

The Molecular, Cellular and Behavioural Effects of Electroconvulsive Stimulation in the Rodent Brain



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DECLARATION

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Summary

Electroconvulsive therapy (ECT) is the most effective treatment available for severe depression however its mechanisms of action have not been fully established. ECT administration is associated with memory deficits. To maintain the antidepressant efficacy of ECT while reducing its associated side effects, the parameters by which ECT is administered have been adjusted. Recently, it has been proposed that reducing stimulus pulse width from brief pulse (0.5 ms) to ultra brief (0.3 ms) pulse may maintain the treatment effect of ECT but reduce the side effects.

Electroconvulsive stimulation (ECS) is the animal model of ECT. Animals were administered a chronic course of either brief pulse, ultra brief pulse or sham ECS, consisting of ten treatments. The cellular, molecular and behavioural effects of the different pulse widths were compared. Brief pulse but not ultra brief pulse ECS induced a significant increase in cell proliferation in the dentate gyrus. Brief pulse ECS treatment resulted in a significant increase in brain derived neurotrophic factor (BDNF) expression in the hippocampus but not the frontal cortex. Ultra brief pulse ECS did not significantly affect BDNF expression in either brain region. Behaviourally, brief pulse ECS treatment induced an effective antidepressant response in the forced swim test by significantly reducing animal immobility time. This result was not obtained in ultra brief pulse treated animals. In summary, brief pulse ECS is more effective at inducing the cellular, molecular and behavioural effects associated with antidepressant treatment than ultra brief pulse ECS. Further study will be required to fully determine whether ultra brief pulse ECS offers an improvement over the side effects associated with ECS treatment.

Proteins are cellular workhorses and control many of the functions carried out in the cell (Strong and Eisenberg, 2007). ECS models an effective antidepressant treatment but the proteomic changes it induces in the hippocampus and frontal cortex have yet to be studied. Therefore, animals were treated with an acute, chronic or chronic + 4 week course of ECS and samples for 2-dimensional Difference in Gel Electrophoresis (2D-DiGE) were collected. The samples were fluorescently labelled and separated in two dimensions according to their isoelectric point and molecular weight. This resulted in the resolution of over 1,000 protein spots in each study. Univariate and multivariate data analysis techniques were employed to determine which protein spots were differentially regulated following treatment with ECS.

Protein spots with a p-value ≤ 0.05 were short-listed for selection and identification. Preparative 2D gels were run and, unlike DiGE, the gels were post-stained. The short-listed proteins were picked from the preparative gels. Due to technical differences between gels, a limited number of significantly differentially expressed protein spots were picked. The selected spots were trypsin digested and identified by Linear Trap Quadrupole-Orbitrap mass spectrometry. This process was conducted for tissue samples collected from the hippocampus and the frontal cortex.

Mass spectrometry identified multiple proteins with several different proteins identified in each proteins spot. In order to organise and categorise the identified proteins, gene ontology (GO) analysis and pathway analysis [for example the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Ingenuity Pathway Analysis (IPA)] were conducted for each study. This highlighted the expression of cytoskeletal-related proteins, in particular, following ECS administration in both the hippocampus and frontal cortex. A number of cytoskeletal-related proteins were selected for further confirmation by semi-quantitative immunoblotting. Actin was statistically differentially expressed following ECS while trends in expression were found between control and ECS samples for other cytoskeletal proteins.

ECS treatment induced a range of subtle changes in cytoskeletal protein expression that may be involved in its mechanisms of action. Future work analysing the proteins identified following ECS treatment will help to elucidate the effects of ECS and potentially of ECT on the brain.

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Abbreviations

°C	degrees celsius	L	Litre
μ	micro	LPS	lipopolysaccharide
μg	microgram	LTQ	linear trap quadrupole
μl	microlitre	M	molar
μm	micron	mA	milli Amp
2D	two dimensional	MAOI	monoamine oxidase inhibitor
5-HIAA	5-hydroxyindoleacetic acid	MAPK	mitogen activated protein kinase
5-HT	5 hydroxytryptamine (serotonin)	max	maximum
Abs	Absorbance	MCM2	minichromosome maintenance protein 2
A	Amplitude	MDD	major depressive disorder
AB	avidin biotin complex	met	methionine
ACTH	adrenocorticotrophin	mg	milligram
Akt	Akt	ml	millilitre
AMU	atomic mass unit	ML	molecular layer
ANOVA	analysis of variance	mm	millimetre
APA	American Psychiatric Association	mRNA	messenger ribonucleic acid
APS	ammonium persulphate	MS	mass spectrometer
BAD	Bcl-2 associated death promoter	msec	millisecond
Bax	Bcl-2 associated protein X	MST	magnetic seizure therapy
Bcl-2	B cell lymphoma 2	mTOR	mammalian target of rapamycin
BDNF	brain derived neurotrophic factor	MVA	multivariate analysis
BrdU	5-Bromo-2'-deoxyuridine	MW	molecular weight
BSA	bovine serum albumin	n	number of animals in study/group
BW	beta coefficient weights	NAc	nucleus accumbens
CA1/CA3	cornu ammonis 1/3	NaOH	sodium hydroxide
Ca ²⁺	calcium ion	NaSSAs	noradrenaline and selective serotonin antidepressant
CamK	Ca ²⁺ /calmodulin dependent kinases	ng	nanogram
cAMP	cyclic adenosine monophosphate	NGF	nerve growth factor
CBT	cognitive behaviour therapy	NIH	novelty induced hypophagia
CHAPS	3-[(3-cholamidopropyl)	NIK	NFκB interacting kinase

	dimethylammonio]-1-propanesulphonate		
cm	centimetre	nk1	tachykinin receptor
COMT	catechol O-methyltransferase	NM	normetanephrine
CREB	response element binding protein	NMDA	N-methyl-D-aspartate
crf1	corticotrophin releasing factor 1	NP-40	nonidet P-40
CRH	corticotrophin-releasing hormone	NT-3	neurotrophin-3
CSF	cerebrospinal fluid	OCT	optimal cutting temperature
Cy	cyanine	OD	optical density
DAB	diamnobenzadine	P	probability
DAG	diacylglycerol	PAGE	poly acrylamide gel electrophoresis
DAVID	database for annotation, visualisation and integrated discovery	PBS	phosphate bufered saline
DBS	deep brain stimulation	PCA	principal components analysis
DEX	dexamethasone	pg	picogram
dH2O	distilled water	pH	acid/base scale
DHEA	dehydroepiandrosterone	pI	isoelectric point
DIGE	difference in gel electrophoresis	Pi-3	phosphatidylinositol-3 kinase
DL DTT	dextrorotary levorotary dithiothreitol	PKA	protein kinase A
DMF	dimethyl formamide	PKC	protein kinase C
DPX	distrene-8 plasticiser xylene	PMT	photomultiplier tube
DSM	diagnostic and statistical manual	PP2A	protein phosphatase 2A
DTT	dithiothreitol	PTSD	post-traumatic stress disorder
DUSP	dual specificity phosphatases	PVDF	polyvinylidene fluoride
ECF-L	eosinophil chemotactic factor-L	Rap-1	ras-related protein 1
ECS	electroconvulsive stimulation	Ras	rast sarcoma GTPase
ECT	electroconvulsive therapy	ROS	reactive oxygen species
EDTA	Ethylenediamine tetraacetic acid	Rsk	ribosomal s6 kinases
ELISA	enzyme linked immunosorbent assay	RT	room temperature
ERK	extracellular regulated kinases	rTMS	repetitive transcranial stimulation
EU	European union	s	seconds

FGF	fibroblast growth factor	SDS	sodium dodecyl sulphate
Flk-1	fetal liver kinase 1	SGZ	subgranular zone
FRL	Flinders resistant line	SNRI	selective noradrenaline reuptake inhibitor
FSL	Flinders sensitive line	SRI	selective reuptake reagent
FST	forced swim test	SSRE	selective serotonin reuptake enhancer
g	gram	SSRI	selective serotonin reuptake inhibitor
GCL	granule cell layer	STEP	striatal enriched phosphatase
GDNF	glial cell line derived neurotrophic factor	TBS	Tris Buffered Saline
GO	gene ontology	TCA	tricyclic antidepressant
GR	glucocorticoid receptor	TEMED	tetramethylenediamine
GSK	glycogen synthase kinase	TMB	tetramethylbenzidine
GWAS	genome wide association studies	TNF	tumour necrotic factor
HCl	hydrochloric acid	Tris	tris(hydroxymethyl) aminomethane
HDRS	Hamilton Depression Rating Scale	Trk	tropomyosin receptor kinase
HIV/AIDS	human immunodeficiency virus/acquired immunodeficiency syndrome	Tx	Treatment
HPA	Hypothalamic-Pituitary-Adrenal	UK	United Kingdom
HRP	horse radish peroxidase	UVA	univariate analysis
IAA	iodoacetamide	v/v	volume per volume
IDO	indoleamine 2,3-dioxygenase	val	valine
IFN	interferon	VC/VS	ventral capsule/ventral striatum
IGF	insulin like growth factor	VEGF	vascular endothelial growth factor
IL	interleukin	VGF	inducible nerve growth factor
IP3	inositol tris phosphate	VTA	ventral tegmental area
IPG	immobilised pH gradient	w/v	weight/volume
KEGG	Kyoto encyclopaedia of genes and genomes	WHO	World Health Organisation
KG	kilogram	WPM	water plus maze
α	Alpha	XCorr	cross correlation
β	Beta	κ	kappa
γ	gamma		

Introduction

1.1 Depression

Major depression is a serious illness that affects more than one hundred and twenty one million people worldwide (World Health Organisation, 2008). The World Health Organisation estimates that depression will be the world's second leading contributor to the global burden of disease by 2020. However, little is known about the pathobiology of the disease. Diagnosis of depression is based on fulfilling clinical criteria set forth in reference texts such as the Diagnostic and Statistical Manual of Mental Disorders-IV (American Psychiatric Association, 1994).

Up to 50% of patients with depression will respond to their first course of antidepressant treatment, however only 50-70% will achieve remission of symptoms, the end goal of treatment (Rush et al. 2003). Patients who are switched to a second, third or fourth new treatment have lower rates of remission and higher relapse rates (Rush et al. 2006). Electroconvulsive therapy (ECT) is a suitable treatment for these patients and those suffering from severe depression. Despite the proven efficacy of ECT (The UK ECT Review Group, 2003) its mechanisms of action still remain to be fully explained.

Elucidating the molecular changes induced by ECT may in turn provide new insight into the neurobiology of depression. Several major hypotheses have been developed to explicate the neurobiology of depression. A summary of these hypotheses and the role of antidepressant treatments, particularly ECT and its animal model electroconvulsive stimulation (ECS) in depression are discussed below.

Studying the molecular effects of existing treatments for depression allows for a greater understanding of the neurobiology of the illness itself and is possibly one of our greatest tools in discovering novel therapeutics to combat depression.

1.1.1 Clinical features of depression

Several criteria must be fulfilled before a diagnosis of depression can be determined. A number of symptoms must be present during the same two-week period and they must demonstrate a change from previous behaviour. The symptoms of depression can include:

- Depressed mood for most of the day
- Anhedonia, a loss of pleasure or interest in almost all activities
- Low energy or fatigue

These three symptoms are most characteristic of depression and are subjective or observable by others and are present nearly every day. Other symptoms are also often present and may be present nearly every day during a depressive period. They include:

- Weight loss or weight gain or an increase or decrease in appetite
- Insomnia or hypersomnia
- Feelings of guilt and worthlessness, low self-esteem or pessimism
- Psychomotor disturbances including agitation or retardation
- Reduced concentration, indecisiveness
- Thoughts of death or suicide, attempts at suicide

One of the symptoms must be a depressed mood or anhedonia. Symptoms that are a result of a general medical condition or induced by a substance (medication or drug of abuse) should not be included in diagnosis. The symptoms should negatively influence social or occupational functioning and result in clinically significant stress. The symptoms should not be a consequence of a recent bereavement (within two months) (American Psychiatric Association, 1994; Wong and Licinio, 2001, 2004). This range of symptoms describes multiple clinical subtypes of depression, which are not clearly biologically separated. These are classified in Table 1-1.

- Onset: early, postpartum or late
- Clinical course: single episode, recurrent, chronic
- Severity: mild, moderate, severe
- In remission: partial or full
- With/without psychotic features: mood congruent or incongruent
- With/without catatonic features
- With atypical features
- With melancholic features
- Seasonal pattern
- Secondary to medical illness

Table 1-1 Classification categories for depression, from Wong and Licinio, 2001

A depression-like syndrome can also arise with medical illnesses including infectious diseases like HIV/AIDS and neurological disorders such as chronic pain and Alzheimer's disease. Different drugs, for example alcohol and anti-inflammatory and analgesic medications such as indomethacin, can also induce the symptoms of depression

(Wong and Licinio, 2004). Due to the complex nature of the disorder, the pathophysiology of depression has been difficult to deduce. Equally, tackling depression therapeutically has been challenging.

The first antidepressant drug therapies were discovered serendipitously. Iproniazid, a monoamine oxidase inhibitor (MAOI), became a treatment for depression based on the clinical effects discovered during the course of its use as a treatment for tuberculosis (Bloch et al. 1954). Imipramine, a tricyclic antidepressant (TCA) and effective treatment for depression, was initially developed for use as an antipsychotic, based on the precursor drug chlorpromazine (Kuhn, 1957; Azima and Vispo, 1958). The development of pharmacotherapies for depression led to increased research into the underlying mechanisms through which the treatments acted.

1.2 Treatment of depression

According to the National Institute for Health and Clinical Excellence (NICE, 2009) guidelines, psychotherapy is recommended to treat mild depression. Pharmacotherapy is not routinely recommended for mild depression unless there is a history of moderate or severe depression or if mild depression persists after other interventions. It is recommended for treatment of moderate or severe depression, possibly in combination with psychotherapy. ECT is recommended for severe, life-threatening depression or when other treatments have failed. Mildly depressed patients have few, if any, symptoms in excess of the five required to make a diagnosis of depression (see 1.1.1). Severe depression is diagnosed when patients have most of these symptoms and experience significant functional impairment. The symptoms or functional impairment associated with moderate depression fall between those diagnosed for mild or severe depression.

1.2.1 Psychotherapy

Various types of psychotherapy are used in the treatment of mild depression with few differences in efficacy between the treatment types compared (Cuijpers et al. 2008). However, cognitive behaviour therapy (CBT) is one of the most commonly recommended and well-established forms of psychotherapy available (Gabbard, 2007a; NICE, 2009).

1.2.1.1 Cognitive behaviour therapy (CBT)

CBT alleviates the symptoms of depression by directing treatment at cognition, behaviour and physiology. Patients apply cognitive restructuring techniques and adaptive thinking strategies to correct negatively distorted thoughts that can underlie depression.

Activity scheduling and social skills are taught to combat the behavioural deficits that can lead to and maintain depression, such as social withdrawal. Agitated and anxious patients learn meditation and relaxation techniques as calming measures (Weissman, 2001). CBT is considered to be an effective treatment for depression with a similar efficacy to pharmacotherapy and improves symptoms better than no treatment (Gabbard et al. 2007b). Meta-analysis has shown that patients receiving CBT were significantly more likely to improve to a point where they were no longer considered clinically depressed than patients receiving other forms of psychotherapy (psychodynamic therapy and supportive therapy) (Churchill et al. 2001). However, a more recent meta-analysis states that, while CBT is an effective treatment, it has only a modest therapeutic effect (Lynch et al. 2010). Both meta-analyses suggest that, due to the low quality of many of the studies included in the meta-analyses, further studies will be required on this subject (Churchill et al. 2001; Lynch et al. 2010). The effects of CBT on depression may be overestimated due to publication bias (Cuijpers et al. 2010). However, CBT is recommended by the NICE guidelines (NICE, 2009) for the treatment of mild depression and in combination with pharmacotherapy for more severe forms of depression.

1.2.2 Pharmacotherapy: summary of different drug classes treatments

Drug treatments for depression can be classed into three major groups (Kandel et al. 2000). Briefly, the monoamine oxidase inhibitors (MAOIs), the first group to be discovered, act by preventing the oxidation of serotonin or noradrenaline. They inhibit the degradation of serotonin and noradrenaline present in a free state in the presynaptic terminal. 5-hydroxyindoleacetic acid (5-HIAA) is the product of serotonin degradation by the enzymes MAO and aldehyde dehydrogenase. Noradrenaline is broken down by catechol O-methyltransferase (COMT) into normetanephrine. Phenelzine and tranylcypromine are examples of MAOIs. Due to the side-effects associated with MAOI use, they are no longer frequently prescribed.

Tricyclic antidepressant (TCA) compounds inhibit the general reuptake of biogenic amines. Serotonin and noradrenaline reuptake into the presynaptic terminal is prevented. The neurotransmitters thus remain in the synapse for longer, resulting in a greater postsynaptic effect. Examples of TCAs include imipramine, amitriptyline and desipramine, which is particularly effective at blocking noradrenaline reuptake. TCAs are most commonly used to treat severe depression.

