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Proteomic analysis of the response of cultured neurons to chemical excitation

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A thesis submitted in partial fulfilment of the requirements for the degree of
Master of Science in Biological Sciences

School of Biological Sciences
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Abstract

Mammalian cells secrete a large number of proteins into the extracellular space, the composition and levels of which change as result of environmental factors. However, the identity of most of these secreted proteins has yet to be determined. Recent advances in proteomic technology now allow the isolation and identification of these secreted proteins. Neuronal cell death and injury as a result of excessive and prolonged activation of excitatory amino acid receptors has been termed excitotoxicity, and has been implicated in mechanisms leading to a variety of disease states, such as Alzheimer's disease. Available evidence suggests that under excitotoxic stress, neurons secrete signals that can alter the function of neurons with which they are connected via synapses or to which they are proximate. Many of these signals act via volume transfer, diffusing via the ECF to act on populations of cells, to induce slow long-lasting effects. The successful isolation and *in vitro* culture of rat cortical cells is reported here, These cultures are 95 % pure for neurons, as determined by immunocytochemistry. They are viable for more than 14 days under B27-supplemented culturing conditions, and are viable for up to a further 3 days under protein-free culturing conditions. Live dead/assays, utilising fluorescein diacetate and propidium iodide, demonstrated that cell survival remained high even after 72 h exposure to 50 μ M kainic acid. Analysis of conditioned medium using 2D-PAGE combined with MALDI-TOF MS demonstrated significant proteolysis of the bovine serum albumin protein. This proteolysis only occurred in the presence of the cortical cells. In addition, immunocytochemistry confirmed that both neurons and glia had taken up the bovine serum albumin. Increasing evidence implicates cerebrovascular dysfunction, in particular the breakdown of the blood-brain barrier, as an early event in the development of Alzheimer's disease. Elevated intracellular Cu^{2+} levels are also seen in the Alzheimer's disease brain, and are thought to be responsible for neuronal cell death through metal-mediated oxidative stress. It is proposed here that the albumin molecule, which binds Cu^{2+} with high-affinity, gains entry to the CNS as a result of BBB dysfunction. Neuronal uptake and processing of the albumin molecule, as demonstrated here, resulting in the intracellular release of Cu^{2+} , may be the mechanism by which the elevated Cu^{2+} levels seen in Alzheimer's disease occur. In addition, initial analysis of the conditioned medium of cortical neurons stimulated with 50 μ M KA for 72 h, suggests that processing of the bovine serum albumin is altered, with some fragments up-regulated and others down-regulated.

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Abbreviations and chemical formulae

A	~	approximately
	A	ampere
	aa	amino acid
	A β	β -amyloid
	Ab	antibody
	ACh	acetylcholine
	α CHC	alpha-cyano-4-hydroxycinnamic acid
	AD	Alzheimer's disease
	Ag	antigen
	AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate
	AOP2	antioxidant protein 2
	AP	action potential
B	BBB	blood-brain barrier
	bFGF	basic fibroblast growth factor
	BPB	bromophenol blue
	BSA	bovine serum albumin
C	$^{\circ}$ C	degrees Celsius
	Ca ²⁺	calcium ion
	cDNA	complementary DNA
	CNS	central nervous system
	CSF	cerebrospinal fluid
	Cu ²⁺	copper (II) ion
D	Da	Dalton
	DMEM	Dulbecco's Modified Eagle's Medium
	DTT	dithiothreitol
E	ECF	extracellular fluid
F	FCS	fetal calf serum
	FD	fluorescein diacetate
	FGF	fibroblast growth factor
	FITC	fluorescein isothiocyanate
G	\times g	relative centrifugal force
	g	gram/s
	GABA	gamma-amino butyric acid
	GFAP	glial fibrillary acidic protein
	GH	growth hormone
	GluR	glutamate receptor
H	h	hour/s
	H ₂ O ₂	hydrogen peroxide
	H ₂ O	water
	HB-A	Hibernate-A medium

	HCl	hydrochloric acid
	HRP	horseradish peroxidase
	HSA	human serum albumin
I	IAA	iodoacetamide
	IEF	isoelectric focusing
	IgG	immunoglobulin G
	iGluR	ionotropic glutamate receptor
	IPG	immobilised pH gradient
K	k	kilo
	KA	kainic acid
L	L	litre
	LDCV	large dense core vesicles
	LDH	lactate dehydrogenase
M	-m	metre or milli
	M	molar
	MALDI	matrix assisted laser desorption ionisation
	MCAO	middle cerebral artery occlusion
	MeCN	acetonitrile
	- μ	micro (1×10^{-6})
	min	minute/s
	mRNA	messenger RNA
	MS	mass spectrometry
	MW	relative molecular weight
	MWCO	molecular weight cut-off
	m/z	mass to charge ratio
N	#	number
	NaCl	sodium chloride
	-n	nano (1×10^{-9})
	NaOH	sodium hydroxide
	NB-A	Neurobasal-A medium
	NBF	neutral buffered formalin
	NCBI	National Centre for Biotechnology Information
	NeuN	neuronal nuclei
	NGF	nerve growth factor
	NH_4HCO_3	ammonium bicarbonate
	NMDA	N-methyl-D-aspartate
	NT	neurotransmitter
O	O_2^\bullet	superoxide
	OH^\bullet	hydroxyl radical
P	PAGE	polyacrylamide gel electrophoresis
	PARP	poly (ADP ribose) polymerase
	PBS	phosphate buffered saline
	PFA	paraformaldehyde
	pH	power of hydrogen

	pI	isoelectric point
	PI	propidium iodide
	ppm	parts per million
R		
	rpm	revolutions per minute
	RT	room temperature (21 °C)
S		
	5HT	serotonin
	SDS	sodium dodecyl sulphate
	SV	small vesicle
T		
	2D	two-dimensional
	TFA	trifluoroacetic acid
	TOF	time of flight
	TTBS	tris-buffered saline with Tween-20
	2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
U		
	UoA	University of Auckland
	US	unstimulated
	UV	ultraviolet
V		
	V	voltage
	VT	volume transmission
	v/v	volume per volume
W		
	WT	wiring transmission
	w/v	weight per volume
	w/w	weight per weight

Company abbreviations

Adobe	Adobe California, USA
Amersham Biosciences	Amersham Biosciences Uppsala, Sweden
Applichem	AppliChem GmbH Darmstadt, Germany
Applied Biosystems	Applied Biosystems California, USA
Axis-Shield	Axis-Shield Group Oslo, Norway
BDH	BDH Laboratory Supplies Poole Dorset, UK
Bio-Rad	Bio-Rad Laboratories California, USA
Brainbits-USA	Brainbits-USA Illinois, USA
Chemicon	CHEMICON International California, USA
Falcon	Falcon Michigan, USA
GraphPad	GraphPad Software Inc. California, USA
Hansen and Rosenthal	Hansen & Rosenthal KG Hamburg, Germany
Invitrogen-GIBCO	Invitrogen Corporation Carlsbad, USA
May and Baker	May and Baker Essex, UK
Medlab	Diagnostic Medlab Auckland, New Zealand
Molecular Probes	Molecular Probes Oregon, USA
Perkin-Elmer	PerkinElmer Life and Analytical Sciences Massachusetts, USA
Promega	Promega Wisconsin, USA
Sartorius	Sartorius AG Goettingen, Germany
Scharlau	Scharlau Chemie Barcelona, Spain
Serva	SERVA Electrophoresis GmbH Heidelberg, Germany
Sigma	Sigma-Aldrich Missouri, USA
Zymed	Zymed Laboratories, Inc. California, USA

Chapter one - introduction

1.1 Intercellular communication in the central nervous system

The neuron is the core unit of the central nervous system. Interconnected neurons make up the communication network that allows an organism to interact with and respond to a diverse range of stimuli. A neuron consists of a cell body or soma, dendrites through which it receives input from neighbouring neurons, and axons with which synaptic connections are made to other neurons (see Figure 1.1) (Berne, 1998).

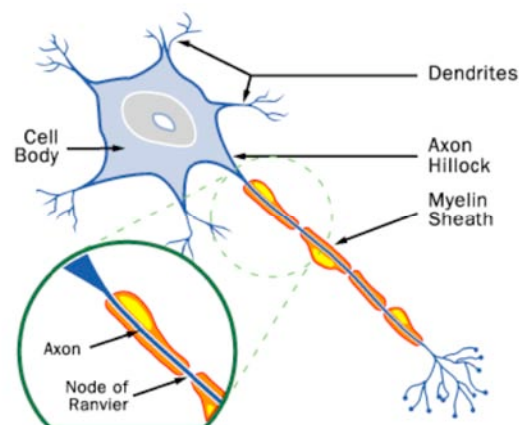


Figure 1.1: Neuron schematic showing the cell body and axon.

Communication between neurons can occur via two main mechanisms, synaptic/wiring transmission and non-synaptic/volume transmission. Originally, it was thought that synaptic transmission was the only route of information transfer but it is now clear that transmission can also occur through the diffusion of signalling molecules as occurs in volume transmission (Merighi, 2002).

1.2 Synaptic / wiring transmission

In wiring transmission signalling occurs through contact sites called synapses. Signalling at synapses can occur electrically, chemically, or through a combination of both mechanisms.

At chemical synapses the postsynaptic-cell and the presynaptic-cell are electrically isolated and signal transduction occurs indirectly through the release of signalling molecules called neurotransmitters (NT) (see Figure 1.2). The NTs diffuse across the synaptic cleft and bind to receptors on the postsynaptic cell evoking a variety of responses depending on the type of NT. NTs can be excitatory or inhibitory by

opening channels to allow the influx of ions that either depolarise the membrane or hyperpolarise it respectively. Excitatory NTs such as acetylcholine (ACh), glutamate (Glut) and serotonin (5HT) open cation channels permeable to Na^+ and Ca^{2+} whilst inhibitory NTs such as GABA and glycine open anion channels permeable to Cl^- . The NT is removed by several mechanisms including degradation, reuptake and diffusion (Matute et al., 2001) .

At electrical synapses, on the other hand, the membranes are physically connected via gap junctions. Gap junctions allow the direct transmission of electrical signals without the use of chemical neurotransmitters (Merighi, 2002).

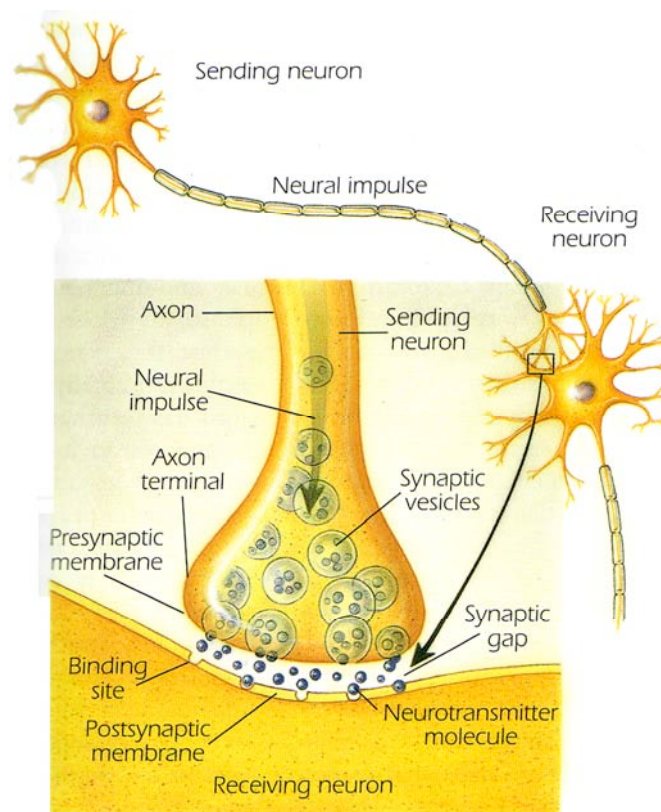


Figure 1.2: A chemical synapse between two connected neurons (Stemberg, 1995).

1.3 Non-synaptic / volume transmission

Volume transmission (VT) occurs by a process of signal diffusion within the brain's extracellular fluid (ECF) in a three-dimensional fashion (Agnati et al., 1995). In contrast to synaptic transmission, VT allows the regulation of populations of neurons that are spatially related rather than physically connected (see Figure 1.3) (Merighi, 2002). VT is not mediated directly by action potentials and induces changes that are slow and of long duration (Merighi, 2002). As a result VT is ideally suited to inducing slow global responses in populations of cells (Agnati et al., 1995). VT is not just restricted to slow responses however, as relatively fast VT can occur (Zoli and Agnati, 1996).

Volume transmission involves long-distance (paracrine) and short-distance (parasympaptic) diffusion of signals in the ECF. Endocrine-like VT allows far-reaching communication in the brain by the use of fluid compartments such as blood and cerebrospinal fluid (CSF) (Agnati et al., 1995). There are also nerve-bundle associated types of VT in which the signal is transferred via small channels that exist outside myelinated axons (Agnati et al., 1995). Two main types of signalling messengers are involved in VT, hydrophilic, as in the case of neuropeptides, and hydrophobic, such as neurosteroids and nitric oxide (Merighi, 2002). Hydrophilic molecules are mainly limited to the ECF whilst hydrophobic molecules can cross cell membranes and enter cells (Zoli and Agnati, 1996). Molecules involved in chemical synapses can also act via VT. One instance where this can occur is if the amount of neurotransmitter released at a synapse overwhelms the reuptake processes. It can then diffuse out of the synapse and into the ECF to act on nearby neurons (Agnati et al., 1995).

Release of the signalling molecules described above is likely to occur via a variety of mechanisms. Much study has been done on the release sites of large dense-core vesicles (LDCV) and small vesicles (SV). LDCVs are known to be released from a range of sites, including parasympaptic, synaptic and non-synaptic domains. The ratio of release from these different sites is likely to change between different neuronal cell populations (Agnati et al., 1995; Zoli and Agnati, 1996). SVs are generally released from the synaptic zone, however they have been shown to be released at non-synaptic sites. Signals can also be secreted into the ECF by non-vesicular mechanisms, such as the reverse

operation of the neurotransmitter reuptake carriers, and both neurons and glial cells are known to release molecules this way (Agnati et al., 1995; Zoli and Agnati, 1996).

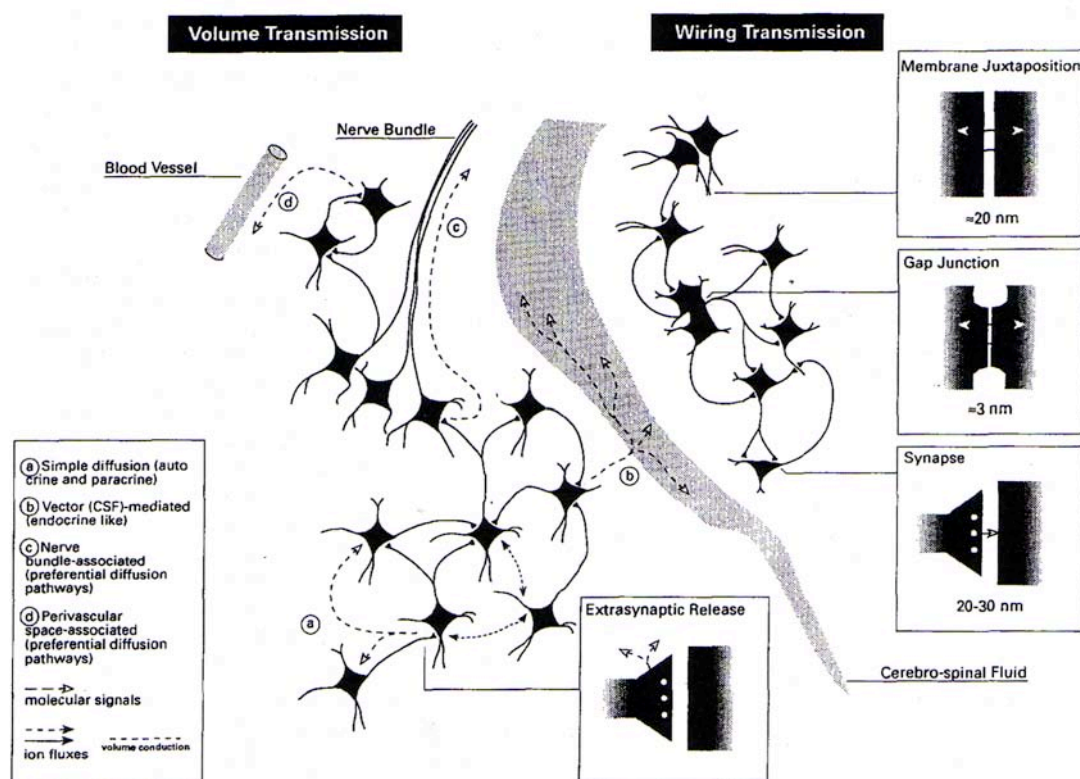


Figure 1.3: Schematic representation of the principle types of intercellular communication in the CNS in the frame of wiring and volume transmission (Agnati et al., 1995).

Neuropeptides, which can act via VT, are frequently stored in LDCVs, but can also coexist with, and be released along with, classical neurotransmitters. Synthesis of neuropeptides occurs *de novo* in the cell body, and no general reuptake mechanisms are thought to exist; instead they are broken down by peptidases (Agnati et al., 1995; Shashoua et al., 2003). Neuropeptides have been shown to be expressed constitutively at low levels, which are then up-regulated in response to changes such as injury. This suggests that neuropeptides may play an important role when the CNS is exposed to stress such as injury and diseases (Shashoua et al., 2003). Communication between neurons, glial and endothelial cells can also occur via diffusion of cytokines and expression of these takes several hours (Abraham et al., 2001).

The role of volume transmission in repair mechanisms in the CNS is being investigated. Studies have shown that VT is involved in energy conservation, and it has been hypothesised that functional recovery after traumatic brain injury may be facilitated by

VT (Zoli and Agnati, 1996; Ridet, 2000). Research into Parkinson's disease has also suggested that the role of VT may be potentiated as a result of lesions in this disease state (Agnati et al., 1995).

1.4 Glutamate and glutamate receptors

The primary excitatory neurotransmitter in the brain is glutamate, making up around one-third of all excitatory synapses (Doble, 1999). As well as mediating many neurologic functions, excitatory neurotransmitters are also important in the developmental plasticity of the brain (Lipton and Rosenberg, 1994). Glutamate actions occur through either ionotropic or metabotropic receptors. Ionotropic receptors elicit their effect through the opening of ion channels whilst metabotropic receptors are linked to G-proteins and result in the activation of second messenger systems.

Ionotropic glutamate receptors (iGluR) have been subdivided into three subtypes on the basis of their selective agonists: AMPA, kainate and NMDA (see Figure 1.4) Each receptor subtype is composed of a different combination of subunits (Alberts, 1994).

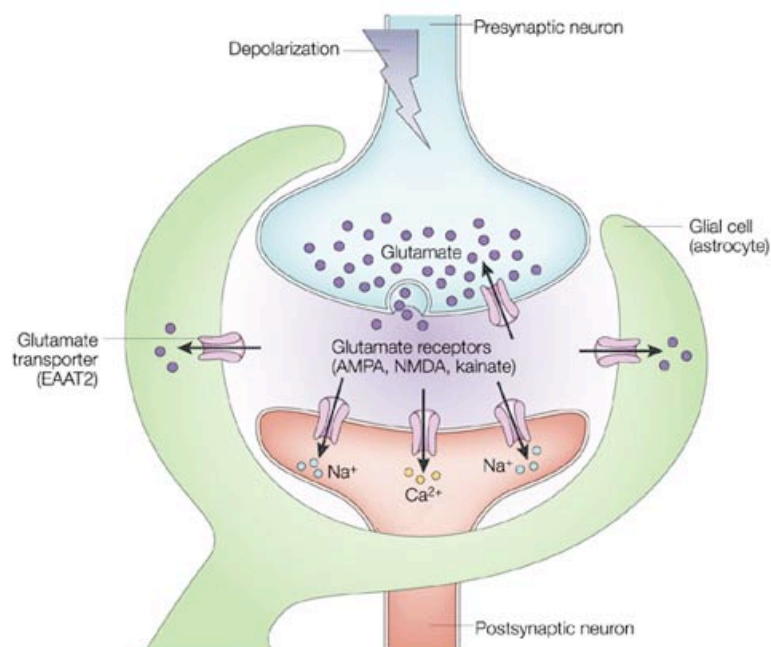


Figure 1.4: Glutamate is released by neurons into the synapse after the arrival of an action potential. The release is carefully controlled and dedicated transporters clear the synapse. Glutamate binds to and opens specific receptor channels (Syntichaki and Tavernarakis, 2003).

Activation of these iGluRs results in an increased permeability of the membrane to the cations Na^+ and Ca^{2+} by the opening of ion-channels. This influx of positively charged ions results in membrane depolarisation. NMDA is the most permeable to Ca^{2+} , the other two have varying degrees of permeability depending on their subunit composition (Lipton and Rosenberg, 1994; Syntichaki and Tavemarakis, 2003).

1.5 Hyperexcitation and excitotoxicity

Overactivation of glutamate receptors (GluR) by excitatory NTs causing cell death has been termed excitotoxicity. This phenomenon was first discovered in retinal cells but has since been found to be a characteristic shared by all neural cells containing GluRs (Alberts, 1994). The excitotoxic hypothesis suggests a central role for the EAAs and their receptors in the neuronal damage as a result of diseases and CNS trauma such as stroke (Schurr et al., 1995) (Lipton and Rosenberg, 1994; Mark et al., 2001).

Elevated levels of excitatory NTs activate iGluRs resulting in increased and prolonged action potential (AP) firing. The Ca^{2+} influx triggers a secondary release of Ca^{2+} from intracellular stores through V-gated Ca^{2+} channels and reverse operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Matute et al., 2001). The resulting increase of Ca^{2+} and K^+ ions further depolarises the neurons, and activates enzymatic and nuclear mechanisms of cell death (Abraham et al., 2001; Schauwecker, 2003). The pivotal role played by GluRs in this process has been demonstrated *in vivo* and *in vitro* with antagonists of GluRs preventing excitotoxicity (Schauwecker, 2003).

Oxidative stress as a result of free radicals plays a role in excitotoxicity, and mitochondria are thought to produce most of the free radicals present in the cell. Studies have suggested that kainic acid encourages free radical production in part by increasing Ca^{2+} uptake into mitochondria. Free radical species such as O_2^\bullet and OH^\bullet are generated by the massive influx of Ca^{2+} into the cells (Yamamoto and Mohanan, 2003).

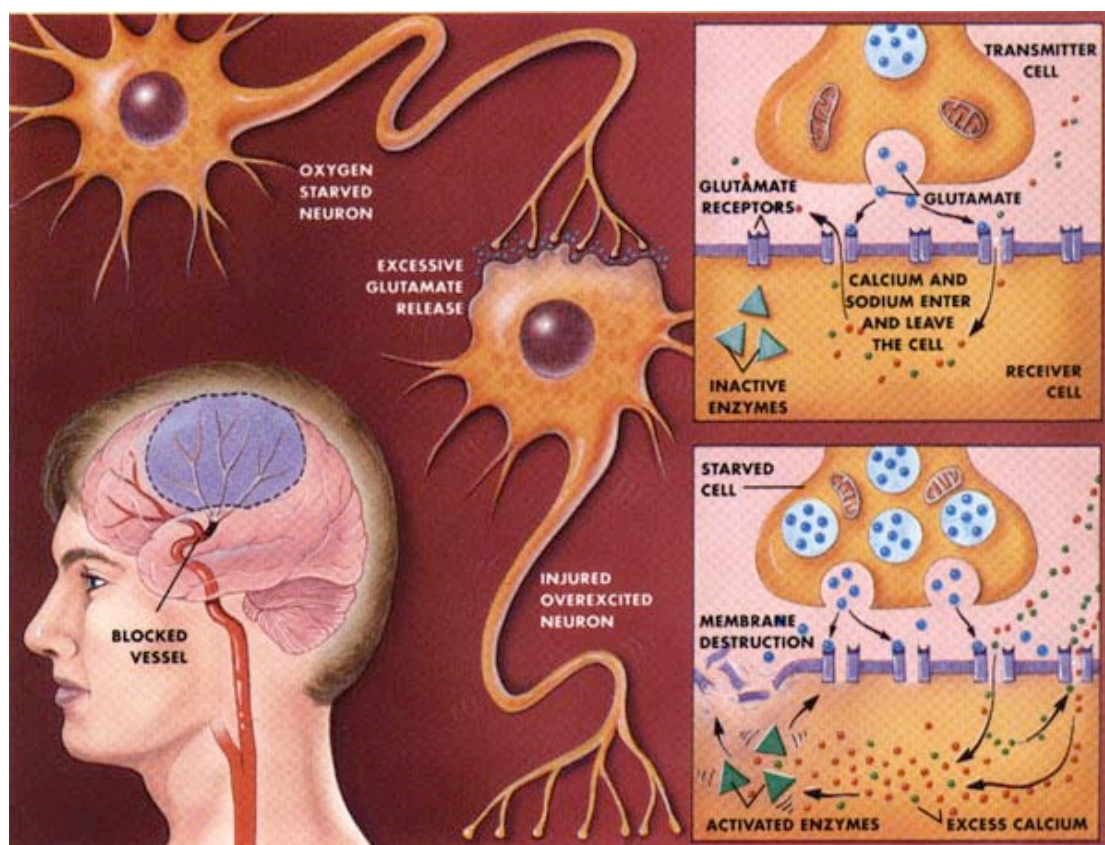


Figure 1.5: Excitotoxicity as a result of stroke. Injured and lysed cells release large amounts glutamate leading to excessive-activation of the GluR (Ariniello, 1994).

Interestingly, different strains of inbred mice show different susceptibility to excitotoxicity. The basis of this is unknown but Schauwecke (2003) suggested that it is not due to differences in composition of the subunits present in the iGluRs (Schauwecker, 2003). The differing ability of neurons to withstand excitotoxicity could be due to differences in their ability to buffer intracellular calcium (Doble, 1999).

As well as being the major excitatory neurotransmitter, glutamate also performs major inhibitory functions. Glutamate maintains inhibition at several excitatory inputs to the cortex and removal of this inhibition, by blockade of the NMDA iGluR, results in excessive excitation of neuron (Pearson et al., 1999)

1.6 Excitotoxicity and disease

Excitotoxicity is implicated in a range of neurodegenerative diseases and conditions such as stroke, epilepsy, Amyotrophic Lateral Sclerosis (ALS), Huntington's disease (HD) and Alzheimer's disease (AD). Elevated glutamate levels can occur for a variety of reasons,

some of which are described below. Increased glutamate release and decreases in the rate of reuptake and breakdown can result in rises in the normally low levels of extracellular glutamate (Mark et al., 2001). Injured neurons can release large amounts, more than the normal cellular reuptake process can handle (Lipton and Rosenberg, 1994). In stroke and ischaemia, cell lysis results in massive rises in extracellular glutamate (see Figure 1.5). Impairment of glutamate reuptake processes is thought to be involved in ALS, a disease involving loss of motor-neurons (Doble, 1999). Studies have shown that β -amyloid, the protein overproduced in AD, sensitises cultured neurons to excitotoxicity. This suggests a possible role of excitotoxicity in this disease state. Inflammatory demyelinating autoimmune diseases such as multiple sclerosis have been linked with excitotoxicity. Injury to oligodendrocytes, the cells which myelinate the axons of neurons, and subsequent demyelination of axons has been demonstrated post GluR stimulation (Matute et al., 2001). The severity and time-course of multiple sclerosis parallel the concentrations of glutamate in the cerebrospinal fluid (CSF) (Matute et al., 2001).

