



<http://researchspace.auckland.ac.nz>

ResearchSpace@Auckland

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage.

<http://researchspace.auckland.ac.nz/feedback>

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form.

Proteomic analysis of the response of cultured neurons to chemical excitation

Ry Yves Tweedie-Cullen

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

School of Biological Sciences
University of Auckland, New Zealand

August 2004



THE UNIVERSITY OF AUCKLAND
NEW ZEALAND

University of Auckland Library thesis consent form

I agree to this thesis being consulted and/or photocopied for the purposes of research or private study provided that due acknowledgment is made where appropriate and that my permission is obtained before any material is published.

Signed:

Ry Tweedie-Cullen

Date:

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.

Acknowledgements

My labmates for all their experimental help and know-how: Tina Lowe, Josh Bradley, Anna Brooks, Kelly Le Fevre, Haylyn Wong, Leo Payne and Teba Al-Haboubi.

My proofreaders: Jess Hayward, Matt Sanders, Ralph Marrett, Christina Buchanan and Sarah Schönberger.

Merylyn and Paul for all their support during the last couple of years of this MSc.

Drs Sarah Schönberger and Christina Buchanan for all their experimental help.

Cynthia Tse for her help during my research and completing the write-up at the end.

Prof. Garth Cooper for supervising me and providing the project and funding.

Protemix Corporation and The Foundation for Research Science and Technology (FRST) for providing me with a scholarship me during this time.

Table of Contents

ABSTRACT	II
ACKNOWLEDGEMENTS	III
LIST OF FIGURES AND TABLES.....	VI
ABBREVIATIONS AND CHEMICAL FORMULAE.....	VII
COMPANY ABBREVIATIONS.....	X
CHAPTER ONE - INTRODUCTION.....	I
1.1 INTERCELLULAR COMMUNICATION IN THE CENTRAL NERVOUS SYSTEM	2
1.2 SYNAPTIC / WIRING TRANSMISSION	2
1.3 NON-SYNAPTIC / VOLUME TRANSMISSION	4
1.4 GLUTAMATE AND GLUTAMATE RECEPTORS.....	6
1.5 HYPEREXCITATION AND EXCITOTOXICITY	7
1.6 EXCITOTOXICITY AND DISEASE.....	8
1.7 PROTEOMICS	10
1.8 AIMS.....	12
CHAPTER TWO - MATERIALS & METHODS.....	13
2.1 PROJECT OVERVIEW AND RESEARCH DESIGN.....	14
2.2 CELL ISOLATION AND CELL CULTURE	15
2.2.1 Introduction.....	15
2.2.2 Poly-D-lysine coating of plates.....	15
2.2.3 Siliconised flame-polished Pasteur pipettes.....	16
2.2.4 Isolation of postnatal primary cortical neurons.....	17
2.3 CHARACTERISATION OF CULTURES.....	21
2.3.1 Introduction.....	21
2.3.2 Dot-blot.....	22
2.3.3 Immunocytochemistry.....	23
2.3.4 Determination of neuron to astrocyte ratio.....	24
2.4 CELL SURVIVAL ASSAYS AND KAINIC ACID STIMULATION	25
2.4.1 Introduction.....	25
2.4.2 Materials.....	25
2.4.3 Protocol.....	26
2.5 COLLECTION OF CONDITIONED MEDIUM AND CYTOSOL FOR ELECTROPHORESIS.....	27
2.5.1 Introduction.....	27
2.5.2 Materials.....	27
2.5.3 Protocol.....	28
2.6 WASHING AND CONCENTRATION OF CONDITIONED MEDIUM FOR ELECTROPHORESIS	29
2.6.1 Introduction.....	29
2.6.2 Materials.....	30
2.6.3 Protocol.....	30
2.7 PROTEIN DETERMINATION VIA REVERSE BIURET	31
2.7.1 Introduction.....	31
2.7.2 Materials.....	31
2.7.3 Protocol.....	32
2.8 TWO-DIMENSIONAL GEL ELECTROPHORESIS	33
2.8.1 Introduction.....	33
2.8.2 Isoelectric focusing.....	34
2.8.3 SDS-PAGE.....	35
2.9 2D GEL STAINING	37
2.9.1 Introduction.....	37
2.9.2 SYPRO Ruby staining.....	37
2.9.3 Colloidal Coomassie staining.....	38
2.10 PROTEIN IDENTIFICATION BY PEPTIDE MASS FINGERPRINTING	39
2.10.1 Introduction.....	39
2.10.2 In-gel trypsin digestion of proteins	40
2.10.3 MALDI-TOF mass spectrometry.....	41