The final main drug class is the selective reuptake inhibitors (SRIs). The selective serotonin reuptake inhibitors (SSRIs) act in much the same way as the TCAs, by inhibiting

the reuptake of neurotransmitter into the presynaptic terminal, but they act specifically to prevent serotonin reuptake. Fluoxetine and citalopram are examples of SSRIs. SSRIs have a superior side effect profile compared to the other two drug classes and are more easily tolerated by patients (Kandel et al. 2000). They are commonly used to treat moderate depression. Other types of SRIs have also been developed to treat depression (Leonard, 2003). Briefly,

- The noradrenaline reuptake inhibitor (NRI) reboxetine selectively inhibits noradrenaline reuptake, however it was shown to be significantly less efficacious clinically than a range of other antidepressant drugs following meta-analysis (Cipriani et al. 2009).
- Selective serotonin and noradrenaline reuptake inhibitors (SNRIs) were developed to combine the efficacy of TCAs with the tolerability of SSRIs and NRIs. Venlafaxine and milnacipran, for example, may be more effective at treating severe depression than SSRIs but do not appear to be as cardiotoxic as the TCAs. However, SNRIs are associated with a higher death rate if taken in overdose than the SSRIs.
- Tetracyclic compounds like mirtazepine, which is also known as a noradrenaline and selective serotonin antidepressant (NaSSA), acts as an α -2 adrenoceptor antagonist and increases noradrenaline release. Mirtazepine acts directly on 5-hydroxytryptamine (5-HT) receptors, acting as a 5-HT_{2A} receptor antagonist. 5-HT_{1A} is indirectly activated by mirtazepine and, like SSRI administration, this results in an anti-anxiety effect. Although mirtazepine is associated with side-effects such as weight-gain, it is more easily tolerated than the earlier-developed tetracyclic antidepressant, mianserin (Leonard, 2003).
- Tianeptine is a selective serotonin reuptake enhancer (SSRE) that is structurally similar but pharmacologically different from the TCAs. It is an effective antidepressant, relieves symptoms of anxiety and has been shown to have good tolerability in comparison to TCAs (McEwen et al. 2010).

The effect of an antidepressant drug treatment may be enhanced by administering it with a non-antidepressant drug, for example lithium (Vieta and Colom, 2011). This is commonly done if patients only partially respond to the initial antidepressant treatment. Meta-analysis has shown that lithium is an effective augmentation therapy but it is only moderately effective at increasing the rate of response to antidepressant treatment in depressed patients (Crossley and Bauer, 2007).

However, despite the research into new pharmacotherapies, the most effective therapy available for depression is not a drug treatment but the physical treatment ECT.

1.2.3 Electroconvulsive therapy

Electroconvulsive therapy (ECT) has been available to patients for over seventy years and is frequently recognised as the most efficacious treatment for severe depression (The UK ECT Review Group, 2003). ECT has achieved remission rates of 75% to 80% in a variety of studies and meta-analyses (Petrides et al. 2001; The UK ECT Review Group, 2003; Husain et al. 2004). The UK ECT Review Group meta-analysis included studies where patients were administered the TCAs imipramine and amitriptyline, the MAOI phenelzine, the SSRI paroxetine, lithium, or a combination of these drugs. The effect of drug treatment and ECT on depression was measured by the Hamilton Depression Rating Scale (HDRS). Pharmacotherapy was found to be significantly less effective at reducing HDRS scores compared to ECT treatment. An average difference of 5.2 points on the HDRS was calculated (The UK ECT Review Group, 2003). ECT treatment effect was superior to sham ECT as well as placebo treatment (Kho et al. 2003; Pagnin et al. 2004).

1.2.3.1 History of ECT and clinical effectiveness

Seizure induction has been used for centuries to treat mental illness; during the 16th century Paracelsus, a Swiss alchemist, chemically induced seizures to “cure lunacy” (Prudic, 2005). However, it was the scientific investigations initiated by Ladislaus von Meduna that led to the development of ECT. In 1934, Meduna induced epileptic seizures in an effort to compensate for the loss of glial cells seen in schizophrenic patients. He successfully induced seizures by administering camphor and later metrazol. Chemical convulsive therapy induced unpleasant sensations in conscious patients during the preictal stage of treatment. As a result, electrically induced seizures replaced chemical seizure induction in the treatment of mental disorders (Abrams, 1997a).

ECT has been used therapeutically since the 1930's. Ugo Cerletti, with others, had studied the induction of epileptic seizures in dogs by electrical currents. Further work was conducted, under Cerletti's direction in the Clinic for Mental and Nervous Diseases in Rome, which led to the development of a safe and reliable method for seizure induction in dogs and pigs. In 1938, the method was refined for use on human patients. The first patient was successfully treated that year and “electroshock” became a new therapy for the treatment of psychopathologies (Accornero, 1988). Shortly after, the use of ECT spread to the United States. By the 1950's it was extensively used to treat a broad range of symptoms and had become a mainstay for the treatment of psychiatric disorders (Abrams, 1997a). The introduction of effective pharmacotherapy for depression during the early 1950s led to a decline in ECT use before a small resurgence in popularity in the 1980's

(Thompson et al. 1994). Abrams estimated that between 1 and 2 million patients worldwide receives ECT each year (Abrams, 1997a). Approximately 100,000 patients receive ECT in the U.S. annually (National Institute of Mental Health, 2008).

It is not yet understood how controlled seizure induction normalise a dysfunctional brain when spontaneous seizures are damaging and even dangerous to the brain and person (Fink, 2009). The mechanisms of ECT remain to be fully elucidated.

1.2.3.2 Side-effects of ECT

ECT is a medically safe and effective treatment for depression. It is mainly used to treat cases of severe depression, usually after multiple drug treatments have been administered but failed. It is recommended for treatment of cases of drug resistant depression (Task Force Report of the American Psychiatric Association, 2001). One reason why ECT is not administered earlier in the course of the illness is due to the range of side-effects associated with treatment. The side-effects range from physical to cognitive deficits and memory loss. Immediately after treatment, patients may experience disorientation, headache or backache. Nausea and vomiting, often a result of the anaesthetic wearing off, may also be experienced. However, these side-effects are short lived and easily treated (Fink, 2009).

Memory loss, whether short term or longer, is reported by patients to be one of the main reasons to seek other forms of treatment before agreeing to ECT as a form of therapy. Cognitive side-effects have been reported by patients since ECT was first introduced (Prudic et al. 2000). In reality, many of the cognitive side-effects of ECT are short-lived and do not have a significant long-term impact on patients. A meta-analysis of eighty-four studies, looking at twenty-four different cognitive variables found that cognitive deficits did not persist among the variables after fifteen days. For the first three days following ECT, there was evidence of significant cognitive impairment. Medium to large deficits were seen in executive functioning in this period. After fifteen days, most variables examined improved to, or beyond, baseline levels (Semkovska and McLoughlin, 2010). Depressed patients had lower performance scores in memory tests conducted before ECT than non-depressed controls. After ECT, the scores were again lower in depressed patients suggesting retrograde amnesia. However, at a three-month follow-up, depressed patient scores had returned to pre-treatment levels. Anterograde memory showed no significant deterioration following ECT. Overall, memory deficits induced by ECT were temporary (Meeter et al. 2011).

Seizure induction is required for ECT to have an antidepressant effect. Treatments with lower, subconvulsive doses of electrical stimulation were not sufficient to induce an antidepressant response in patients (Ulett et al. 1956). In rodents, subconvulsive seizure induction did not induce sprouting of mossy fibres, the axons of dentate gyrus granule cells, while electroconvulsive stimulation (ECS) did (Lamont et al. 2001). However, a more recent study has manipulated a subconvulsive electrical stimulation protocol and may have reduced ECS-associated cognitive side-effects while still having an antidepressant effect (Gersner et al. 2009).

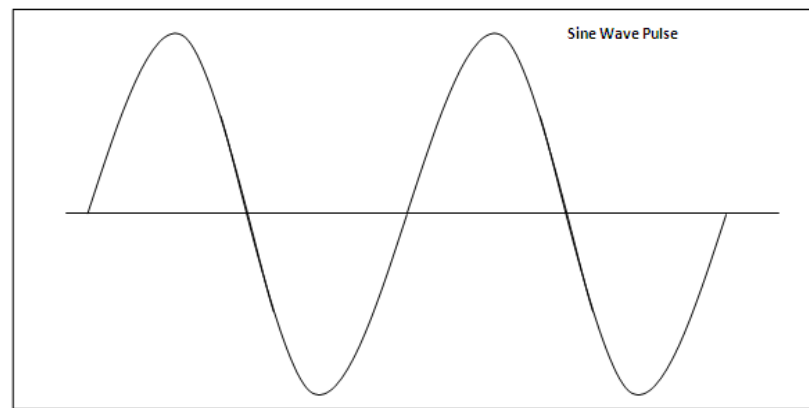
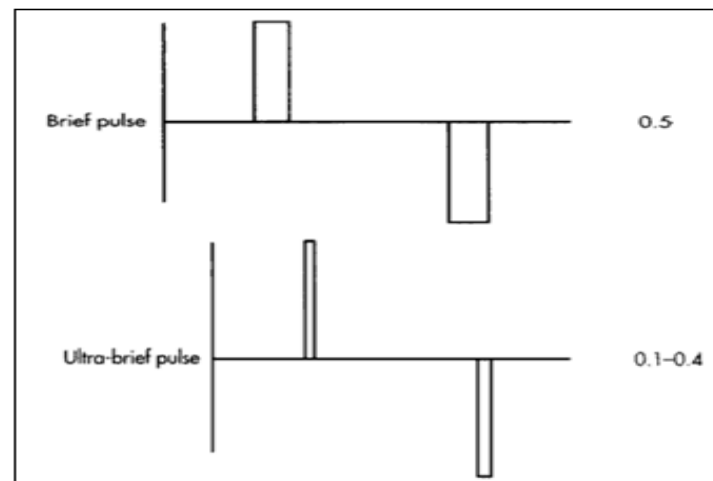
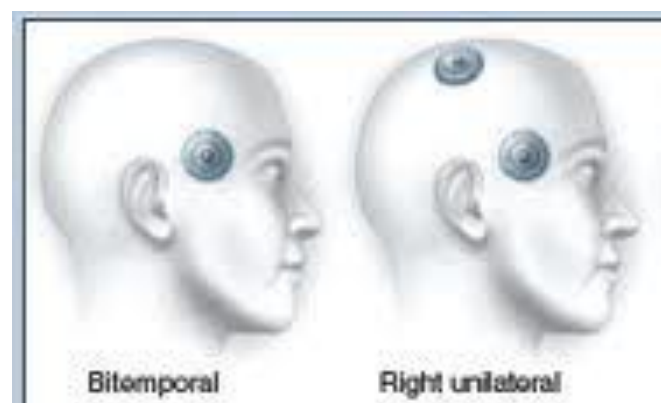
The mortality rate associated with ECT is low. Patients treated with ECT in a psychiatric hospital were less likely to die of natural causes than their fellow in-patients (Munk-Olsen et al. 2007). In Texas, there were two to ten deaths per 100,000 sessions of ECT over a five-year period (1993-1998). The mortality rate associated with anaesthesia use was two per 100,000, based on 48,852 sessions of ECT (Shiwach et al. 2001). In the U.S. death by lightning is six times more likely than by ECT (Abram, 1997b).

The public perception of ECT as a frightening and damaging treatment arises from its inaccurate representation in the media and a history of poor treatment practice (Payne and Prudic, 2009). Despite the side-effects associated with this treatment, ECT is an effective treatment for severe depression and may be the most suitable therapeutic option available for patients with treatment resistant depression.

1.2.3.3 Modifications to ECT to improve side effect profile

ECT has been refined in an effort to reduce the associated side-effects. Treatment parameters that have been modified include pulse waveform, electrode placement and stimulus pulse width.

ECT was traditionally delivered in sine-wave form (Figure 1-1(A)) which was replaced by brief pulse delivery (Figure 1-1 (B)) when it was determined that brief pulse ECT induced fewer side-effects (Weiner et al. 1986). Sine wave ECT delivered almost seven times the current (coulombs per second) of brief pulse ECT. Brief pulse ECT has a typical pulse width of 0.5ms – 1.5 ms while a sine wave had a phase period of 8.33ms (Sackeim et al. 2008). The excess inefficient energy of sine wave ECT may explain its poor side effect profile even compared to brief pulse ECT.

(A)**(B)****(C)****Figure 1-1 Treatment parameters for ECT**

(A) Sine wave bidirectional pulse form **(B)** Brief pulse and ultra brief pulse ECT. Brief pulses range from approximately 0.5ms-1.5ms in width; ultra brief pulses are normally up to 0.3ms wide. **(C)** Bilateral and unilateral electrode placement, from (Lisanby, 2007).

Another modification to the ECT administration protocol is to deliver ECT unilaterally instead of bilaterally (Figure 1-1(C)). Bilateral ECT is administered via electrodes placed fronto-temporally on both sides of the head. Unilateral ECT is administered via electrodes placed on the non-dominant hemisphere. One electrode is placed temporally and one is placed ipsilateral to the vertex of the head on the same hemisphere (Fink, 2009). Unilateral ECT must be delivered at a higher intensity than bilateral ECT to be an effective antidepressant treatment (Sackeim et al. 2000; Kellner et al. 2010). Typically, unilateral ECT is delivered at 6 times seizure threshold while bilateral ECT is delivered at 1.5 times seizure threshold (Sienaert et al. 2009, 2010). Seizure threshold is determined by repeatedly administering incremental subconvulsive stimulation until the smallest electrical dose required to induce a tonic-clonic seizure of a minimum acceptable duration is obtained (Abrams, 1997a).

However, unilateral ECT does not appear to be as effective an antidepressant treatment as bilateral ECT (Sackeim et al. 1993; Rosa et al. 2006). Unilateral ECT may result in fewer cognitive side-effects than bilateral ECT. Deficits in memory for events and details, impersonal memories, are more apparent in bilateral than unilateral ECT (Lisanby et al. 2000). However, some authors report little difference in the memory deficits induced by unilateral or bilateral ECT (Kellner et al. 2010).

Few preclinical studies have been conducted to examine the effect of unilateral or bilateral ECS on the rodent brain, with a single study looking at the effects of electrode placement on dopamine release (McGarvey et al. 1993) in which bilateral ECS was found to induce greater dopamine release than unilateral ECS. No studies comparing the antidepressant efficacy of bilateral compared to unilateral ECS have been conducted to date.

Another mechanism to improve the side-effect profile of ECT is the suggestion that delivering the charge in ultra brief instead of brief pulse form may reduce the cognitive side-effects while maintaining therapeutic efficacy (Figure 1-1 (B)). An ultra brief pulse is typically no more than 0.3ms wide while brief pulse ECT is normally delivered in 0.5ms-1.5ms wide pulses (Niemantsverdriet et al. 2011; Roepke et al. 2011). Ultra brief pulse ECT treatment produced less anterograde and retrograde amnesia. Ultra brief pulse unilateral ECT memory deficits were significantly less severe than those deficits induced by brief pulse and ultra brief pulse bilateral ECT (Sackeim et al. 2008). Ultra brief pulse unilateral ECT appeared to result in fewer cognitive side-effects especially in relation to retrograde autobiographical memory and visual and verbal information retention than did standard unilateral ECT (Loo et al. 2008).

While some clinical studies have proposed that ultra brief pulse ECT is as efficient as brief pulse ECT (Sackeim et al. 2008; Niemantsverdriet et al. 2011) others suggest that this treatment results in a slower, less effective clinical response (Loo et al. 2008; McCormick et al. 2009).

The side-effects of ECT can be disturbing for patients and coupled with the stigma attached to the treatment may prevent its use in clinically appropriate situations. Investigating the parameters of ECT has lead to an improvement in the side-effect profile. However, further study is required to minimise the negative effects associated with this treatment while maintaining its therapeutic efficacy.

1.2.4 Other physical therapies for depression

1.2.4.1 Repetitive transcranial magnetic stimulation (rTMS)

rTMS involves passing a high-intensity electrical current through an electromagnetic coil that is placed on the scalp. This is turned on and off to generate repetitive pulses. The magnetic pulses are focused on specific brain regions to induce an electrical stimulation and therapeutic effect (Howland et al. 2011). The dorsolateral prefrontal cortex is commonly targeted as it implicated in neuropsychiatric disorders. rTMS appears to be a less effective treatment for depression when compared to ECT (Eranti et al. 2007).

1.2.4.2 Magnetic seizure therapy (MST)

rTMS can be used to administer a seizure if it is delivered in very high frequency intensities; this is called magnetic seizure therapy (MST). MST can produce seizures in specific brain regions, reducing the spread of seizure to other areas and may reduce the cognitive side-effects associated with seizure induction. MST could provide a substitute for ECT, however, its antidepressant effects have yet to be investigated (Howland et al. 2011).

1.2.4.3 Deep brain stimulation (DBS)

Deep brain stimulation (DBS) involves implanting electrodes into the brain to stimulate specific subcortical regions. The potential therapeutic effects of DBS were first investigated in the subgenual anterior cingulate area of treatment-resistant depressed patients (Mayberg et al. 2005). DBS was found to have a significant antidepressant effect in the small number of patients involved in the pilot study. Later pilot trials also targeting the subgenual anterior cingulate (Lozano et al. 2008) and ventral capsule/ventral striatum (Malone et al. 2009) found that DBS was generally well tolerated and resulted in remission

rates of approximately 30% (Lozano et al. 2008; Malone et al. 2009). Other brain regions that may act as future targets for DBS have been identified (Hauptman et al. 2008). The results from larger scale randomised controlled studies will be required to determine the efficacy of DBS as a treatment for depression (Howland, et al. 2011).

Other physical therapies are being developed as potential treatments for depression including vagus nerve stimulation. However, these therapies are currently experimental and are not commonly used to treat depression. Large-scale randomised, controlled studies must be conducted and replicated before physical therapies other than ECT can be introduced as reliable antidepressant treatments.

1.3 Genetics of depression

Depression does not appear to be associated with a single gene of major effect. Instead, it may be a polygenic disorder with multiple genes of small effect, in combination with environmental influences, contributing to the development of depression (Crow, 2011).

Depression is a familial disorder with a relative risk of first-degree relative inheritance of 2.8 compared to the general population (Sullivan et al. 2000). Meta-analysis of twin and adoption studies of depression has shown the inheritance of depression is between 31-43% (Sullivan et al. 2000). In comparison, the heritability of schizophrenia is approximately 70% to 80% (Tsuang et al. 2001). The high levels of heritability associated with these disorders cannot be explained by the genes that, to date, have been identified to contribute to the disorders (Crow, 2011).