The adult brain is able to recover remarkably well from a range of insults. In the surrounding regions and those remote to a lesion site, the plasticity of the brain is increased through changes in membrane properties and receptor expression of the neurons. Plasticity is the ability of the brain to alter existing connections, to generate new connections and to make use of pre-existing but latent connections (Bütefisch et al., 2003). This increased plasticity can be utilised by the brain to restore functions lost as a result of the injury (Witte, 1998). The degree of this plasticity and the extent to which the brain can regrow axons and neurons is of much interest (Witte, 1998).

From the Greek meaning "shocked throughout" diaschisis is a term first coined by Von Monakow in 1914 (Von Monakow, 1969; Feeney and Baron, 1986). He used the term to describe the phenomenon in which lesions in one region of the brain caused changes in remote but interconnected brain regions. It is now a more generally used term and includes all remote effects of brain injury (Andrews, 1991). These remote effects usually take the form of alterations in blood flow, metabolism and neuronal activity (Reinecke et al., 1999). The underlying cause or function of these changes is not completely clear but they may be important for restoration of function and could also be an underlying cause of hyperexcitability resulting in diseases such as epilepsy, seen in many patients

post stroke (Reinecke et al., 1999; Hokfelt et al., 2000). It is likely however, that many of these changes are due to the action of secreted factors acting via volume transmission. Diaschisis is one of many theories relating to the recovery of function following brain injuries such as stroke (Feeney and Baron, 1986). Recovery utilising diaschisis could involve axon growth and postsynaptic receptor expansion (Feeney and Baron, 1986). Long-term changes in excitatory and inhibitory neurotransmitter systems has been suggested as the process by which this occurs (Bütefisch et al., 2003). Indeed decreases in GABA and AMPA, and increases in NMDA receptor density have been shown in studies of brain lesion induced hyperexcitability. These receptor changes result in increases of excitatory systems relative to inhibitory systems (Witte, 1998; Bütefisch et al., 2003). One possible mechanism is depression of the excitatory transcallosal fibres connecting the two brain hemispheres (Thajeb et al., 2001).

1.7 Proteomics

The term “proteome” first appeared in literature in 1995 and is defined as the ‘total protein complement of a cell (Humphery-Smith et al., 1997). The analysis of expressed proteins at a global level has been termed proteomics (Gygi et al., 2000). Many disease states result in changes in the levels of different proteins such as hormones and peptides. Proteomics allows the characterisation of these disease-specific changes and the identification of disease-markers, which can be used for drug development (Rohlf, 2000). 2D-PAGE is ideally suited to the investigation of secreted proteins. Such proteins are soluble by nature, and the secreted fraction is generally free of high-abundance proteins allowing better resolution of lowly-expressed proteins (Kubota et al., 2003; Shaw and Riederer, 2003).

Measurement of the changes in the expression of multiple proteins provides a powerful strategy for the identification of events in complex pathophysiological mechanisms. Thus, proteomics allows for the identification of novel proteins that play key roles in the pathogenesis of neurodegenerative diseases such as schizophrenia and Alzheimer’s disease.

A combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) are typically used when investigating a cell’s proteome. 2D-

PAGE allows the separation and visualisation of proteins, which can then be identified by MS (Gygi et al., 2000).

Protein separation using 2D-PAGE is the basis of proteome studies and its ability to separate complex protein mixtures remains unsurpassed (Humphery-Smith et al., 1997). Multidimensional gel separation reduces the amount and number of peptides introduced into the mass spectrometer. 2D-PAGE separates proteins by their molecular weight (MW) and isoelectric point (pI). Separation in the first dimension occurs by isoelectric focusing and in the second dimension by their molecular weight using sodium dodecylsulphate (SDS) gel electrophoresis (Brewer, 1997; Humphery-Smith et al., 1997).

Mass spectrometry is the second stage in proteomic studies. Proteins that have been isolated are digested using a protease such as trypsin, into peptides. Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) is used to identify proteins by what is known as peptide-mass mapping or peptide-mass fingerprinting. The peptide is ionised and the mass to charge ratio (m/z) is calculated by measuring the flight time of each ionised peptide. Using this procedure, proteins are identified by comparing the experimental peptide masses with databases containing calculated masses for large numbers of previously sequenced proteins (Pandey and Mann, 2000; Aebersold and Mann, 2003).

1.8 Aims

The main aims of this research were to:

- Establish primary neuronal cell cultures using cortical tissue from postnatal male rats
- Characterise cortical cultures using immunocytochemistry and light microscopy, to determine the proportion of neuronal and non-neuronal cells
- Investigate the *in vitro* survival of neurons under normal culturing conditions, and in the presence chemical stimulation, using live/dead assays and a measure of membrane integrity
- Establish conditions which allow neuronal survival in the absence of added proteins, i.e. protein-free culturing conditions
- Analyse the conditioned medium of cultured neurons using a combination of 2D-PAGE and MALDI-TOF MS
- Analyse the conditioned medium of cultured neurons exposed to excitatory chemical stimuli using a combination of 2D-PAGE and MALDI-TOF
- Identify proteins that are differentially expressed in chemically-stimulated and non-stimulated neurons

Chapter two – materials and methods

2.1 Project overview and research design

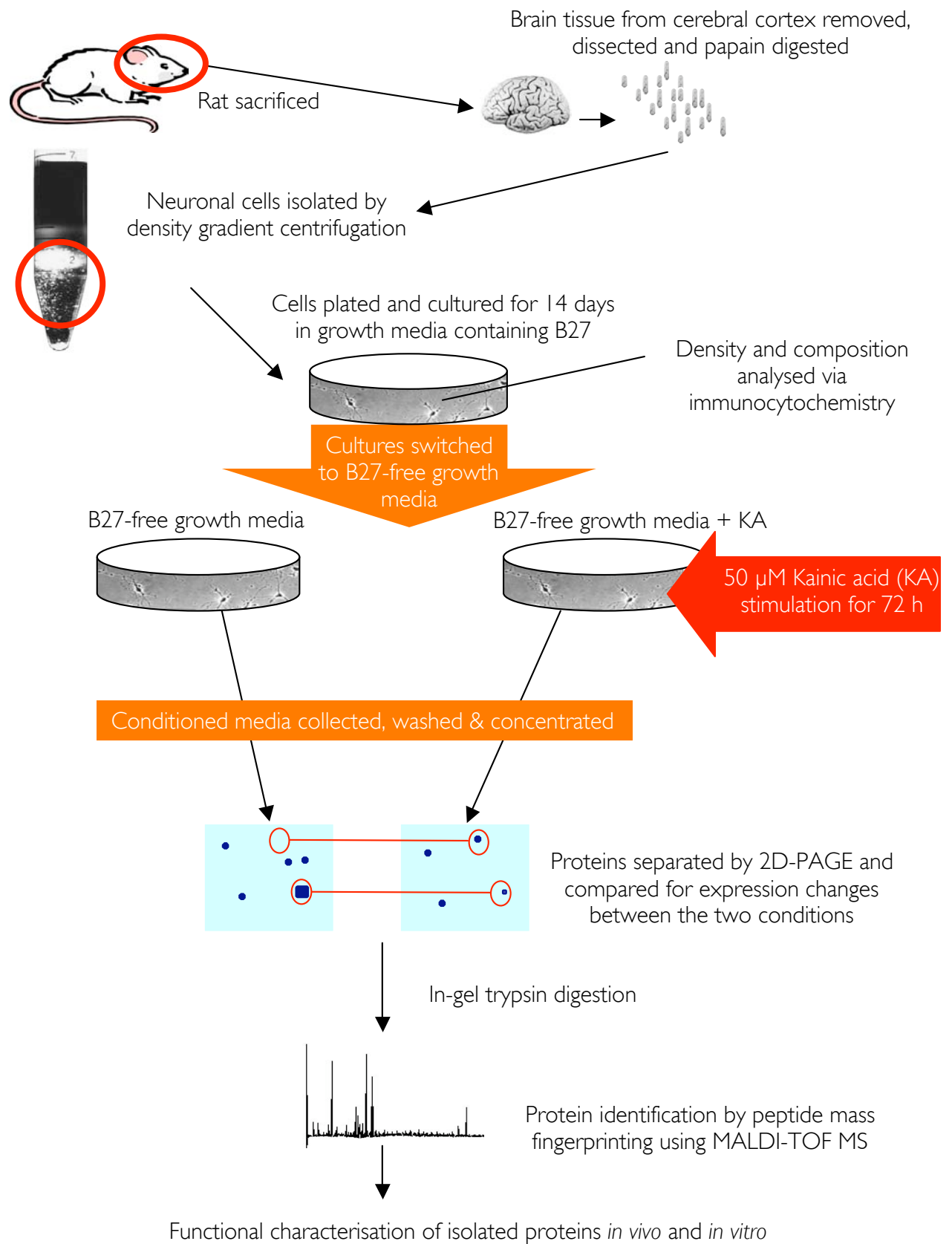


Figure 2.1: Diagram detailing the overall protocol followed in this research.

2.2 Cell isolation and cell culture

2.2.1 Introduction

Methodologies for isolating neuronal cells vary in the literature (Wang and Cynader, 1999; Bledi et al., 2000; Tasker et al., 2002), however, a methodology by Brewer et al. (1993) was chosen. This method was advantageous because first it included steps to enrich for neurons, secondly the culturing medium was optimised for neurons, and thirdly it has been shown to be successful for both postnatal and adult neurons (Brewer et al., 1993; Brewer and Price, 1996; Kivell et al., 2001). Cultures of embryonic neurons are common in the literature, but are problematic for several reasons: embryonic brains produce less tissue and hence less cells, cells may not be fully differentiated, the procedure involves sacrificing the mother, and extra ethical approval above and beyond that granted for the current project would be required. Consequently, it was decided to use tissue from 21-day old postnatal rats.

After cells have been isolated from the rat brains, subsequent *in vitro* cell culture is dependent upon proper attachment of the cells to the growth surfaces (Bledi et al., 2000). Tissue culture plates are therefore coated with reagents such as poly-D-lysine, a positively charged polymer, or biologically derived adhesive molecules such as collagen, prior to cell plating. Without proper attachment, any manipulation such as medium changes or washing, will cause substantial loss of cells (Bledi et al., 2000). Poly-D-lysine is frequently used when culturing primary neuronal cell types and has been shown to provide attachment superior to poly-L-lysine (Kivell et al., 2000). The glass pipettes used for tissue dissociation were flame-polished and coated with Sigmacote, a siliconising agent, to minimise damage to the cells. Sigmacote forms a covalent hydrophobic silicone layer on the glass, preventing clotting of blood and adhesion of cells.

2.2.2 Poly-D-lysine coating of plates

2.2.2.1 Materials

Sterile media filter (Sartorius)

Poly-D-lysine hydrobromide (Sigma)

13 mm coverslips (Scientific supplies)

2.2.2.2 Protocol

A stock solution of 1 mg/ml poly-D-lysine was first prepared by dissolving 100 mg of poly-D-lysine powder in 100 ml of milli-Q-H₂O; 10 ml aliquots were stored at -20°C until used. Stock solutions were sterile filtered and diluted ten-fold in milli-Q-H₂O, to 0.1 mg/ml, prior to use. In a laminar flow hood, enough solution was pipetted to cover the surface of each well or flask. Approximate volumes are shown in Table 2.1. Where necessary coverslips were autoclaved and placed 1 per well in a 12-well plate, within the sterile environment of a laminar flow. Poly-D-lysine coating of the coverslips and wells was then performed as below.

Table 2.1: Volume of 0.1 mg/ml poly-D-lysine added to each well/flask.

T-75 flask	5 ml per flask
6-well plate	1 ml per well
12-well plate	0.5 ml per well

The solution was evenly distributed over the surface of the well or flask by gentle agitation and incubated for 1 h in the laminar flow hood. The liquid was removed and the coated surfaces washed twice with milli-Q-H₂O before being left exposed overnight in the laminar flow-hood at RT to dry.

2.2.3 Siliconised flame-polished Pasteur pipettes

2.2.3.1 Materials

Sigmacote (Sigma)

9 inch flint glass Pasteur pipettes (Chase Scientific Glass, Inc.)

Bunsen burner

2.2.3.2 Protocol

Tips of Pasteur pipettes were flame-polished by being held briefly in the flame of a Bunsen burner, to reduce the opening to around 0.8-0.9 mm and smooth it. Sigmacote was pipetted up and down twice through the flame-polished Pasteur pipettes and allowed to drain. The silicon coating was left to dry and harden in a 50°C warmer overnight. All Pasteur pipettes were autoclaved immediately prior to use.

2.2.4 Isolation of postnatal primary cortical neurons

2.2.4.1 Ethical approval

All procedures were fully approved by the University of Auckland (UoA) animal ethics committee. Initial investigative studies to develop and optimise cell isolation protocols were performed under ethics application **AEC/06/2003/RI42**. All subsequent experiments were performed under ethics application **AEC/10/2003/RI84**. Copies of the approvals for both ethics applications are contained in the Appendix.

2.2.4.2 Animals

Albino male Wistar rats (*Rattus norvegicus*) were used for all experiments performed as part of this research. The majority of rats were born and raised in the Animal Facility in the School of Biological Sciences, UoA, some were also sourced from the Auckland Animal Unit based at the Auckland School of Medicine, UoA. Rats were raised on a 12/12 h light/dark cycle, at an average RT of 21 °C. The animals were fed *ad libitum* on Teklad diet 2018. Teklad 2018 is a rodent diet containing 18 % protein and 5 % fat. It is a fully vegetarian diet containing no animal protein or fishmeal. The Appendix contains a printout of the exact contents of this feed. Rats were sacrificed at day 21, the day of weaning, and on average weighed 75 g. Brain tissue was removed and processed as described below. Approximately 2.5 g of tissue was obtained from each brain.

2.2.4.3 Materials

Sterile media filter (Sartorius MiniSart)

Neurobasal-A (Invitrogen-GIBCO)

GlutaMax (Invitrogen-GIBCO)

B27 (Invitrogen-GIBCO)

Nycoprep Universal (Axis-Shield)

Hibernate-A (Brainbits USA)

Basic fibroblast growth factor (bFGF) (Invitrogen-GIBCO)

Streptomycin sulphate (Serva)

Penicillin-G (Sigma)

70 µm tissue strainer (Falcon)

Papain *papaya Latex* (Sigma)

Growth medium

- Neurobasal-A (Invitrogen-GIBCO)
- 2 % B27 (Invitrogen-GIBCO)
- 0.5 mM GlutaMax (Invitrogen-GIBCO)
- 50 units penicillin-G (Sigma)
- 0.05 mg/ml streptomycin sulphate (Serva)
- 5 ng/ml bFGF (Invitrogen-GIBCO)

Dissection medium

- Hibernate-A (Brainbits USA)
- 2 % B27 (Invitrogen-GIBCO)
- 0.5 mM GlutaMax (Invitrogen-GIBCO)

Prorbital temperature controlled shaker (Forman Scientific)

Eppendorf centrifuge 5415D (Eppendorf)

2.2.4.4 Protocol

Six 21-day old male Wistar rats were anaesthetised using halothane and sacrificed by cervical dislocation. Skin on the head of the animal was cut and the skull removed to reveal the brain. Brains were then removed from each of the six rats and placed in a 35 mm dish containing 4 ml of Hibernate-A supplemented with 2 % B27 and 0.5 mM GlutaMax at 4°C (dissection medium). The surgical steps above were performed in a non-sterile environment.

Prior to all sterile manipulations, the surfaces of the laminar flow hood were wiped with 70 % ethanol and exposed to ultraviolet light for 20 min. The six brains in dissection medium were transferred to the sterile laminar flow hood, in which all subsequent steps were performed. Three brains were removed from the first dish and placed in a second 35 mm dish containing 1 ml of dissection medium. The cerebral cortex was dissected from both lobes of each brain (Figure 2.2) and minced into a fine paste using scalpels. This was repeated for the remaining three brains.

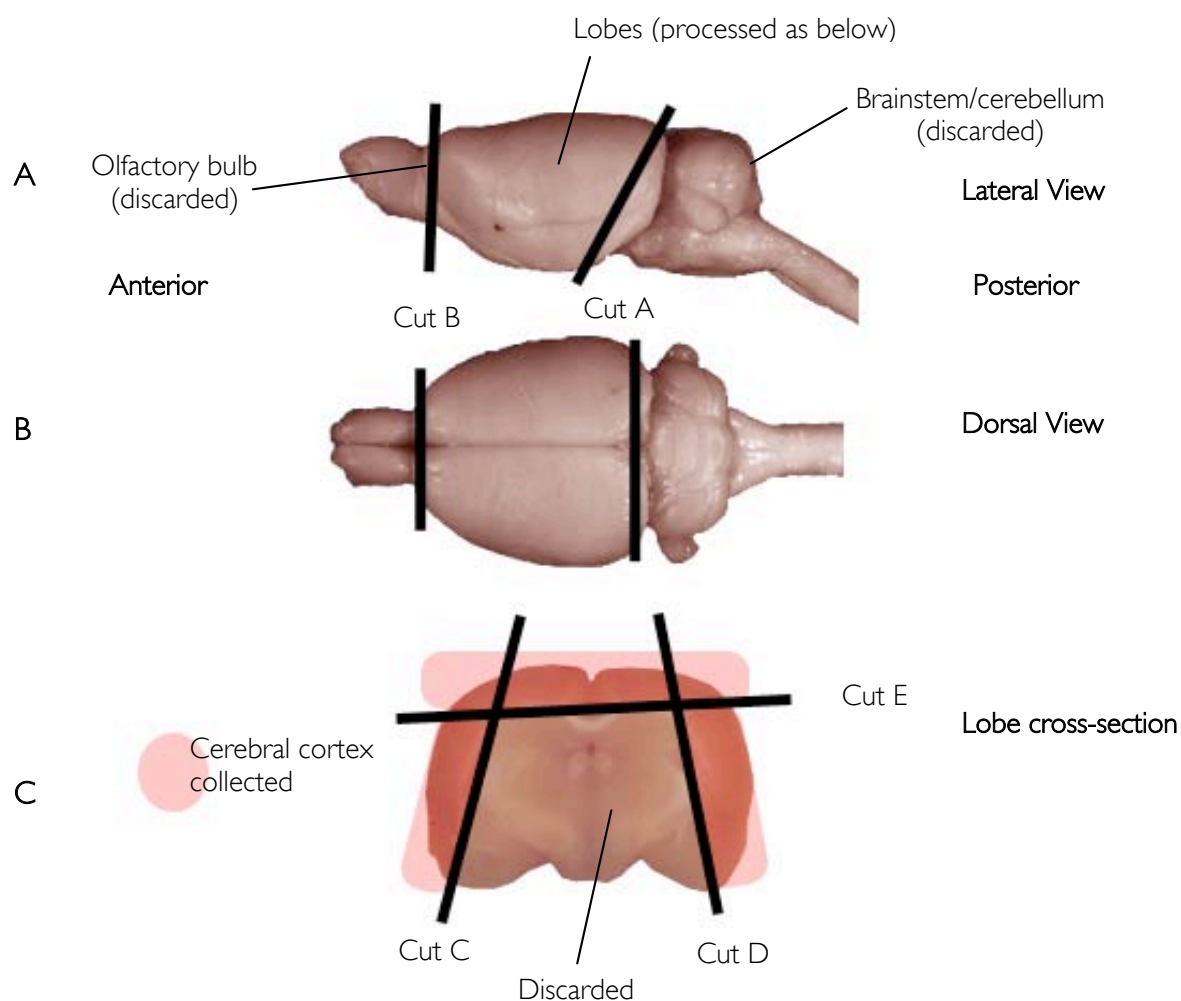


Figure 2.2: Diagram showing approximate location of brain regions isolated. The posterior and anterior regions of each isolated brain were removed leaving the two lobes of the brain (A and B). The outer layer, being the cerebral cortex (shown in pink highlight), was then removed and processed (C).

The tissue paste from each dish was transferred into two separate 50 ml falcon tubes and 1 ml of the dissection medium was added. The tubes were placed in an orbital shaker for 8 min, with the shaker set to a temperature of 30°C and a rotation speed of 220 rpm, which was sufficient to suspend the tissue pieces. After shaking for 8 min, 6 ml of papain at 30°C was added.

A papain solution was prepared by mixing 200 μ l of papain stock into 5.8 ml of Hibernate-A, to give a working concentration of \sim 31 units/ml. The papain solution was warmed for 5 min at 37°C and then filter-sterilised using a sterile 0.2 μ m cell-culture filter. The papain solution was used within 30 min of preparation. All tissue digestions were performed in protein-free Hibernate-A medium without B27 to ensure that the proteins present in B27 did not compete with the tissue as a protease substrate.

The tissue and papain mixture was then incubated for 30 min in the orbital shaker with the same settings, 30°C and 220 rpm. After the 30 min incubation, tubes were centrifuged at 200 × g for 2 min at 4°C and the papain supernatant discarded. To the pellet was added 2 ml of 30°C Hibernate-A supplemented with 2 % B27 (HB-A/B27); the tubes were gently shaken and allowed to sit for 5 min at RT. The tissue solutions were quickly triturated around 30 times with a sterile siliconised flame-polished Pasteur pipette, applied to a 70 µm tissue-filter using the same pipette and then allowed to pass through. A wash buffer consisting of 1 ml of HB-A/B27 was added to clear the membrane of any remaining cells. The tissue-filter containing debris and particulate matter was discarded.

The cell suspension (~3-4 ml) was then applied to the top of a Nycoprep 1.15 g/cm³ gradient in a 15 ml centrifuge tube. The Nycoprep gradient was made in four steps: 35 % (3 ml), 25 % (2 ml), 20 % (2 ml) and 15 % (3 ml) Nycoprep in HB-A/B27 (see Figure 2.3). The gradient was formed by underlaying each layer using a Pasteur pipette, starting with the least dense solution (15 %). The gradient and cell suspension was then centrifuged at 800 × g for 15 min at 4°C in an Eppendorf centrifuge.

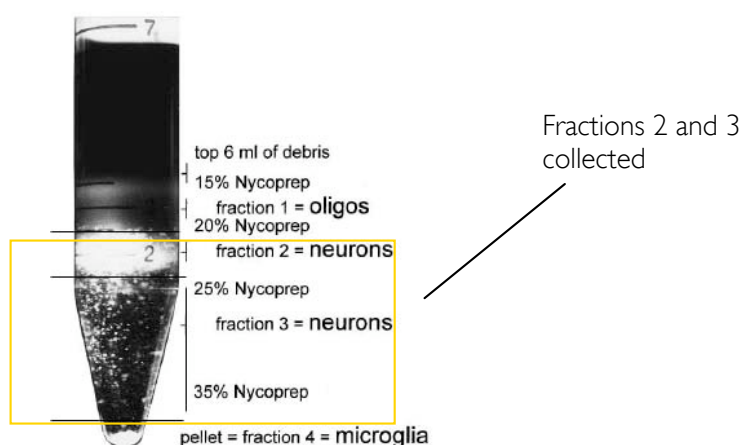


Figure 2.3: Fractions 2 and 3 which comprised the main neuron-containing bands were both collected and pooled prior to plating.

Fraction 2 contained the densest band of fragments and cells, appearing as a white band, with fraction 3 below. Both fraction 2 and 3 were enriched for neurons. Everything above fraction 2 was discarded. Fraction 1 has been shown to be enriched

for oligodendrocytes (Brewer, 1997). The pellet at the bottom of the tube appeared to consist of red blood cells and was also discarded. Fractions 2 and 3 from each separate gradient were collected with a siliconised Pasteur pipette into a solution of 50 ml HB-A/B27 and 15 ml NBA/B27/GlutaMax.

This cell suspension was then plated on poly-D-lysine coated plates and left for ~3 h. After this time the majority of live cells had attached to the poly-D-lysine substrate and the floating debris could be washed off using two 10 ml washes of 37°C HB-A/B27. After washing, growth medium was added at the volumes in Table 2.2. The cells were maintained at 37°C in a humidified atmosphere with 5 % CO₂.

Table 2.2: Volume of growth medium added after washing to each well/flask.

T-75 flask	18 ml per flask
6-well plate	1 ml per well
12-well plate	0.5 ml per well

Half of the medium was removed every 3-4 days and replaced with fresh medium to equal the original volume. For medium changes, the bFGF concentration in the fresh medium was double the original concentration (i.e. 10 ng/ml) so that the final concentration was the same, assuming that the old bFGF was totally consumed.

2.3 Characterisation of cultures

2.3.1 Introduction

Primary cultures were characterised using immunocytochemistry. Neurons and astrocytes present in the cultures were identified using antibodies raised against the neuron-specific protein neuronal nuclei (NeuN), and the astrocyte specific glial fibrillary acidic protein (GFAP), respectively. A third antibody, raised against bovine serum albumin, was used to investigate whether this protein was present intracellularly. Dot-blots provide a method to confirm the reactivity of an antibody, in this case whether a provided antibody was reactive against the bovine serum albumin protein.

2.3.2 Dot-blot

2.3.2.1 Materials

Rabbit anti-bovine serum albumin (provided by Dr. Christina Buchanan)

Hyperfilm ECL mix (Amersham Biosciences)

50 % reagent A

50 % reagent B

Lumi-light western blotting substrate (Roche)

Ovalbumin (Sigma)

Albumin, bovine fraction V (BSA) (GIBCO)

Kodak automatic film processor (Kodak Corp.)

Hybond ECL nitrocellulose membrane (Amersham Biosciences)

Tris-buffered saline with Tween-20 (TTBS)

500 mM sodium chloride

20 mM tris, pH 7.5

1 % Tween-20

2.3.2.2 Protocol

1 μ l of four solutions of 0.1, 10, 100 μ g/ μ l and 1 ng/ μ l BSA was spotted onto four small pieces of nitrocellulose paper. In a small sealed container the nitrocellulose was blocked by incubating overnight in 4 % ovalbumin in TTBS at 4°C with agitation. The nitrocellulose was washed briefly twice, then once for 15 min and twice for 5 min with TTBS at RT, before being incubated for 1 h in the primary antibody, rabbit anti-BSA, or TTBS as the control. Three concentrations of the primary antibody were used: neat, 1:50 and 1:100. After incubating for 1 h at 37°C the nitrocellulose was washed again as before and incubated for 1 h in the secondary antibody, goat anti-rabbit HRP labelled IgG, diluted 1:1000. The nitrocellulose was washed again as before, drained on filter paper and placed protein-blot side up on a sheet of transparency. Hyperfilm ECL mix (240 μ l) was pipetted onto each piece of nitrocellulose, incubated for 1 min in the dark and removed by blotting on the edge with filter paper. The nitrocellulose was sandwiched between two sheets of transparency and exposed to film for 1-5 min in a film cassette, the exposed film was then processed using an automatic Kodak film processor. If the antibody is reactive to the BSA then black spots should be visible on the film corresponding to where the BSA was applied to the nitrocellulose, and the

control should show no signal. Lack of signal is an indication that the antibody is not immunoreactive to the substrate.