2.10.4	Database identification of proteins.....	42
CHAPTER THREE - RESULTS & DISCUSSION.....		43
3.1	ISOLATION AND CULTURE OF NEURONAL CELLS.....	44
3.1.1	Primary cell culture.....	44
3.2	CHARACTERISATION OF CULTURES.....	45
3.3	CELL SURVIVAL ASSAYS.....	49
3.3.1	Survival in B27-free conditions.....	49
3.3.2	Survival after kainic acid exposure.....	52
3.3.3	Live/dead assay, comparison to LDH as a measure of viability.....	52
3.4	2D-PAGE ANALYSIS OF CONDITIONED MEDIUM FROM UNSTIMULATED NEURONS.....	56
3.4.1	Processing of conditioned medium.....	56
3.4.2	2D gels of conditioned medium from unstimulated cultures.....	58
3.4.3	2D gels of conditioned medium from unstimulated cultures; addition of thiourea.....	59
3.5	MALDI-TOF MS IDENTIFICATION OF PROTEINS.....	60
3.6	2D GEL OF B27 SUPPLEMENT; COMPARISON TO CONDITIONED MEDIUM.....	62
3.7	TRYPTIC PEPTIDE COVERAGE OF THE BSA MOLECULE.....	64
3.8	BSA GEL SPOT PATTERNS IN THE LITERATURE.....	65
3.8.1	Cleavage and proteolysis.....	66
3.8.2	Addition of thiourea to rehydration buffer changes gel pattern.....	67
3.9	BOVINE SERUM ALBUMIN IS INTERNALISED BY NEURONS AND GLIA.....	69
3.10	ALBUMIN IN THE BRAIN.....	70
3.10.1	Gene expression and access.....	70
3.10.2	Neuronal uptake and effects.....	71
3.10.3	Astrocyte uptake and effects.....	72
3.10.4	Albumin binds metal ions and other factors.....	73
3.11	A PROPOSED ROLE FOR ALBUMIN AS A Cu^{2+} CARRIER IN ALZHEIMER'S DISEASE.....	74
3.12	2D-PAGE ANALYSIS OF KAINIC ACID STIMULATED CORTICAL NEURONS.....	76
3.12.1	Processing of conditioned medium.....	76
3.12.2	50 μM kainic acid stimulation for 72 h.....	77
3.12.3	10 μM kainic acid stimulation for 24 h.....	78
3.12.4	Gel pattern changes after kainic acid stimulation.....	78
3.12.5	Kainic acid induced cellular changes.....	79
3.12.6	Diaschisis.....	80
3.13	GENERAL ISSUES.....	81
3.13.1	Confirmation of protein secretion.....	81
3.13.2	Overlap of the cytosol and secretome.....	82
3.13.3	Difficulties with protein identification.....	83
3.14	CONCLUSIONS.....	83
3.15	FUTURE.....	85
APPENDIX.....		86
A.1	ETHICAL APPROVALS.....	87
A.1.1	Ethics application number 2 approval.....	87
A.1.2	Ethics application number 1 approval.....	87
A.2	COMPOSITION OF NEUROBASAL-A MEDIUM.....	88
A.3	COMPOSITION OF HIBERNATE-A MEDIUM.....	89
A.4	COMPOSITION OF TEKLAD 2018 VEGETARIAN RODENT DIET.....	90
A.5	SEQUENCES OF BSA MOLECULES.....	92
BIBLIOGRAPHY.....		93