1.3.1 Genetic studies: causative gene effect

To date, no single causative gene has been identified for depression. A range of genetic studies have been conducted to find genes that may be involved in depression. Linkage studies have identified genes of interest, including genes that code for neurotrophin receptors (Verma et al. 2008). The serotonin transporter, *SLC6A4*, was implicated by a study looking at a sample of American and Dutch pedigrees with two or more affected siblings (Middeldorp et al. 2009). However, these findings were not confirmed. Association studies may be more effective at detecting multiple genetic variants that have a moderate effect, as is likely the case in depression, than linkage studies that are more effective at detecting genes that follow Mendelian transmission or have a strong effect.

Meta-analyses of candidate gene studies highlighted single nucleotide polymorphisms (SNPs) in multiple genes, including *SLC6A4*, as being statistically significantly associated to depression (Lopez-Leon et al. 2008).

Genome wide association studies (GWAS) for depression have implicated other potentially interesting genes. These studies typically did not find genes that were statistically significant at the field standard “genome wide significance” probability level of $p \leq 5.0 \times 10^{-8}$ (Shyn and Hamilton, 2010). The GWASs were quite comprehensive and compared SNPs in large studies often containing at least 1,000 case subjects and controls (Sullivan et al. 2009; Muglia et al. 2010; Schosser et al. 2011). Genes emphasised in GWASs included those that encoded for presynaptic proteins (Sullivan et al. 2009), cell cycle proteins (Muglia et al. 2010), glutamate receptors (Muglia et al. 2010; Shyn et al. 2011) and transcription factors (Shi et al. 2011; Shyn et al. 2011). Although these genes do not generally achieve levels of statistical significance in the GWASs, in combination with the knowledge derived from the current literature base, GWAS highlight these genes as being potentially relevant in the study of depression or antidepressant treatment. A recent GWAS of patients displaying suicidal behaviour again indicated that many genes of small effect contributed to depressed behaviour (Schosser et al. 2011).

A polymorphism in the gene for the neurotrophin brain derived neurotrophic factor (*Bdnf*), a molecule of particular interest in the study of depression, has been implicated in depression by genetic studies (Rybakowski, 2008; Kanellopoulos et al. 2011). While *Bdnf* has been implicated as a candidate gene (Kao et al. 2011) several studies and meta-analyses have failed to replicate this finding (Chen et al. 2008b; Lopez-Leon et al. 2008).

Multiple genes may play a role in depression. To date, replication of the results of genetic studies appears to be poor, with contradictory results of findings quite common (Karg et al. 2011). However, different types of genetic analysis have repeatedly uncovered a number of genes that may prove to be particularly interesting in the study of depression. These genes include neurotransmitter receptors, neurotrophins and genes involved in the stress response system.

1.3.1.1 Pharmacogenomic studies

Pharmacogenomics, the study of how an individual's genes affect their response to drugs, may provide further insight into the genes involved in depression. A recent meta-analysis has implicated genes that code for *Bdnf* and various serotonin receptors including the serotonin transporter *SLC6A4*, in the response to antidepressant treatment (Kato and Serretti, 2010). Analysis of genes that may be associated with the hypothalamic pituitary

adrenal (HPA) axis and depression found significant associations of response to antidepressants and recurrence of depressive episodes with SNPs in *FKBP5* (Binder et al. 2004). *FKBP5* is a co-chaperone of the glucocorticoid receptor and may be involved in antidepressant action that restores negative HPA axis feedback (Pariante and Miller, 2001). Corticotrophin releasing hormone receptor 1 (*CRHR1*) SNP analysis revealed that variability in this gene influenced patient response to SSRI treatment and increased risk of early onset of the first depressive episode (Papiol et al. 2007). A SNP in *CRHR1* was reproducibly associated and linked to suicide attempters who were exposed to low levels of stress (Wasserman et al. 2008).

Cytochrome P450 (CYP) describes a class of enzymes responsible for the metabolism of many drugs. Polymorphisms in members of this family, for example CYP2C19, lead to different rates of metabolism of antidepressant drugs (Porcelli et al. 2011). An ultra rapid metabolising isoform of this cytochrome (CYP2C19*17) reduces the concentration of escitalopram in patient plasma by half while deleting this gene increases escitalopram concentration by approximately six-fold (Rudberg et al. 2008). Therefore, patients with the CYP2C19*17 isoform would require a higher escitalopram dosage to reach a similar level of drug exposure to non-ultra rapid metaboliser isoform carriers (Rudberg et al. 2008).

A range of genes of small effect that are potentially involved in antidepressant response, the HPA axis and neurotrophic mechanisms have been implicated by different studies. They provide interesting research targets in the effort to study the mechanisms of depression. Genes that affect response to antidepressant therapy may offer insight into improving and creating more focused treatments for depression. However, the impact of environmental influences in combination with genetic risk factors in the onset of depression must also be considered.

1.3.2 Gene-environment interaction

Gene-environment interactions are also risk factors for depression. The way an individual responds to the environment is influenced by their genes. Evidence for the impact of gene-environment interactions in depression came from individuals with a polymorphism in the serotonin transporter gene *SLC6A4* (Caspi et al. 2003). Those with copies of the short serotonin transporter allele were more likely to suffer from depression in relation to stressful life events than those with copies of the long allele of the serotonin transporter (Caspi et al. 2003). The short serotonin transporter allele was associated with reduced transcriptional efficiency (in the promoter region) than the long allele (Lesch et al.

1996). A recent meta-analysis supported the hypothesis that the polymorphism in *SLC6A4* may moderate the relationship between stress and depression (Karg et al. 2011).

Further evidence for a gene-environment effect came from a SNP in the *FKBP5* gene. Individuals with a polymorphism in this gene that were exposed to childhood physical abuse had a significantly increased susceptibility to adult depression (Appel et al. 2011). The *FKBP5* gene codes for a co-chaperone protein that regulates the glucocorticoid receptor (GR). Glucocorticoids and the GR are involved in the HPA axis, the body's stress response system (see section 1.5.2).

A combination of childhood adversity and a polymorphism in the *Bdnf* gene lead to an increased risk for depression (Gerritsen et al. 2011). The polymorphism was a substitution of the amino acid methionine (met) for valine (val) at codon 66 (Val/Met instead of Val/Val). Individuals with the *Bdnf* Met-allele who experienced childhood adversity had significantly reduced grey matter in the anterior cingulate cortex of the prefrontal cortex. Met-allele carriers with no history of childhood adversity or Val/Val allele individuals did not show reduced grey matter volume (Gerritsen et al. 2011).

In conclusion, taken together the above studies suggest a combination of environment and genetic make-up may thus be required for the onset of depression. The polygenicity of depression suggests that further genes of small effect are yet to found. A more complete understanding of the gene profile of depression could provide new targets for antidepressant treatments and lead to a greater understanding of the neurobiology of depression.

1.4 Functional anatomy of depression

1.4.1 The neural circuitry and neuroanatomy of depression

The symptoms of depression may arise from brain regions that are no longer functioning normally. These brain areas can be targeted for study in an effort to elucidate their role in depression. However, depression likely results from the interaction of several brain areas. Another difficulty in pinpointing which parts of the brain are involved in depression comes from the lack of pathology distinct to depression. For example, Alzheimer's disease, a neurodegenerative disorder, is associated with functional and structural brain abnormalities that can aid in diagnosis (Herholz et al. 2007) but psychiatric conditions such as depression are diagnosed by the presence of clinical symptoms only. As a result, treatments for depression, including ECT, tend to affect the whole brain. Treatments that target specific brain regions can be successful, although the reasons why

may not be understood, as knowledge about the circuitry and physiological processes involved is incomplete (Murray et al. 2011).

Several brain regions are valid areas of study for their role in depression. The hippocampus and frontal cortex are most commonly implicated in depression as many of the cognitive aspects of depression, memory impairment, hopelessness, guilt, doom and suicidality may result from perturbations in these areas (Nestler et al. 2002).

Meta-analysis of magnetic resonance imaging (MRI) studies showed that depressed patients have a reduced hippocampal volume in comparison to controls (Campbell et al. 2004b; McKinnon et al. 2009). Imaging studies have revealed that depressed non-remitting patients have reduced hippocampal volume even at a one-year follow-up (Frodl et al. 2004). A relatively small hippocampal volume in patients can result in a poorer clinical outcome than in patients with a larger hippocampal volume (Frodl et al. 2008a). Depressed patients also have reduced grey matter volume, in brain regions including the hippocampus, indicating increased vulnerability to morphological changes during depressive episodes (Frodl et al. 2008b). Structural differences in the subiculum and CA1 extending into the CA2-3 subfields of the hippocampus of depressed patients compared to controls have been detected (Cole et al. 2010). These deformations may underlie the memory deficits associated with depression; hippocampal atrophy is associated with memory loss in depression (Hickie et al. 2005). The subiculum region in the hippocampus is specifically implicated in episodic memory retrieval (Eldridge et al. 2005). The structural neuroanatomy of the hippocampus in depression has potential value as a prognostic marker of response to antidepressant treatment in depressed patients (Costafreda et al. 2009), further emphasising the role of the hippocampus in depression.

The pre-frontal cortex (PFC) is also affected by depression. The dorsolateral (dlPFC) and ventromedial (vmPFC) regions are particularly studied in depression, possibly due to their roles in executive and emotional functions respectively. Lesioning studies found that vmPFC lesioned patients had lower levels of depression while patients with lesions of the dlPFC had significantly higher levels of depression (Koenigs et al. 2008). This finding was in line with the results from imaging studies, which suggested vmPFC hyperactivity and dlPFC hypoactivity of the brains of depressed patients at rest (Koenigs and Grafman, 2009). These two brain regions appear to have opposing actions, but are strongly implicated, in depression (Koenigs and Grafman, 2009). Reductions in grey matter volume in the frontal cortex have been found in depressed patients (Drevets and Price, 2005). Metabolism is increased in frontal cortex structures, an increase which

correlates positively with depression severity in patients (Drevets and Price, 2005; Hasler et al. 2008).

Brain imaging studies have also implicated the amygdala, among other structures, in depression (Smith et al. 2011). However, there are also contradictions in these studies. Amygdala volume was found to be both increased and decreased in depressed patients compared to controls in different studies (Drevets et al. 2004). Meta-analysis concluded that the effect of depression on amygdala volume remains to be fully established (Campbell et al. 2004b). The amygdala shows higher resting levels of metabolism in depressed patients (Drevets, 2001) which is normalised by antidepressant treatment (Drevets et al. 2002). Glial cell densities (Bowley et al. 2002), specifically oligodendrocytes (Hamidi et al. 2004), were significantly lower in the amygdala of depressed patients compared to controls. The role of the amygdala in depression remains to be fully elucidated.

Recent meta-analyses of structural neuroimaging studies have implicated the hippocampus, frontal cortex, amygdala and many other brain regions in depression (Arnone et al. 2011; Kempton et al. 2011). Although a number of brain regions appear to contribute to depression, meta-analysis of the multitude of imaging studies conducted on this subject will allow for the discrimination of brain areas that may be of specific interest and importance in depression research.

Overall, a range of brain regions appear to be involved in depression. The interaction of these areas may begin to explain the complex pathology of depression. As no single region defines the causes or manifestation of depressive disorder, a complex linked network of interacting brain areas may explain the varied and multifaceted pathogenesis of depression.

1.5 Depression, monoamines, stress and the immune system

Depression is a heterogeneous disorder which may contribute to the difficulty in finding antidepressant treatments with widespread efficacy in the population. Currently available antidepressants achieve mixed response rates and relatively low remission levels in depressed patients (section 1.1). A lack of reliable biomarkers, measurable biological features that signify the presence of a disease state, mean there are few validated targets for the treatment of depression. The majority of currently available antidepressants were developed based on the monoamine hypothesis of depression (Schildeckraut and Kety, 1967). However, overreliance on treating the monoaminergic systems has hindered the development of other antidepressants. Novel antidepressants are currently being

investigated. However, much work remains to be done before such treatments (for example ketamine) can be reliably introduced as mainstream antidepressants.

Other hypotheses to explain depression have developed, spurred by deficits in the monoamine hypothesis. One of the downstream effects of increased monoamine release is the upregulation of neurotrophic factors. Much research has been conducted to elucidate the pathways that activate neurotrophins. Neurotrophins play a role in upregulating and promoting neurogenesis and neuroplasticity, which led to the development of the neuroplastic hypothesis of depression.

A summary of the main hypotheses of depression, as well as the impact of the neuroimmune and stress response systems that may play a secondary role in depression, will be discussed below. Figure 1-2 reviews the impact of antidepressant treatment on the factors that may contribute to depression.

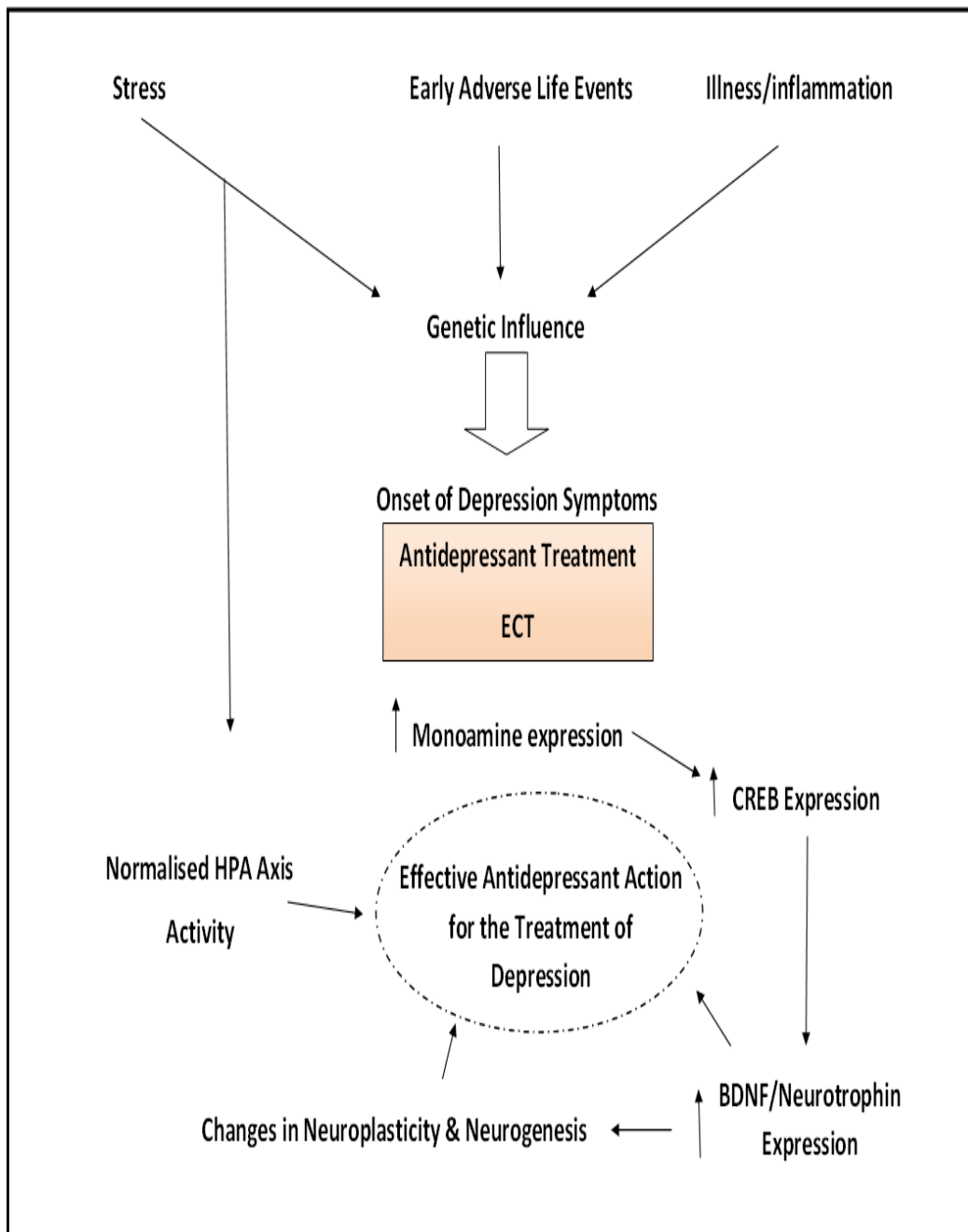


Figure 1-2 Possible interaction of factors that may induce antidepressant effect following ECT or antidepressant drug administration

BDNF, brain derived neurotrophic factor; CREB, cAMP-regulated response element binding protein; HPA axis, hypothalamic-pituitary adrenal axis; ECT electroconvulsive therapy

1.5.1 The monoamine hypothesis of depression

When it was first discovered that antidepressant drugs increased monoamine, particularly noradrenaline and serotonin, function in the brain (section 1.1.1), it led to the search for the defect in the monoamine system that could account for depression (Iversen, 2005). The “Monoamine Hypothesis of Depression”, the idea that depression was a result of a monoamine deficiency in the brain that could be reversed by antidepressant administration, was formulated (Schilderkraut and Kety, 1967). The hypothesis stimulated research and led to the discovery of new antidepressants, for example the SSRIs.

However, studies conducted to find evidence for the monoamine hypothesis often yielded disappointing results (Leonard, 2003; Iversen, 2005). Briefly, initial studies found abnormal increases in beta adrenoceptors and 5-HT_{2A} receptor density in the brains of depressed suicide victims; increases that were normalised by antidepressant treatment. Other indicators of monoamine-system abnormalities in depression were seen with decreases in the levels of the 5-HT metabolite 5-HIAA in the brains of suicide victims and the cerebrospinal fluid (CSF) of severely depressed patients. However, many of the studies that implicated the noradrenaline and serotonergic systems in depression were not replicated or were conducted with small numbers of patients. More recent studies have revealed other limitations of the monoamine hypothesis including studies that found increases in noradrenaline levels in the CSF and plasma of depressed patients (Veith et al. 1994; Wong et al. 2000; Wong and Licinio, 2001).