2.3.3 Immunocytochemistry

2.3.3.1 *Materials*

Mouse anti-neuronal nuclei monoclonal antibody (NeuN) (Chemicon)

Rabbit anti-gial fibrillary acidic protein polyclonal antibody (GFAP) (Zymed)

Rabbit anti-bovine serum albumin (Molecular Probes)

Normal donkey serum (provided by J. Bradley)

Donkey anti-mouse Texas red (Jackson)

Goat anti-rabbit FITC (Jackson ImmunoResearch)

Albumin, bovine fraction V (GIBCO)

Ovalbumin (Sigma)

Cityfluor (Agar Scientific)

Phosphate buffered saline (PBS)

137 mM sodium chloride (BDH)

3 mM potassium chloride (Merck)

9 mM sodium phosphate (BDH)

2 mM potassium dihydrogenphosphate (BDH)

Adjusted to pH 7.4 with HCl

4 % paraformaldehyde (PFA) (BDH)

90 % ethanol (BDH) and 10 % glacial acetic acid (BDH)

Neutral buffered formalin (NBF)

10 % v/v formalin (BDH)

26 mM sodium dihydrogenorthophosphate (BDH)

46 mM disodium orthophosphate (BDH)

Adjusted to pH 7.0 with HCl

50 % acetone (BDH) and 50 % methanol (BDH)

2.3.3.2 *Protocol*

Cells were grown on poly-D-lysine coated coverslips for 14 days in culture. The medium from each well was removed and the cells washed three times with PBS. After transferring the coverslips to a 24-well plate and washing twice more with PBS, the cells were fixed. Four fixing protocols were initially investigated: 1) 4 % PFA, 2) 90 % ethanol

+ 10 % glacial acetic acid, 3) NBF, all for 20 min at RT and 4) 50 % acetone + 50 % methanol for 20 min at -20°C . NBF was used for all subsequent staining procedures. After two 5 min washes to rinse the cells free of fixative, the cells were permeabilised with 0.5 % Triton X-100 and 0.1 % sodium citrate in PBS for 5 min. After two 5 min PBS washes the coverslips were blocked in 5 % normal donkey serum in PBS for 1 h at 37°C and then washed once for 2 min with PBS. Coverslips were incubated overnight at 37°C with 150 μl of 1 of the following primary antibodies: rabbit anti-GFAP (diluted 1:50), mouse anti-NeuN (diluted 1:50), rabbit anti-BSA (diluted 1:50), or 150 μl of PBS as a control. Antibody dilutions for GFAP and NeuN were made in 10 % BSA in PBS, while the antibody for BSA was diluted in 10 % ovalbumin in PBS. Slides were washed 3 times with PBS for 10 min prior to incubation with the secondary antibody for 4 h at RT. The secondary antibodies for NeuN and GFAP, donkey anti-mouse Texas red and goat anti-rabbit FITC respectively, were also diluted 1:50 in 10 % BSA in PBS. The secondary antibody for BSA, goat anti-rabbit FITC, was diluted in 10 % ovalbumin in PBS (see Table 2.3). The coverslips were then washed twice in PBS for 10 min and mounted on glass slides using Cityfluor for viewing.

Table 2.3: The primary and secondary antibodies used to target either astrocytes, neurons or bovine serum albumin.

Target	Primary antibody	Secondary antibody
Neurons	1:50 mouse anti-NeuN	1:50 donkey anti-mouse Texas red
Astrocytes	1:50 rabbit anti-GFAP	1:50 goat anti-rabbit FITC
BSA	1: 50 rabbit anti-BSA	1:50 goat anti-rabbit FITC

Staining was observed using a Zeiss AxioSkop microscope and recorded using Zeiss AxioCam software.

2.3.4 Determination of neuron to astrocyte ratio

Cells staining positive to either NeuN or GFAP were counted as neurons or astrocytes respectively, in cultures double-stained for both antibodies. Three randomly selected representative culture fields were counted and the ratio calculated.

2.4 Cell survival assays and kainic acid stimulation

2.4.1 Introduction

Two methods to determine cell viability were used, a live/dead assay utilising fluorescein diacetate (FD) and propidium iodide (PI), and a test of cytoplasmic integrity by means of lactate dehydrogenase (LDH).

FD can readily cross cell membranes, but is rapidly hydrolysed to the impermeant fluorescent dye fluorescein once inside the cell. Consequently, fluorescein accumulates in live cells but not dead cells, whose membrane is not intact (Banker and Goslin, 1997). Propidium iodide is commonly used as a vital dye in cell culture and labels the nucleus in cells that lack an intact membrane. PI is impermeable to cells that have an intact membrane, but in dying cells where membrane integrity is compromised it gains access to the nucleus. The resulting complex of PI and DNA renders the nucleus highly fluorescent (Brana et al., 2002). Cell counts of FD and PI stained cells are then performed to determine the ratio of live to dead cells.

LDH is a cytosolic enzyme that catalyses the reduction of pyruvate into lactate. LDH is not secreted, but cells that have lost membrane integrity release LDH into the surrounding medium. The level of LDH released into the medium has been shown to correlate with the number of dead cells and when cell survival is high, very little or no LDH can be detected (Legrand et al., 1992). This technique has been successfully used to quantify excitotoxic neuronal death in culture (Klingman et al., 1990; Deupree et al., 1996).

2.4.2 Materials

Kainic acid (KA) (Sigma)

Propidium iodide (Sigma)

Fluorescein diacetate (Sigma)

Acetone (BDH)

Zeiss AxioVert S100 (Zeiss)

Incubation buffer

- 154 mM sodium chloride (BDH)
- 5.6 mM potassium chloride (Merck)
- 5.6 mM glucose (BDH)
- 8.6 mM HEPES (BDH)
- 1 mM magnesium chloride (BDH)
- 2.3 mM calcium chloride (BDH)
- Adjusted to pH 7.4

B27-free growth medium

- Neurobasal-A medium (Invitrogen-GIBCO)
- 0.5 mM GlutaMax (Invitrogen-GIBCO)
- 50 units/ml penicillin-G (Sigma)
- 0.05 mg/ml streptomycin sulphate (Serva)
- 5 ng/ml bFGF (Invitrogen-GIBCO)

2.4.3 Protocol

2.4.3.1 *Transfer of cultures to B27-free conditions*

Neurons were grown in 12-well plates and used after 14 days in culture. For experiments looking at survival in B27-free growth medium, existing medium was removed, and the cells and the flask were washed with 1 ml of B27-free growth medium and left to incubate for 1 h in 1 ml of the same medium. This medium was removed and the cells and flask washed once more in 1 ml B27-free growth medium before 1 ml of the same medium was added. Comparisons were also made to cells growing in the presence of B27 supplement, and for these the medium was replaced with new growth medium.

2.4.3.2 *Lactate dehydrogenase analysis*

Medium was collected at 6, 12, 24, 30 and 96 h time points. Medium samples were also collected from all KA exposed cultures used for live/dead assays at the indicated times in section 2.4.3.3. Samples were sent to Medlab for LDH analysis. The LDH measurement was normalised to units per 10^4 cells, to allow comparisons between cultures. The total number of neurons per dish was calculated from the ratio between the area of the dish and the area of the representative culture fields counted using a digital-microscope (see section 2.4.3.3).

2.4.3.3 *Fluorescein diacetate/propidium iodide live/dead assay*

The method used was adapted from that described by Tasker et al. (2002). FD and PI were dissolved at 1000 × working concentrations: 5 mg/ml in acetone and 50 mg/ml in milli-Q-H₂O, respectively. Kainic acid was added to the B27-free growth medium for the indicated time and concentration: 0 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM, 500 μM, 1000 μM for 24 h; 10 μM for 12, 18, 24 and 36 h; 50 and 100 μM for 24, 30, 48 and 72 h, all were done in triplicate. B27-free growth medium was then removed and cultures were incubated for 5 min with 1 ml incubation buffer, to which the vital stain FD was added to give a final concentration of 5 μg/ml. The staining mixture was then aspirated and replaced with fresh incubation buffer, to which PI was added to give a final concentration of 50 μg/ml. Cultures were examined for neurotoxicity under fluorescent light. Live cells stain a bright green colour in the cell body and neurites, while dead cells do not retain any fluorescein diacetate, and their nuclei can be stained red by the propidium iodide. Photographs of three randomly selected representative culture fields in each well were taken, and live and dead cells in these fields counted.

Images were taken using Zeiss Axiocam software 2.0 for Mac OS 9 connected to a Zeiss AxioVert S100 microscope. Data was analysed using GraphPad Prism v.4.0a for Mac OS X.

2.5 Collection of conditioned medium and cytosol for electrophoresis

2.5.1 Introduction

The B27 supplement contains proteins such as BSA and insulin at concentrations much higher than that of cell-produced proteins. The cultures were switched to B27-free growth medium so that ideally only cell-produced proteins would be present in the conditioned medium. The process involved washing the plates and cells to remove the existing B27-containing medium.

2.5.2 Materials

PBS

Kainic acid (Sigma)

Growth medium

Neurobasal-A medium (Invitrogen-GIBCO)

2 % B27 (Invitrogen-GIBCO)

0.5 mM GlutaMax (Invitrogen-GIBCO)

50 units/ml penicillin-G (Sigma)

0.05 mg/ml streptomycin sulphate (Serva)

5 ng/ml bFGF (Invitrogen-GIBCO)

2.5.3 Protocol

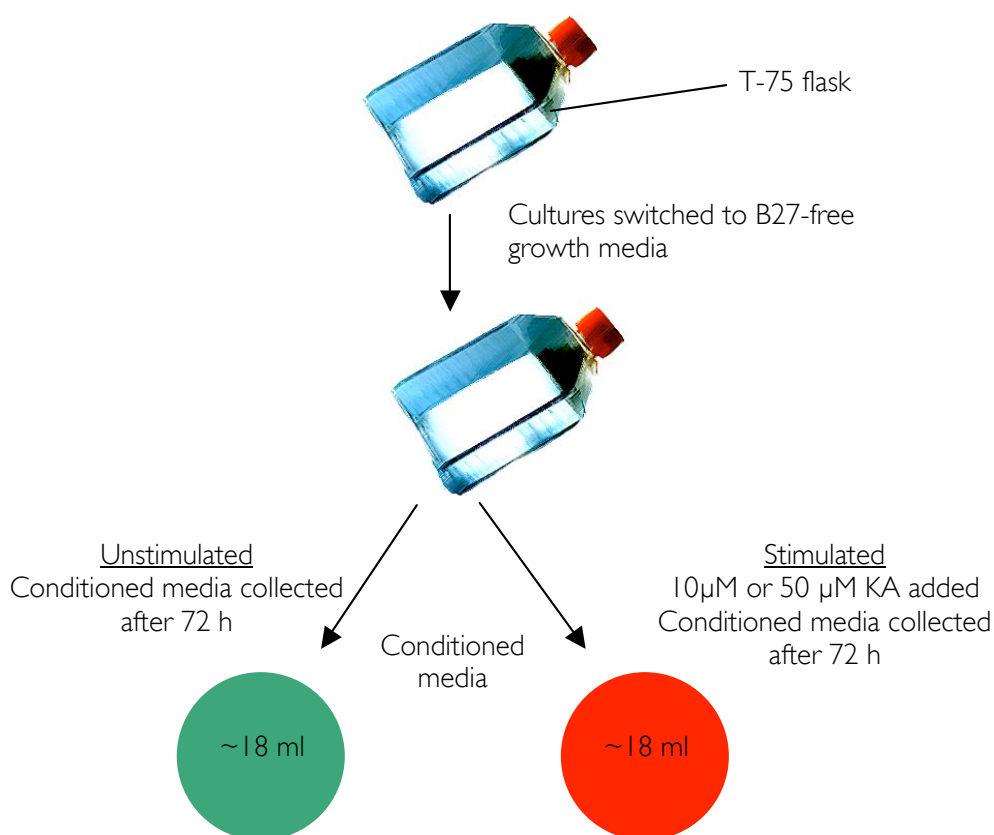


Figure 2.4: Optimised conditions for medium collection. Cultures were switched to B27-free growth medium and grown in the presence (stimulated) or absence (unstimulated) of 50 µM KA. Conditioned medium was collected after 72 h.

2.5.3.1 Transfer of cultures to B27-free conditions

Existing medium was removed, the cells and the flask were washed with 10 ml of B27-free growth medium and left to incubate for 1 h in a further 10 ml of the same medium. This medium was removed and the cells and flask washed once more in 10 ml B27-free growth medium before 18 ml of the same medium was added.

2.5.3.2 *Unstimulated cultures*

The cells were left to grow in this medium for 3 days before the conditioned medium was collected and processed as described below (see Figure 2.4). 500 μ l of conditioned medium was also collected and the LDH level quantitated as described in Section 2.4.3.2.

2.5.3.3 *Kainic acid stimulation*

Kainic acid was added to the B27-free growth medium at a concentration of either 10 μ M or 50 μ M. The cells were left to grow in this medium for 3 days before the conditioned medium was collected and processed as described below (see Figure 2.4). Conditioned medium (500 μ l) was also collected and the LDH level quantified as described previously.

2.5.3.4 *Collection of cytosol*

The cells were washed three times in 20 ml PBS and then scraped in 20 ml PBS. These cells were then centrifuged at 3,000 rpm for 5 min at 4°C and the supernatant removed. The remaining cell pellet was then lysed in 1 ml milli-Q-H₂O, freeze-thawed quickly twice, sonicated to aid in lysis and then spun down at 14,000 rpm for 10 min at 4°C. The cytosol containing supernatant was kept for future analysis and the cellular debris pellet discarded.

2.6 Washing and concentration of conditioned medium for electrophoresis

2.6.1 Introduction

Conditioned medium must be processed prior to 2D-PAGE. Samples must have a high protein concentration and be free of salt and other contaminating factors such as ionic detergents, nucleic acids, lipids etc. If these factors are not removed, they can affect the electrophoresis and make it difficult to obtain reproducible results (Jiang et al., 2004). The main techniques for desalting and concentrating samples are protein precipitation and ultrafiltration (Jiang et al., 2004). Protein precipitation techniques, although much faster than ultrafiltration, have been shown to result in higher protein loss, a significant issue when dealing with the very limited protein content of conditioned medium (Dahl et al., 2003). Ultrafiltration using a low molecular weight cut-off (MWCO) membrane is

time consuming and more expensive, but results in the recovery of practically all the protein in the starting material (Jiang et al., 2004). As the proteins of interest in these studies are likely of low abundance and the starting material is limited, ultrafiltration was used.

2.6.2 Materials

20 ml Vivaspin 5 kDa MWCO ultrafiltration column (Vivascience)

0.05 % v/v triton X-100 (BDH)

Speedvac protein concentrator (Savant)

Eppendorf centrifuge 5415D (Eppendorf)

2.6.3 Protocol

2.6.3.1 *Removal of cells and cellular debris*

On the day of collection conditioned medium was centrifuged at $1,300 \times g$ for 5 min to pellet cells. After transferring the supernatant to a new tube, it was spun at $13,000 \times g$ for 1 h to pellet cellular debris and organelles. The supernatant was again removed and stored by freezing at -80°C in glass vials until subsequent processing.

2.6.3.2 *Ultrafiltration*

The conditioned medium supernatant was applied to the top well of a Vivaspin ultrafiltration column and centrifuged at 3700 rpm and 4°C until the liquid had reduced to 1 ml, this on average took 1 h. Milli-Q- H_2O at 4°C was added to equal the original volume of conditioned medium, $\sim 17\text{-}18$ ml, and the column centrifuged again until 1 ml remained. This wash sequence was repeated 10 times followed by a final wash with 18 ml 0.05 % Triton X-100 in milli-Q- H_2O . The column was centrifuged until the volume had reached $\sim 700 \mu\text{l}$, this spin took significantly longer than the previous washes due to the presence of the detergent. The protein levels were quantitated as described in section 2.7. Further concentration was performed using a speedvac protein concentrator.

2.6.3.3 *Speedvac protein concentrator*

The volume of retentate from the Vivaspin ultrafiltration column was required for 2D-PAGE was further concentrated using the speedvac until dry.

2.7 Protein determination via reverse biuret

2.7.1 Introduction

It is necessary to determine the protein concentration in a sample in order to load the same amount in each 2D-PAGE gel. This is essential for valid gel to gel comparisons to be made. The reverse biuret reaction is a modified version of the biuret reaction for protein determination and has increased sensitivity (Matsushita et al., 1993). In the reverse biuret reaction, colour is generated by the reduction of excess cupric ions, i.e. those not bound by protein. The colour is inversely proportional to the protein concentration. The reaction is independent of the composition of the protein, an advantage over many other protein determination methods (Matsushita et al., 1993; Sapan et al., 1999). Amersham Biosciences also produce a product, the 2D-Quant Kit, which makes use of the reverse biuret reaction. The advantage of this kit is that the solutions are readymade and quality controlled so that concentrations determined at different times are comparable.

The technique has two protocols, 'protein in solution' and 'precipitated protein'; the latter is suitable for protein samples that are in rehydration buffer (see section 2.8) or other buffers which would otherwise interfere with the assay.

2.7.2 Materials

Precipitant

10 % trichloroacetic acid (Sigma)

5 M sodium chloride (BDH)

Co-precipitant

1 % sodium deoxycholate (BDH)

Copper solution

1.6 mM tartaric acid (BDH)

0.6 mM copper sulphate (BDH)

0.6 M sodium hydroxide (BDH)

Colour reagent A

1.4 mM ascorbic acid (BDH)

Colour reagent B

65 mM bathocuproine disulphonic acid disodium salt (BDH)

Working reagent

100 parts colour reagent A

1 part colour reagent B

Protein standard

2 mg/ml BSA (GIBCO)

SpectraMAX 440 96-well plate-reader (Molecular Devices)

2.7.3 Protocol

2.7.3.1 *Determination of precipitated protein*

This protocol was used when samples were in buffers, such as rehydration buffer, that contained components that would interfere with the protein assay. Duplicate tubes containing 0, 5, 10, 15, 20 and 25 μl of a 2 mg/ml BSA standard solution and 5-10 μl of sample/s diluted to approximately 0.5-50 $\mu\text{g}/\mu\text{l}$ were prepared. To each tube was added 500 μl of precipitant, the tube briefly vortexed and the sample left to incubate for 2-3 min at RT. To this 500 μl of co-precipitant was added, the tube briefly vortexed again and centrifuged at $>10,000 \times g$ for 5 min. After decanting the supernatant, the tubes were briefly re-centrifuged and any remaining supernatant removed. Copper solution (100 μl) was added to each tube and vortexed vigorously to dissolve the precipitate; to this 500 μl of working-reagent was added, mixed by inversion and incubated at RT for 15 min. 250 μl from each tube was pipetted into a 96-well plate, using milli-Q- H_2O as a blank and the absorbances read at 485 nm, using a SpectraMAX 440 spectrophotometer. SoftMAX Pro software was used to generate a linear standard curve and to determine the protein concentration of the samples.

2.7.3.2 *Determination of protein in solution*

Sample solutions free of any substances that could interfere with the assay were analysed directly in solution. Standards of BSA at concentrations of 0, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 mg/ml were prepared. Duplicate tubes containing 5 μl of each standard or 5 μl of the sample/s diluted to approximately 0.25-2.0 mg/ml were prepared. Copper solution (100 μl) was added to each tube and briefly vortexed, followed by 500 μl of working reagent. This was mixed by inversion and incubated at RT for 15 min. As above, 250 μl from each tube was pipetted into a 96-well plate, using milli-Q- H_2O as a blank and the absorbances read at 485 nm, using a SpectraMAX 440

spectrophotometer. SoftMAX Pro software was used to generate a linear standard curve and to determine the protein concentration of the samples.

2.8 Two-dimensional gel electrophoresis

2.8.1 Introduction

Two-dimensional gel electrophoresis separates proteins by their isoelectric point (pI) in the first dimension using isoelectric focusing (IEF), followed by SDS-PAGE in the second dimension, in which proteins are separated by their molecular weight (MW) (see Figure 2.5). Prior to this, samples must be prepared by converting the proteins from their native state into a form suitable for IEF and SDS-PAGE, while still maintaining their native charge and MW. This process typically involves solubilisation, disaggregation, denaturation and reduction (Shaw and Riederer, 2003). The rehydration buffer contains components to denature, disaggregate, reduce and solubilise proteins, and is applied to the sample prior to IEF. IEF is performed using precast gels that contain an immobilised pH gradient (IPG strips). Before the second dimension is run, focused IPG strips are equilibrated in two buffers which reduce and alkylate the protein, and which also contain SDS, a negatively charged molecule, which denatures and binds to proteins. The resulting additional negative charge is directly proportional to the MW of the protein. Consequently, separation occurs exclusively by MW when electrophoresis is performed in the second dimension (Schägger and Von Jagow, 1987).

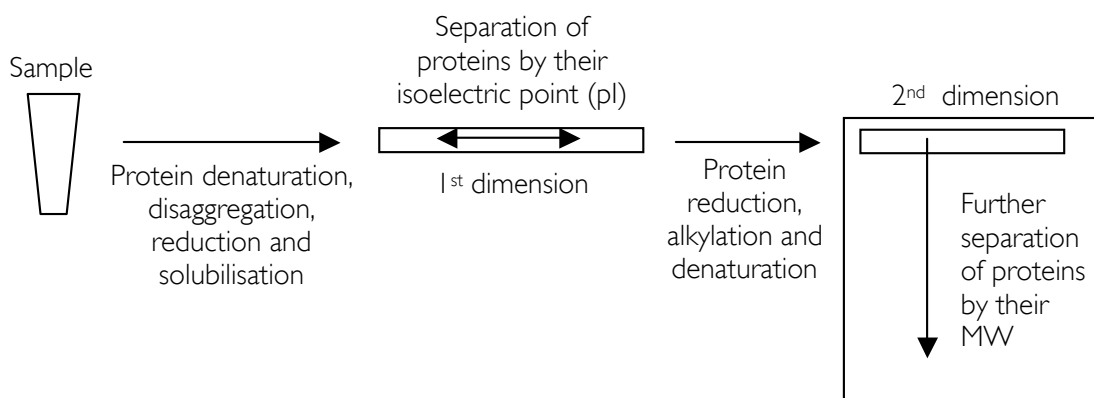


Figure 2.5: The three main steps of two-dimensional gel electrophoresis are unfolding and linearisation of the proteins, separation of the proteins by their pI, and then separation by MW.

2.8.2 Isoelectric focusing

2.8.2.1 Materials

Immobiline DryStrip gels 18 cm pH 3-10 NL (IPG strip) (Amersham Biosciences)

Immobiline DryStrip Reswelling Tray (Amersham Biosciences)

Bio-Rad Protean IEF Cell (Bio-Rad)

Ondina oil (Hansen and Rosenthal)

Urea rehydration buffer

9 M urea (Amersham Biosciences)

2 % w/v CHAPS (AppliChem)

1 % v/v Pharmalyte 3-10 for IEF (Amersham Biosciences)

50 mM dithiothreitol (DTT) (AppliChem)

Trace bromophenol blue (BPB) (May and Baker)

Thiourea and urea rehydration buffer

7 M urea (Amersham Biosciences)

2 M thiourea (Amersham Biosciences)

2 % w/v CHAPS (AppliChem)

1 % v/v Pharmalyte 3-10 for IEF (Amersham Biosciences)

50 mM DTT (AppliChem)

Trace BPB (May and Baker)

2.8.2.2 Protocol

The rehydration buffer was made in the following order. Urea and thiourea were made up to 8 ml with milli-Q-H₂O and dissolved with vigorous mixing and warming in a water bath set at 37°C. Desiccated DTT was allowed to come to RT before using, the remaining reagents were added and a small amount of BPB added to give a strong blue colour. The solution was made up to 10 ml with milli-Q-H₂O and mixed thoroughly; aliquots of 0.5 ml were stored at -20°C until use. The amount of dried protein sample required for 2D-PAGE was resolubilised in rehydration buffer (see Table 2.4).

Table 2.4: The volume of rehydration buffer and amount of protein used to rehydrate the IPG strips.

Strip size	Volume of rehydration buffer	Amount of sample protein
7 cm	125 µl	100 µg
18 cm	340 µl	250-300 µg

Samples in rehydration buffer were vortexed vigorously every 10 min over a period of 1 h, during which time samples were left at RT. The samples were then centrifuged at $>10,000 \times g$ for 5 min to pellet any insoluble material. The supernatant was removed and pipetted evenly along the well of a rehydration tray. An IPG strip was removed from the -20°C and placed gel-face down in the well and gently moved back and forth until the whole gel surface was wet. A small volume of ondina oil ($\sim 2\text{-}3$ ml) was applied over the top of the IPG strip and it was left for 16 h to fully rehydrate at RT. Rehydrated strips were removed from the tray, rinsed with milli-Q- H_2O and blotted on moistened filter paper. The strip was placed gel face down in an IEF tray, with its positive end at the anode. Moistened filter wicks were placed at each end between the electrode and the strip and 2-3 ml of ondina oil applied to cover the strip.

Strips were focused on the protean IEF cell according to manufacturer's instructions (Bio-Rad), according to the following parameters.

Voltage: rapid ramp to 10,000 V

Current: 50 μA limit per strip

Length: 60,000 V h

The filter wicks were changed several times in first few hours or until the voltage was above ~ 3000 V. After 60,000 V h, focused strips were removed immediately, wrapped in cling film and stored at -80°C prior to 2D-PAGE in the second dimension.

2.8.3 SDS-PAGE

2.8.3.1 *Materials*

ExcelGel SDS XL 12-14 gradient gels (Amersham Biosciences)

ExcelGel SDS buffer strips (Amersham Biosciences)

Multiphor II Electrophoresis Unit (Amersham Biosciences)

Multitemp II Thermostatic Circulator (Amersham Biosciences)

EPS 3500 XL power supply (Amersham Biosciences)

Electrode wicks (Bio-Rad)

Filter paper (Whatman 32 cm 4 Qualitative)

Ondina oil (Hansen and Rosenthal)

Tris-HCl

0.5 M tris (GIBCO)

Adjust to pH 6.8 with HCl

DTT equilibration buffer

5 M urea (Amersham Biosciences)

0.05 M tris-HCl

30 % v/v glycerol (Scharlau)

0.03 M SDS (BDH)

0.05 M DTT (AppliChem)

Iodoacetamide (IAA) equilibration buffer

5 M urea (Amersham Biosciences)

0.05 M tris-HCl

30 % v/v glycerol (Scharlau)

0.03 M SDS (BDH)

0.2 M IAA (Sigma)

Trace BPB (May and Baker)

2.8.3.2 Protocol

ExcelGel gradient gels were used in the second dimension, with the Multiphor II flatbed system, according to manufacturer's instructions (Amersham Biosciences). Focused IPG strips were equilibrated in DTT equilibration buffer for 15 min at RT, followed by IAA equilibration buffer for 15 min at RT. SDS-PAGE was carried out at 15°C according to the following electrophoretic parameters.

When running one gel:

Step 1	200 V	20 mA	20 W	40 min
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At the end of step 1, IPG strip removed and buffer strip repositioned.

Step 2	800 V	40 mA	40 W	2 h 40 min
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When running two gels:

Step 1	200 V	40 mA	40 W	40 min
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At the end of step 1, IPG strips removed and buffer strips repositioned.

Step 2	800 V	80 mA	80 W	2 h 40 min
--------	-------	-------	------	------------

Following electrophoresis, gels were stained with SYPRO Ruby, digitised and subsequently over-stained with Colloidal Coomassie, see following section.