List of figures and tables

Figure 1.1: Neuron schematic showing the cell body and axon.....	2
Figure 1.2: A chemical synapse between two connected neurons.....	3
Figure 1.3: Forms of intercellular communication in the brain.....	5
Figure 1.4: The glutamate receptor and glutamate release.....	6
Figure 1.5: Excitotoxicity as a result of stroke.....	8
Figure 2.1: Diagram of the protocol followed in this research.....	14
Figure 2.2: Diagram of the brain regions isolated.....	19
Figure 2.3: The fractions collected after gradient separation of the dissociated cortical cells.....	20
Figure 2.4: Optimised conditions for medium collection.....	28
Figure 2.5: The three main steps of two-dimensional gel electrophoresis.....	33
Figure 2.6: Summary of the method employed to identify proteins previously separated by 2D-PAGE....	40
Figure 2.7: Four sample spots were spotted around each calibration spot; triangulation for near-point calibration.....	41
Figure 3.1: Phase-contrast microscopy of primary rat cortical cells after 14 days in culture.....	45
Figure 3.2: Immunocytochemistry of primary cultures with anti-NeuN antibodies.....	47
Figure 3.3: Immunocytochemistry of primary cultures with anti-NeuN and anti-GFAP antibodies.....	48
Figure 3.4: Photomicrographs of the live/dead assay shortly after exposure to kainic acid.....	53
Figure 3.5: Graph of data in Table 3.3. Neuronal survival after exposure to kainic acid for 24 h.....	54
Figure 3.6: Graph of data in Table 3.4. Neuronal survival after exposure to kainic acid for up to 72 h.....	55
Figure 3.7: 2D-PAGE of protein from conditioned medium of unstimulated cortical neurons after 72 h of growth.....	58
Figure 3.8: 2D-PAGE of protein from conditioned medium of unstimulated cortical neurons after 72 h of growth.....	58
Figure 3.9: 2D-PAGE of protein from conditioned medium of unstimulated cortical neurons after 72 h of growth.....	59
Figure 3.10: 2D-PAGE of protein from conditioned medium of unstimulated cortical neurons after 72 h of growth.....	59
Figure 3.11: Spots identified as bovine serum albumin by MALDI-TOF MS.....	60
Figure 3.12: 2D gel of the B27 medium supplement.....	62
Figure 3.13: Comparison between the B27 supplement and conditioned medium from unstimulated cortical neurons.....	63
Figure 3.14: Tryptic peptides identified by MS as BSA, aligned to show the sequence coverage of the peptides across the full BSA molecule.....	64
Figure 3.15: Protein from conditioned medium rehydration with and without 2 M thiourea.....	68
Figure 3.16: Photomicrographs of cortical cells stained with anti-BSA antibodies.....	69
Figure 3.17: Protein from conditioned medium of neurons stimulated for 72 h with 50 μ M kainic acid....	77
Figure 3.18: Protein from conditioned medium of neurons stimulated for 72 h with 50 μ M kainic acid....	77
Figure 3.19: Protein from conditioned medium of neurons stimulated for 24 h with 10 μ M kainic acid....	78
Figure 3.20: Comparison between the conditioned medium of unstimulated cortical neurons and cortical neurons stimulated for 72 h with 50 μ M kainic acid.....	78
Table 2.1: Volume of 0.1 mg/ml poly-D-lysine added to each well/flask.....	16
Table 2.2: Volume of growth medium added to each well/flask.....	21
Table 2.3: The primary and secondary antibodies used to target either astrocytes, neurons or BSA.....	24
Table 2.4: The volume of rehydration buffer and amount of protein used to rehydrate the IPG strips....	34
Table 3.1: Composition of B27 medium supplement for neuronal cultures.....	50
Table 3.2: LDH levels in the conditioned medium of cultured neuronal cells.....	51
Table 3.3: LDH levels and live/dead assay results for cortical neurons exposed to 0-1000 μ M kainic acid for 24 h.....	54
Table 3.4: LDH levels and live/dead assay results for cortical neurons exposed to 50 μ M or 100 μ M kainic acid for 72 h.....	55
Table 3.5: The protein concentration of the purified and concentrated conditioned medium.....	57
Table 3.6: The identities of protein spots as determined using MS.....	61
Table 3.7: The protein concentration of the washed and concentrated conditioned medium.....	76

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
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