Acutely, antidepressant administration leads to an increase in monoamine levels, affecting the neurotransmitter systems within hours of treatment but therapeutic effects are not seen for several weeks (Wong and Licinio, 2001). The delay in effect cannot be explained by a simple increase in monoamine levels; the antidepressant effect is brought about by other mechanisms (Leonard, 2003).

1.5.1.1 Rapid response antidepressants

Unlike traditional antidepressants, ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, leads to a rapid onset of antidepressant effect. Thirty minutes after ketamine administration mice displayed a significant reduction in immobility in the forced swim test (FST), a test of antidepressant efficacy (Autry et al. 2011). This behavioural effect lasted for one week. The sustained antidepressant response is most likely due to changes induced in synaptic plasticity and not sustained receptor blockade, as ketamine has a half-life of two and a half to three hours. The neurotrophin BDNF was also required for the fast-acting antidepressant response. BDNF knockout animals did not display reduced

immobility in the FST as control animals did (Autry et al. 2011). The mammalian target of rapamycin (mTOR) pathway has been implicated in ketamine's rapid antidepressant affect (Li et al. 2010). Activation of mTOR signalling led to an increase in synaptic proteins and spine number in the prefrontal cortex. mTOR signalling blockade prevented ketamine induced synaptogenesis and behavioural response in the FST (Li et al. 2010).

ECT is considered a fast acting antidepressant. However, while patients may initially respond to ECT within one week (54% of patients in trial), at least two weeks of treatment were required for patient remission (34% of patients in trial), while the majority of patients went into remission following up to four weeks of treatment (65% of patients in trial) (Husain et al. 2004). Response to ECT is more rapid than response to pharmacotherapy (Folkerts et al. 1997).

ECT administered in combination with ketamine anaesthetic resulted in a significantly lower Hamilton Depression Rating Scale (HDRS) score earlier in treatment compared to ECT administered with other anaesthetic agents (Okamoto et al. 2010). A combination of both therapies may result in a more rapid onset of therapeutic effect providing earlier relief for patients.

Despite the inconsistencies in the time from antidepressant administration to the onset of therapeutic effect, the monoamine hypothesis has guided antidepressant and depression research for several decades (Iversen, 2005). Monoamines undoubtedly play a role in antidepressant action but further hypotheses are required to explain depression and the mechanisms of antidepressant action.

1.5.2 The stress response and HPA-axis hypothesis of depression

Stressful life events can precipitate major depression and influence its onset and course (Kessler, 1997; Kendler et al. 1999; Pine et al. 2002). However, the majority of people do not become depressed after a stressful experience. Exposure to extremely stressful events, such as combat, can result in post-traumatic stress disorder (PTSD), a disorder distinct from depression (Nestler et al. 2002). The response to stress shares many features with depression due to their action on similar brain circuits (Gold and Chrousos, 1999). Therefore, while the development of depression can be related to exposure to stressors in susceptible individuals (Caspi et al. 2003), stress exposure alone is not sufficient to cause depression (Nestler et al. 2002).

1.5.2.1 The HPA axis

The brain reacts to acute and chronic stress by activation of the HPA axis (Figure 1-3). Hypothalamic paraventricular nuclei neurons secrete corticotrophin-releasing hormone (CRH) stimulating the synthesis and release of adrenocorticotrophin (ACTH) from the anterior pituitary. ACTH in turn stimulates the adrenal cortex and the release of glucocorticoids (Nestler et al. 2002). Glucocorticoids profoundly affect behaviour by interacting directly with different brain regions, including the HPA axis. They are responsible for feedback inhibition on CRH in the hypothalamus and ACTH in the pituitary (Pariante and Lightman, 2008). The HPA axis is controlled by the hippocampus and amygdala, which have inhibitory and excitatory influences respectively.

1.5.2.2 HPA axis dysfunction in depression

HPA axis dysfunction is a biological feature in many depressed patients (Gold and Chrousos, 2002). Hyperactivity of the HPA axis is commonly detected in depressed patients. As the HPA axis is activated in response to stress, a major precipitating factor to depression, the mechanisms underlying HPA axis abnormality are of interest. HPA axis dysfunction is no longer considered to be just an epiphenomenon of this disorder (Pariante, 2003). A greater understanding of this stress response system and its central role in depression may be essential to understand the neurobiology of depression.

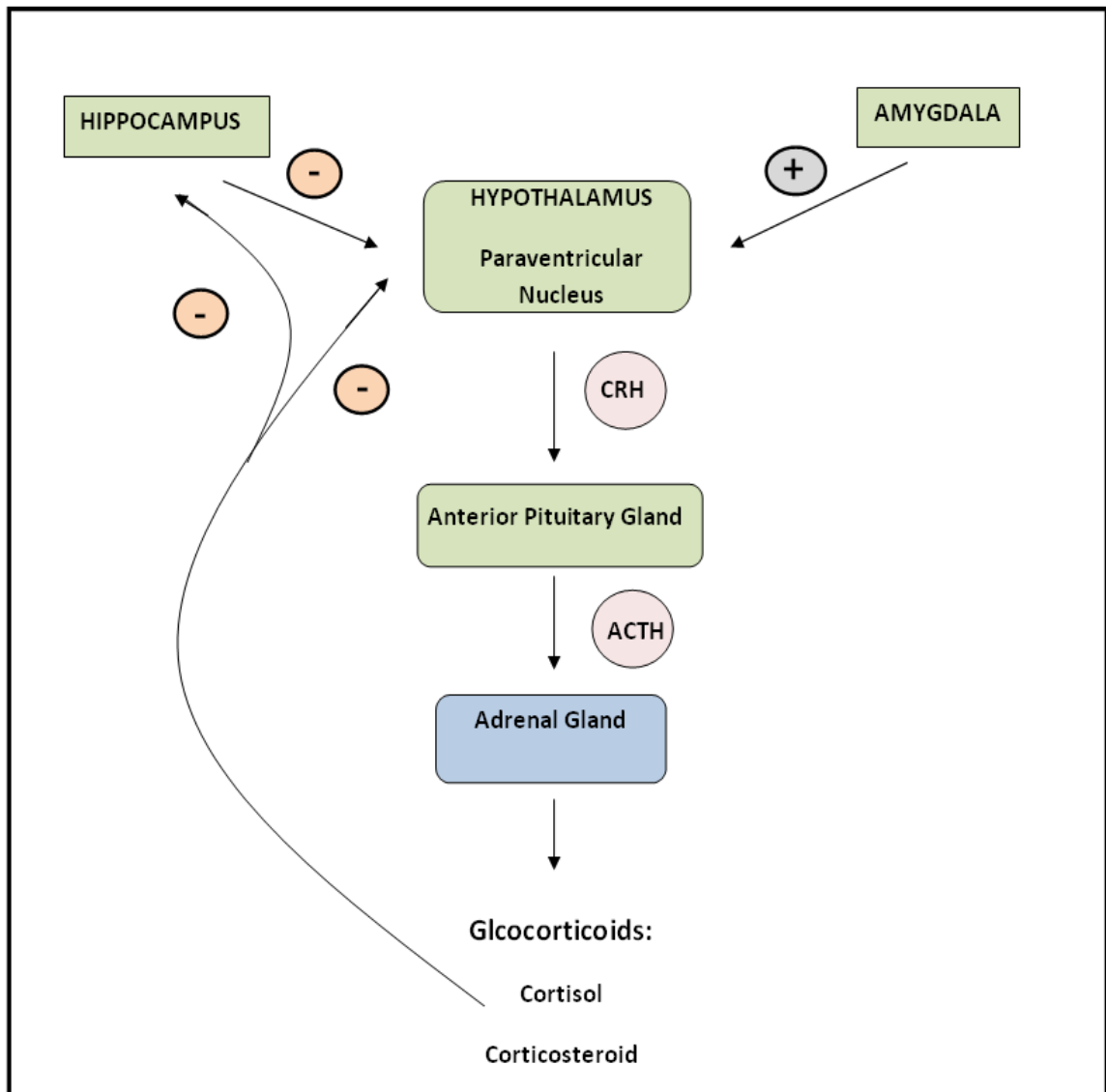


Figure 1-3 The HPA axis

Glucocorticoid release from the adrenal glands forms a negative feedback loop which may be disregulated in depression. The HPA Axis is also regulated by the hippocampus and amygdala.

HPA, hypothalamic pituitary adrenal axis; CRH, corticotrophin releasing hormone; ACTH, adrenocorticotropin.

Impaired glucocorticoid negative feedback may be related to hyperactivity of the HPA axis. Cortisol, an endogenous glucocorticoid, is present in significantly higher levels in plasma of depressed patients compared to controls (Rubin et al. 1996). Waking salivary cortisol levels are also higher in depression (Bhagwagar et al. 2005). CRH levels in CSF are higher in depressed patients (Nemeroff et al. 1984). In dexamethasone/corticotrophin-releasing hormone (DEX/CRH) tests (a sensitive challenge test of HPA axis function), patients with depression release significantly more ACTH and cortisol compared to matched controls, suggesting an alteration in glucocorticoid feedback regulation (Heuser et al. 1994). The hypersecretion of cortisol can be resistant to feedback suppression by dexamethasone, a potent synthetic corticosteroid which depresses ACTH secretion (Kandel et al. 2000).

1.5.2.3 Changes in volume of HPA axis glands

There is also some evidence that pituitary and adrenal gland volume is increased in depressed patients (Kessing et al. 2011). The increase seen in pituitary volume may provide evidence of impairment in the HPA axis; inefficient negative glucocorticoid feedback on the pituitary cells that produce ACTH leads to an increase in the number of those cells (Pariante et al. 2005). In healthy control subjects, adrenal volume correlates positively with dexamethasone suppressed salivary cortisol and total salivary cortisol (Golden et al. 2007) suggesting that increased adrenal gland volume may indicate subclinical hypercortisolism (Kessing et al. 2011). While studies have shown an increase in adrenal gland volume associated with depressed patients compared to controls (Nemeroff et al. 1992; Rubin et al. 1996), it has also been argued that there is no real relationship between adrenal gland size and measures of the HPA axis function in depression; factors other than ACTH may play a role in increasing adrenal gland size but not increasing corticoid production (Rubin et al. 1996).

1.5.2.4 Antidepressants normalise HPA axis dysfunction

Antidepressant treatment is associated with resolving the impaired negative feedback on the HPA axis by glucocorticoids (Heuser et al. 1996; Pariante, 2006).

Glucocorticoid action is mediated by the glucocorticoid receptor (GR). GR levels were reduced in the brains of depressed patients' post-mortem (Webster and Carlstedt-Duke, 2002). GR function is also impaired in depression (Pariante, 2004). Antidepressant treatment normalises this effect. For example, citalopram, an SSRI, has been shown to increase HPA axis negative feedback by glucocorticoids (Pariante et al. 2004).

1.5.2.4.1 Effect of ECT on the HPA axis in depression

The changes in the HPA axis in depression were normalised by ECT treatment (Yuuki et al. 2005). Treatment with ECT decreased the pituitary-adrenocortical response of depressed patients, which were increased compared to controls, in the DEX/CRH test (Kunugi et al. 2006). Higher value results in the DEX/CRH test in depressed patients could be used to detect patients at greater risk of relapse than patients who had better-improved cortisol response (Aubry et al. 2007). A meta-analysis study determined the mean effect of antidepressant treatment on pre- and post- treatment cortisol levels in patients was not significantly different between ECT and pharmacotherapy (McKay, 2010). In animals, ECS treatment increased neuronal activation and endothelial cell proliferation in the paraventricular, supraoptic and ventromedial nuclei of the hypothalamus. An angiogenic response to ECT may counteract hypothalamic dysfunction in depression (Jansson et al. 2006).

1.5.2.5 Summary

The HPA axis is dysregulated in a proportion of depressed people (Mello et al. 2007). However, antidepressant treatment appears to normalise HPA axis dysfunction. Changes in the volume of structures of the HPA axis have been investigated in imaging studies to further determine their potential role in depression. The role of volume changes in the HPA axis is not yet well understood. While HPA axis dysfunction may contribute to depression in some patients its overall contribution to the aetiology of depression is yet to be fully elucidated.

1.5.3 Depression and the immune system

Pro-inflammatory cytokines induce the symptoms of depression, as well as sickness, in patients who are physically ill but do not have a previous history of psychiatric disorders (Dantzer et al. 2008). A role for cytokines in depression was first suggested in the macrophage theory of depression (Smith, 1991). The risk of developing depression was increased in patients receiving the cytokines interferon (IFN) or interleukin (IL)-2, which were initially administered to boost the immune system (Denicoff et al. 1987; Renault et al. 1987).

Evidence for cytokine induced depression was also found in animal models. Animals treated with lipopolysaccharide (LPS), a component of bacterial outer membrane that elicits a strong immune response in animals, had increased immobility in the FST (Frenois et al. 2007). A reduction in the time spent immobile in this test is indicative of an

antidepressant effect. Pre-treatment with antidepressants reversed the decreased intake of sucrose solution in LPS treated rats (Yirmiya et al. 1999), suggesting a reversal in anhedonia-like behaviour. Activation of the immune system by pro-inflammatory cytokines may cause depression symptoms.

Among several potential mechanisms of cytokine-induced depression (discussed Dantzer et al. 2008) is the modulation of serotonergic systems in the brain, which have been implicated in depression (Dunn et al. 2005). IFN γ induces an enzyme, indoleamine 2, 3-dioxygenase (IDO) which degrades tryptophan. IFN α and IL-2 have a similar, but lesser, effect on IDO. Tryptophan is an amino acid that is required for serotonin synthesis. Lower levels of tryptophan are associated with depression (Coppen and Wood, 1978). It was suggested that decreased tryptophan availability in the brain may limit serotonin synthesis and lead to depression (Wichers and Maes, 2004) however further evidence is required to demonstrate this.

The immune system may also modulate the HPA axis in depression. The HPA axis is upregulated by several cytokines, particularly IL-1 (Besedovsky et al. 1986). HPA axis activation is also consistently seen in depressed patients (Gold and Chrousos, 2002). However, while cytokines and immune activation are involved in depression symptoms in some patients, they do not appear to be essential mediators of depression.

1.6 Neurotrophic hypothesis of depression

The neurotrophic hypothesis proposes that deficient neurotrophic support contributes to the onset of depression. Antidepressants reverse this deficit, potentially resolving the depressive symptoms (Nestler et al. 2002). Brain derived neurotrophic factor (BDNF) is of particular interest in the study of the neurotrophic hypothesis. Stress decreases BDNF expression levels (Smith et al. 1995) in the rodent dentate gyrus and pyramidal cell layers of the hippocampus. BDNF expression was increased in these areas by chronic administration of antidepressants including MAOIs, SSRIs, TCAs, NaSSAs and ECS. Antidepressant administration also prevented stress induced decreases in BDNF levels (Nibuya et al. 1995). Increased BDNF expression was seen in post-mortem human hippocampus samples in patients treated with antidepressants compared to those who were not (Chen et al. 2001b). Evidence suggests that BDNF plays an integral role in depression and antidepressant treatment.

1.6.1 Antidepressant induced CREB activation

As discussed in section 1.4 above, treatment with ECT and the major antidepressant classes acutely increased synaptic monoamine expression concentrations. However, it is often several weeks before an antidepressant effect is seen. Increased monoamine levels leads to the activation of post synaptic receptors and downstream signalling cascades. One of the main transcription factors targeted by these signalling pathways following antidepressant treatment is cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). CREB activation is important for antidepressant-drug target gene regulation (Conti et al. 2002). CREB was upregulated in response to chronic antidepressant treatment, suggesting it was a common target of antidepressants, including ECS (Nibuya et al. 1996).

CREB is activated by different signalling pathways (Figure 1-3) including the cAMP-protein kinase A (PKA) pathway and the Ca^{2+} /calmodulin dependent kinase (CamK) signalling cascade. Synaptic neurotransmitter levels are upregulated following antidepressant treatment. This leads to activation of the cAMP- PKA cascade and an antidepressant response (Pittenger and Duman, 2008). A number of noradrenaline (β -adrenergic receptor (β AR)) and serotonin (5-HT₄, 6, 7) receptors couple directly to the cAMP-PKA pathway. These receptors are activated by antidepressants and increase intracellular cAMP levels and PKA activation. Downstream, CREB phosphorylation and activation occurs.

CREB is also activated by Ca^{2+} /CamK, induced by Ca^{2+} via receptors such as the 5-hydroxytryptamine 3 (5HT₃) receptor, α 1-adrenergic and 5-HT₂ receptors (Duman et al. 1999) that are not directly coupled to the cAMP cascade (Sheng et al. 1990).

Therefore, different antidepressants activate cAMP or Ca^{2+} signalling and may induce different responses, depending on the sets of genes that are expressed by CREB. Three different serine residues in CREB (Ser133, Ser142, Ser143) are phosphorylated following Ca^{2+} influx. Other stimuli induce phosphorylation of a single serine (Ser133). Selective regulation of CREB phosphorylation triggers gene expression (Kornhauser et al. 2002; Blendy, 2006).

One of the best-studied targets of CREB activation is the neurotrophin BDNF. BDNF has been strongly implicated in the action of antidepressants and the pathophysiology of depression since its upregulation by CREB, following antidepressant treatment, was identified (Duman et al. 1999; Manji et al. 2001; Manji et al. 2003).