2.9 2D gel staining

2.9.1 Introduction

SYPRO Ruby protein gel stain is a recently designed fluorescent stain for proteomic studies and has many advantages over other stains such as Colloidal Coomassie or silver (Lopez et al., 2000). SYPRO Ruby and silver are 10-100 fold more sensitive than Colloidal Coomassie or standard Coomassie; Colloidal Coomassie's lower protein detection limit is around 5 ng, silver staining and SYPRO Ruby have comparably lower limits of ~0.5 ng (Berggren et al., 2002). Compared with SYPRO Ruby, the staining procedure for silver is complex with multiple-steps, which can lead to increased variability between gels. Most significantly, unless gluteraldehyde is omitted from the formulation, silver stained spots cannot be used for peptide mass fingerprinting, since proteins are covalently modified. However, the omission of gluteraldehyde leads to significantly decreased sensitivity and higher background (Berggren et al., 2002). Both SYPRO Ruby and Colloidal Coomassie are compatible with peptide mass fingerprinting, since neither stain covalently modifies proteins. SYPRO Ruby's linear dynamic range is orders of magnitude better than that of silver and Colloidal Coomassie, meaning quantitative differences in protein expression can be more reliably detected (Lopez et al., 2000). The staining period is not critical for SYPRO Ruby, so gels can be left overnight without any risk of over-staining (Berggren et al., 2002). However, being a fluorescent dye, spots stained with SYPRO Ruby are not visible to the naked eye. In order to excise spots for MS identification, the gels must be over-stained with Colloidal Coomassie.

2.9.2 SYPRO Ruby staining

2.9.2.1 Materials

Fixing and destaining solution

10 % v/v methanol (BDH)

7 % v/v glacial acetic acid (BDH)

SYPRO Ruby protein gel stain (Molecular Probes, BIO-RAD)

FujiFilm FLA-3000 (FujiFilm Corp.)

2.9.2.2 Protocol

Following electrophoresis, the gels were immediately placed in fixing solution for 1 h at RT with agitation, then stained with SYPRO Ruby for 19 h at RT in the dark with agitation, and then destained for 1 h at RT in the dark with agitation. Gels were given a final milli-Q-H₂O wash prior to image acquisition and were subsequently stored in milli-Q-H₂O in light-proof containers.

2.9.2.3 Imaging

A FujiFilm fluorescent image analyser FLA-3000 was used for image capture. The glass stage was wiped clean with 70 % ethanol and milli-Q-H₂O prior to use. The gel was placed gel-face down on the stage and the plastic backing wiped clean of oil and streaks using milli-Q-H₂O. The glass stage was placed inside the FLA-3000 and a digital image captured at a wavelength of 473 nm with a 580 nm filter. The resulting image was manipulated in Adobe Photoshop to increase the contrast and label the MW and pI.

2.9.3 Colloidal Coomassie staining

2.9.3.1 Materials

Colloidal Coomassie solution

1.2 mM Coomassie brilliant blue R250 (Serva)

34 % w/v methanol (BDH)

1.3 M ammonium sulphate (BDH)

3 % w/v orthophosphoric acid (BDH)

Destaining solution

1 % glacial acetic acid (BDH)

2.9.3.2 Protocol

Colloidal Coomassie was made in the following order. Coomassie powder was dissolved in methanol before ammonium sulphate, and then orthophosphoric acid were added. The solution was made to volume with milli-Q-H₂O. Ammonium sulphate was dissolved by vigorous mixing. Gels were stained for 16-24 h at RT with agitation, rinsed thoroughly in milli-Q-H₂O, then destained in 1 % acetic acid for 1 h at RT to enhance

staining and remove background staining. Gels were stored at 4°C in milli-Q-H₂O in sealed bags.

2.10 Protein identification by peptide mass fingerprinting

2.10.1 Introduction

After separation by 2D-PAGE, individual protein spots can be identified using a procedure called peptide mass fingerprinting. Excised proteins are digested into peptides using proteases such as trypsin. Peptides are easier to elute and analyse by MS than full-length polypeptides, and provide more mass information. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) MS is used to gain mass information for all the peptides. The protein can then be identified by comparing the experimental peptide masses with databases containing theoretical masses for large numbers of previously sequenced proteins (Pandey and Mann, 2000; Aebersold and Mann, 2003).

In MALDI-TOF mass spectrometry, a portion of the digested sample is dried in excess matrix solution on a stainless steel mass spectrometry plate and inserted into the mass spectrometer. Short pulses of ultraviolet laser light are emitted which are strongly absorbed by the matrix, resulting in desorption and ionisation of the sample and matrix. An electric field accelerates the sample ions, which subsequently collide with an electron multiplier detector. Because the velocity of each ion is proportional to the mass to charge ratio ($m/z^{1/2}$), the mass of each ion is proportional to the time of flight (TOF) taken to reach the detector (Schönberger, 2001; LoPachin et al., 2003).

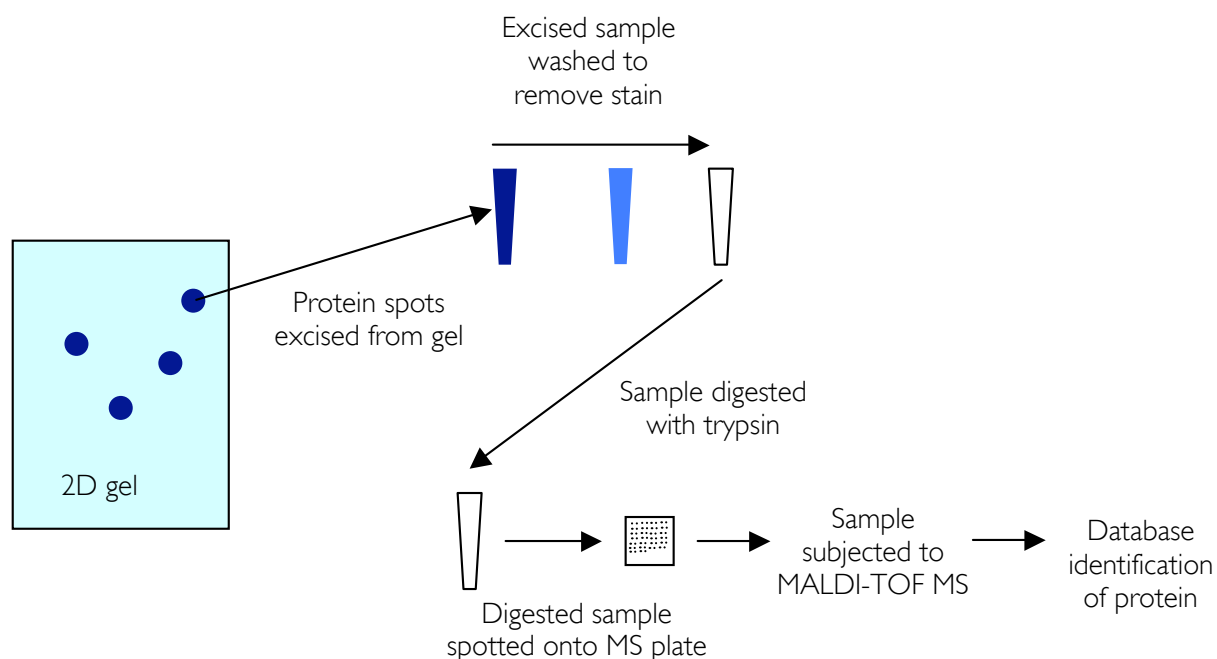


Figure 2.6: Summary of the method employed to identify proteins previously separated by 2D-PAGE.

2.10.2 In-gel trypsin digestion of proteins

2.10.2.1 Materials

Ammonium bicarbonate (NH_4HCO_3) (BDH)

50 mM NH_4HCO_3

Adjusted to pH 7.8

Sequencing grade trypsin (Promega)

15 ng/ μl dissolved in 25mM NH_4HCO_3

Sequencing grade trifluoroacetic acid (TFA) (Applied Biosystems)

Acetonitrile (MeCN) (Scharlau)

Speedvac protein concentrator (Eppendorf)

2.10.2.2 Protocol

Spots were excised, diced finely and destained with 50 % MeCN in 25 mM NH_4HCO_3 . Samples were incubated at 37°C for 10 min with agitation, then briefly centrifuged to allow the supernatant to be removed and replaced with fresh 50 % MeCN in 25 mM NH_4HCO_3 . This wash was repeated until no blue colour, from the Coomassie stain, was visible. The samples were dried in a speedvac protein concentrator and the pellets rehydrated with 8 μl of a 15 ng/ μl trypsin solution (or sufficient solution to cover gel

pieces). The sample and trypsin solution were briefly centrifuged to the bottom of the tube, covered and incubated at 37°C. After incubating for 16 h, 8 µl of a 50 % MeCN and 1 % TFA solution in milli-Q-H₂O was added. Tubes were sonicated for 20 min and stored at -80°C until analysis by MS was performed (see Figure 2.6).

2.10.3 MALDI-TOF mass spectrometry

2.10.3.1 Materials

Matrix solution

10 mg/ml α -cyano-4-hydroxycinnamic acid (α -CHC) matrix (Hewlett Packard)

50 % v/v MeCN (Scharlau)

1 % v/v TFA (Applied Biosystems)

Calibration mixture

1 x v/v cal-2 MS calibration mixture (Applied Biosystems)

10 mg/ml α CHC (Hewlett Packard)

Stainless steel 10x10 MS place plate (Voyager MALDI-TOF plate) (Applied Biosystems)

Voyager-DE Pro mass spectrometer (Applied Biosystems)

2.10.3.2 Protocol

Trypsin digested samples were dried in the speedvac protein concentrator and then resolubilised in 3 µl of a 50 % MeCN and 1 % TFA solution in milli-Q-H₂O. The volume of sample and matrix loaded depends on the MALDI-TOF plate used. The plate used here required that 1 µl of sample and 1 µl of matrix solution was loaded onto each sample spot, while 0.5 µl of calibration mix and 0.5 µl of matrix solution was loaded on each calibration spot (see Figure 2.7).

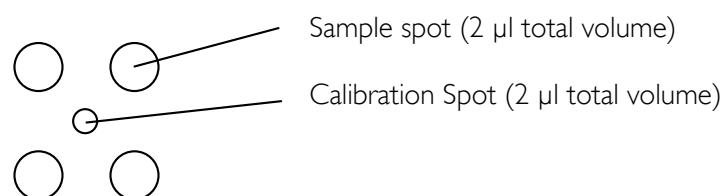


Figure 2.7: Four sample spots were spotted around each calibration spot, the machine was calibrated on the calibration spot before the four surrounding samples were analysed, this is known as triangulation for near-point calibration.

After the spots had air-dried, the samples were analysed on a Voyager-DE Pro mass spectrometer. The machine was calibrated three times on each calibration spot prior to analysis of the four surrounding sample spots. The spectra for each spot were deisotoped for deuterium adducts after the baseline was adjusted and background noise removed. The mass lists then exported for submission to protein databases.

2.10.4 Database identification of proteins

2.10.4.1 Internet addresses

Mascot peptide mass fingerprinting tool

<http://www.matrixscience.com>

MS-Fit peptide mass fingerprinting tool

<http://prospector.ucsf.edu>

SwissProt protein database

<http://au.expasy.ch>

2.10.4.2 Protocol

Peptide masses were initially submitted to the SwissProt database by MS-Fit under the following conditions: species, mammalia; peptide masses, monoisotopic, cysteines modified by carbamidomethylation; mass tolerance, \pm 50-200 ppm; enzyme, trypsin; maximum missed cleavages, 1; minimum peptide matches, 4; possible modifications, oxidation of M and acrylamide modified cysteines; pI, all; MW, 1,000 Da to 100,000 Da; P factor, 0.4.

MS-Fit results are ranked by the MOWSE score, which takes into account the MW of each peptide relative to the MW of the whole protein, and also missed cleavages, which contribute a P factor to the score.

Results from Mascot were used to confirm those gained from MS-Fit, using the following conditions: database Swissprot; taxonomy, mammalia; enzyme, trypsin; missed cleavages, 1; variable modifications, carbamindomethyl (C) and oxidation (M); peptide tolerance, \pm 50-200 ppm, mass values, $[M+H^+]$; monoisotopic.

Mascot scores are a measure of the statistical significance of a match.

Chapter three – results and discussion

3.1 Isolation and culture of neuronal cells

3.1.1 Primary cell culture

Due to the difficulty of isolating and culturing primary cortical neurons, media and supplements specifically developed for neuronal cells were used at all stages. Hibernate-A medium (see Appendix for composition) supplemented with B27 was used during the dissection and dissociation of the brain tissue. This medium has been shown to allow refrigerated storage of undissociated brain pieces for several days without loss of neuronal survival (Brewer and Price, 1996; Kivell et al., 2000). This feature of the medium was highly desirable as it removed the time constraints, allowing the specific removal of cortical regions, complete dissociation of the tissue pieces, and gradient enrichment of neurons prior to the cells being plated. Techniques not utilising Hibernate-A generally recommend that no more than 1-2 h pass between tissue removal and plating of the cells (Buse, 1985; Kivell et al., 2000). Neurobasal-A medium was used for all stages of cell culture and experimentation. Neurobasal-A is a bicarbonate buffered medium based on Dulbecco's Modified Eagle's Medium (DMEM) and has been optimised for growth of fetal and postnatal neurons from a variety of brain regions (Brewer et al., 1993; Kivell et al., 2000). This was supplemented with B27, basic fibroblast growth factor (bFGF), GlutaMax, penicillin and streptomycin. GlutaMax was used instead of glutamine because the latter can breakdown to form ammonia, a substance which is neurotoxic to neurons (Kivell et al., 2000). For normal neuronal development to occur a group of neurotrophic proteins must be present in the extracellular environment. These factors, which include NGF and FGF, promote survival, growth, morphological plasticity and synthesis of proteins for neuronal differentiation (Lee et al., 2000). bFGF, used in these studies, has been found to be extremely neurotrophic to cultured neurons and is found at high levels in the developing rat brain *in vivo* (Fernandez-Sanchez and Novelli, 1993). It has been shown that glial cell proliferation is inhibited in cell cultures grown in serum-free medium, resulting in purer populations of neurons. This has the added advantage that drugs to inhibit glial cell division, which can also reduce the viability of the neurons (Marriot et al., 1995; Kivell et al., 2000; Moldrich et al., 2001), need not be added.

After 14 days in culture (DIC) a confluent population of cells, shown in Figure 3.1, was observed. Morphologically, the cells appeared to have the characteristics of neurons, with large cell bodies and neurite-like projections to surrounding cells.

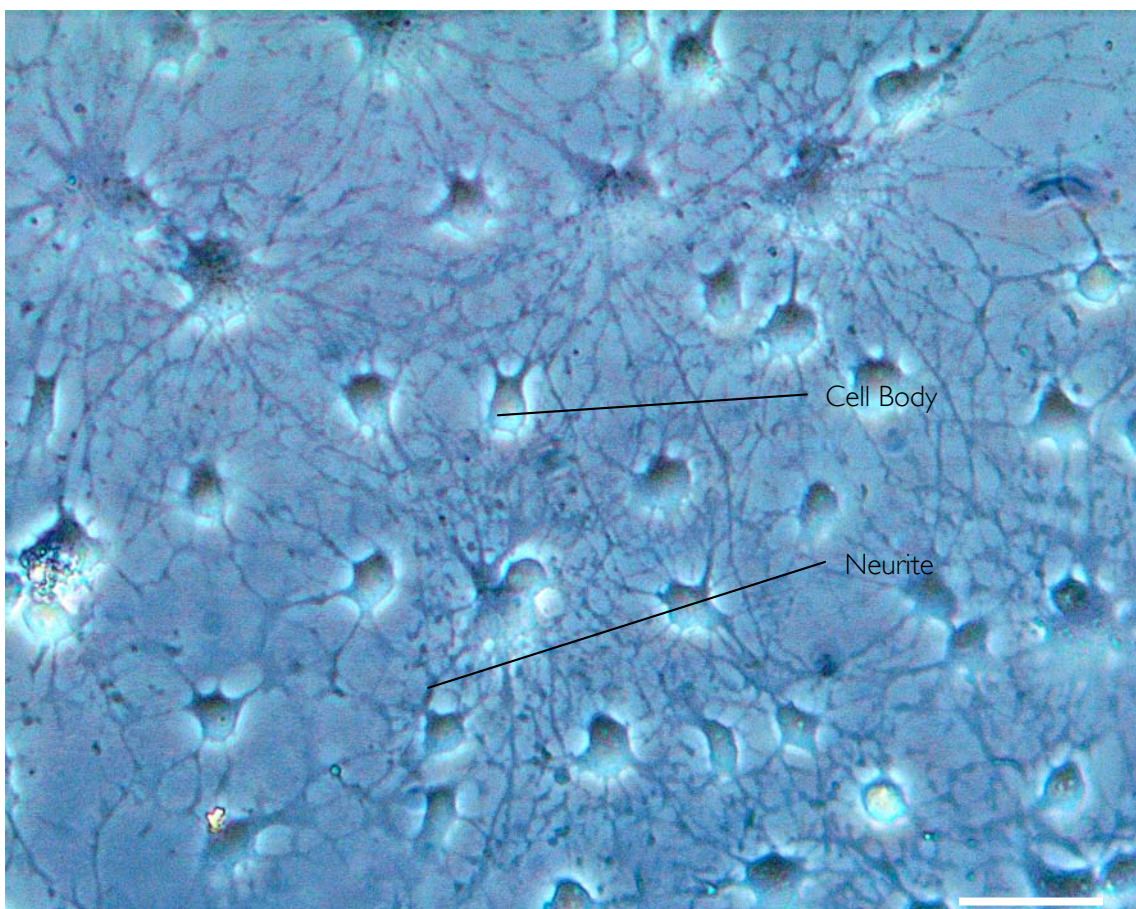


Figure 3.1: Phase-contrast microscopy of primary rat cortical cells after 14 days in culture. A cell body and neurite are indicated. Scale bar = 30 μm .

Embryonic and early postnatal rats are the traditional sources of tissue for the establishment of neuronal and glial cell cultures. This is because as the cells develop, extensive axonal and dendritic arbors form, making the cells much harder to dissociate from one another, and increasing the likelihood of damaging the cells. The connective tissue is also easier to remove at this age (Banker and Goslin, 1997). This was observed here—brain tissue from 4-5 month old rats failed to produce significant numbers of cells, while the same technique applied to postnatal 21-day old rats was successful.

3.2 Characterisation of cultures

Visually inspecting cells to determine their type is difficult and time consuming, and when cells are at high-density it is often not possible to differentiate between individual

cells. The use of immunocytochemistry, such as that used here, allows a non-biased method of cell identification (Banker and Goslin, 1997). Cytoskeletal proteins are generally expressed at high levels throughout the cell and are desirable antibody targets when their expression is cell-specific (Banker and Goslin, 1997).

NeuN is expressed in neurons of the CNS and PNS and has been shown to be an excellent marker for neurons in primary cultures (Mullen et al., 1992; Mertz et al., 2002). Extensive staining of the cell body and axons by the anti-NeuN antibody was observed in these studies (See Figure 3.2). Glial fibrillary acidic protein (GFAP) is a cytoskeletal intermediate filament subunit that is expressed in type 1 and 2 astrocytes. Astrocytes are one of the most common non-neuronal cell types found in primary cultures (Banker and Goslin, 1997). The use of GFAP to quantify the proportion of astrocytes in primary cultures is common in the literature (Kivell et al., 2000; Velasco et al., 2003). Double-staining, in which two primary antibodies are used at the same time (see Figure 3.3), allows the ratio of cell types in a mixed culture to be determined and gives confirmation that cells that stain for a neuronal marker do not stain for a non-neuronal marker, and vice versa (Banker and Goslin, 1997). No co-expression of these two markers was detected in any instance when cells were double-stained using both anti-NeuN and anti-GFAP antibodies (see Figure 3.3). In addition, no cells were observed that didn't stain for either. These experiments confirmed that most cells were neuronal, and demonstrated that some astrocytes were also present.

Prior to application of the primary antibody, a fixative is applied to the cells. Fixatives cross-link, oxidise, or denature proteins, thereby rendering them insoluble so that they remain in the tissue (Larsson, 1988). This makes the cells or tissue suitable for immunocytochemistry. Four fixative solutions were initially trialled and compared. Neutral buffered formalin (NBF) gave the best result, and was used for all immunocytochemistry shown here (see Figures 3.2, 3.3 and 3.16).

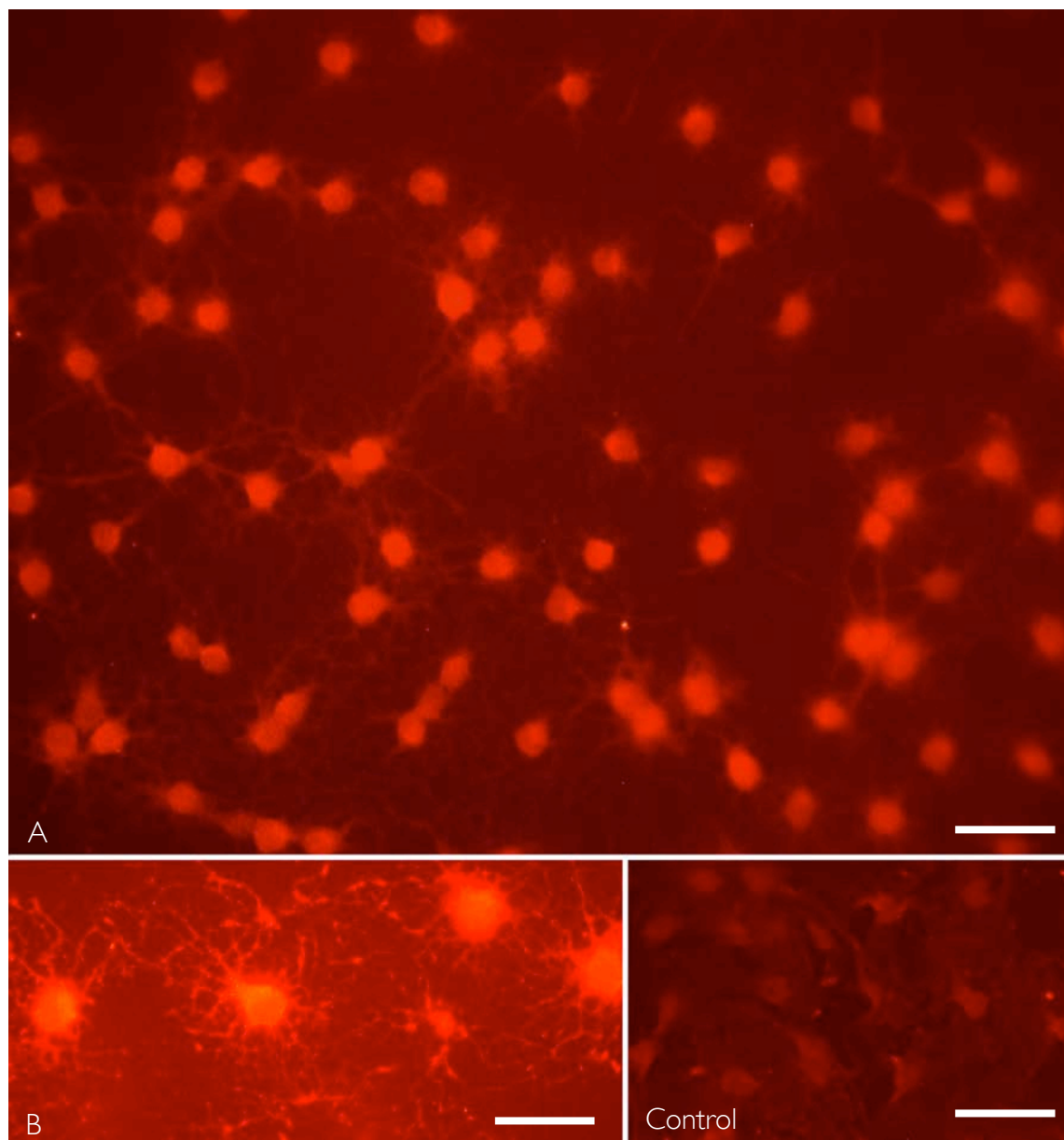


Figure 3.2: Immunocytochemistry of primary cultures. Ultraviolet photomicrographs showing staining of the cultures with anti-NeuN antibodies in red. Strong staining of the cell bodies and neurites (A and B) confirms the identity of these cells as neurons. Omission of the primary anti-NeuN antibody in the control eliminated staining. Scale bar A, control = 30 μm ; B = 15 μm .

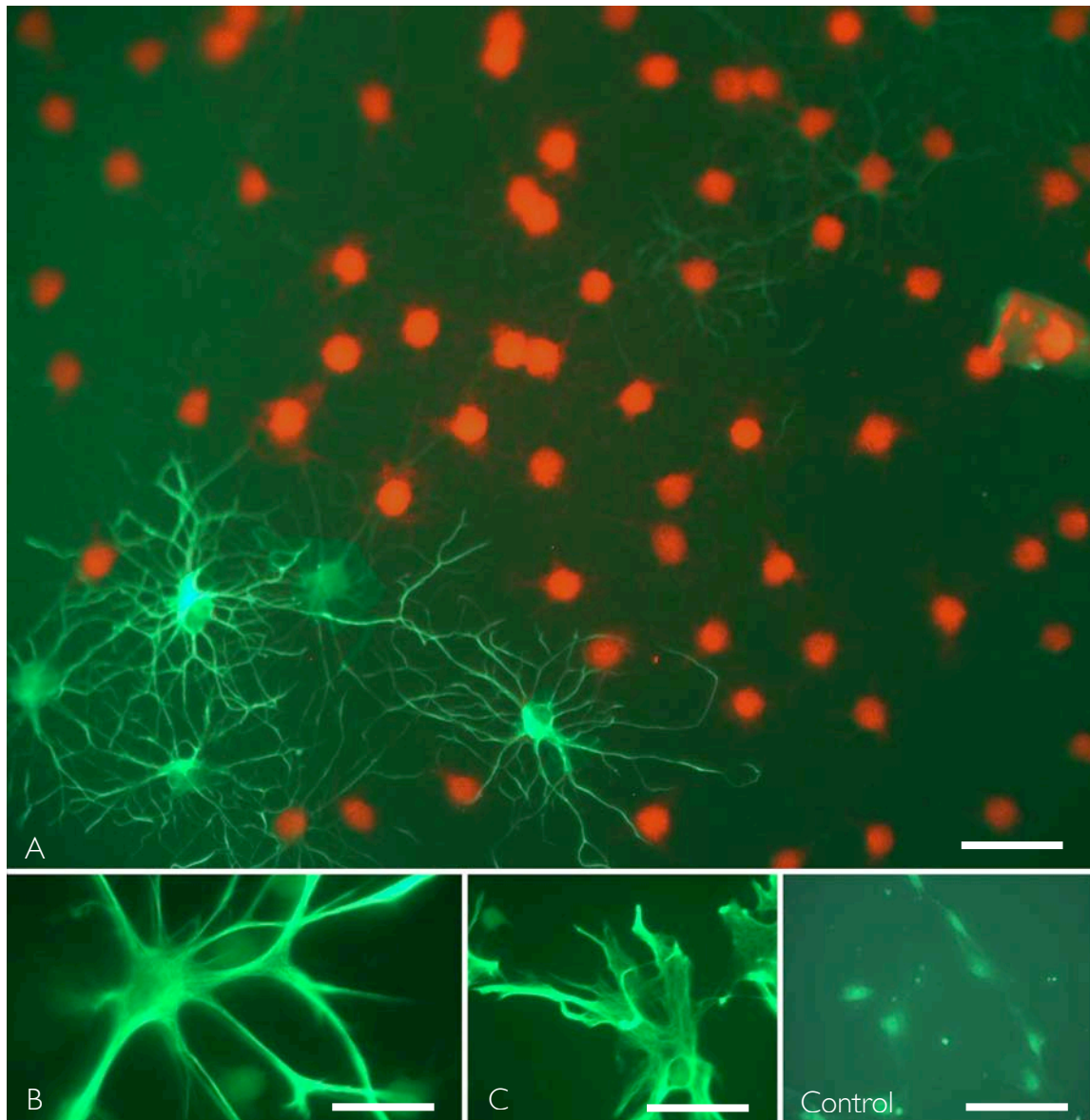


Figure 3.3: Ultraviolet photomicrographs showing labelling of neurons with anti-NeuN (red) and astrocytes with anti-GFAP (green). Image A shows cultures double-stained for the two antibodies; Adobe Photoshop was used to overlay the images. Images B and C show staining of astrocytic processes and neurites. Omission of the primary anti-GFAP antibody in the control eliminated staining. Scale bar A, control = 30 μm ; B, C = 15 μm .

