that simply a re-introduction of glucose, initiates the depolarisation of the cell causing an influx of calcium and a fast sequestration of excess $[Ca^{2+}]_i$ by ER and mitochondria as well as an increase in the activity in calcium-binding proteins. Alternatively, could the reduction of $[Ca^{2+}]_i$ after 24 hours of recovery reflect the increase in activity of calcium-dependent processes, such as the removal of damaged mitochondria or other intracellular organelles?

It is possible that NF-κB is involved in the recovery of cells after hypoglycaemic toxicity. Although data show no NF-κB activation within the early phase of recovery, NF-κB could switch on genes during early hypoglycaemia when energy levels are not completely depleted, which later preserves the cell's integrity when the cells return to normal metabolic functioning. For example, NF-κB could induced an overexpression of Mn-SOD, to counter-balance the potential ROS production upon recovery.

It is clear that a new hypothesis is required that is consistent with the data obtained in this study. When the extracellular source of glucose is removed, the activity of the TCA cycle decreases and ATP levels fall. The membrane remains at resting potential and VGCC and EAA receptors are therefore closed. The ATP source required to maintain the resting potential is provided by the existing concentration of ATP left after glucose removal and alternative substrates such as glutamate. The cells falls into a suspended animation state where energy demand is reduced but with sufficient amount of energy to keep the membrane polarised but not to initiate active processes, namely apoptosis. However, this disruption of internal energy homeostasis triggers ATP-independent mechanisms resulting in the activation of endonucleases and fragmentation of DNA. During early hypoglycaemia, when energy levels are still relatively high, genes are upregulated in the

event of further cellular damage, such as calcium-buffering proteins and possibly DNA repair systems. However, following transcription of these genes the cell's energy begins to fall below a specific threshold and consequently these new proteins are not utilised until energy levels rise. On the re-introduction of glucose, ATP levels rise and cytosolic calcium is buffered via the previously up-regulated calcium buffering genes, metabolism within the mitochondria begins to function normally, all respiratory processes normalise, DNA repair begins, and hence, normal cellular functioning ensues.

This idea could be termed a neuroprotective mechanism employed in striatal neurones against energy deprivation. It is reasonable to suggest that neurones, because of their post-mitotic nature require complex protective mechanisms and the state of a suspended animation is possibly one of them. Indeed, it is known that in most cases damage in hypoglycaemic patients is reversible and irreversible neuronal damage occurs only in extreme prolonged situations (Herold *et al.*, 1985), suggesting that cells exposed to hypoglycaemia have effective innate neuroprotective systems preventing irreversible cell loss. These results also have clinical implications in that the therapeutic window for the treatment of profound hypoglycaemia may be prolonged. Together with further research into the mechanism of cellular recovery from glucose deprivation and DNA fragmentation, it is possible that the outcome of pathological conditions associated with energy deprivation can be improved.

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Appendices.

Media Formulations

Table 1 Dulbecco's Modified Eagle Medium (1X), Liquid (low glucose)

Component	mg/L
$CaCl_2.2H_2O$	264.0
$Fe(NO_3).9H_2O$	0.10
KCL	400.0
$MgSO_4.7H_2O$	200.0
NaCl	6400.0
$NaHCO_3$	3700.0
$NaH_2PO_4.2H_2O$	141.0
D-Glucose	100.0
Phenol Red	15.0
Sodium Pyruvate	110.0
L-Arginine.HCl	84.0
L-Cysteine	48.0
L-Glutamine	580.0
Glycine	30.0
L-Histidine HCL.H_2O	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-Lysine.HCl	146.0
L-Methionine	30.0
L-Phenylalanine	66.0
L-Serine	42.0
L-Threonine	95.0
L-Tryptophan	16.0
L-Tyrosine	72.0
L-Valine	94.0
D-Ca pantothenate	4.0
Choline Chloride	4.0
Folic Acid	4.0
i-Inositol	7.20

Nicotinamide	4.0
Pyridoxine HCl	4.0
Riboflavin	0.40
Thiamine HCl	4.0

Table 2 Dulbecco's Modified Eagle Medium (1X), Liquid (no glucose)

Component	mg/L
$CaCl_2$ (anhyd.)	200.0
$Fe(NO_3).9H_2O$	0.10
KCL	400.0
$MgSO_4$ (anhyd.)	97.67
NaCl	6400.0
$NaHCO_3$	3700.0
$NaH_2PO_4.H_2O$	125.0
Phenol Red	15.0
L-Arginine.HCl	84.0
L-Cystine.2HCl	63.0
L-Glutamine	584.0
Glycine	30.0
L-Histidine HCL.H_2O	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-Lysine.HCl	146.0
L-Methionine	30.0
L-Phenylalanine	66.0
L-Serine	42.0
L-Threonine	95.0
L-Tryptophan	16.0
L-Tyrosine (disodium salt)	104.0
L-Valine	94.0
D-Ca pantothenate	4.0
Choline Chloride	4.0
Folic Acid	4.0
i-Inositol	7.20
Nicotinamide	4.0
Pyridoxine HCl	4.0
Riboflavin	0.40
Thiamine HCl	4.0