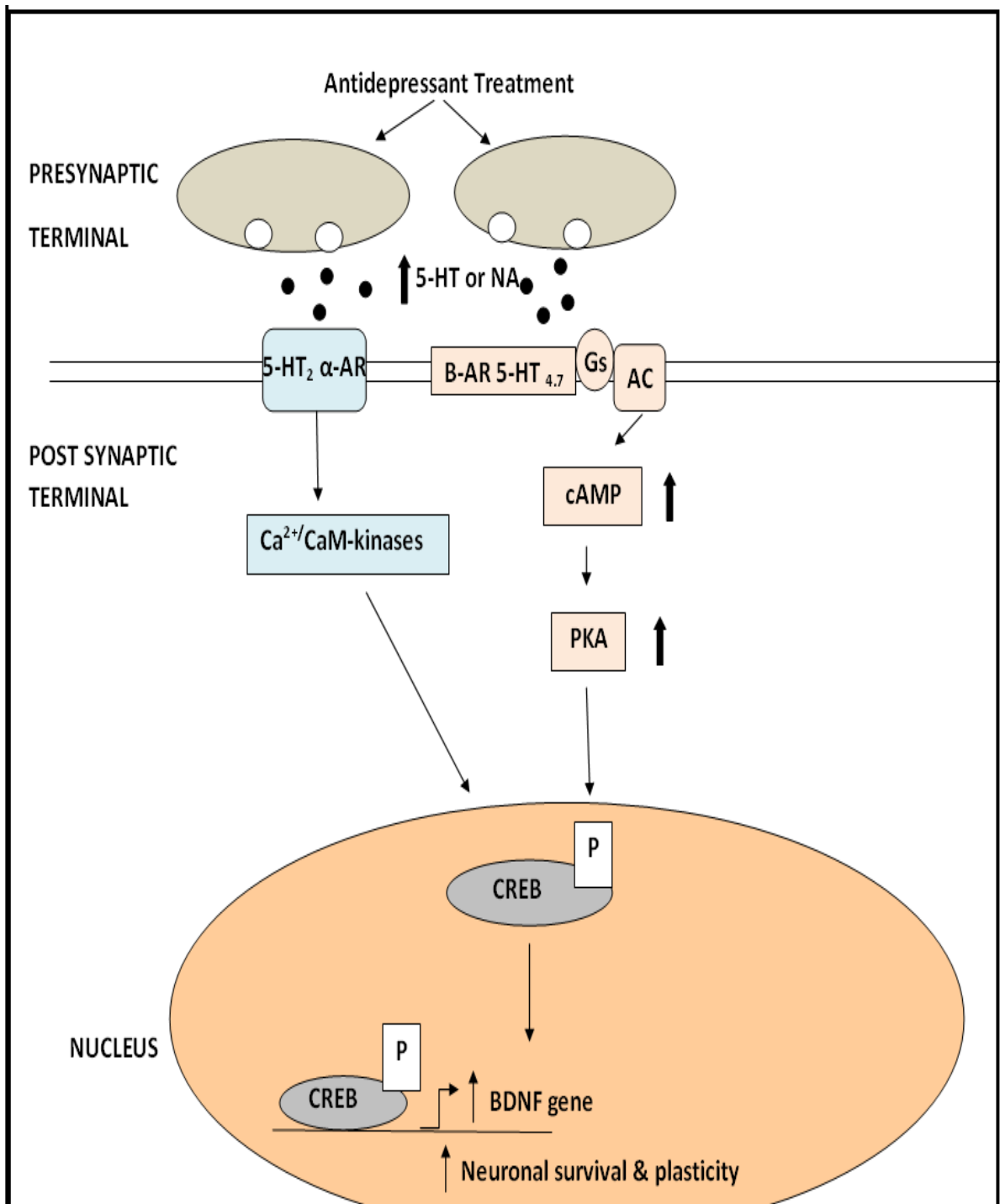


Figure 1-4 Antidepressant activation of CREB

Activation of the cAMP cascade or Ca²⁺ dependent kinases by ECS and antidepressant treatment leads to CREB activation. In turn, CREB activates BDNF, neurotrophins and other gene targets. The targets activated downstream can differ depending on the mode of CREB activation.

CaMK, calmodulin dependent kinases; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; P, phosphate; AC, adenylyl cyclase; AR, adrenergic receptor; NA, noradrenaline; Gs, G protein signal

1.6.2 Brain derived neurotrophic factor (BDNF)

BDNF is a member of the nerve growth factor family of neurotrophic factors that includes nerve growth factor (NGF) and neurotrophin-3 (NT-3). BDNF is a 27kDa basic protein of non-covalently linked 13.5kDa subunits (Rosenthal et al. 1991). The BDNF gene has one 3' exon that codes for the proBDNF protein and at least eight 5' noncoding exons, each with its own promoter that generates a transcript. Each BDNF transcript consists of a 5' exon spliced to the protein coding exon (Figure 1-5) (Timmusk et al. 1993; Pattabiram et al. 2005; Aid et al. 2007). Multiple 5' exons allow for greater regulatory diversity and may explain the differential expression of splice variants of BDNF in different brain regions (Liu et al. 2006).

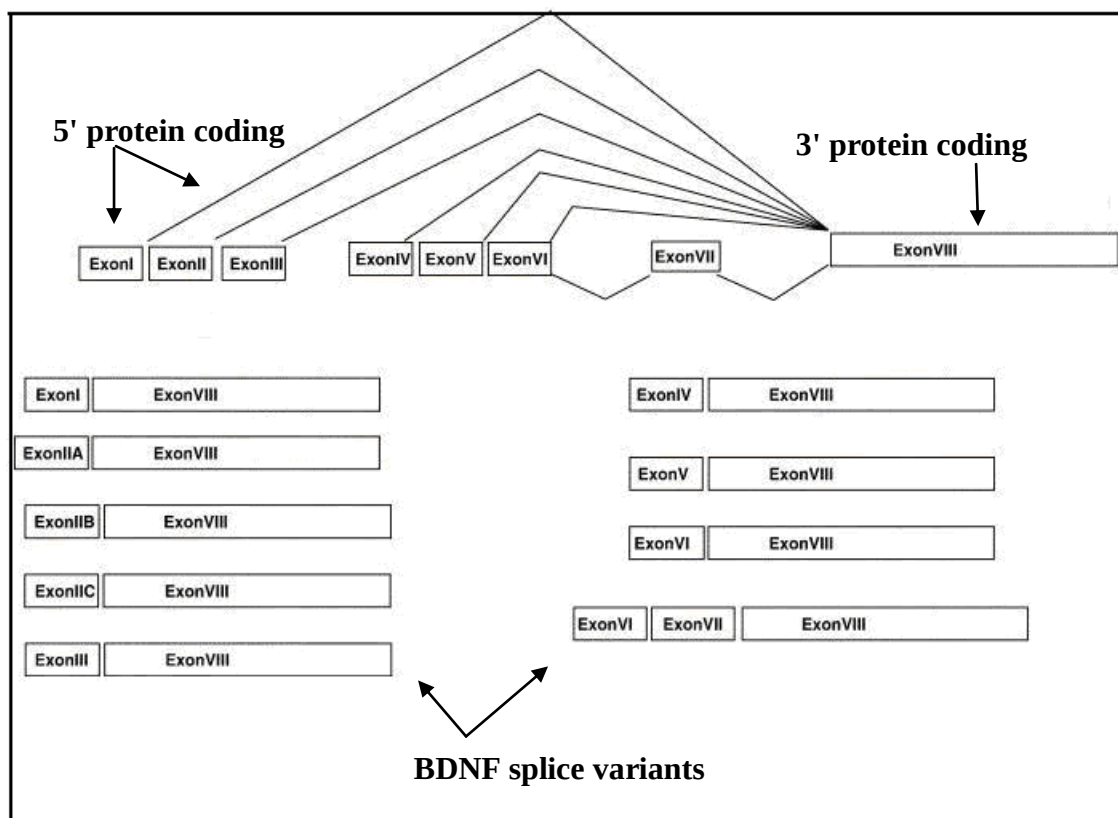


Figure 1-5 Rat BDNF gene structure and splicing variants adapted from Liu et al. 2006

BDNF is synthesised in the endoplasmic reticulum as a 32kDa precursor protein (proBDNF). ProBDNF is converted to the 13.5kDa mature form of BDNF and dimerises to form the 27kDa basic protein (Thomas and Davies, 2005). BDNF is widely expressed in the hippocampus and cerebral cortex (Ernfors et al. 1994; Murer et al. 2004). Like the other members of the NGF family, BDNF signals through the tropomyosin receptor kinase (Trk) family of receptors, tyrosine kinase receptors that dimerise upon ligand binding.

Specifically, BDNF signals through TrkB. All members of the NGF family also signal through p75 neurotrophic receptors (p75^{NTR}) (Murer et al. 2001). BDNF activates many signalling pathways through its receptors. In the developing brain, BDNF modulates neuronal survival while in the mature brain BDNF plays a role in structural and synaptic plasticity and functioning as well as continuing to contribute to cell survival (Huang and Reichardt, 2001).

1.6.3 Stress decreases neurotrophin expression in depression

A potential mechanism by which stress plays a role in the pathophysiology of depression may be related to altered regulation of BDNF expression. Neurotrophins are essential for neuronal survival and maintenance (Huang and Reichardt, 2001). Altered neurotrophic expression may contribute to hippocampal atrophy seen in depressed patients as it may result in modifications to networks involved in the symptoms of depression (Schmidt and Duman, 2007). BDNF mRNA levels are decreased in the hippocampus by different stressors in animal models (Duman and Monteggia, 2006). The immobilisation stress model (Smith et al. 1995; Nair et al. 2007), chronic unpredictable stress (Bland et al. 2007) and footshock (Rasmusson et al. 2002) models all altered BDNF mRNA expression in the hippocampus.

Glucocorticoids are important mediators of stress-related downregulation of BDNF in the hippocampus (Schmidt and Duman, 2007). Corticosterone, when administered peripherally to rats, decreased expression of BDNF mRNA and protein (Schaaf et al. 1997; Schaaf et al. 1998). Adrenalectomy increased BDNF expression in the hippocampus (Chao et al. 1998) but did not block all stress-induced decreases in BDNF (Smith et al. 1995). Other, non-glucocorticoid factors, may relate neurotrophin responses to stress. Interleukin 1 (IL-1) receptor antagonist infusion, for example, blocked decreases in BDNF expression brought about by the social-isolation induced stress paradigm, where social animals like rats are housed individually (Barrientos et al. 2003).

Other neurotrophins may also regulate the detrimental effects of stress in the hippocampus. Vascular endothelial growth factor (VEGF), which increased neuronal proliferation (Jin et al. 2002; Cao et al. 2004), and its receptor fetal liver kinase-1 (Flk-1) have been shown to be decreased in the hippocampus of chronically stressed rats (Heine et al. 2005). Other authors also report a decrease in VEGF in response to stress and increased expression following antidepressant drug and ECS treatment (Warner-Schmidt and Duman, 2008b).

1.6.4 Antidepressant treatments increase neurotrophin expression

1.6.4.1 Evidence from gene expression microarray studies

Neurotrophins, in particular BDNF, are potential targets for antidepressants, mediating their therapeutic effect. Gene expression microarray studies found that ECS significantly upregulated a number of neurotrophic factors in the hippocampus. The expression of BDNF, VEGF and inducible nerve growth factor (VGF) were increased both acutely and chronically in the hippocampus of rats treated with ECS (Newton et al. 2003; Altar et al. 2004). NGF expression levels were also increased (Madsen et al. 2003; Newton et al. 2003). A study looking specifically at the dentate gyrus of the rat hippocampus also found increases in BDNF (Ploski et al. 2007). Signalling pathways implicated by gene expression studies in the antidepressant action of ECS and potentially ECT included the mitogen activated protein kinase (MAPK) pathway (section 1.5.7). Genes involved in neurogenesis, neuronal plasticity and neurite outgrowth were also heavily implicated in the antidepressant action of ECS in the hippocampus and frontal cortex (Altar et al. 2004).

1.6.4.2 BDNF mRNA and protein changes

BDNF mRNA has been shown to be upregulated robustly in the hippocampus, particularly the granule cell layer of the dentate gyrus, following chronic ECS administration (Zetterstrom et al. 1998).

Chronic ECS effectively upregulates the acute induction of exons I, II and IV mRNAs in the hippocampus while treatment with the antidepressants tranylcypromine and desipramine enhanced exon II and III mRNAs. Antidepressant treatments differentially regulated BDNF transcription suggesting varied signalling mechanisms may control BDNF mRNA regulation (Dias et al. 2003).

ProBDNF, a precursor protein, is cleaved to form mature BDNF. Secreted proBDNF can activate the p75 receptor, resulting in neuronal apoptosis (Teng et al. 2005). Others have reported chronic ECS increased BDNF protein concentration in the frontal cortex as well as the hippocampus (Angelucci et al. 2003; Altar et al. 2003).

1.6.4.3 Other neurotrophin mRNA and protein levels

The expression levels of other types of neurotrophins are also upregulated by antidepressant treatment. Chronic ECS induces increases in fibroblast growth factor-2 (FGF-2) and NGF mRNA in the frontal cortex. NGF mRNA was also increased in the hippocampus. Acute ECS was effective at increasing mRNA levels of these neurotrophins

in the same regions, suggesting induction and the ability to sustain neurotrophin levels in the action of chronic ECS (Kondratyev et al. 2002).

NGF protein levels are increased in the frontal cortex and hippocampus respectively, following chronic ECS, while in the same study glial cell line derived neurotrophic factor (GDNF) concentrations were decreased in the hippocampus (Angelucci et al. 2002). In a genetic animal model of depression, the Flinders Sensitive Line/Flinders Resistant Line (FSL/FRL), chronic ECS increased NGF protein concentrations in the hippocampus of FSL but not FRL animals and decreased NGF concentration in the frontal cortex (Angelucci et al, 2003).

1.6.4.4 Serum and plasma BDNF following ECT and ECS treatment

Meta-analyses revealed that serum and plasma BDNF levels are significantly reduced in depressed patients (Bocchio-Chiavetto et al. 2010). In patients administered ECT, serum BDNF levels were significantly higher one month after ECT treatment than at baseline or directly after ECT treatment (Bocchio-Chiavetto et al. 2006). Patient plasma levels of BDNF were also significantly higher post ECT treatment (Marano et al. 2007; Piccinni et al. 2009). Serum levels of BDNF following ECS administration may be indicative of brain tissue levels. BDNF crosses the blood brain barrier and measuring serum BDNF, allowing for a time delay, could provide the means to measure changes in brain tissue neurotrophin levels (Sartorius et al. 2009).

1.6.5 Antidepressant effect of BDNF

BDNF is important for antidepressant action. It is induced by many antidepressant treatments. Several studies have been conducted to determine whether BDNF itself may have potential as an antidepressant treatment.

BDNF infusion into the rat brain increased activity in monoaminergic systems and behavioural models, demonstrating antidepressant properties (Celada et al. 1996; Siuciak et al. 1997). A direct bilateral infusion of BDNF into the dentate gyrus produced an antidepressant effect that lasted for at least ten days after a single infusion (Shirayama et al. 2002). Conversely, an infusion of BDNF into the midbrain ventral tegmental area (VTA) resulted in a depression-like effect, an opposite effect to that seen in the hippocampus (Eisch et al. 2003). Although BDNF had antidepressant properties in the hippocampus, in the VTA its actions had a different effect. Stress increased levels of BDNF and local knockout of BDNF from the VTA had an antidepressant effect (Berton et al. 2006). Targeting of specific BDNF-induced signalling proteins may be a more effective approach

in developing antidepressant therapies than creating drugs that promote BDNF functioning in different brain regions (Berton and Nestler, 2006) due to its pro- and antidepressant effects.

When administered peripherally, BDNF had a significant antidepressant effect in animal behavioural models and on a cellular and molecular level. In tests of antidepressant response, immobility times were decreased in the FST (depressed animals spend less time trying to escape than non-depressed animals) and latency to drink was reduced in the novelty-induced hypophagia test (depressed animals take longer to drink when placed in new surroundings) (Schmidt and Duman, 2010). Chronically stressed (chronic unpredictable stress paradigm) mice treated peripherally with BDNF consumed significantly more sucrose than chronically stressed animals treated with saline. In the elevated plus maze (a test of anxiety that measures the amount of time an animal spends in the exposed, open arms of the plus maze), animals administered peripheral BDNF had increased entry into open arms (Schmidt and Duman, 2010).

BDNF administered peripherally also increased levels of hippocampal neurogenesis and hippocampal levels of BDNF and phosphorylated CREB. Peripheral BDNF may be of significance as a biomarker of depression as well as playing a role in signalling, cell proliferation and behaviour (Schmidt and Duman, 2010). This provides further evidence that BDNF contributes to the therapeutic action of antidepressant treatment.

1.6.6 Evidence for the role of BDNF from post-mortem studies

To investigate the effects of BDNF in the human brain during depression and in response to antidepressant treatment, post mortem tissue of depressed patients has been studied. Post-mortem, BDNF mRNA and protein expression levels were decreased in the hippocampus and prefrontal cortex of depressed suicide subjects compared to matched controls (Dwivedi et al. 2003c). Expression levels of full length TrkB were also significantly reduced (Dwivedi et al. 2003a).

Antidepressant treated patients had higher levels of BDNF expression in the hippocampus, dentate gyrus and hilus, compared to controls who did not receive antidepressant treatment (Chen et al. 2001b). Hippocampal expression levels of BDNF and NT-3 protein and prefrontal cortex levels of BDNF were significantly decreased in drug-free suicide victims compared to non-suicide controls. Neurotrophin levels of suicide subjects that were drug treated did not differ significantly from non-suicide controls suggesting that treatment with antidepressants normalised neurotrophin levels (Karege et al. 2005b).

In addition to decreased levels of BDNF in depressed patient brains, plasma and serum levels of BDNF expression were also reduced in depressed patients compared to controls (Karege et al. 2005a; Aydemir et al. 2006; Lee and Kim, 2008; Matrisciano et al. 2009). Different classes of antidepressant modulated BDNF levels differently though all were effective in reducing depression symptoms. Increases in BDNF levels were sustained for up to six months suggesting a role in the maintenance of antidepressant action (Matrisciano et al. 2009). Neurotrophins, particularly BDNF appear to play an important role in the efficacy of antidepressant treatment and in depressive illness in humans.

1.6.6.1 Neurotrophins and neuroplastic changes

Neuroplasticity is a general term that describes the brain's ability to adapt and remodel with experience. The adult brain is not hard-wired and incapable of responding to the environment. Neuroplastic changes can include neurogenesis, dendritic remodelling and synapse turnover (McEwen et al. 2011). Neurotrophin action has been implicated in various forms of neuroplasticity, particularly in the formation of new neurons, neurogenesis.