As discussed above, it has been shown that the use of Neurobasal-A supplemented with B27 allows the establishment of almost pure neuronal cell populations. In contrast, more traditional approaches using media supplemented with serum typically have much higher proportions of glial cells, as much as 46 % in some studies (Deupree et al., 1996; Velasco et al., 2003). Glial cells grow well in media containing serum proteins and albumin for one, has been shown to stimulate mitosis (Nadal et al., 1995; Dziegielewska et al., 2000). For instance, when fetal calf serum (FCS) is added to neural cell progenitors, non-neural cells become dominant (Dziegielewska et al., 2000). The previous findings are consistent with the cultures achieved here, in which approximately 95 % of cells were characterised as neuronal, as determined by cell counting.

Astrocytes play important roles in the CNS and are one of the most abundant glial cell types. *In vitro* studies have demonstrated diverse roles for astrocytes in culture, for instance they have been shown to aid in neuronal attachment and growth, secreting adhesion and ECM proteins, as well as a range of growth factors and cytokines (Wang and Cynader, 1999; Tabernero et al., 2002a). However, many of their functions are mediated through the release of amino acids and proteins into the extracellular fluid. Using a similar approach to that employed here, a recent study was able to identify over 30 proteins secreted into the conditioned media of cultured astrocytes (Lafon-Cazal et al., 2003). As neurons are the cells of interest here, proteins secreted by astrocytes would further complicate the analysis of the conditioned media. While the inclusion of greater numbers of astrocytes and other glial cells would more accurately mimic the conditions *in vivo*, it would be very difficult to identify the cell type responsible for any identified secreted proteins in a mixed culture.

3.3 Cell survival assays

3.3.1 Survival in B27-free conditions

Cells are typically cultured in the presence of serum, such as FCS or horse serum. Serum is used in cell culture as it contains factors that aid in cellular attachment, neurite outgrowth and cell survival, amongst others (Wang and Cynader, 1999). A defined supplement called B27 (see Table 3.1), optimised for the culture of neurons, was used in these studies instead of serum. However, when studying the protein composition of conditioned medium, it is necessary to work with serum/supplement free media. The

cell-produced proteins are at much lower concentrations than that of the serum/supplement proteins and would otherwise be masked (Volmer et al., 2004). B27 contains proteins such as insulin, BSA and transferrin (see Table 3.1). The presence of these proteins poses several problems: they can severely limit the amount of non-abundant proteins that can be loaded on a 2D gel, the spots they produce may mask or displace other spots, leading to incorrect pI/MW measurements. This can lead to a failure to identify what could be proteins of interest (Shaw and Riederer, 2003).

In these studies no serum was used at any stage of the culturing process. The use of defined medium such as Neurobasal-A (See Appendix for composition) and defined supplements such as B27 allows for complete control of all culturing conditions *in vitro*, and eliminates the variability inherent in the use of serum (Banker and Goslin, 1997).

Table 3.1: Composition of B27 medium supplement for neuronal cultures (Brewer et al., 1993)

Biotin	Selenium
L-carnitine	T3 (triiodo-L-thyronine)
Corticosterone	DL- α -tocopherol (vitamin E)
Ethanolamine	DL- α -tocopherol acetate
D(+)-galactose	<i>Proteins</i>
Glutathione (reduced)	Albumin, bovine serum (fraction V)
Linoleic acid	Catalase
Linolenic	Insulin
Progesterone	Superoxide dismutase
Putrescine	Transferrin
Retinyl acetate	

While it is necessary to remove the B27 supplement when investigating the conditioned medium, this results in non-optimal conditions, and can result in increased cell-death and the subsequent release of cytoplasmic proteins by cell lysis. The levels of many cytoplasmic proteins, especially 'housekeeping' proteins, are not only at levels far greater than those of secreted proteins, but their presence in the conditioned medium makes it difficult to determine what is actually secreted. It was necessary therefore, to determine how long cultured cortical cells remained viable in B27-free growth medium. Growth medium was composed of Neurobasal-A medium and additives such as antibiotics and

growth factors (see Chapter 2: Section 2.2). Over a four-day period LDH levels were found to remain unchanged in cultures grown in B27-free growth medium and were comparable to those of cultures grown in B27 supplemented growth medium. LDH is a cytosolic protein and its level in the surrounding medium correlates with the number of dead cells. Cells do not secrete LDH, but loss of membrane integrity, which occurs after cell death, results in its release (Klingman et al., 1990; Legrand et al., 1992; Uliasz and Hewett, 2000).

Table 3.2: LDH levels in the conditioned medium of neuronal cells grown in either B27-free growth medium or growth medium containing B27. LDH levels were the same in both conditions throughout the 96 h period, indicating that cell survival was no lower when B27 was removed.

Time (hr)	LDH (units/litre/ 10^4 cells)	
	B27 present	B27-free
6	10, <5	5, <5, <5, <5
12	<5, <5	6, <5, <5, <5
24	<5, <5	14, <5, <5, <5
30	<5, <5	<5, <5, 6, <5
96	<5, <5	5, <5, <5, <5

The amount of LDH released by dead cells can be significantly different depending on the cell-type (tumour or primary) and cell number or density (Jurisic, 2003). As would be expected, the number of cortical cells surviving the isolation, plating and culturing protocol used here varied, and consequently some cultures had higher cell densities than others. So that the LDH values would be comparable between cultures, the LDH values from Medlab diagnostics, in units/litre, were normalised to units per litre per 10^4 cells (units/litre/ 10^4 cells). Cells also release LDH spontaneously, despite being healthy (Konjevic et al., 1997). The LDH levels in Table 3.2 most likely represent this background level of spontaneous release.

Typically, total LDH, which represents all the LDH present intracellularly and extracellularly, is also determined by total cell lysis. This value represents 100 % cell death, and comparing experimental values to this allows an estimation of the neuronal survival in that experiment (Klingman et al., 1990; Konjevic et al., 1997). This was not done here, however, and was not required. Cell-survival was high under normal B27-

containing culturing conditions, as determined by visual inspections and the lack of dead-floating cells in the medium. Because LDH levels under these normal conditions were the same as those under B27-free conditions, it is reasonable to conclude that cell survival was also the same under both conditions, demonstrating that the cortical cultures remained viable under culturing conditions that did not include B27, for at least 96 h. Based on these results, conditioned medium was collected from unstimulated cultures (no kainic acid exposure), after 72 h of growth in B27-free growth medium. 2D gels of this conditioned medium are shown in Figures 3.6 and 3.7 (see Section 3.4).

3.3.2 Survival after kainic acid exposure

3.3.2.1 Source and action of kainic acid

Kainic acid is a naturally occurring excitatory amino acid that acts via the kainite ionotropic GluR subtype. It has been extensively used as an excitotoxin in experimental research on neurodegeneration (Novelli et al., 1988; Black et al., 1995; MacManus et al., 1997; Uliasz and Hewett, 2000; Tasker et al., 2002)

Up until the year 2000, only one supplier of kainic acid (KA) was available, however, this source is no longer available. Three different companies now provide KA, but all three have different sources and purification techniques from one another and are also different to the sole source prior to year 2000. Subtle differences do exist between the effects of the four brands, and it has been recommended that they not be used interchangeably (Tasker et al., 2002). For the studies detailed here, a synthetically produced form of KA sold by Sigma was used.

3.3.3 Live/dead assay, comparison to LDH as a measure of viability

The ratio of live (green) to dead (red) cells was used to determine the proportion of cells that survive KA exposure at a variety of concentrations (see Figure 3.4). This was also compared to the LDH measurements to see whether they correlated in these studies. LDH has the advantage over live/dead assays in that it should be representative of all cells that have died, including ones that are floating in the medium. Live/dead assays, on the other hand, are limited to cells still adhered to the dish.

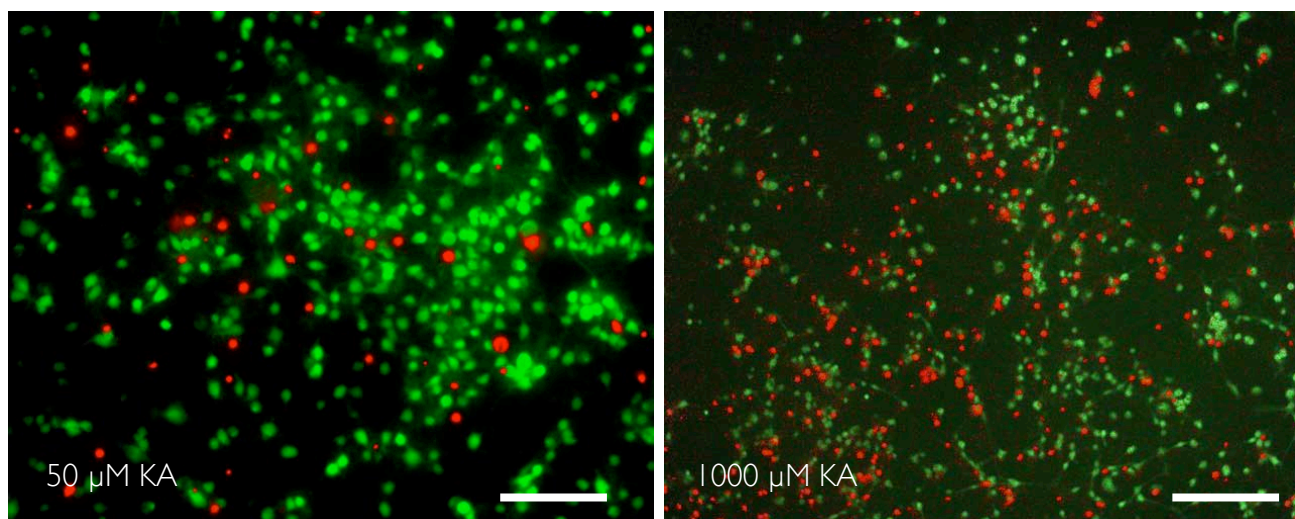


Figure 3.4: Photomicrographs of the live/dead assay shortly after exposure to 50 μM KA (left) and 1000 μM KA (right) for 24 h. Cells stained green with fluorescein diacetate are viable, while cells stained red with propidium iodide are dead. Scale bar 50 μM KA = 100 μm ; 1000 μM KA = 200 μm .

Neuronal survival in these studies is higher than that previously reported by Tasker et al. (2002) in a similar experiment comparing three brands of KA. In that study greater than 80 % of neurons were killed after 24 h exposure to 1000 μM KA, compared with approximately 60 % in this study (see Figure 3.5 and Table 3.3). The difference may be due to the culturing conditions used. Tasker et al. (2002) cultured neurons in serum supplemented medium. Velasco et al. (2003) have shown that cortical neurons grown in Neurobasal-A medium supplemented with B27 are significantly more resistant to excitotoxic cell death than those grown in serum containing medium. The researchers theorised that this was likely due to antioxidants, such as selenium and vitamin E, present in the B27 supplement. The assays performed here used neuronal cultures that had been switched to B27-free Neurobasal-A growth medium prior to application of KA. It is likely, however, that residual antioxidants would still be present intracellularly and on the culture dish. In addition, the growth factor bFGF was present in the medium during the assays and this has also been shown to increase survival after GluR mediated excitotoxicity in neuronal cell cultures (Fernandez-Sanchez and Novelli, 1993). It should be noted that neuronal survival in mixed culture such as those here would be slightly overestimated by this assay, as astrocytes are not injured nor do they die following KA exposure (Uliasz and Hewett, 2000). The proportion of astrocytes, estimated at 5 %, is low enough that this overestimation will be negligible and was consequently ignored. The aim of these live/dead assays was to determine a level of exposure of KA sufficient to activate large numbers of GluRs but at the same time have minimal cell death. In this

respect, the increased survival of neurons grown in Neurobasal-A medium with the antioxidant rich B27 supplement and added bFGF is highly advantageous.

Table 3.3: Neurons were exposed to varying levels of KA for 24 h. LDH levels were measured and a live/dead assay performed to determine the level of cell death at each KA concentration. Neuronal survival can be seen to drop off at KA concentrations of greater than 100 μM ; this was not reflected in the LDH levels however.

KA conc. (μM)	LDH (units/litre/ 10^4 cells)	Neuronal survival (%)
0	8.7, 5.7, 10.7	95.1, 96.5, 92.8
5	12.4, 18.9, 11.3	93.5, 91.0, 96.0
10	3.2, 7.4, 3.9, 7.0, 5.9, 7.9	93.5, 94.0, 81.0, 96.6, 96.0, 95.1
20	17.6, 8.0, 16.8	92.0, 91.0, 91.4
50	8.4, 13.8, 10.4	94.6, 94.9, 96.2
100	5.5, 7.0, 6.0	93.7, 98.0, 97.0
500	10.3, 7.8, 8.0	69.0, 82.9, 75.0
1000	22.1, 12.4, 18.6	48.6, 33.9, 39.8

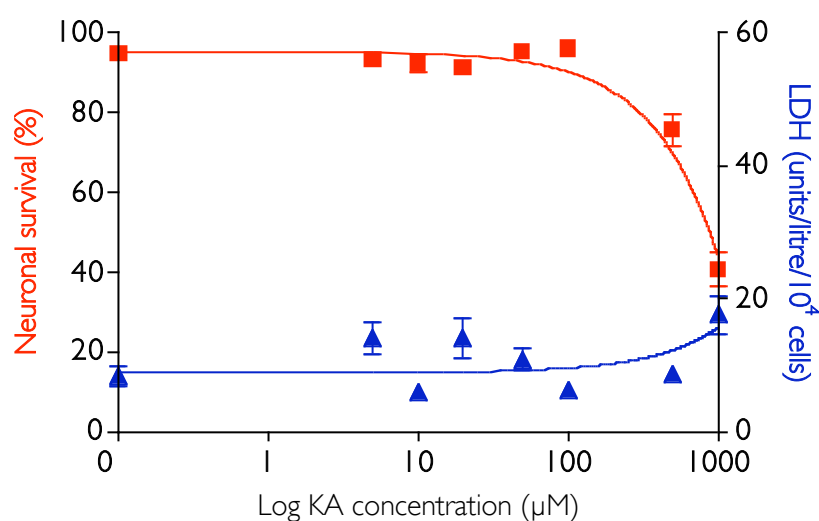


Figure 3.5: Graph of data in Table 3.3. Neuronal survival after exposure to KA ranging from 0 μM to 1000 μM for 24 h. Expressed as means \pm SE. Neurotoxicity is expressed as percentage neuronal survival as determined by live/dead assay (left axis and in red), and the LDH level at each KA concentration (right axis and in blue). Lines show linear regression fits to the data.

The initial experiments with 24 h KA exposure demonstrated that neuronal survival dropped off rapidly at concentrations greater than 100 μM (see Figure 3.5). KA concentrations of 50 μM and 100 μM were investigated further to determine neuronal survival over 2-3 days of exposure. Neuronal survival was similar under both KA

concentrations until ~30 h. After this point cell death was triggered by 100 μM KA but not by 50 μM KA (see Figure 3.6 and Table 3.4). Conditioned medium from unstimulated neurons grown in B27-free growth medium was collected after a 72 h growth period. To allow comparisons between the KA stimulated and unstimulated states, the growth periods in B27-free medium needed to be the same. As demonstrated here, the cultures could withstand 50 μM KA exposure for at least 72 h; consequently, this was the exposure length and concentration chosen.

Table 3.4: Neurons were exposed to 50 μM and 100 μM KA for the indicated lengths of time. LDH levels were determined and live/dead assays performed to determine the level of cell death at each KA concentration. Neuronal survival dropped off after ~30 h 100 μM KA exposure.

Time (h)	LDH (units/litre/ 10^4 cells)		Neuronal survival (%)	
	50 μM KA	100 μM KA	50 μM KA	100 μM KA
0	4.1, 3.7, 3.7	5.0, 2.9, 3.8	93.4, 97.4, 96.2	92.4, 95.6, 95.57
18	no data	3.6, 5.0, 7.7	no data	98.2, 98.9, 90.8
24	8.4, 13.8, 10.4	6.1, 7.0, 5.5	95.0, 94.6, 96.2	93.7, 98.0, 90.1
48	3.2, 3.3, 2.9	6.8, 5.8, 8.5	98.6, 96.8, 97.4	86.0, 73.5, 80.6
72	3.9, 3.5, 3.7	12.3, 9.6, 6.0	98.5, 97.8, 96.2	54.9, 85.2, 89.6

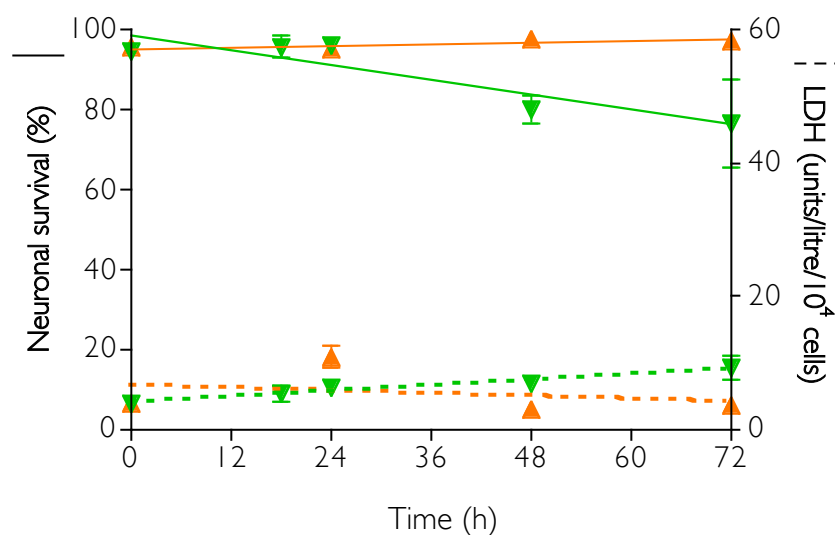


Figure 3.6: Graph of data in Table 3.4. Neuronal survival (unbroken lines) and LDH levels (broken lines) after exposure to 50 μM KA (orange) and 100 μM KA (green) for 72 h. Values expressed as mean \pm SE. Lines show linear regression fits to the data.

LDH measurements as shown here (see Figures 3.5 and 3.6) do not show good correlation with the results from the live/dead assays. Cell death was induced after 24 h

exposure to KA concentrations greater than 100 μM , and after 30 h with 100 μM KA exposure, but this was not reflected in the LDH levels. In both instances, LDH values remained static in the range of 5-15 units/litre/ 10^4 cells. In comparison, the live/dead assay results correlated well with what would be expected based on the literature (Tasker et al., 2002). The disparity in the LDH results suggests issues with how this was collected and analysed. Several factors were probably involved. First, media samples were not processed immediately after collection. Instead they were refrigerated and sent to Medlab diagnostics in batches for analysis. Samples could therefore sit for several days in storage. LDH is an unstable molecule, even when stored at 4°C, as shown by Mi (2002) who found that LDH activity dropped to 70 % after only 4 days in storage at 4°C. It is very likely that a large proportion of the LDH present in media samples had reduced activity by the time it was tested. Secondly, the testing as done by Medlab diagnostics, which is primarily a medical diagnostic service, is likely not to have the accuracy needed here. In confirmation of this, one sample divided into triplicates was analysed by Medlab diagnostics as having 5, 8 and 11 units/litre; and their minimum detection limit is only 5 units/litre. Thirdly, the density of cells in the cultures studied here is comparatively low compared to that possible with cell-lines. Consequently the levels of released LDH are in all likelihood low, and, based on the data here, too low for Medlab diagnostics to accurately determine.

3.4 2D-PAGE analysis of conditioned medium from unstimulated neurons

3.4.1 Processing of conditioned medium

A minimum of two refills and respins ('washes') of the Vivaspin 5 kDa MWCO 20 ml ultrafiltration column is required to remove ~98 % of salts in the conditioned medium (manufacturer's instructions Vivascience). In these studies eleven washes were performed for each sample of conditioned medium. The use of ultrafiltration, rather than protein precipitation, to remove salts and concentrate the protein, not only yields higher protein recovery (Sickmann et al., 2000), but has also been shown to give improved 2D-PAGE resolution (Hammack et al., 2003; Jiang et al., 2004). These eleven washes resulted in a dilution of 4000 x of salts and other contaminants in the medium. Because the protein content of the conditioned medium was expected to be low (Dahl et al., 2003), in preliminary studies the conditioned medium from three T-75 flasks was

combined. Each T-75 flask typically contained ~16-18 ml of medium, so in total ~54 ml of medium was combined. After washing and concentrating to a volume of ~700 μ l, the protein concentration was determined to be ~10 μ g/ μ l. This was the case for both of the samples US A and US B (see Table 3.5). The 2D gels for both US A and US B used 250 μ g of protein from this concentrated medium (see Figures 3.6 and 3.7). These initial experiments demonstrated that the medium from one T-75 contained more protein than the 250 μ g needed to run one large-format SYPRO Ruby-stained 18 cm 2D gel. Consequently, all subsequent gels used the concentrated and purified medium from only one T-75 flask.

Table 3.5: The protein concentration of the purified and concentrated conditioned medium. For both US A and US B, ~54 ml of conditioned medium was used, only ~16 ml of conditioned medium was used for US C.

Sample	Volume of conditioned medium	Protein conc. after concentrating
US A	~ 54 ml (3 x T-75)	~ 10 μ g/ μ l
US B	~ 54 ml (3 x T-75)	~ 10 μ g/ μ l
US C	~ 16 ml (1 x T-75)	~ 2.0 μ g/ μ l

2D-PAGE of US C in rehydration buffer containing thiourea gave a good number of protein spots, therefore the aim was to also do 2D-PAGE of US A and US B in rehydration buffer containing thiourea. Due to the limited amount of sample and the difficulty of separating medium-derived proteins by 2D-PAGE, it was not possible to get a 2D gel of each sample, which would be able to be compared by densitometric analysis. US A and US B (see Figures 3.6 and 3.7 respectively) when run without urea, look very similar. It is reasonable to expect therefore, that US A when run in rehydration buffer containing thiourea would look like US B #2 (see Figure 3.9).

3.4.2 2D gels of conditioned medium from unstimulated cultures

US A

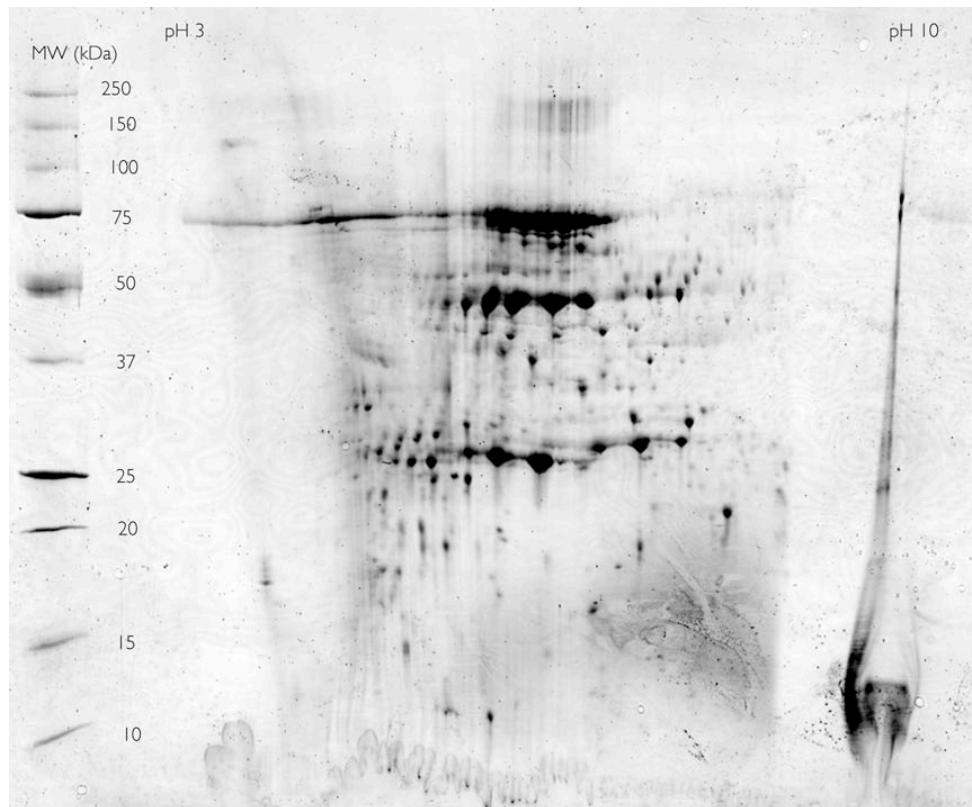


Figure 3.7: Protein from conditioned medium of unstimulated cortical neurons after 72 h of growth (250 μ g of protein from sample US A run in rehydration buffer without thiourea).

US B

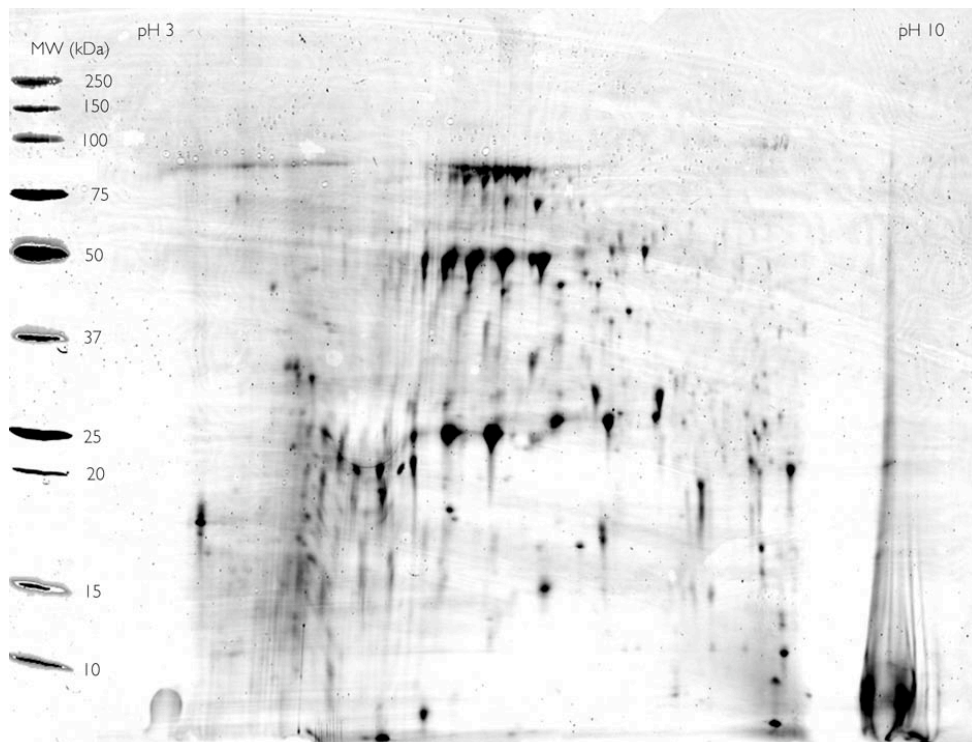


Figure 3.8: Protein from conditioned medium of unstimulated cortical neurons after 72 h of growth (250 μ g of protein from sample US B run in rehydration buffer containing 7 M urea).

3.4.3 2D gels of conditioned medium from unstimulated cultures; addition of thiourea

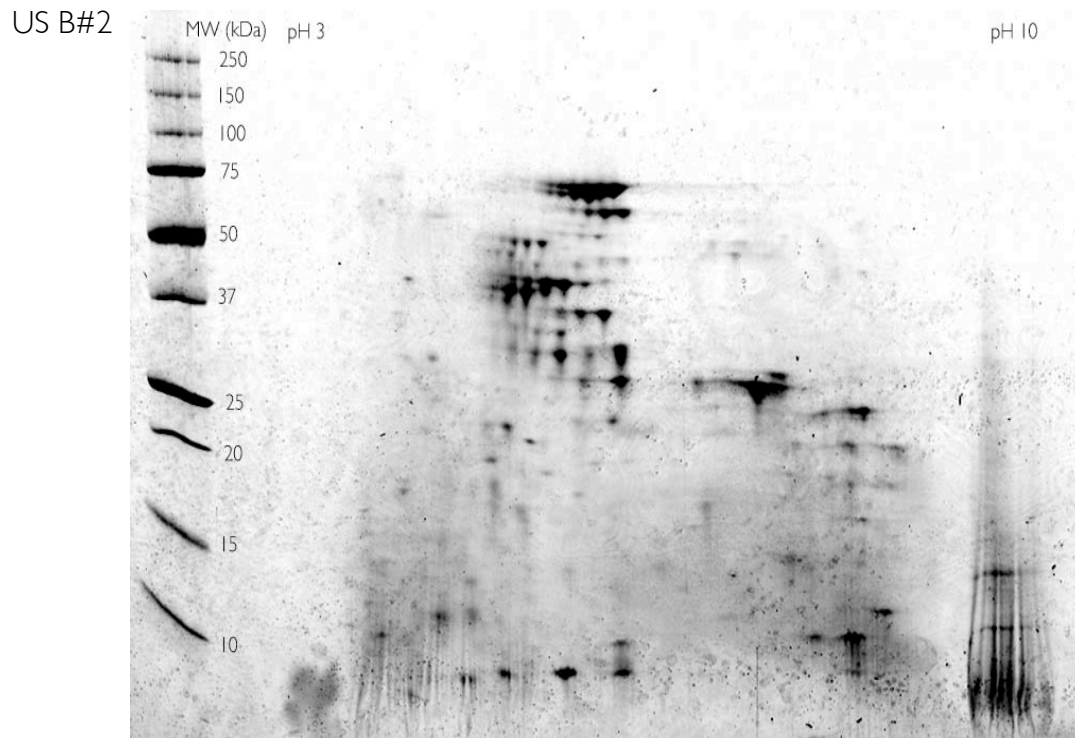


Figure 3.9: Protein from conditioned medium of unstimulated cortical neurons after 72 h of growth (250 μ g of protein from sample US B (see Figure 3.9) run in rehydration buffer containing thiourea).

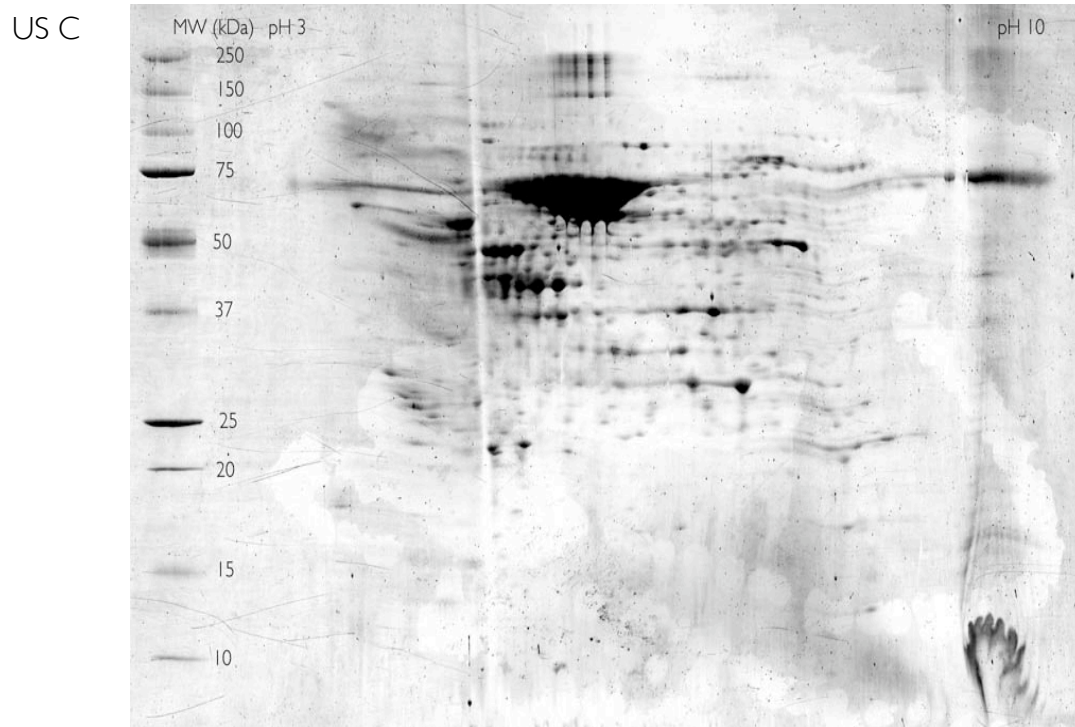


Figure 3.10: Protein from conditioned medium of unstimulated cortical neurons after 72 h of growth (500 μ g of protein from sample US C run in rehydration buffer containing 7 M urea and 2 M thiourea).

3.5 MALDI-TOF MS identification of proteins

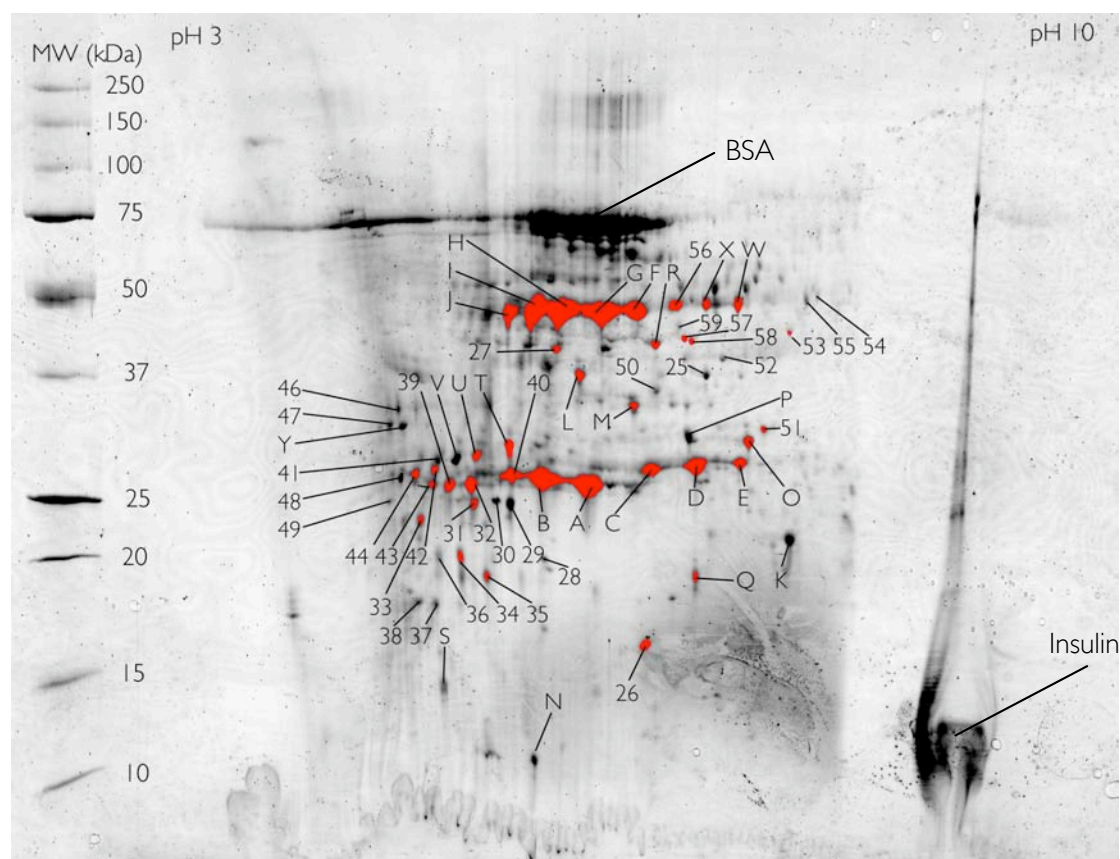


Figure 3.11: 2D-PAGE image of protein from conditioned medium of unstimulated cortical neurons. Spots identified as bovine serum albumin are indicated in red, numbers/letters indicate all spots for which MS was performed. Spots labelled insulin and BSA were not processed as they matched their respective MW/pI and have been identified in separate studies.

Discrete spots visible after overstaining with Colloidal Coomassie were excised, digested, and MALDI-TOF MS analysis was performed. Fifty-nine spots were processed, and thirty-three were identified, all of which were bovine serum albumin (see Table 3.6). The dominant band at ~69 kDa and the long vertical trail at pI ~10, were not processed as they matched the MW/pI and pattern of BSA (Sanchez et al., 1995) and insulin (pers. comm. C. Buchanan) respectively, identified in other studies, and were assumed to be such (see Figure 3.11). Several samples were unable to be identified despite having good spectra, suggesting that the protein was perhaps not in the database. However, analytical 2D-PAGE, as used here, is generally not capable of supplying sufficient amounts of low abundance proteins for characterisation with MS, without combining samples from multiple gels (Davidsson et al., 2001). This was not done here, however, due to time constraints. Proteins with low MW are also difficult to

identify as they usually only yield a few proteolytic fragments, making database searching difficult and inconclusive (Sickmann et al., 2000).

The images shown here were stained with SYPRO Ruby, and some spots visible in these gels could not be seen when overstained with Colloidal Coomassie. These spots would have contained very low levels of protein <5 ng, the detection limit of Colloidal Coomassie (Berggren et al., 2002).

Table 3.6: The identities of protein spots as determined using MS. The spot number/letter, in the left hand column, corresponds to those in Figure 3.10. Only spots for which a positive ID was gained are listed.

Spot	Protein	Species	Swiss-Prot accession no.	Sequence Coverage (%)	Theoretical MW (kDa)	Estimated MW from gel (kDa)	theoretical pI
A	Serum albumin	Bovine	P02769	23	66429	28	5.7
B	Serum albumin	Bovine	P02769	22	66429	28	5.7
C	Serum albumin	Bovine	P02769	12	66429	29	5.7
D	Serum albumin	Bovine	P02769	16	66429	30	5.7
G	Serum albumin	Bovine	P02769	19	66429	46	5.7
H	Serum albumin	Bovine	P02769	19	66429	47	5.7
I	Serum albumin	Bovine	P02769	22	66429	47	5.7
J	Serum albumin	Bovine	P02769	16	66429	46	5.7
L	Serum albumin	Bovine	P02769	28	66429	38	5.7
M	Serum albumin	Bovine	P02769	16	66429	35	5.7
O	Serum albumin	Bovine	P02769	23	66429	32	5.7
Q	Serum albumin	Bovine	P02769	9	66429	21	5.7
R	Serum albumin	Bovine	P02769	10	66429	42	5.7
T	Serum albumin	Bovine	P02769	8	66429	31	5.7
U	Serum albumin	Bovine	P02769	7	66429	30	5.7
W	Serum albumin	Bovine	P02769	25	66429	47	5.7
Y	Serum albumin	Bovine	P02769	23	66429	33	5.7
26	Serum albumin	Bovine	P02769	13	66429	16	5.7
27	Serum albumin	Bovine	P02769	24	66429	69	5.7
31	Serum albumin	Bovine	P02769	16	66429	30	5.7
32	Serum albumin	Bovine	P02769	13	66429	28	5.7
33	Serum albumin	Bovine	P02769	12	66429	25	5.7
35	Serum albumin	Bovine	P02769	8	66429	21	5.7
39	Serum albumin	Bovine	P02769	13	66429	28	5.7
40	Serum albumin	Bovine	P02769	13	66429	29	5.7
42	Serum albumin	Bovine	P02769	18	66429	30	5.7
43	Serum albumin	Bovine	P02769	14	66429	29	5.7
44	Serum albumin	Bovine	P02769	15	66429	28	5.7
51	Serum albumin	Bovine	P02769	8	66429	33	5.7
53	Serum albumin	Bovine	P02769	9	66429	43	5.7
56	Serum albumin	Bovine	P02769	8	66429	47	5.7
57	Serum albumin	Bovine	P02769	18	66429	29	5.7
58	Serum albumin	Bovine	P02769	17	66429	29	5.7

3.6 2D gel of B27 supplement; comparison to conditioned medium.

The abundance of BSA on the 2D-PAGE gels lead to the initial assumption that poor washing of the cells and flask, when switching the culture to B27-free growth medium, could lead to large amounts of albumin remaining. The B27 medium supplement contains proteins such as BSA, insulin and transferrin, amongst others (see Table 3.1).

As expected, several B27 proteins such as BSA and insulin were present in the conditioned medium. These proteins are at high levels in the B27 supplement and associate with the cell surface and the extracellular matrix, making them difficult to fully remove (Lim and Bodnar, 2002). However, the large number of BSA spots present appeared unusual. In order to rule out the B27 supplement as the sole source of the albumin, it was analysed on a 2D gel (see Figure 3.12). Figure 3.13 is a comparison between the B27 gel and that of conditioned medium from unstimulated neurons.

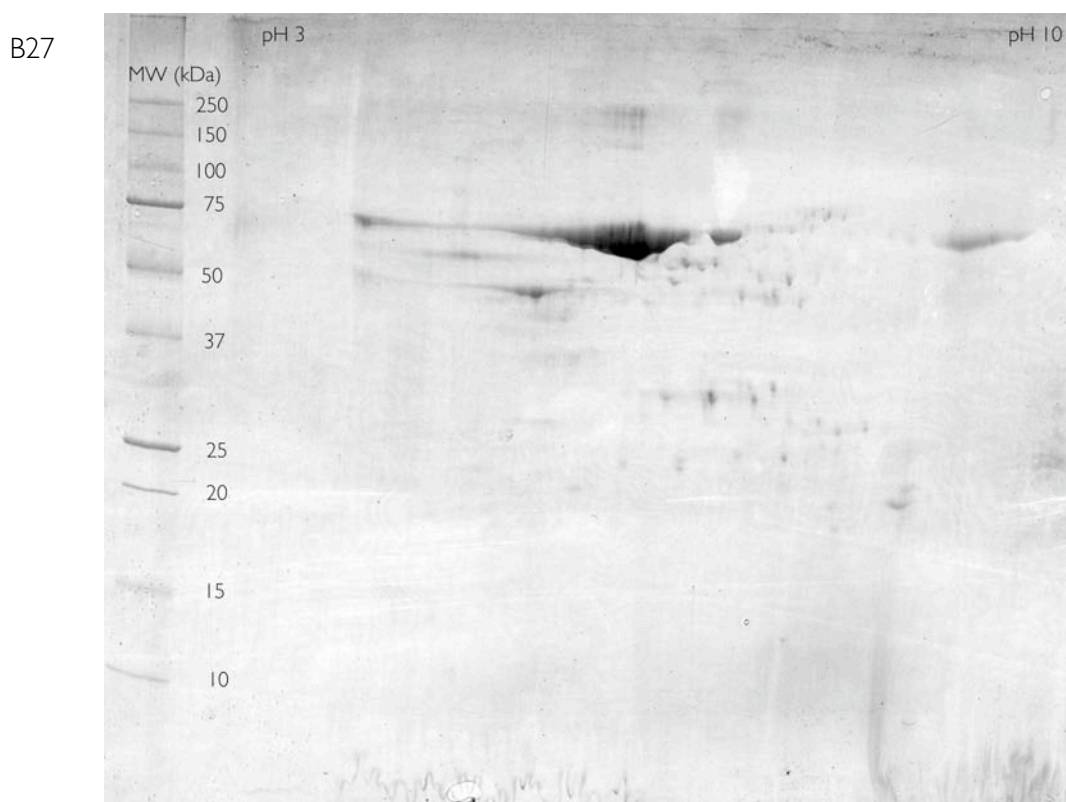


Figure 3.12: 2D gel of the B27 medium supplement (275 μ g of protein run in rehydration buffer without thiourea).

Clear differences in the gel pattern of the B27 supplement compared to that of conditioned medium from unstimulated neurons is shown in Figure 3.13. The four red ellipses indicate areas that contain obvious differences. The primary similarity between the two is the abundant BSA at ~66 kDa. Most of the smaller spots also appear to be at a higher abundance, indicated by a darker spot, in the conditioned medium than in the B27 supplement.

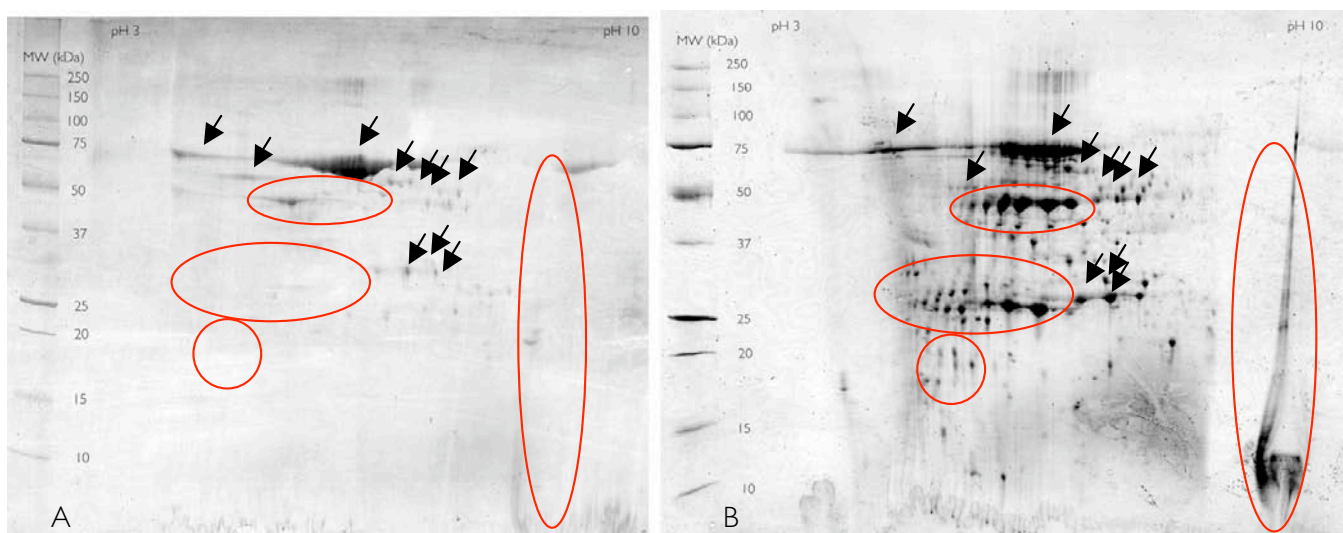


Figure 3.13: Comparison between 2D gels of B27 supplement (A) and protein from conditioned medium of unstimulated cortical neurons (B). The main areas of difference are circled red, and dominant spots appearing in both gels are indicated with arrows.

A large amount of what was been assumed to be insulin appeared on all the 2D gels (see Figures 3.7, 3.8 and 3.9 3.10). However, it was not as apparent in the B27 supplement 2D gel, indicating that the concentration of insulin in the conditioned medium is higher than in the B27 supplement.

Insulin and receptors for insulin are found throughout the CNS (Gerozissis, 2004). *In vivo*, insulin in the CNS can be either derived peripherally, from the β -cells of the pancreas, or locally, synthesised by neurons. Depolarisation of neurons, in addition to inducing the release of neurotransmitters, also results in the exocytosis of insulin. There is evidence that insulin is stored in synaptic vesicles, within nerve endings, and is mobilised upon excitation (Wei et al., 1990; Gerozissis, 2004). It is likely therefore, that there are three sources for the insulin present in the conditioned medium. Some insulin is likely to remain adhered to the cell dish and cells despite washing. Neurons internalise insulin from the environment and release it upon neural activity, and evidence thus far

suggests neurons also produce and secrete insulin. However, it was not experimentally determined what the ratio was in the conditioned medium analysed in these studies.

3.7 Tryptic peptide coverage of the BSA molecule

For protein identification by MALDI-TOF MS, gel spots are excised and digested, using the protease trypsin, into tryptic peptides. Trypsin cleaves L-amino acid peptide bonds adjacent to positively charged residues lysine and arginine (Hedstrom, 2001). A unique set of peptides is generated whose masses are then detected using MS, and compared to databases of theoretical digests to identify the protein. In Figure 3.14 each black segment represents a tryptic peptide identified using MS that was matched to the BSA protein. Each row represents an individual gel spot identified as BSA, as shown in Figure 3.11 and Table 3.6.

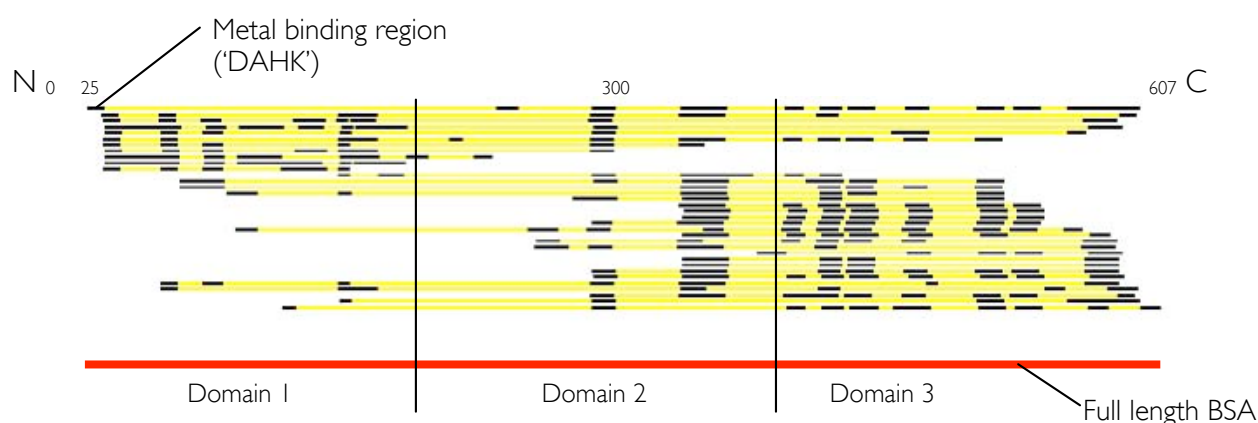


Figure 3.14: Tryptic peptides identified by MS as BSA, aligned to show the sequence coverage of the peptides across the full BSA molecule. Black segments represent tryptic peptides and yellow segments represent theoretical coverage, formed by joining the peptides. See the Appendix for the sequence and alignment. The size of the full length BSA molecule is indicated in red and the N-terminal metal binding region is indicated.

If each gel spot contained the same portion of the BSA molecule, the area of coverage by the tryptic peptides should be very similar for all spots. However, while some spots have a similar coverage pattern, the pattern is not uniform for all spots. For instance some only have coverage at the N-terminal or the C-terminal region, and others have coverage over the full-length of the BSA molecule. Assuming each gel spot only contained one BSA fragment, and that the tryptic peptides have coverage across the entire sequence of the fragment, joining them (shown in yellow in Figure 3.14) gives an indication of the BSA fragment each gel spot represents. Visually it is clear that several

different fragments are present in the gel, as expected based on the range of MW and pI values (see Figure 3.11). The BSA molecule contains three homologous domains. Domain 1, from amino acid (aa) 25 to aa 204 (180 aa long) containing the metal binding region. Domain 2 = from aa 211 to aa 396 (186 aa long) and domain 3 = from aa 403 to aa 594 (192 aa long) (see Figure 3.14) (Carter and Ho, 1994). These domains do not appear to correspond to any of the fragments.

3.8 BSA gel spot patterns in the literature

Multiple BSA gel spots of varying pI and MW have been observed in other studies also analysing the conditioned medium of cultured cells. Lim and Bodnar (2002) observed 41 discrete spots identified as BSA, with MW(kDa)/pI ranging from 20.9/5.2 to 76.5/5.9, all of which were different to BSA's predicted MW/pI of 66/5.7. Their study was investigating secreted factors from a cultured murine embryonic fibroblast cell line. The conditioned medium collection protocol was very similar to that used in these studies; cells were washed twice before being grown in serum-free medium, debris and cells were removed prior to concentration and washing via ultrafiltration, and the sample was then dried and re-suspended in rehydration buffer. Lim and Bodnar suggested that either degradation of the BSA had taken place during desalting and concentration steps, or that the protein was processed by the cells. Other proteins were generally found in only 1-2 discrete spots, and no BSA band at 66 kDa, representing the full-length protein, was observed. If the BSA banding pattern was indeed due to degradation, it is interesting that all of the full-length BSA was degraded and yet no other protein seemed to have been degraded to the same extent. A paper comparing the main protein preparation techniques prior to 2D-PAGE, being protein precipitation and ultrafiltration, did not show degradation of BSA after any of the procedures. BSA remained as a dominant band at 66 kDa (Jiang et al., 2004). This suggests that the gel pattern of BSA seen in this study cannot be solely due to degradation during the ultrafiltration process. In a similar study by Kubota et al. (2003), using protein precipitation, three separate BSA spots were identified, along with a large BSA band at 66 kDa. The same spots were observed in conditioned medium from both differentiated and undifferentiated osteoclasts, but were up-regulated in the differentiated cells. The authors suggested it was as a result of protease activity being up-regulated in osteoclast differentiation (Kubota et al., 2003). The BSA fragmentation

was the same in both cases, suggesting that if proteases were the cause, they only cleaved at 2-3 sites within the BSA molecule. Lafon-Cazal et al. (2003) analysed the conditioned medium from primary cultures of murine astrocytes and their 2D gels did not display any albumin, of murine or bovine origin. Analysis of mouse CSF did show a large band of murine serum albumin at 66 kDa, with no other albumin spots present (Lafon-Cazal et al., 2003). Their study also used protein-precipitation when processing the conditioned medium. Taberbero et al. (2003) demonstrated astrocytic endocytosis of BSA and this was also demonstrated in this study (see Figure 3.16). Dahl et al. (2003) cultured hippocampal progenitor cells and analysed the conditioned medium. No mention of BSA was made in the paper and gel images were not included, so it is unknown whether they observed BSA fragmentation. No other papers have been found investigating the protein composition of conditioned medium from cultured CNS cells, be they glial or neuronal.

3.8.1 Cleavage and proteolysis

Several artefactual processes could have played a part in the generation of the multiple BSA protein spots of varying MW and pI present in the 2D gels in this study, and these are discussed below.