TrkB and BDNF signalling are required for antidepressant induced survival of newborn dentate gyrus granule cells. In BDNF heterozygote knockout mice and transgenic dominant-negative TrkB receptor mice, immature neuron survival rates in the dentate gyrus were decreased (Sairanen et al. 2005). Antidepressant induced increase in neuronal proliferation is blocked by the deletion of TrkB from neural progenitor cells (Li et al. 2008). The neurotrophin VEGF and its receptor Flk1, signalling also increased the rate of proliferation of neural progenitor cells and increased hippocampal neurogenesis (Jin et al. 2002; Wada et al. 2006). VEGF has been shown to promote neurite outgrowth, which may be mediated by the MAPK pathway (Khaibullina et al. 2004). FGF2 and GDNF neurotrophic receptors were also upregulated in response to antidepressant drug treatment and ECS (Chen et al. 2001a; Mallei et al. 2002).

Overall, enhanced BDNF and TrkB signalling in the depressed brain may gradually lead to an antidepressant response (Groves, 2007). It has been proposed that depression does not result from a deficit of hippocampal or cortical BDNF, but induction of BDNF may be essential in neuronal network plasticity and adaptation in depressed individuals in response to antidepressants (Castren and Rantamaki, 2010). Therefore, there is much evidence to suggest that antidepressant drugs are responsible for changes in neuronal plasticity and that neurotrophin, especially BDNF, signalling may mediate this response (Krystal et al. 2009).

The discovery that BDNF was a target of CREB activation (Duman et al. 1999) and that BDNF supported peripheral neuron survival during development (Huang and Reichardt, 2001) initially focused attention on the potential role of BDNF in depression and antidepressant action. As discussed above, there is very strong evidence for altered neurotrophin levels in depressed human patients and in animal studies of depression.

BDNF itself has even been shown to act as antidepressant in certain brain regions (Shirayama et al. 2002). The downstream effects of neurotrophic factors may explain their role in depression. The signalling pathways induced by neurotrophins and the neuroplastic changes that are activated in response to increased neurotrophin levels are discussed below.

1.6.7 BDNF, neurotrophins and signalling pathways

Neurotrophins are involved in plasticity and neurogenesis, important factors in depression and antidepressant treatment. Following antidepressant treatment, BDNF and other neurotrophins activate signalling pathways which bring about these plastic changes, potentially mediating an antidepressant response. G-protein coupled signalling and Trk receptor phosphorylation and activation lead to the upregulation of several established signalling cascades (Figure 1-6) involved in promoting neuroplasticity and in controlling cell survival.

1.6.7.1 Signalling pathways induced by antidepressant treatment

MAPK is induced following Ras activation by Trk phosphorylation. MAPKs activate ribosomal S6 kinases (Rsk) downstream which in turn activate CREB and other transcription factors (Huang and Reichardt, 2001). CREB regulates genes whose products are required for neuronal survival (Bonni et al. 1999; Riccio et al. 1999). The MAPK pathway is activated in response to chronic antidepressant treatment (Mercier et al. 2004; Kodama et al. 2005). Treatment with an inhibitor of the kinase mitogen activated protein kinase kinase (MEK) blocked signal transduction through the MAPK pathway and the antidepressant effect of desipramine and sertraline in the FST (Duman et al. 2007). The MAPK pathway was important for synaptic plasticity induction in various brain regions including the hippocampus, amygdala and cortex (Giovannini, 2006). In studies of depressed suicide victims, decreased MAPK signalling was seen post-mortem and may contribute to the pathophysiology of depression (Dwivedi et al. 2006a; Dwivedi et al. 2006b; Dwivedi et al. 2007).

1.6.7.1.1 Cell survival pathways

Neurotrophic factors promote cell survival by, at least in part, inhibiting cell death signalling cascades (Riccio et al. 1999). Neurotrophins inhibit apoptotic cascades by activating MAPK and phosphatidylinositol-3 kinase (PI3 kinase) pathways. The MAPK signalling pathway may have prevented cell death by inducing CREB and increasing anti-apoptotic Bcl-2 expression (Bonni, 1999; Finkbeiner, 2000).

PI-3 kinase activates Akt (see Figure 1-4), a serine/threonine protein kinase that controls proteins involved in cell survival, including Bcl-2 associated death promoter (BAD) protein. Phosphorylated BAD associates with 14-3-3 proteins, a family of signalling proteins, and is thus prevented from promoting apoptosis (Datta et al. 1997). Glycogen synthase kinase 3- β (GSK3- β) is negatively regulated by Akt; its upregulation promotes apoptosis in cell culture (Chin et al. 2005). PI-3 kinase and its signalling pathways could be activated by Ras dependent or independent mechanisms (Huang and Reichardt, 2001).

1.6.7.2 p75 neurotrophin receptor

p75^{NTR} can bind all neurotrophins with similar affinity (Dechant and Barde, 2002). p75^{NTR} is a tumour necrotic factor (TNF) type receptor that activates several different signalling pathways. It is involved in cell survival related pathways. p75^{NTR} activates the NF κ B signalling path (Arch et al. 1998; Huang and Reichardt, 2001). The NF κ B pathway is implicated in neuronal plasticity and synaptic transmission (O'Neill and Kaltschmidt, 1997). Jun kinase signalling is activated by p75^{NTR} receptor activation via protein 53 (p53) and is required by p75^{NTR} for apoptotic mediation in neurons (Huang and Reichardt, 2001).

p75^{NTR} signalling appears to induce opposite effects to Trk receptors. Pro-BDNF and pro-NGF bind to p75^{NTR}, activating it and inducing neuroplastic changes including neuronal atrophy, apoptosis and dendritic pruning (Lee et al. 2001b; Lu et al. 2005; Zagrebelsky et al. 2005). It has been proposed that TrkB signalling, via mature BDNF activation has an antidepressant response while pro-BDNF induced p75^{NTR} signalling would have a pro-depressant effect (Martinowich et al. 2007).

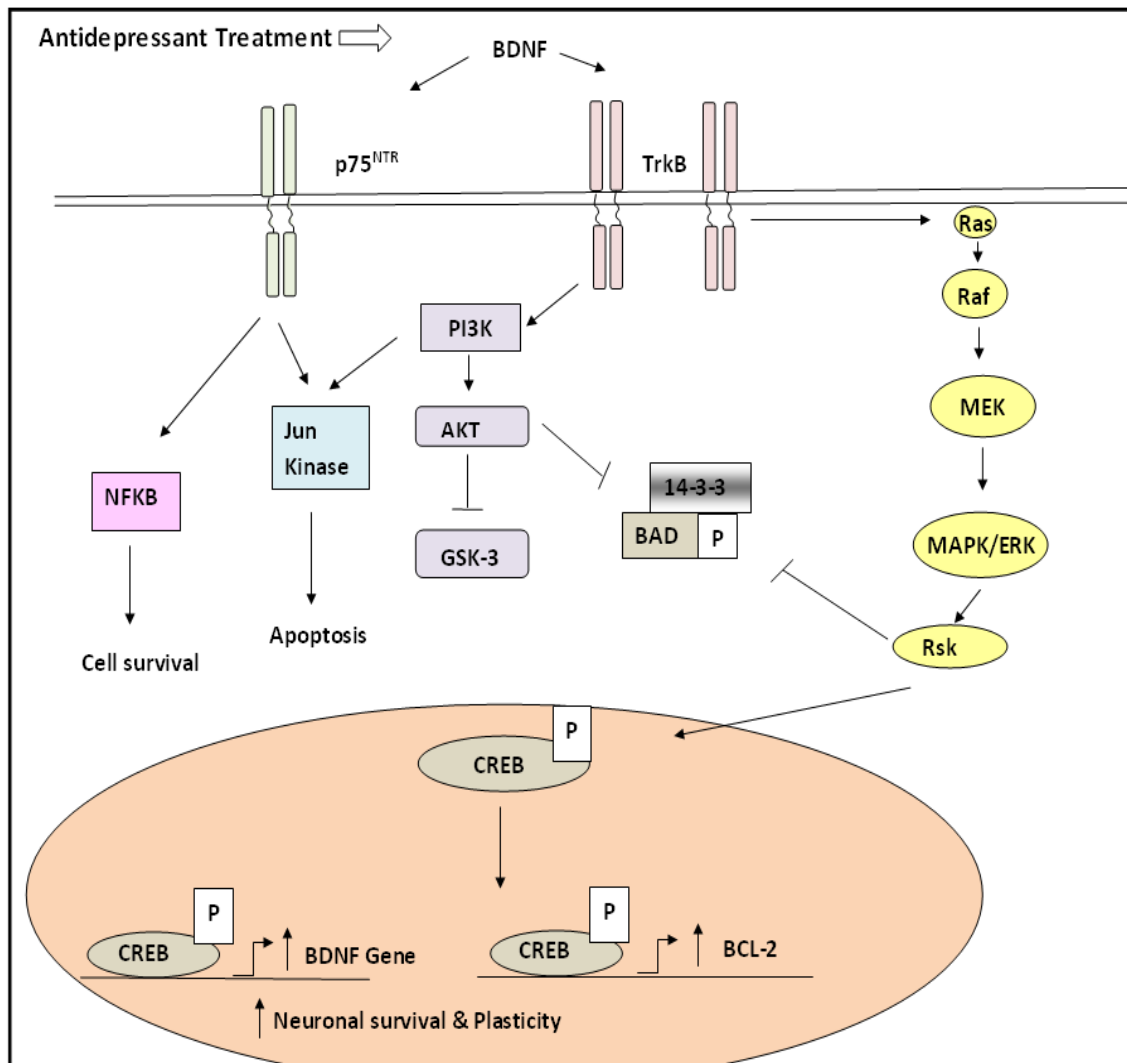


Figure 1-6 Signalling pathways that can mediate the response to antidepressant administration

The activation of Trk receptors by neurotrophins activates various signalling pathways, including MAPK and PI3K. These converge to mediate CREB activation and transcription of neurotrophins and their receptors, leading to downstream regulation of various forms of neuroplasticity. ERK, extracellular signal regulated kinase; MAPK, mitogen activated protein kinase; BDNF brain derived neurotrophic factor; CREB, cAMP response element binding; BAD, Bcl-2 associated death promoter; Rsk, ribosomal s6 kinase; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal regulated kinase; GSK-3, glycogen synthase kinase 3; AKT, family of non specific serine/threonine protein kinases; Raf, proto oncogene serine/threonine protein kinase; Ras, rat sarcoma; PI3K, phosphatidyl inositol-3 kinase; NFκB, nuclear factor kappa light chain enhancer of activated B cells; JNK, c-Jun N-terminal kinase; p75^{NTR}, p75 neurotrophic receptor; TrkB, tropomyosin receptor kinase B.

Neuronal plasticity does not solely describe processes that result in structural increases such as neurogenesis and synaptogenesis. These mechanisms are balanced by programmed neuronal death or synapse retraction or pruning. However, research into depression has mainly focused on the positive (matureBDNF-TrkB) aspects of plasticity rather than the negative (proBDNF-p75^{NTR}). Much work has yet to be done to fully understand this bidirectional interaction (Lu et al. 2005) and the role it may play in depression.

In summary, upregulation of monoamine levels by antidepressants upregulates the cAMP and Ca²⁺ signalling cascades (Figure 1-4). This increases CREB, BDNF, and other neurotrophin levels, further upregulating signalling pathways (Figure 1-6) and inducing changes in cell proliferation and neural plasticity. Recognising the signalling pathways activated by ECS and antidepressant treatment will aid understanding of downstream antidepressant processes and may provide new targets for study. Upregulation of neurotrophin expression, in particular BDNF, in response to antidepressant treatment provides a solid background for the study of neurogenesis and neuroplastic changes in the brain in depression and in response to antidepressant treatment (Duman and Monteggia, 2006).

As discussed earlier (section 1.5.6.1), BDNF may be responsible for mediating cortical plasticity activation following antidepressant administration (Castren and Rantamaki, 2010). Therefore, a greater understanding of the plasticity changes induced by treatment may be critical to determine the mechanisms of antidepressant action and the underlying neurobiology of depression.

1.7 Adult neurogenesis, stress and depression

1.7.1 The discovery that new neurons form in the adult brain

New neurons are now known to arise in the adult brain of many species, including humans (Altman and Das, 1965; Gould et al. 1999; Gage, 2000; Duman et al. 2001). Neurogenesis has been detected in the subventricular zone and subgranular zone, giving rise to new granule cells in the olfactory bulb and the hippocampus respectively. In the hippocampus, progenitor cells in the sub granular zone proliferate, forming postmitotic daughter cells. These cells differentiate and migrate into the granule cell layer. They mature, spreading processes into the molecular layer and the pyramidal layer (Figure 1-6) (Markakis and Gage, 1999; Gage, 2000; Gould et al. 2000). The maturing neurons are similar to the existing granule cells (van Praag et al. 2002).

Approximately 80% of the newly formed cells differentiate into neurons (Scott et al. 2000; Rao and Shetty, 2004). The remaining cells differentiated into astrocytes, oligodendrocytes and endothelial cells (Warner-Schmidt et al. 2008). The rate of neurogenesis in the rodent brain (6% of the total size of the granule cell population generated each month) (Cameron and McKay, 2001), may be of functional significance in the hippocampus. Neurogenesis and other forms of synaptic plasticity have been implicated in depression.

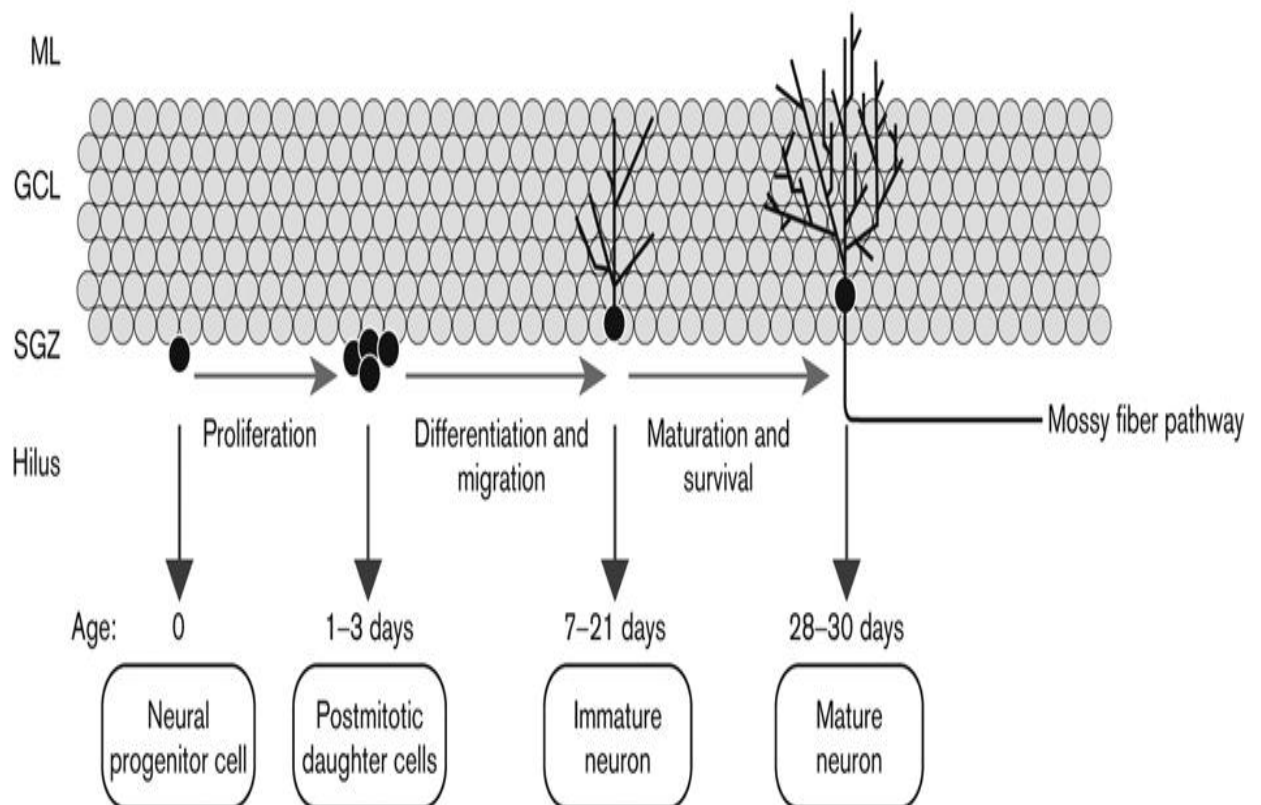


Figure 1-7 The maturation and migration of progenitor cells in the subgranular zone to mature neurons in the granule cell layer

SGZ, subgranular zone; GCL, granule cell layer; ML, molecular layer from Schmidt & Duman 2007.

1.7.2 Neuronal atrophy and antidepressant treatment in depression

1.7.2.1 Evidence from brain imaging studies

Brain imaging studies have demonstrated that patients with depression often have decreased hippocampal volume (Bremner et al. 2000; Frodl et al. 2002; Arnone et al. 2011; Kempton et al. 2011), although some authors have not reported reduced hippocampal volume but a change in hippocampus shape in depressed patients (Posener et al. 2003) . Others have reported no change in hippocampal volume (Vakili et al. 2000; von Gunten et al. 2000) however these studies were conducted in different depressed patient population types. The different findings between studies may be due to a range of factors including patient gender, patient's response to antidepressant treatment and the exact hippocampal regions that were analysed. Studies that controlled for sex and age differences have repeatedly shown decreased hippocampal volumes in older patients (Sheline et al. 1999; Sheline et al. 2000). Volumetric changes may occur after repeated episodes of depression in aged patients (Schmidt and Duman, 2007).