During isoelectric focusing, proteins have been observed to cleave off fragments and then refocus at altered pI (Schönberger, 2001). In a paper by Langen et al. (1999) 400 gel spots were shown to be actually only 180 different brain proteins, and 44 gel spots identified from 2D gels of the human brain came from actually only 28 different proteins (Schönberger, 2001).

Proteolysis by extracellular proteases could also in part explain the presence of BSA fragments of lower MW seen in these gels. Proteases could have acted during the ultrafiltration washing stage, as no inhibitors were added, and also during IPG strip rehydration, which occurred at RT over a ~16 h period. The presence of urea in the rehydration buffer does not prevent the action of all proteases—some are very resistant to denaturation even in 9 M urea, and studies have shown sample proteolysis after only two hours in rehydration buffer (Olivieri et al., 2001; Castellanos-Serra and Paz-Lago, 2002). However, it cannot be assumed that artefactual degradation is occurring until further studies are done. Indeed, most of the BSA fragments in this study

were reproducible between the 2D gels (see Figures 3.6 and 3.7), arguing against this possibility. Additionally, proteolysis only occurred in the presence of the cortical neurons, what is not known is whether the proteolysis occurred intracellularly or extracellularly.

In addition some of the different gel spots could represent aggregation complexes of lower MW fragments, or complexes with other proteins in solution, although the denaturing conditions used during sample preparation should minimise this. The migration of proteins is also affected by post-translational modifications such as phosphorylation and glycosylation (Davidsson et al., 2001). These changes which determine protein trafficking, function and turnover (LoPachin et al., 2003), change the MW and charge of the protein.

3.8.2 Addition of thiourea to rehydration buffer changes gel pattern

Urea, a chaotrope, is always used in rehydration buffers for first dimension IEF when run under denaturing conditions. Urea denatures proteins by disrupting non-covalent and ionic bonds and has a neutral charge, meaning it remains in the gel during IEF and does not migrate (Molloy, 2000; Shaw and Riederer, 2003). The addition of thiourea, another powerful chaotrope, to the rehydration buffer, has been shown to increase the solubilisation of proteins, particularly hydrophobic membrane proteins (Rabilloud, 1998; Molloy, 2000; Shaw and Riederer, 2003). Thiourea is usually used at a concentration of 2 M in conjunction with 7-9 M urea, the latter required to dissolve the relatively insoluble thiourea (Molloy, 2000). Higher concentrations of thiourea are not generally used as this results in loss of resolution at acidic pH values (<4.5) (Musante et al., 1998), probably due to thiourea's inhibition of SDS-protein binding, leading to poor transfer to the second dimension (Molloy, 2000).

Initial 2D gels of conditioned medium used urea at a concentration of 9 M, however some banding was observed at pIs of ~3 and ~8, which was thought to be possibly due to poor solubilisation and/or protein precipitation (see Figures 3.6 and 3.7). To investigate the effect of thiourea on this, these samples were rerun in 7 M urea with 2 M thiourea (see Figure 3.9). The addition of thiourea to the rehydration buffer dramatically changed the gel pattern, but did not appear to change the amount of protein present or the banding. The biggest changes were seen in the pI shifts of the

proteins in the 25-75 kDa mass range, indicated by the two red lines in Figure 3.15. Some of the spots appear to have shifted to lower pI values in the presence of thiourea.

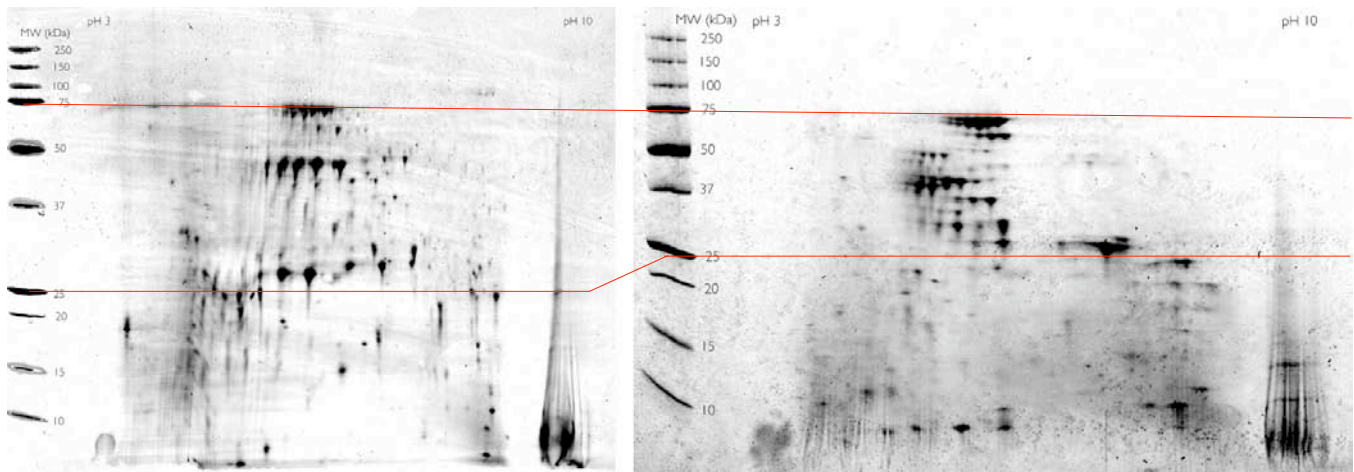


Figure 3.15: Protein (250 μg) from conditioned medium of sample US A in 9 M urea rehydration buffer (left) and in 7 M urea rehydration buffer with 2 M thiourea (right). The gel pattern changed dramatically with the addition of thiourea, especially between 25 and 75 kDa as indicated by the two red lines.

The reason for these shifts is not clear. It is possible that the proteins achieved better solubilisation allowing them to move to their true pI. In addition, thiourea has been shown to improve the solubilisation of lipids, which can also affect the resolution of 2D gels. Lipids bind proteins via hydrophobic interactions, affecting their charge, and hence their pI, and MW (Shaw and Riederer, 2003). Thiourea has also been shown to be highly effective against proteolysis (see Section 3.8.1) (Castellanos-Serra and Paz-Lago, 2002) when present in the rehydration buffer. This could explain some of the changes in gel patterns. However, since the apparent MWs have either not changed or in some cases appear to have shifted to a lower MW, the reverse of what would be expected if proteolysis was being inhibited, this seems unlikely.

3.9 Bovine serum albumin is internalised by neurons and glia

Cortical cultures were grown for two weeks on glass coverslips as previously described, before being washed and fixed using NBF. Antibodies for BSA were used to determine whether BSA could be detected intracellularly (see Figure 3.16).

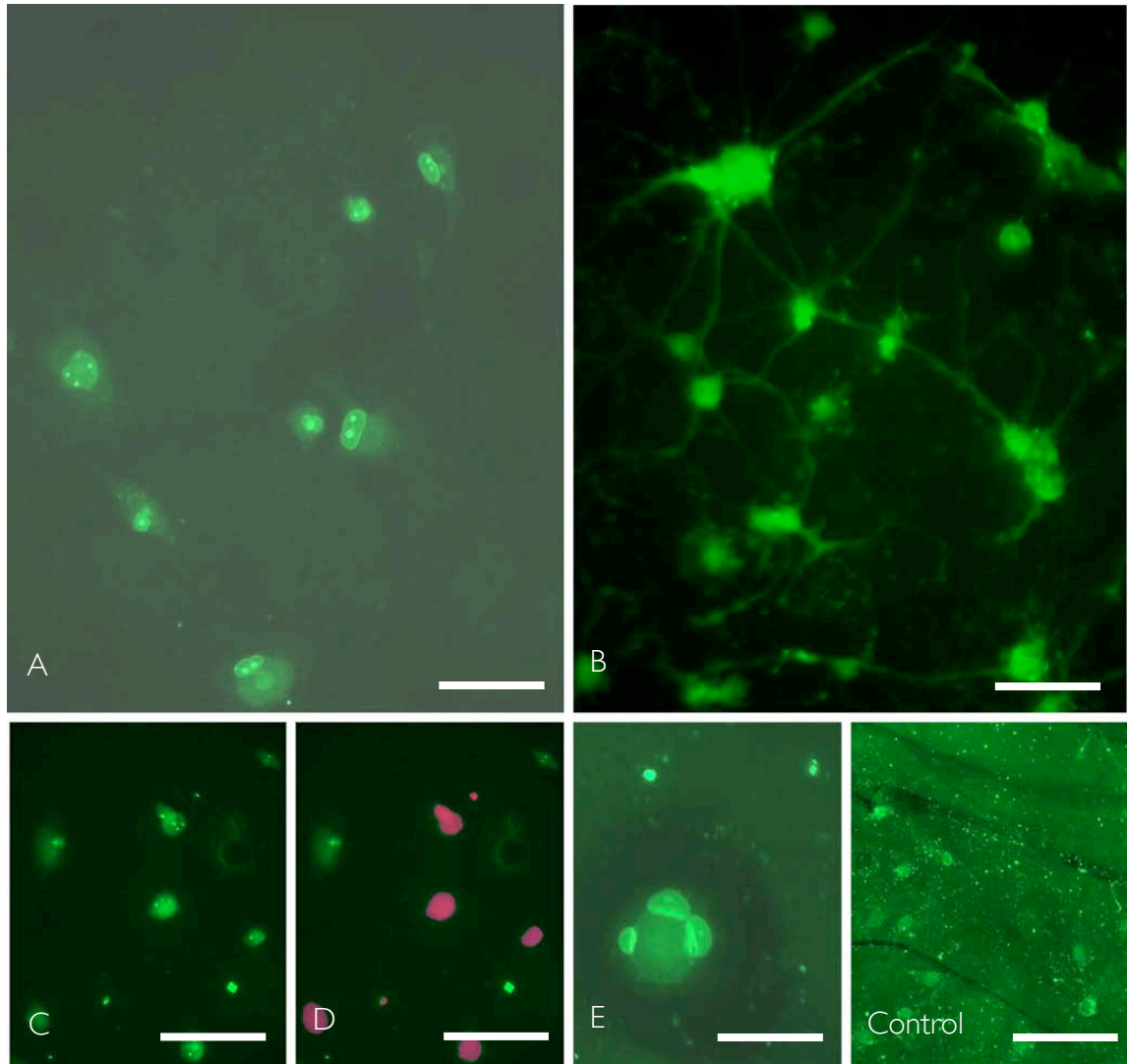


Figure 3.16: Staining with anti-BSA antibodies (green) showed that both neurons and astrocytes had internalised BSA. A, E: Diffuse staining of the whole cell body was seen along with discrete units of stain in small 'balloon-like' structures. B, C: Neuronal cells stained for BSA. D: Neuronal cells in C double-stained with anti-NeuN (cyan). Omission of the primary anti-BSA antibody in the control eliminated staining. Scale bar A, B, D = 15 μ m, C, D, control = 30 μ m.

Morphologically, cells positive for BSA appeared to be both neurons (Figure 3.16 B) and astrocytes (Figure 3.16 A). As the anti-GFAP and anti-BSA antibodies were both raised in rabbit, it was only possible to double-stain for BSA and NeuN. Triple-staining, by including the anti-GFAP antibody, was not possible. Double-staining (Figure 3.16 C and

D) showed that not all the cells that stained for BSA stained for NeuN, indicating that both glia and neurons had taken up BSA. This is in agreement with the literature, as BSA has been shown to accumulate intracellularly in both astrocytes and neurons (Tabernero et al., 1999; Tabernero et al., 2002b). While it cannot be assumed that the cells not staining for NeuN are astrocytes, in earlier experiments characterising the cell populations present no cells were observed that didn't stain for either NeuN or GFAP (see Section 3.2). Thus it is very likely that the NeuN-negative cells are astrocytes.

These results demonstrate that the BSA present in the 2D gels is not merely due to inadequate washing, nor that components were contaminated with BSA during the MALDI-TOF MS process. It would be expected that some of the BSA present was left behind when the cultures were switched to B27-free growth medium; the difficulty, however, is determining which of the spots these are. Initial attempts at 2D-PAGE gel of the cytosol were unsuccessful; further work is therefore needed in this area. Analysing the BSA content of the cells by 2D-PAGE would indicate which proteins in this study represent non-internalised BSA. Non-internalised BSA is considered to be the BSA that remained attached to the culture plate and was not removed by washing.

3.10 Albumin in the brain

3.10.1 Gene expression and access

Albumin is an important serum protein, serving as a carrier of ions and hormones, and is one of the main proteins that contribute to colloidal osmotic pressure in the vasculature (Tabernero et al., 1999; Dziegielewska et al., 2000; Gum et al., 2004). Albumin, along with other serum proteins, is also adsorbed into intestinal cells and broken down to provide amino acids (Baracos, 2004). In the immature rat CNS, albumin is present at very high levels relative to the adult CNS. This is likely due to a combination of specific uptake and *in situ* synthesis (Dziegielewska et al., 2000; Tabernero et al., 2002a). Albumin is one of several serum proteins for which specialised transport mechanisms exist that are only active in the immature brain (Habgood et al., 1992). Albumin is transferred from the blood to the CSF via a small proportion of epithelial cells in fetal and early postnatal life (Habgood et al., 1992; Dziegielewska et al., 2000). While the albumin gene is not expressed in the adult rat CNS (Moos, 1995), *in situ* hybridisation in brain tissue with albumin cDNA, has shown that all neuronal cells express albumin

during fetal development and up until the fifth week after birth (Poliard et al., 1988). Because the presence of albumin in the CNS is developmentally regulated, it may play an important role in neural cell differentiation (Tabernero et al., 2002b). In contrast, in the adult brain the intracellular presence of this protein comes exclusively from plasma (Moos, 1995), and only those cells which span the blood-brain barrier (BBB) will have exposure to significant amounts of serum protein (Dziegielewska et al., 2000). Such cells are also only exposed to serum proteins over a limited area of their cell surface (Dziegielewska et al., 2000).

3.10.2 Neuronal uptake and effects

Immunocytochemical studies by Moos et al. (1991) have shown that exogenous human albumin injected into the tongues of rats is taken up by peripheral axon terminals of nerve motor neurons with projections beyond the BBB. Both rat and human albumin were seen co-localised within the cytoplasm of individual neurons. They suggested that both human and rat albumin were taken up by endocytosis and transported in organelles associated with the endosomal-lysosomal system (Moos et al., 1991). Molecular signals such as growth factors gain access to the CNS from outside the BBB by uptake in the axonal terminals and subsequent retrograde axonal transport. This is carried out by motor neurons, many of which project past the BBB (Moos, 1995). Protein-bound substances like fatty acids and essential metals are perhaps the reason for plasma protein uptake in motor neurons (Moos, 1995). Interestingly, studies have shown that rats administered human serum albumin following focal-cerebral ischaemia show significant protection of the cortex (Remmers et al., 1999). Cortical neurons in these treated animals with preserved structural features, were shown to have taken up albumin. However, it is not yet known whether high intracellular concentrations of albumin are specifically beneficial for the cortical neurons (Remmers et al., 1999). Studies by Tabernero et al. (2002b) showed that albumin uptake by cultured neurons was an active process sensitive to temperature, with uptake dropping when the temperature was lowered from 37°C to 4°C. In addition, Moos et al. (1995) proposed uptake to be non-specific as uptake/transport was dose-dependent.

Albumin has been shown to increase both the amount of glutamate exported to the extracellular medium and also the synthesis of glutamate by neurons (Tabernero et al., 2002b). While high levels of glutamate can induce excitotoxicity in the adult brain, they fails to do so in the immature brain during development (Sperber et al., 1991;

Tabernero et al., 2002b). Instead, glutamate at low doses has a trophic effect, promoting neurite outgrowth and increased neuronal survival (Tabernero et al., 2002b). It is thought that a basal level of GluR activation is required for normal development (Lee et al., 2000). It is likely that the trophic effects of albumin shown in studies by Tabernero et al. (2002b) are brought about by its ability to induce glutamate synthesis and release. The syntheses of growth factors, such as NGF, are induced by GluR activation. Inhibition of NGF's receptor, TrkA, eliminated the trophic effects of albumin, providing strong evidence that TrkA mediates albumin's effects (Tabernero et al., 2002b). Additionally, cells grown in the absence of any added factors died by apoptosis, but this was inhibited by the presence of albumin. Inhibition of the TrkA receptor was able to block the effect of albumin (Tabernero et al., 2002b), demonstrating that underactivation of the GluR prevents normal development.

3.10.3 Astrocyte uptake and effects

Albumin promotes the uptake of calcium by cortical astrocytes into sub-cellular stores. This potentiates calcium spike trains, by helping to refill stores between spikes. This effect may be involved in the response of astrocytes to damage in the CNS (Nadal et al., 1996). In primary cultures of astrocytes, albumin has been shown to regulate pyruvate dehydrogenase by free fatty acid sequestration (Tabernero et al., 1999). This may play a role immediately after birth by allowing the utilisation of glucose and lactate, the main metabolic substrates available during this time (Tabernero et al., 1999). It is known that albumin can regulate the spread of calcium waves and the rate of astrocyte proliferation (Tabernero et al., 2002b). Studies by Tabernero et al. (2002a) have shown that albumin induces the synthesis and release of oleic acid, which acts to induce neuronal differentiation. Their results suggested the presence of an albumin-binding protein located on the astrocytic plasma membrane involved in albumin uptake. Brief treatment with proteases prevented internalisation, suggesting that a protein acting as a receptor mediates the process. It is likely that the process involves receptor-mediated endocytosis (Tabernero et al., 2002a). Inhibitors which prevent vesicular trafficking from the ER to the golgi, and from the golgi to the plasma membrane, block albumin release by astrocytes (Tabernero et al., 2002a). Juurlink and Devon (1990) have shown that astrocytes possess receptors for albumin and that it is endocytosed in uncoated pits—this has also been shown for transferrin. The grey matter is compartmentalised into a series of interconnecting winding and narrow channels by astrocytic and neuronal

processes. The translocation of albumin, often bound to fatty acids and other important molecules, across and between astrocytes could be a mechanism to aid in the movement of these molecules through the brain (Juurlink and Devon, 1990). The transfer could also occur between astrocytes and neurons and indeed neurons have been shown to take up and transport albumin to the cell body.

3.10.4 Albumin binds metal ions and other factors

The role of this albumin uptake is unclear. It may have a nutritive function, since albumin can bind vitamins, metals and growth factors (Moos et al., 1991). Exposure to heavy metals has been suggested to play a role in various CNS diseases. Uptake of heavy metals into axon terminals and retrograde transport has been previously shown (Arvidson, 1985), suggesting a link between plasma proteins and such diseases (Moos et al., 1991).

HSA in CSF is $\sim 3.7 \mu\text{M}$ compared with $588 \mu\text{M}$ in the plasma (Gum et al., 2004). Consequently post-stroke, HSA levels would rise substantially. This could help to protect neurons against oxygen-free radicals in the extracellular space (Gum et al., 2004). HSA is a potent antioxidant probably due to its ability to bind copper and other transition metals (Gum et al., 2004). The binding of copper to proteins has been shown to block its ability to generate hydroxyl radicals (Gum et al., 2004). HSA maintains colloidal osmotic pressure in the vasculature, binds fatty acids, some drugs and metabolites, and has cation binding sites. It acts as a free radical scavenger and as a chelator of transition metals (Gum et al., 2004).

Albumin contains the binding ability to accommodate a variety of chemical substance, and it contains the largest pool of complexed metals ions in the blood plasma (Zatta et al., 2003). Metal ions such as copper and aluminium are able to bind to two sites on the albumin molecule (Zatta et al., 2003). The metal-binding region at the N-terminus of the molecule (see Figure 3.14), termed the DAHK region, can bind transition metals and copper specifically (Bar-Or et al., 2001; Gum et al., 2004). Plasma proteins may play a role in transporting metal ions into the CNS (Moos, 1995). Albumin has the ability to bind metals and retrograde transport of these metals has been observed and has been shown to be toxic (Moos, 1995). Aluminium has been shown to be a neurotoxic agent (Zatta et al., 2003).

3.1.1 A proposed role for albumin as a Cu^{2+} carrier in Alzheimer's disease

Increasing evidence suggests that cerebrovascular disorders, such as the breakdown of the BBB, are early events in the development of sporadic Alzheimer's disease (AD) (Skoog et al., 1998; Chaney et al., 2003; Iadecola, 2004; Sadowski et al., 2004). Risk factors and contributors to cerebrovascular disorders include brain injury such as stroke and trauma, diseases such as diabetes and hypertension, and the ageing process (Chaney et al., 2003; Iadecola, 2004).

The BBB refers to the vascular bed of the CNS and consists mainly of vascular endothelia surrounded by a layer of astrocytes (Mulder et al., 2001; Banks, 2004). It maintains homeostasis in the CNS by controlling and restricting the transfer of molecules between the blood and the extracellular fluid (Banks, 2004). Two main mechanisms allow blood-borne substances to enter the CNS. Small, lipid soluble molecules can diffuse across the capillary membranes, while carrier-mediated transport systems exist for non-lipophilic molecules such as glucose and insulin (Juurlink and Devon, 1990; Banks, 2004).

The accumulation of the amyloid- β ($\text{A}\beta$) peptide in the brain and blood vessels is a characteristic feature of AD (Iadecola, 2004). The 'amyloid plaster hypothesis' proposes that these vascular amyloid deposits act as an initial mechanism to seal breaches of the BBB (Roher et al., 1993; Chaney et al., 2003). Initial deposits would therefore be advantageous, helping to maintain CNS homeostasis by preventing toxic agents and plasma proteins entering (Mulder et al., 2001). However, over time, growth of these deposits would lead to the obstruction of the vascular system and further breakdown of the BBB, preventing oxygen and nutrient delivery as seen in AD (Roher et al., 1993; Chaney et al., 2003). Albumin in the CSF is derived from circulating albumin in the serum. The ratio of CSF albumin to serum albumin is an accepted measure of BBB function and permeability (Skoog et al., 1998; Sadowski et al., 2004). Increased CSF albumin is an indication of impaired BBB function. Using albumin as an indicator, a positive relationship between the degree of BBB disturbance and the progression of AD has been shown in studies of the aged with and without AD. In addition, BBB abnormalities occur early in the disease before the onset of clinical dementia (Wada, 1998; Sadowski et al., 2004). CSF albumin levels were also shown to increase with age in non-demented individuals but not to the same extent as in AD (Skoog et al., 1998),

demonstrating that vascular disorders are involved in the etiology of AD, and not simply a pathological feature. During development and after events such as hypoxia, or breakdown of the BBB, albumin gains access to the brain (Lu et al., 1999; Tabernero et al., 1999; Yao et al., 2003).

Increasing evidence suggests that metal, and in particular copper-mediated oxidative stress, plays an important role in the development of AD (Capanni et al., 2004; Marlatt et al., 2004). With ageing, copper levels increase in the brain, and have been found to be significantly increased in the brain in AD (Lovell et al., 1998; Capanni et al., 2004). High concentrations can be found in pathological markers of AD, such as the extracellular A β plaques, and in the A β oligomers in the cytoplasm of neurons (Rosenberg, 2000; Strausak et al., 2001; Marlatt et al., 2004). A β contains metal ion binding sites. Copper binding destabilises the protein, greatly accelerating aggregation, and consequently A β deposition (Capanni et al., 2004). Redox reactions occur upon the binding of the metal ion Cu²⁺ to A β . The oxidation of Cu²⁺ to Cu⁺, is accompanied by the reduction of O₂ to H₂O₂ (Capanni et al., 2004). Fenton chemistry between the reduced Cu⁺ ions and H₂O₂, results in the production of highly reactive hydroxyl radicals (Marlatt et al., 2004). The formation of hydroxyl radicals by this means is the likely mechanism of oxidative damage seen in AD (Capanni et al., 2004; Marlatt et al., 2004).

A mechanism is proposed, by which BBB dysfunction leads to the increased entry of serum albumin complexed with Cu²⁺. As shown here and in other studies, albumin is endocytosed by both astrocytes and neurons. The uptake of albumin bound to copper could be one mechanism to explain the raised intracellular levels of Cu²⁺ seen in AD and other neurodegenerative diseases. Albumin maintains low levels of free copper in the body by complexing with the metal (Capanni et al., 2004). The first four amino acids of the N-terminus of albumin form an extremely tight binding site for copper (Bar-Or et al., 2001). However, the processing of albumin by neurons, as shown in these studies could be a mechanism by which copper is released, thus allowing copper binding to A β , leading to the formation of hydroxyl radicals and oxidative damage as seen in AD and other neurodegenerative diseases.

3.12 2D-PAGE analysis of kainic acid stimulated cortical neurons

3.12.1 Processing of conditioned medium

For each of KA A, KA B and KA C, the conditioned medium from one T-75 flask (~17 ml) was washed and concentrated to a volume of ~700 μ l. The protein concentration of in this volume for each sample is shown in Table 3.7. Although the protein concentration in these samples was lower than that of the unstimulated neuron conditioned medium, enough protein was present for a SYPRO Ruby-stained large-format gel.

Table 3.7: The protein concentration of the washed and concentrated conditioned medium. For both all three KA samples ~17 ml of conditioned medium was used.

Sample	Volume of conditioned medium	Protein conc. After concentrating
KA A	~ 17 ml (1 x T-75)	~0.6 μ g/ μ l
KA B	~ 17 ml (1 x T-75)	~0.6 μ g/ μ l
KA C	~ 17 ml (1 x T-75)	~1.5 μ g/ μ l

The 2D gels run using the protein from this conditioned medium are shown in Figures 3.16, 3.17 and 3.18.

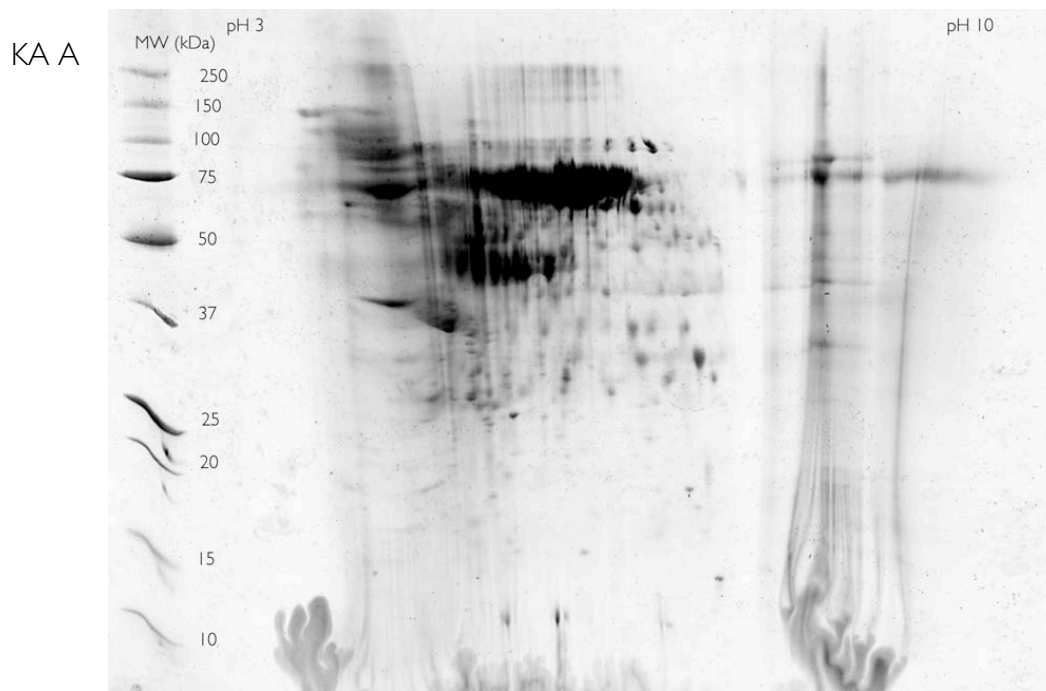
3.12.2 50 μ M kainic acid stimulation for 72 h

Figure 3.17: Protein from conditioned medium of cortical neurons stimulated for 72 h with 50 μ M KA (415 μ g of protein run in rehydration buffer containing 7 M urea and 2 M thiourea).

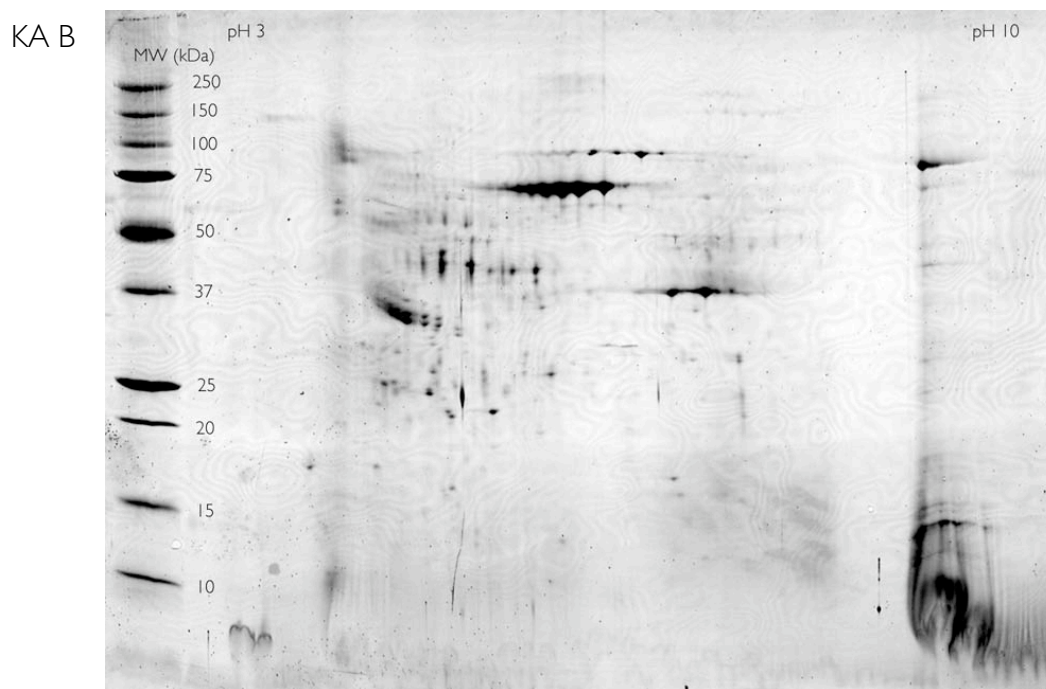


Figure 3.18: Protein from conditioned medium of cortical neurons stimulated for 72 h with 50 μ M KA (280 μ g of protein in rehydration buffer containing thiourea).

Too much protein was loaded for the 2D gel of sample KA A (see Figure 3.17) and as a result the 2D-PAGE was not entirely successful, the image appearing blurred and smeary. The primary cause is likely the large the large band of protein at MW \sim 75 kDa, this is probably bovine serum albumin.

3.12.3 10 μ M kainic acid stimulation for 24 h

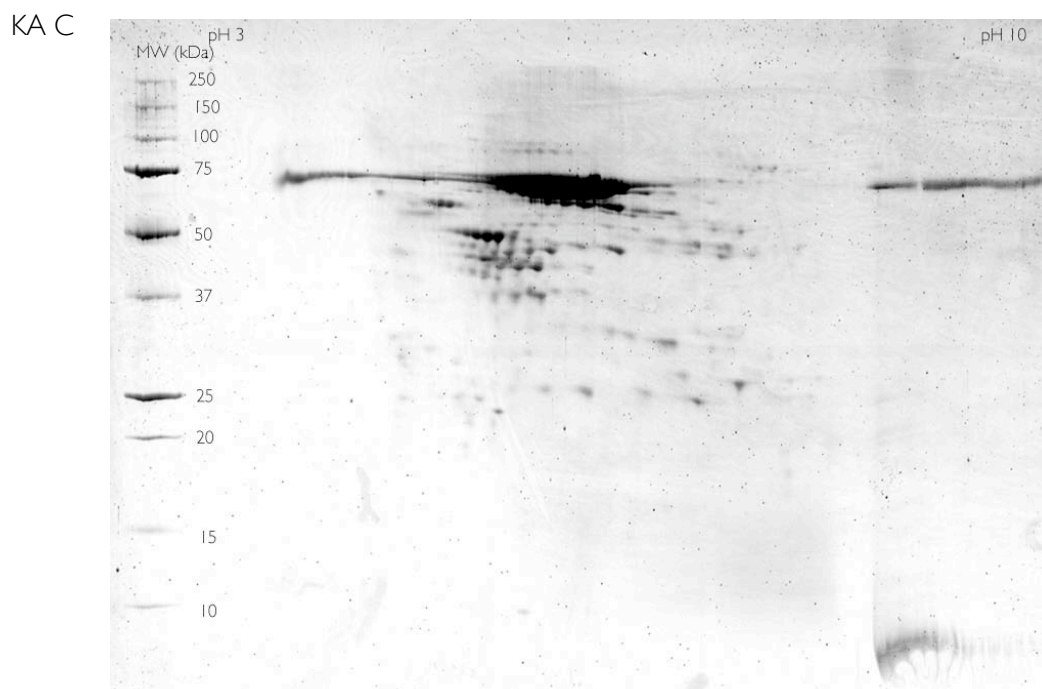


Figure 3.19: Protein from conditioned medium of cortical neurons stimulated for 24 h with 10 μ M KA (305 μ g of protein in rehydration buffer containing thiourea).

3.12.4 Gel pattern changes after kainic acid stimulation

Only half the amount of protein was separated on the KA stimulated gel, to prevent saturation with 66 kDa albumin band. It is clear that the levels of some proteins remain the same (red arrowhead) while others are up-regulated (black arrowhead) or down-regulated (blue arrowhead) suggesting the possibility that albumin is being processed differently.

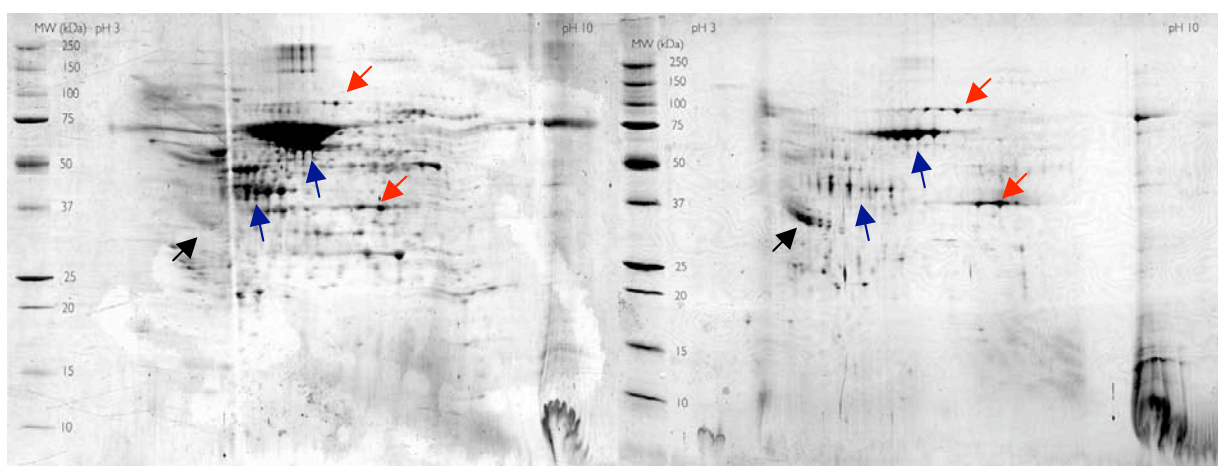


Figure 3.20: Protein (500 μ g) from conditioned medium of unstimulated cortical neurons (left) in comparison with protein (250 μ g) from conditioned medium of KA stimulated cortical neurons (right).

The 2D gel KA C (Figure 3.19), which used protein from conditioned medium of cortical neurons stimulated with only 10 μ M KA for 24 h, resembles that of US B #2 (Figure 3.9), rather than KA B (Figure 3.9). Suggesting that a certain level and length of KA stimulation is required before albumin processing is altered, and that 10 μ M KA for 24 h is below this level. However, until more 2D gels of KA stimulated neurons are performed, and the spots on the KA gels are identified using MS, it is not possible to make conclusions with any certainty.

3.12.5 Kainic acid induced cellular changes

The pharmacological and morphological changes following KA exposure have been well studied. However, the effect of KA on protein expression is not well understood (Krapfenbauer et al., 2001a). To our knowledge, no studies have been undertaken to look at the changes in protein secretion of cortical neurons following KA exposure. Most studies have looked at changes by analysing brain lysate, rather than specific cell compartments or secreted components (Kaneda and Meada, 1994; Krapfenbauer et al., 2001b; Krapfenbauer et al., 2001a).

A study looking at changes in brain protein levels following KA exposure in rats used a proteomic approach of 2D-PAGE coupled with MS (Krapfenbauer et al., 2001a). The cytosolic proteins albumin and alpha-enolase were found to be mainly present in the cytosol. The authors concluded their study by showing changes in the regulation of HSPs, neuronal death, cytoskeletal disruption, and mitochondrial rearrangement. Decreased expression of neurofilament L and M indicates neuronal loss, and increased expression of dihydropyrimidinase-related proteins is indicative of repair processes, therefore it is likely these protein changes reflect repair processes activated in the surviving population of cells (Morrison et al., 2002). Injured neurons die by an active mechanism of cell death (Krapfenbauer et al., 2001a; Morrison et al., 2002) and protein synthesis inhibitors have been shown to reduce this neuronal loss following KA injury (Joashi et al., 1999). A subsequent study by Krapfenbauer et al. (2001b) of the changes in low-abundance brain proteins following KA injury in rats, showed that protein changes were observable. These changes indicated the induction of apoptosis, the HSP system, the antioxidant response, and repair mechanisms, although the mechanism of action was unclear they concluded. They concluded low abundance gene products were of interest in proteomic studies, due to their probable involvement in disease-

related changes, and that their altered levels of modifications may carry significant biological information (Krapfenbauer et al., 2001b).

Proteomic studies have previously shown that antioxidant protein 2 (AOP2) is present in the conditioned medium of cultured astrocytes and is therefore likely secreted (Lafon-Cazal et al., 2003). It is up-regulated in the brains of rats treated with KA (Krapfenbauer et al., 2001a). It is likely therefore, that neuronal cells would also secrete AOP2. AOP2, is a member of the thiol-specific antioxidant family of proteins known to reduce reactive oxygen species (Sparling and Phela, 2003). Reactive oxygen species are generated by over-activation of the GluR by KA. This induces the expression of antioxidants such as AOP2 (Krapfenbauer et al., 2001a; Sparling and Phela, 2003).

KA is less neurotoxic on developing neurons (1-2 DIC) *in vitro* and has been shown previously to be quite low compared with mature neurons (14 DIC) (Maes et al., 1999). NGF is known to be neurotrophic via its activation of the TrkA receptor. TrkA is a gene-product that is known to be rapidly and transiently induced by environmental factors (Lee et al., 2000). NGF release and TrkA expression has been shown to occur just minutes after KA exposure, via a calcium dependent process. The released NGF then acts in a paracrine/autocrine fashion on TrkA receptors, helping protect neurons from the KA induced neurotoxicity (Lee et al., 2000).

3.12.6 Diaschisis

In addition to the area of primary injury, cell death and synaptic reorganisation have also been observed in brain regions distant to the initial site of damage (Joashi et al., 1999). These changes in regional neuronal activity, brain perfusion and metabolism in an area remote from the diseased/lesioned cortical area is called diaschisis (Thajeb et al., 2001). Apoptosis is thought to play a role in the cell death observed in diaschisis (Joashi et al., 1999), and studies have also shown axonal sprouting and the formation of new cortical connections in areas undergoing diaschisis (Kawamata et al., 1997; Carmichael et al., 2004). Focal cortical strokes in rats produced areas of cortical hypometabolism, which were larger than, and distinct from, the regions of cell-death and damage (Kawamata et al., 1997). These regions showed increased levels of growth-promoting proteins (GAP-40 and tenascin), supporting the idea that diaschisis is part of a process of reorganisation and reconnection after cortical injury (Kawamata et al., 1997). The idea that excitotoxicity, as occurs after cortical injury and in various disease conditions, results in

changes in neuronal metabolism and protein secretion is supported by the studies here. Preliminary 2D-PAGE analysis of the conditioned medium of cortical neurons stimulated with KA, demonstrated changes in the processing of albumin (see Figure 3.20). Some albumin fragments appeared to be up-regulated whilst others appeared to be down-regulated. Further investigation would likely reveal other secreted proteins that could be involved in the changes involved in diaschisis. In addition, changes in the ECF, cellular swelling, cell loss or proliferation can all effect the movement of VT signalling molecules within the CNS post-injury or damage (Zoli et al., 1999). Switches from short- to long-distance VT signalling are also thought to be a compensatory mechanism in various diseases states (Zoli et al., 1999), and have been observed in the dopamine and serotonin systems (Bjelke et al., 1994; Zoli et al., 1999). However, as so many changes overlap after an injury, including oedema, vascular changes, intracranial pressure, cell death, dispersions of toxic waste products, glial proliferation and changes in neurotransmitter levels. The complexity of all these changes makes it difficult to single out any one change as the causal factor for any transient event (Finger et al., 2004).

3.13 General issues

3.13.1 Confirmation of protein secretion

A problem encountered in proteomic studies of conditioned medium is demonstrating that identified proteins are truly secreted proteins. Cell death and subsequent lysis is difficult to overcome in cell culture and many proteins in the conditioned medium are likely to be cytosolic in origin. This issue has been tackled in different ways by other researchers studying the protein content of conditioned medium.

To identify proteins released by the vesicular pathway, Lafon-Cazal et al. (2003) applied brefeldin A (BFA), an inhibitor of secretory vesicle assembly, to primary cultures of astrocytes. Comparisons showed that many spots were absent on 2D gels after BFA treatment. Differential analysis of total cell-lysate compared with conditioned medium was also used to show that another group of proteins was significantly enriched in the conditioned medium. This was given as evidence of their secretion, suggesting that astrocytes secreted large amounts of protein through non-vesicular means (Lafon-Cazal et al., 2003). Immunostaining was used in a similar study in an attempt to confirm

identified proteins were secreted. One of the proteins seemed to be located in vesicles in the cytosol, while the other had a cytosolic and membrane distribution (Dahl et al., 2003). This demonstrates the difficulty of attempting to show secretion as the source of protein in the conditioned medium when the protein is present in the cytosol. The possibility still remains that cell lysis is the source. Most studies also rely on the fact that measures of cell viability such as LDH and live/dead assays indicate cell death is low and that correspondingly cell lysis should be minimal.

3.13.2 Overlap of the cytosol and secretome

The term secretome describes all protein components released or specifically secreted by the cell into the conditioned medium. Protein components released by lysed or dead cells are not considered part of the secretome (Volmer et al., 2004). In this study care was taken to minimise cell death to avoid intracellular or cytosolic proteins overwhelming protein secreted by the neuronal cells. However, even if no cells were to lyse, a certain overlap between the cytosol and secretome could be expected and has previously been observed. In a study by Volmer et al. (2004) on colon carcinoma cells, proteins expected to be intracellular were found in conditioned medium. These proteins had also previously been found in exosomes. The authors suggested that the overlap was due to the secretion by exosomes from the carcinoma cells into the conditioned medium (Volmer et al., 2004). Exosomes are small vesicles thought to be involved in antigen presentation. Their protein composition has been studied using proteomic technology and been shown to include cytoskeletal proteins, chaperones, adhesion, and signalling molecules amongst others (They et al., 2001). Different cell types have been shown to secrete exosomes including red blood cells, platelets, lymphocytes, dendritic cells, intestinal epithelial cells, and various cancer cell lines (Raposo et al., 1996; They et al., 2001; Van Niel et al., 2001). Similarities between the conditioned medium of cultured astrocytes and the protein composition of dendritic cell exosomes, has been given as evidence that the presence of cytosolic proteins in the conditioned medium could be due to their release by the exosome secretory pathway (Lafon-Cazal et al., 2003). However, until it has been shown that this pathway exists in astrocytes, the presence of such cytosolic proteins could also be due to cell death and lysis. No studies have yet investigated the presence of exosome-like vesicles in neurons. No cytosolic proteins were identified in this study, however, as no proteins other than BSA were identified this cannot be taken to mean they were not present.

3.13.3 Difficulties with protein identification

As encountered in these studies, it is often difficult to determine the identity of proteins using mass spectrometry. Failure can be for a variety of reasons which include:

- Comigration resulting in a mixture of proteins in one spot
- Low abundance proteins that do not provide sufficient signal
- Modifications to the protein
- The protein is not present in the database and homology searches are not always accurate (Nicolls et al., 2003).

In addition, the recovery of protein from in-gel digestion is less than 60 %, making low-abundance proteins even more difficult to detect (LoPachin et al., 2003). Low molecular weight proteins are also difficult to detect as they only generate a few tryptic fragments, generally not enough for identification (Sickmann et al., 2000).

3.14 Conclusions

The use of cell isolation techniques and medium developed by Brewer et al. (1996) enabled the establishment of almost pure cortical cultures from 21-day old postnatal male Wistar rats. Immunocytochemical techniques utilising GFAP and NeuN demonstrated that approximately 96 % of cells were neuronal, the remainder being astrocytes.

Live/dead assays utilising PI and FD, demonstrated that cell death was activated after 24 h exposure to KA at concentrations above 100 μ M, and after exposure to 100 μ M KA for 72 h. Survival under KA exposure was greater in these studies than previously seen. The presence of antioxidants from the B27 supplement, the lack of serum in culturing conditions, and the use of bFGF in the medium is proposed to be responsible. Although previously used to successfully investigate KA mediated cell death, LDH levels in these studies were found to be an unreliable marker of cell death. No correlation between cell death, as detected by PI, and the level of LDH was shown. It is likely that this was due to insufficient sensitivity in LDH quantitation.

Cortical neuronal cultures were grown in the absence (unstimulated) or presence of 50 μ M KA (stimulated), for a period of 72 h. The resulting conditioned medium was analysed using a combination of 2D-PAGE for protein separation and MALDI-TOF MS for protein identification. The protein BSA was found at unexpected levels in the

conditioned medium of unstimulated neurons. 2D-PAGE maps showed fragmentation of the albumin molecule into at least 33 spots, all of varying MW/pl. Immunocytochemistry confirmed that BSA was internalised by both the neurons and astrocytes in the cultures, in agreement with previous studies. Comparisons with the B27 supplement demonstrated that this extent of fragmentation only occurred after exposure to the neurons, raising the possibility that after internalisation the BSA was processed by the neurons and/or astrocytes.

The emerging view is that cerebrovascular dysfunction plays a role in the pathogenesis of neurodegenerative diseases such as AD. The deterioration of the BBB, allowing the entrance of serum molecules into the CNS, has been linked to the etiology of AD, and a positive correlation between the degree of dementia and the level of albumin in the CNS has been demonstrated. Serum albumin carries the largest pool of complexed metal ions in the blood plasma (Zatta et al., 2003).

It is proposed here that breakdown of the BBB, as occurs in ageing and after CNS trauma, allows the access of albumin to the CNS. The albumin-copper complex acts as a mechanism to deliver copper to the CNS and uptake of albumin by neurons allows access of copper to the cytoplasm of neurons. Cu^{2+} is linked to oxidative damage in AD through its ability, in conjunction with $\text{A}\beta$, to produce hydroxyl free radicals. The fragmentation of albumin by neurons could be a mechanism by which copper is released from the albumin molecule. It is not clear what role the uptake and processing of albumin by neurons and astrocytes plays in the undiseased state.

2D-PAGE maps of conditioned medium from cultures stimulated with 50 μM KA, showed distinct changes in the gel pattern when compared with maps of unstimulated cultures. Significantly, many of the BSA proteins identified were not visible, raising the possibility that cellular changes induced by KA exposure may have altered or inhibited the pathway by which albumin was being processed.

3.15 Future

Several possible strategies could be employed in the future to advance the studies performed here:

- Several products exist to deplete albumin from the conditioned medium, this could be used to allow the identification of other secreted proteins/factors present in the conditioned medium
- Other studies have successfully enriched for low-abundance protein by separating the protein sample, using ion-exchange chromatography, into several smaller pools, each containing a subset of proteins. Allowing for greater amounts of each subset to be loaded when performing 2D-PAGE.
- Further investigation into the cellular location of the albumin proteolysis is needed; these studies could not conclusively show whether it occurred intracellularly or extracellularly. One strategy would be to cool the cells, this has been shown to minimise the neuronal uptake of albumin.
- Initial attempts at 2D-PAGE of the cytosol of neurons were unsuccessful in these studies. Achieving this would not only show whether the albumin fragments are present intracellularly but would also help differentiate between secreted and intracellular proteins.
- Additional conditioned medium samples of cortical neurons stimulated with kainic acid need to be analysed via 2D-PAGE, and the separated protein spots identified using MALDI-TOF MS.
- Differential analysis using computer programmes between kainic acid stimulated and unstimulated conditioned medium samples, would confirm or deny the initial observations of changes in the BSA processing, and allow the identification of other differentially regulated secreted proteins.

Appendix

A.1 Ethical approvals

A.1.1 Ethics application number 2 approval:

From: Chris Thoreau

Date: 28 November 2003

Subject: Re: AEC/10/2003/R184 - Continued studies of the proteome of the brain.

This protocol is now approved.
Approval date 19 November 2003
Expiry date 19 November 2006.

A.1.2 Ethics application number 1 approval

From: Chris Thoreau

Date: 30 July 2003

Subject: AEC/06/2003/R142 - Proteomic analysis of the response of cultured neurons to chemical excitation

Herewith confirmation that the response and modified protocol has been assessed as acceptable and this application is now approved.

Approval date 8 July 2003
Expiry date 8 July 2006

Chris Thoreau, AEC Secretary, Operations Manager,
Faculty Services, FM&HS, The University of Auckland, Private Bag 92019,
Auckland, New Zealand

A.2 Composition of Neurobasal-A medium

From Brewer et al. (1993)

Component	Concentration (μM)
<i>Inorganic salts</i>	
CaCl ₂ (anhydrous)	1,800
Fe(NO ₃) ₃ .9H ₂ O	0.2
KCl	5,360
MgCl ₂ (anhydrous)	812
NaCl	51,300
NaHCO ₃	26,000
NaH ₂ PO ₄ .H ₂ O	900
<i>Other components</i>	
D-glucose	25,000
Phenol red	23
HEPES	10,000
Sodium pyruvate	230
<i>Amino acids</i>	
L-alanine	20
L-arginine.HCl	400
L-asparagine.H ₂ O	10
L-cysteine	5
Glycine	400
L-histidine.HCl.H ₂ O	200
L-isoleucine	800
L-leucine	800
L-lysine.HCl	5
L-methionine	200
L-phenylalanine	400
L-proline	67
L-serine	400
L-threonine	800
L-tryptophan	80
L-tyrosine	400
L-valine	800
<i>Vitamins</i>	
D-Ca pantothenate	8
Choline chloride	28
Folic acid	8
i-Inositol	40
Niacinamide	30
Pyridoxal.HCl	20
Riboflavin	1
Thiamine.HCl	10
Vitamin B12	0.2

A.3 Composition of Hibernate-A medium

From [http://www.brainbitsllc.com/Hibernate\(1\).html](http://www.brainbitsllc.com/Hibernate(1).html)

Component	Concentration (μM)
<i>inorganic salts</i>	
CaCl ₂	1800
Fe(NO ₃) ₃	0.25
KCl	5360
MgCl ₂	812
NaCl	89000
NaHCO ₃	880
NaH ₂ PO ₄	906
ZnSO ₄	0.67
<i>other compounds</i>	
D-glucose	25000
phenol red	23
MOPS	10,000
sodium pyruvate	227
choline chloride	28
i-inositol	40
<i>vitamins</i>	
D-Ca pantothenate	8
folic acid	8
niacinamide	30
pyridoxal	20
riboflavin	1
thiamine	10
B12	0.2
<i>amino acids</i>	
L-alanine	22
L-arginine	483
L-asparagine	5.5
L-cysteine	7.7
L-glutamine	503
glycine	400
L-histidine	200
L-isoleucine	802
L-leucine	802
L-lysine	798
L-methionine	201
L-phenylalanine	400
L-proline	67
L-serine	400
L-threonine	798
L-tryptophan	78
L-tyrosine	398
L-valine	803

A.4 Composition of Teklad 2018 vegetarian rodent diet

Ingredients

Ground wheat, ground corn, wheat middlings, soybean meal, corn gluten meal, soybean oil, calcium carbonate, dried brewers yeast, dicalcium phosphate, iodised salt, L-lysine, DL-methionine, choline chloride, niacin, vitamin A acetate, biotin, pyridoxine hydrochloride, thiamine mononitrate, vitamin D3 supplement, folic acid, menadione sodium bisulphite complex (source of vitamin K activity), vitamin E supplement, vitamin B12 supplement, riboflavin, calcium pantothenate, ferrous sulphate, magnesium oxide, manganous oxide, zinc oxide, copper sulphate, calcium iodate, cobalt carbonate, chromium potassium sulphate.

Partial Proximate Analysis

Crude Protein	0.19 %
Crude Oil	0.06 %
Crude Fiber	0.04 %

Other Components

Ash	0.06 %
Nitrogen-Free Extract	0.55 %
Carbohydrate	0.57 %
Starch	0.41 %
Sugar	0.05 %
Digestible Energy	3.40 kcal/g
Metabolisable Energy	3.30 kcal/g

Vitamins

Vitamin A	15.4 IU/g
Vitamin D3	1.54 IU/g
Vitamin E	101 mg/kg
Menadione (K3)	51 mg/kg
Thiamine (B1)	16.5 mg/kg
Riboflavin (B2)	14.9 mg/kg
Niacin (Nicotinic Acid)	41.2 mg/kg
Pyridoxine (B6)	18.5 mg/kg
Pantothenic Acid	33 mg/kg
Cyanocobalamin (B12)	0.08 mg/kg
Biotin	0.3 mg/kg
Folate	3.34 mg/kg
Choline	1120 mg/kg
Beta Carotene	2.47 mg/kg
Inositol	1455 mg/kg

Minerals

Calcium	1.01 %
Phosphorus	0.65 %
Sodium	0.23 %
Potassium	0.68 %
Chloride	0.4 %
Magnesium	0.2 %
Zinc	77 mg/kg
Manganese	118 mg/kg
Copper	15.21 mg/kg
Iodine	11.55 mg/kg
Iron	226 mg/kg
Selenium	0.2 mg/kg

Cobalt	0.63 mg/kg
Chromium	0.53 mg/kg
<i>Amino Acids</i>	
Arginine	1.06 %
Methionine	0.35 %
Cystine	0.33 %
Histidine	0.47 %
Isoleucine	0.85 %
Leucine	0.94 %
Lysine	0.92 %
Phenylalanine + Tyrosine	1.60 %
Threonine	0.67 %
Tryptophane	0.20 %
Valine	0.95 %

A.5 Sequences of BSA molecules

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