Some studies that looked at hippocampal volumetric changes in young and middle aged patients have reported no significant differences in volume (Hastings et al. 2004). While there is some controversy over the role of reduced hippocampal volume in depression, a recent meta-analysis has shown that hippocampal volume was significantly reduced in a first episode depression patients compared to controls (Cole et al. 2011). In contrast, antidepressants can reduce the hippocampal atrophy seen in depressed patients (Sheline et al. 2003).

However, other brain regions were also reported to show a reduced volume in depression including the prefrontal cortex and anterior cingulate gyrus (van Tol et al. 2010; Desmyter et al. 2011). Reductions in glial cell density and neuronal size were also reduced in the frontal cortex (Cotter et al. 2001; Cotter et al. 2005).

Reduced volume of brain structures like the hippocampus is not unique to depression and has been detected in several other neuropsychiatric disorders (Heckers and Konradi, 2010; Lisy et al. 2011). Equally, antidepressants can reduce the hippocampal atrophy seen in patients suffering from post traumatic stress disorder (Vermetten et al. 2003). Therefore, while a reduction in the volume of brain structures in depression has been advocated as a biomarker by some (Cole et al. 2011), it is unlikely to act alone as a sufficient biomarker for this disorder.

Further evidence is required to determine if hippocampal atrophy is associated with depression. It is not yet known if reduced hippocampal volume contributes to the aetiology of depression or if hippocampal atrophy is a consequence of the disorder.

1.7.2.2 Neuronal atrophy induced by cell signalling changes in humans

The cell signalling changes that may induce hippocampal atrophy seen in some patients with depression have been investigated in post-mortem studies. There is evidence that PKA and cAMP undergo altered expression in depressed patients (de Rooij et al. 1998). Studies from depressed suicide victims show a decrease in expression of these signalling molecules in the hippocampus compared to healthy controls, suggesting that the cAMP-PKA signalling pathway may be altered in depression (Dwivedi et al. 2003b; Dwivedi et al. 2004; Dwivedi et al. 2006b).

The MAPK pathway (Figure 1-6) was also implicated in depression by human post mortem studies. Extracellular regulated kinase 1 (ERK 1) and 2 (ERK 2) mRNA and protein levels and p44/42 MAP kinase expression were decreased in the hippocampus of depressed suicide victims. The upstream activator of the ERK pathway, B-Raf also showed reduced expression (Dwivedi et al. 2001; Dwivedi et al. 2006a; Dwivedi et al. 2007; Dwivedi et al. 2009). The signalling cascades induced by antidepressant administration (see Figure 1-6) may play a role in neuronal atrophy in the hippocampus in depressed patients.

1.7.3 Stress decreases neurogenesis

Stress has a profound effect on neurogenesis. Cell proliferation in a range of animals has been significantly reduced following stress induction by different protocols: for example footshock in the rat (Malberg and Duman, 2003; Vollmayr et al. 2003), prenatal stress in the rhesus monkey and rat (Lemaire et al. 2000; Coe et al. 2003) and chronic mild stress in the mouse. Decreased neurogenesis is associated with a behavioural response several days after exposure to stress (Malberg and Duman, 2003) suggesting the reduction in neurogenesis is not just an acute effect.

However, animal models of stress and depression have limitations. Changes in neurogenesis do not always follow depression-like behaviour in animals, as a reported dissociation between decreased hippocampal cell proliferation and learned helplessness behaviour in a model of learned helplessness (inescapable foot shock) shows (Vollmayr et al. 2003). Although stress downregulated cell proliferation in the dentate gyrus, it did not correlate with the development of the behavioural response to the model. The decreased cell proliferation associated with stress may be involved in the hippocampal atrophy seen in depression (Duman, 2004). In humans, there is a causal association between stress and the onset or exacerbation of depression (Kendler et al. 1999; Gold and Chrousos, 2002) though this association may diminish with repeated depressive episodes.

TCA, SSRI and SSRE administration have all been shown to block the effects of social, chronic mild stress and maternal separation stress in rodents (Czeh et al. 2001; Lee et al. 2001a; van der Hart et al. 2002; Alonso et al. 2004). Antidepressant treatment increased neurogenesis in normal animals as well as blocking the effects of stress on neurogenesis in the adult brain.

Overall, decreased neurogenesis does not appear to result in depression (Henn and Vollmayr, 2004). It is possible that neurogenesis is an epiphenomenon rather than an aetiological factor associated with depression. However, neurogenesis does appear to be required for an antidepressant effect to be induced following treatment with ECT and drug therapies (Dranovsky and Hen, 2006).

1.7.4 Antidepressant treatment increases neurogenesis in the hippocampus

The importance of neurogenesis in depression can also be seen from antidepressant treatment studies. Chronic administration of SSRI, TCA, MAOI and other antidepressant drug treatments have been shown to increase hippocampal neurogenesis in the rodent brain (Malberg et al. 2000; Nakagawa et al. 2002; Santarelli et al. 2003). This may represent a common target of antidepressant treatments as different classes of drugs robustly induce neurogenesis. Chronic ECS also induces hippocampal neurogenesis (Madsen et al. 2000; Malberg et al. 2000; Scott et al. 2000; Hellsten et al. 2002). In a study with nonhuman primates, ECS significantly increased neurogenesis in the brains of bonnet monkeys (*Macaca radiata*) (Perera et al. 2007), an interesting result given the greater relevance of nonhuman primate studies to human studies.

Other treatments, including oestrogen, dehydroepiandrosterone (DHEA) and exercise, that have an antidepressant effect can also effectively induce neurogenesis (Tanapat et al. 1999; Karishma and Herbert, 2002; van Praag et al. 2002). Antidepressant treatment not only increased the number of new cells but also increased the number that survived at a four week time point after antidepressant drug treatment (Malberg et al. 2000; Nakagawa et al. 2002). Antidepressants appear to block the effects of stress and potentially depression on hippocampal atrophy.

1.7.5 Is neurogenesis required for an antidepressant effect?

Therefore, although neurogenesis may not play a role in depression itself, it appears to be important for effective antidepressant treatment. Blocking cell proliferation by irradiating the rat hippocampus also blocked antidepressant-induced neurogenesis in the

hippocampus (Santarelli et al. 2003). This demonstrated that antidepressant treatment required neurogenesis to induce a response. A similar effect was seen in nonhuman primates (Perera et al. 2011). Bonnet monkeys were treated with an SSRI, fluoxetine, which stimulated neurogenesis. Neurogenesis was ablated by irradiation, which abolished the therapeutic effect of fluoxetine suggesting that neurogenesis was required for the therapeutic effect of fluoxetine. Neurogenesis may have a similar role in the treatment of depression in humans given the similarities between nonhuman primates and humans (Perera et al. 2011).

Antidepressant administration has been repeatedly shown to increase neurogenesis in the hippocampus although chronic administration appears to be required (Madsen et al. 2000; Malberg et al. 2000; Larsen et al. 2007). Antidepressant treatments have no effect on neurogenesis levels in the subventricular zone, their efficacy seems to be specific to the subgranular zone (Malberg et al. 2000; Santarelli et al. 2003). Equally, other commonly prescribed psychotropic drugs, for example the antipsychotic haloperidol, which have no antidepressant effect, increased neurogenesis in the subventricular zone but not in the subgranular zone (Halim et al. 2004; Wang et al. 2004). Antipsychotic drugs that are also effective antidepressants, for example olanzapine, led to increased levels of neurogenesis in the dentate gyrus (Kodama et al. 2004). However, when neurogenesis was blocked by the cytostatic agent MAM in rodents administered fluoxetine or imipramine, therapeutic efficacy was not impacted (Bessa et al. 2009).

These results suggest that an increase in neurogenesis contributed to, and may be required for, an antidepressant treatment response although other mechanisms may be at work.

1.7.5.1 How might neurogenesis bring about an antidepressant effect?

Recent studies have investigated how the presence of new neurons may bring about an antidepressant effect in the brain. Stress impairs both the ability of the hippocampus to regulate brain areas involved in the HPA axis activity and hippocampal neurogenesis. Ablating hippocampal neurogenesis alone did not impair the HPA axis but neurogenesis was required for antidepressant treatment to reinstate the ability of the hippocampus to regulate the HPA axis. This suggests that in a depressed brain the formation of new neurons allowed a dysfunctional hippocampus regain control of the stress system, enabling recovery (Surget et al. 2011). Hippocampal granule neurons regulate the normal neuroendocrine and behavioural response to stress (Snyder et al. 2011). Adult-born neurons buffer the response to stress, an effect that is reduced in depression due to a stress

induced decrease in neurogenesis (Snyder et al. 2011). Coping with social stress, an essential mechanism employed by animals (squirrel monkeys) living in social groups, also encouraged hippocampal neurogenesis (Lyons et al. 2010).

Neurogenesis is also required for efficient synaptic transmission at perforant pathway synapses onto hippocampal dentate gyrus granule cells (Massa et al. 2011). Ablating neurogenesis showed that long term potentiation (LTP) and long term depression (LTD), well established forms of neural plasticity, required neurogenesis. Restoring neurogenesis also restored the impaired hippocampal bidirectional synaptic plasticity. Neurogenesis may be a key influence on the neuroplastic processes in the dentate gyrus (Massa et al. 2011). BDNF signalling may provide a mechanism through which this proceeds as it has been proposed that mature BDNF promotes LTP through TrkB signalling while proBDNF promotes LTD via p75^{NTR} signalling (Lu et al. 2005).

However, antidepressants also work via neurogenesis-independent mechanisms. SSRI induced increases in animal food intake were not affected by hippocampal irradiation in the novelty suppressed feeding test (NSF) suggesting some behaviours induced by antidepressants do not require neurogenesis and may be modulated by distinct mechanisms (Surget et al. 2008; David et al. 2009). One study suggested that neurogenesis was not required for antidepressant action but other forms of neuronal plasticity, dendritic remodelling and synaptic contacts, may form the basis of the antidepressant response (Bessa et al. 2009).

Neuroplasticity, whether in the form of neurogenesis or synaptic or dendritic plasticity, appears to be essential for the induction of an antidepressant effect following treatment with ECT or pharmacotherapy.

1.7.6 Synaptic plasticity

There are forms of neuroplasticity, other than neurogenesis, that may underlie the action of antidepressants and their effect on depression. While alterations in neurogenesis have been implicated in depression and antidepressant action, these effects were limited to the hippocampus (discussed above 1.7.5). However, synaptic plasticity, the ability of synapses to change in strength, and dendritic arborisation were impaired in the hippocampus and prefrontal cortex by stress, a trigger of depression (Sousa et al. 2000; Cook and Wellman, 2004; Cerqueira et al. 2007). Antidepressant treatment worked to reverse these effects (Sairanen et al. 2007). Increased mRNA levels of synaptic remodelling proteins have been found following antidepressant treatment in the rat hippocampus and prefrontal cortex.

1.7.6.1 Dendritic spines

Spines are small, synapse-containing protrusions, found along dendrites. Synapse formation and removal as well as changes in spine density and morphology allow for neuronal connections and remodelling. Changes to spines can have significant effects on synapse function and plasticity in the brain. These changes may underlie a range of neuropsychiatric disorders including depression (Penzes et al. 2011). Spines can be defined by their shape. Mushroom spines have a large head and narrow neck, thin spines have a smaller head and a narrow neck and stubby spines have no obvious differentiation between head and neck (von Bohlen Und Halbach, 2009). Spine densities were unchanged in animals administered antidepressants, subjected to chronic mild stress protocols or their controls. However, spine morphology, the proportion of mushroom-shaped and thin spine types, varied in the different groups, suggesting small but significant changes in spine function. BDNF can differentially influence spine morphology. Under normal conditions BDNF increased the proportion of stubby spines but following blocked miniature synaptic transmission BDNF increased the proportion of thin spines (Tyler and Pozzo-Miller, 2003).

1.7.6.2 The effect of ECS and antidepressant treatment on synaptic plasticity

Corticosterone or inescapable footshock induced the loss of spine synapses in the dentate gyrus, CA1 and CA3 regions of the hippocampus, effects that were reversed by desipramine treatment (Hajszan et al. 2009). ECS significantly increased the total number of synapses, spine synapses and the perforated (perforated synapses have discontinuous post-synaptic densities (Calverley and Jones, 1990; Nikonenko et al. 2002) and nonperforated subtypes of spine synapses in the CA1 region of the hippocampus (Chen et al. 2009). As shaft synapses were unchanged in this study, spine synapses appear to contribute to the total spine number increases seen. The volume of the dentate gyrus granule cell layer and hilus were significantly larger in ECS treated animals (Chen et al. 2009).

Rearrangement of spines may allow for ECS induced synaptic plasticity. Fluoxetine and imipramine also induced hippocampal synaptogenesis in CA1 (Hajszan et al. 2005; Chen et al. 2008a). Synaptic remodelling following antidepressant treatment may represent a common mechanism of action (Chen et al. 2009).

There is robust evidence to suggest that changes in neuronal plasticity, in its varied forms, may be an important aspect of the response to antidepressant treatment.

The study of neuroplasticity provides a potentially fruitful avenue for research into depression and antidepressants.

1.8 Proteomics

Proteomics is the science that studies the complement of proteins expressed by a biological system (Wilkins et al. 1996). Neuroproteomic studies, which look at nervous system proteomes, have impacted positively on the study of neuropsychiatric disorders (Taurines et al. 2011).

Technological advancements over several decades have resulted in proteomic methodologies that contribute significantly to our understanding of the proteome. A brief background to the history and methodologies of proteomic analysis, particularly the neuroproteome, is discussed below.

Protein separation by gel electrophoresis techniques were the earliest and most widely used methods. Two-dimensional electrophoresis (2DE) was first developed in 1975-76 and represented the start of large scale protein analysis (O'Farrell et al. 1975; Klose et al. 1976). In combination with mass spectrometry, gel separation techniques allow the simultaneous separation and identification of hundreds of proteins in an experiment.

The first 2DE neuroproteomic study looked at cerebrospinal fluid (CSF) and detected approximately 300 protein spots (Coldman et al. 1980) which increased to 1,000 spots with 248 proteins identified by 1992 (Yun et al. 1992). The first proteomic profiling of mammalian (mouse) brain was conducted in 1981 (Klose et al. 1981). Improvements in technology, including increased sensitivity of mass spectrometry techniques, have led to the identification of over 7,700 protein spots in the mouse brain. This represented 34% of the predicted mouse proteome (Wang et al. 2006).

Proteomic studies of psychiatric disorders were first conducted in 1999 to compare the hippocampal proteome of Alzheimer's disease and schizophrenic patients (Edgar et al. 1999). Proteomic profiling was also used to study the molecular mechanisms of the effects of drugs. Initially, the effects of kainic acid on brain protein levels were studied in 2001 (Krapfenbauer et al. 2001). More recently, proteomic techniques were first applied to the study of synaptic plasticity in the hippocampus proteome.(McNair et al. 2006).

1.8.1 Other proteomic methodologies

An alternative to the 2DE-mass spectrometry method is the shotgun proteomic or direct mass spectrometry approach. Typically, a combination of chromatographic and tandem mass spectrometry is used to identify proteins. While this approach does not

provide direct information on intact proteins as 2DE does (Martins-de-Souza et al. 2009) it is a more accurate protein quantitation method. Liquid chromatography was first combined with mass spectrometry in a gel-free proteomic technique in 1995, leading to the development of high-throughput proteomics (Appella et al. 1995). Later developments in mass spectrometry allowed for the identification of post-translational modifications and in the first case, a study of the phosphoproteome (Ficarro et al. 2002).

Non-mass spectrometry based technologies were also developed to study the proteome. Protein arrays are a chip-based methodology similar in principle to DNA microarrays. They allow thousands of proteins to be tested based on an antibody-based array the size of a microscope slide. However in practice there have been many delays in the development and application of this technology which has limited its use (Bayes & Grant, 2009). Genetic based methods using recombinant DNA techniques to fluorescently tag proteins have also been developed to study the proteome. Fluorescent tags allow protein-protein interactions to be investigated, particularly by fluorescence energy resonance transfer (FRET) which indicates the proximity between two molecules by manipulating donor fluorophore and acceptor fluorophore labelled molecules (Bayes & Grant, 2009; Giepmans et al. 2006).

The large quantities of data obtained from gel and mass-spectrometry proteomic methods require suitable bioinformatic resources to obtain meaningful information from proteomic experiments. The first proteomic database, SWISSPROT, was developed in 1986 with the purpose of providing a free, comprehensive resource of protein sequence and functional information. There is no standard methodology for all steps in bioinformatic analysis following the generation of mass spectrometry data. However the most common approach involves protein classification and annotation (Bayes & Grant, 2009). Lists of proteins originating from mass spectrometry analysis are collated and annotated, typically according to their function, using public databases of protein information. Enriched annotation terms in protein sets are selected using statistical techniques. The most commonly used annotation for enrichment analysis is the gene ontology (GO) database (Kumar & Mann, 2009). Protein-protein interactions and canonical pathway analysis of protein lists can also be conducted using online databases or software, for example the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database and Ingenuity Pathway Analysis (IPA) which are discussed further in Section 2.2.20.2.

Proteomic techniques applied to neuropsychiatric disorders, particularly depression, have yielded substantial insight into these disorders. However, proteomic technologies have also been employed in biomarker discovery, the identification of changes in proteins

that can act as markers of a disease or its progression. CSF and plasma are often the samples of choice for neuropsychiatric biomarkers (Huang et al 2006). Thorough cataloguing of the CSF proteome has made this a particularly useful substrate for biomarker research in patients. However, current proteomic-based biomarker studies are conducted on a small scale which must be expanded to obtain statistically valid results (Bayes & Grant, 2009).

The development of proteomics in recent years is largely due to the improvements in biological mass spectrometry technology and bioinformatic resources. Neuroproteomics has been established as a powerful discovery tool for uncovering predicted, hypothetical or new proteins that were not previously known to be involved with specific neurobiological processes (Bayes et al. 2009; Taurines et al. 2011).

1.9 Summary

Depression is a complex, debilitating, neuropsychiatric disorder with a lifetime prevalence of ~17% (Kessler et al. 2003). A wide range of genetic and environmental factors can increase the risk of developing depression. Multiple genes have been implicated in depression but no single candidate “depression” gene has been found. Several brain regions and biological networks have been shown to play a role in depression including the neuroimmune system, neuroendocrine system and the monoaminergic neurotransmitter systems. The potential influence of so many biological systems contributes to the complexity of depression. Further study is required to elucidate through which of these systems ECT primarily acts or whether a combination of factors contributes to its mechanism of action.

The focus of antidepressant research has been on the monoaminergic system, in particular the development of drugs that increase serotonin and noradrenaline in the synapse. While these drugs can be effective at treating depression, ECT is currently the most efficacious antidepressant treatment available for depression.

ECT treatment has a significant impact on the expression of neurotrophins, which have a role in neuroplasticity and cell proliferation in the brain. Neuroplastic changes appear to be an important factor in the ability of antidepressants to induce a therapeutic effect. Although a decrease in neurogenesis is no longer considered to contribute directly to the development of depression, neurogenesis appears to be required for an antidepressant response (Santarelli et al. 2003). Other forms of neuroplasticity are now

being investigated for their potential role in inducing an antidepressant response (Bessa et al. 2009).

Investigating the molecular changes induced by ECS may provide insight into the mechanisms of action of antidepressant treatment that may in turn be informative about the neurobiology of depression.

1.10 Aims and objectives

The aim of the work presented in this thesis is to further elucidate the molecular changes induced by ECT and newer forms of ECT, using its animal model electroconvulsive stimulation (ECS). The objectives are:

- To determine whether modifying stimulation parameters of ECS will maintain treatment efficacy and reduce the cognitive deficits associated with this antidepressant treatment.
- To detect protein changes in the frontal cortex and hippocampal proteomes following administration of acute and chronic ECS. The longer-term effects of ECS on the proteome will also be examined in chronically treated animals sacrificed four weeks after the final treatment of ECS.
- To identify proteins using mass spectrometric techniques, that are statistically significantly different in animals treated with ECS.
- To confirm the presence and expression level changes of identified proteins of interest.

Materials & Methods

Unless otherwise stated, all solutions were prepared with MilliQ ultrapure water or distilled water. When required, solutions were autoclaved for fifteen minutes at 121°C at 15 pounds per square inch or filtered through a 0.22 µm pore filter (Millipore). General laboratory techniques were adapted from “Practical Skills in Biomolecular Sciences” (Reed et al. 2003).

2.1 Materials

2.1.1 Animals

Sprague Dawley Rats

Harlan, UK

2.1.2 ECS equipment

ECS pulse generator 57800

Ugo Basile, Italy

Padded ear clip electrodes

Ugo Basile, Italy

2.1.3 Behavioural equipment

The swim tank and four arm maze used in behavioural experiments were custom made.

2.1.4 Immunohistochemistry

2.1.4.1 Reagents and chemicals

5-Bromo-2'-deoxyuridine (BrdU)	(Sigma Aldrich, Ireland)
Avidin SP-2001	(Vector Laboratories Inc. Ca, USA)
Biotin SP-2001	(Vector Laboratories Inc. Ca, USA)
Distrene-80 plastiser xylene (DPX) mountant	(Fluka, Germany)
Hydrogen peroxide 3%	(Fisher Scientific, UK)
Isopentane	(Sigma Aldrich, Ireland)
Mayer's Hematoxylin solution	(Sigma Aldrich, Ireland)
Normal rabbit serum	(Dako, Denmark)
Optimal cutting temperature (OCT)	(TissueTek Inc., USA)
SigmaFast DAB tablets	(Sigma Aldrich, Ireland)
Streptavidin biotin/horseradish peroxidase (AB/HRP)	(Vector Laboratories Inc. Ca, USA)
solution A, Avidin DH solution	
solution B biotinylated enzyme	
Urethane	(Acros Organics, Germany)

Vectastain Elite PK-6100 Standard ABC kit	(Vector Laboratories Inc.CA, USA)
Xylene 3 & 4	(Fisher Scientific, UK)

2.1.4.2 Equipment

Cryostat CM1850	(Leica Microsystems, Germany)
Cryostat microtome blades C-35	(Feather Safety Razor Co., Ltd. Ireland)
Superfrost plus glass slides 25 x 75 x 1,0mm	(Menzel Glaser, UK)
Twin frost microscope slides ground 0.8- 1,0mm	(Fisher Scientific, Ireland)
Coverglass 22mm x 50mm x 0.17mm	(Menzel Glaser,UK)
pH meter UB-5	(Denver Instruments, USA)
Pump, variable flow	(Fisher Scientific, Ireland)
Olympus BX51 light microscope and DP72 camera attachment	(Olympus Corporation, UK)
Cell B Soft imaging systems imaging software	(Olympus Corporation, UK)

2.1.4.3 Antibodies

Rat monoclonal (BU1/75(ICR1)) to BrdU ab6326	(Abcam, Cambridge, UK)
Rabbit anti-rat biotinylated secondary antibody BA- 4001	(Vector Laboratories Inc., USA)
Rat anti ECF-L (Clone 281926) MAB2446	(R&D Systems, UK)

2.1.4.4 Recipes

Saline Solution

0.9% sodium chloride (NaCl) dissolved in distilled water
Solution was autoclaved before use.

Bromodeoxyuridine (BrdU) solution

40mg/ml BrdU dissolved in heated 0.9% saline

Tris(hydroxymethyl)aminomethane buffered saline (TBS) solution

0.05M Tris-HCl

0.15M NaCl

Final pH7.6

Citric acid buffer

10mM citric acid

0.05% Tween 20

Final pH 6

Normal rabbit serum solution

10% normal rabbit serum diluted in TBS

3,3'-Diaminobenzidine (DAB) tablets

SigmaFast DAB tablet set (Sigma Aldrich) DAB tablet: urea hydrogen tablets (1:1) were dissolved in distilled water according to manufacturer's instructions

Phosphate buffered saline (PBS) solution

80mM sodium phosphate monobasic

20mM sodium phosphate dibasic

100mM sodium chloride

pH 7.4

Paraformaldehyde solution

0.1M PBS was heated to 60°-65°C

4% paraformaldehyde was added slowly and the solution was maintained at ~65°C until dissolved. Solution was cooled, filtered and stored at 4°C

Sucrose solution

30% sucrose in distilled water

Antibodies

Antibodies were diluted as required in TBS (see methods section for dilutions)

2.1.5 Enzyme Linked ImmunoSorbent Assay (ELISA)**2.1.5.1 Reagents**

Brain derived neurotrophic factor (BDNF)

sandwich ELISA kit (Chemikine, Chemicon, Millipore, USA)

EDTA.Na² (Sigma Aldrich, Ireland)

Sodium azide (Sigma Aldrich, Ireland)

Triton X-100	(Sigma Aldrich, Ireland)
Tris HCl	(Sigma Aldrich, Ireland)
Protease inhibitors: AEBSF 104mM, Aprotinin 80µM, Bestatin 4mM, E-64 1.4mM, Leupeptin 2mM, pepstatin A 1.5mM	(Sigma Aldrich, Ireland)
BSA	(Sigma Aldrich, Ireland)
Sodium chloride	(Sigma Aldrich, Ireland)

All reagent supplied in the ELISA kit were prepared as per the manufacturer's instructions.

2.1.5.2 Equipment

Handheld homogeniser & disposable pestles	(Sigma Aldrich, Ireland)
Plate reader ELX800	(BioTek Instruments Inc., USA)

2.1.5.3 Recipes

Tris HCl solution

100mM Tris base
pH 7

Wash buffer

10X 100ml wash buffer solution added to 900ml distilled water as per manufacturer's
(Chemicon, Millipore) directions

Homogenisation buffer

100mM Tris HCl pH 7
2% BSA
1M sodium chloride
4mM EDTA.Na²
2% Triton X-100
0.1% sodium azide
10% protease inhibitors

2.1.6 Proteomic analysis

2.1.6.1 Reagents and chemicals

Cyanine dye Cy3 25nmol	(GE Healthcare, Ireland)
Cyanine dye Cy5 25nmol	(GE Healthcare, Ireland)
Acetonitrile	(Fisher Scientific, UK)
Agarose	(Amersham Biosciences, UK)
Ammonium bicarbonate	(Sigma Aldrich, Ireland)
Ammonium sulphate	(Sigma Aldrich, Ireland)
Bromophenol blue	(Sigma Aldrich, Ireland)
Butanol	(Sigma Aldrich, Ireland)
3-((3-cholamidopropyl)dimethylammonio)-1-propanesulphonate (CHAPS)	(USC, USA)
Coomassie Brilliant Blue R-250	(Sigma Aldrich, Ireland)
DL Dithiothreitol (DTT)	(USC, USA)
Dimethylformide (DMF)	(Sigma Aldrich, Ireland)
Gelseal ¼ oz tube	(GE Healthcare, Ireland)
Glycerol	(USC, USA)
Glycine	(USC, USA)
Hydrochloric acid	(Sigma Aldrich, Ireland)
Iodoacetamide	(Sigma Aldrich, Ireland)
IPG pH4-7 strips	(GE Healthcare, Ireland)
Lysine	(Sigma Aldrich, Ireland)
MBO Amberlite MN-1	(Supelco, PA, USA)
Pharmalyte	(Sigma Aldrich, Ireland)
PlusOne silver stain kit	(GE Healthcare, Ireland)
PlusOne strip cover fluid	(GE Healthcare, Ireland)
Potassium ferricyanide	(Sigma Aldrich, Ireland)

Sequencing grade modified trypsin porcine lyophilized & trypsin resuspension dilution bufferv5111	(Promega Corporation, UK)
Sodium dodecyl sulphate	(USC, USA)
Sodium thiosulphate	(Sigma Aldrich, Ireland)
Tris base	(USC, USA)
Urea	(USC, USA)

2.1.6.2 Equipment

1mm low fluorescence glass plates	(Amersham Biosciences, UK)
2ml micro screw cap sterile cryogenic tubes	(Simport, Canada)
Centrifuge 5417C	(Eppendorf, USA)
Cuvettes, disposable	(Sarstedt, Ireland)
Gel casting rig	(Amersham Biosciences, UK)
Glass plate 12-place rack	(Amersham Biosciences, UK)
GS-800 calibrated densitometer	(Biorad Laboratories Inc., CA, USA)
Immobiline DryStrip reswelling tray	(Amersham Biosciences, UK)
Innova 2300 platform shaker	(New Brunswick Scientific, USA)
IPGphor EttanDALT	(Amersham Biosciences, UK)
Microson ultrasonic cell disruptor XL	(Misonix, USA)
Multitemp III (cooling unit)	(Biorad Laboratories Inc., USA)
Paper wicks	(GE Healthcare, Ireland)
pH Test strips pH 6-9	(Whatman, USA)
Plastic buffer inserts for tank	(Amersham Biosciences, UK)
ProteanPlus dodeca cell gel tank	(Biorad Laboratories Inc., CA, USA)
Spectrophotometer	(Biorad Laboratories Inc., CA, USA.)
Spilfyter delicate-task wipes 4.5" x 8"	(NPS Corp, WI,USA)
Typhoon 9410 variable mode imager	(Amersham Biosciences, UK)
Variable speed pump	(Biorad Laboratories Inc., Ca, USA)

2.1.6.3 RecipesDifference in Gel Electrophoresis (DiGE) compatible lysis buffer

9.5M Urea

2% CHAPS

20mM Tris pH 8.0

Urea was mixed with 0.5g of Amberlite for 10 minutes and filtered, aliquoted and stored at -20°C

2X dilution buffer

9.5M Urea

2% CHAPS

2% DTT

1.6% Pharmalyte

Urea was mixed with 0.5g of Amberlite for ten minutes and filtered. Aliquoted and stored at -20°C

Lysine solution

10mM Lysine

Rehydration buffer

8M Urea

0.5% CHAPS

0.2% DTT

0.2% Pharmalyte

Grains of bromophenol blue

Urea was mixed with 0.5g of Amberlite for ten minutes and filtered. Aliquoted and stored at -20°C

Stock equilibration buffer

6M Urea

30% Glycerol

2% SDS

0.05M TrisHCl Buffer (pH 8.8)

Equilibration buffer A

1% DTT to stock equilibration buffer

Equilibration buffer B

2.5% iodoacetamide to stock equilibration buffer

Sodium dodecyl sulphate (SDS) electrophoresis buffer: 10X stock solution

25mM Tris Base

192mM Glycine

0.1% (w/v) SDS

Agarose sealing solution

1% agarose in 1X SDS Electrophoresis buffer

Agarose was dissolved by heating in microwave. Grains of bromophenol blue were added until a light blue colour was obtained.

Tris HCl buffer

1.5M Tris Base, to pH8.8 with HCl

Ammonium persulphate (APS) solution

10% (w/v) ammonium persulphate in distilled water

Ammonium bicarbonate stock solution

200mM ammonium bicarbonate

Potassium ferricyanide solution

30mM potassium ferricyanide

Sodium thiosulphate solution

100mM sodium thiosulphate

2.1.6.3.1 Silver Staining

PlusOne silver staining kit: solutions were prepared according to the manufacturer's (GE Healthcare) instructions. *Italicised components were supplied in the staining kit.*

Fixative solution

10% acetic acid

40% ethanol

50% water

Sensitising solution

75ml ethanol

10ml 5% sodium thiosulphate

17g sodium acetate

165ml water

Silver stain solution

25ml silver nitrate

225ml water

Developing solution

6.25g sodium carbonate

100µl formaldehyde

250ml water

Stop solution

3.65g EDTA

250ml water

2.1.6.3.2 Coomassie blue stainingFixing solution

10% (v/v) acetic acid

10% (v/v) methanol

40% (v/v) ethanol

40% water

Sensitising solution

1% (v/v) acetic acid
10% (w/v) ammonium sulphate
89% water

Coomassie blue stain

0.125% coomassie blue R-250
5% (v/v) acetic acid
45% (v/v) ethanol
50% water

Destain solution I

5% (v/v) acetic acid
40% (v/v) ethanol
55% water

Destain solution II

3% (v/v) acetic acid
30% (v/v) ethanol
67% water

Storage solution

5% (v/v) acetic acid
95% water

2.1.6.3.3 Trypsin preparation

The trypsin (sequencing grade modified v5111, Promega) was prepared as follows: a vial of lyophilised trypsin (20ug) was resuspended in 100ul of the accompanying resuspension buffer. This was added to 900ul of 25mM ammonium bicarbonate giving a final concentration of 20ng/μl.

2.1.7 Semi-quantitative immunoblotting

2.1.7.1 Reagents and chemicals

Re-Blot plus strong	(Millipore, USA)
Immobilon Western blot	(Millipore, USA)
chemiluminescent HRP substrate:	
Peroxide solution	
Luminol reagent	
Supersignal molecular weight protein ladder	(Thermo Fisher Scientific, USA)
Protease inhibitor cocktail	(Sigma Aldrich, Ireland)
AEBSF 104mM, Aprotinin 80μM, Bestatin 4mM, E-64 1.4mM, Leupeptin 2mM, pepstatin A 1.5mM	
Igepal CA630 (NP-40)	(Fluka, Germany)
Protogel stacking buffer	(National Diagnostics, USA)
Immobilon-P transfer membrane	(Millipore, Ireland)
polyvinylidene fluoride (PVDF) 0.45μm	
Non-fat dried milk powder	(Marvel, Ireland)
Whatman filter paper 3	(Whatman, USA)

2.1.7.2 Equipment

Casting stand, buffer dam & gaskets	(Biorad Laboratories Inc., USA)
Heating block QBD2	(Grant Engineering, Ireland)
Intelligent dark box Las3000	(Fujifilm, UK)
Mini PROTEAN 3 system glass plates short plates	(Biorad Laboratories Inc., USA)
Mini PROTEAN 3 system glass plates spacer plates with 1.0mm spacers	(Biorad Laboratories Inc., USA)
Mini see-saw rocker SSM4	(Stuart, Bibby Scientific Ltd., UK)
Mini-PROTEAN system 10 well comb	(Biorad Laboratories Inc., USA)
Mini-PROTEAN tetra cell	(Biorad Laboratories Inc., USA)
ReadyGel loading guide 10 well	(Biorad Laboratories Inc., USA)
Soniprep 150 sonicator	(MSE, UK)
Square petri dish	(Sterilin, Ltd, UK)
Trans blot SD cell	(Biorad Laboratories Inc., USA)

2.1.7.3 Antibodies

Anti-Spectrin alpha chain (nonerythroid), clone AA6 monoclonal antibody	(Millipore, UK)
Calponin 3 (H-55), rabbit polyclonal antibody sc-28546	(Santa Cruz Biotechnology, USA)
Goat anti-Rabbit IgG, (H & L) HRP Conjugate AP307P	(Millipore, UK)
Monoclonal anti- β -Actin in mouse A5441 clone AC-15	(Sigma Aldrich, Ireland)
Polyclonal rabbit anti-GFAP Z0334	(Dako, Germany)
Rabbit anti-Mouse IgG (H+L) HRP secondary antibody	(Millipore, UK)
Rabbit Anti-NAP-22 polyclonal antibody AB9304	(Millipore, UK)
Rabbit anti-Neurogranin polyclonal antibody AB5620	(Millipore, UK)
Rabbit polyclonal to CamKII β ab34703	(Abcam, Cambridge, UK)
Rabbit polyclonal to CRMP2 ab36201	(Abcam, Cambridge, UK)
Rabbit polyclonal to GAPDH ab9485	(Abcam, Cambridge, UK)
Rabbit polyclonal to Tropomodulin 2 ab104219	(Abcam, Cambridge, UK)
RhoB (119), rabbit polyclonal antibody, sc-180	(Santa Cruz Biotechnology, USA)

2.1.7.4 RecipesSDS solution

10% SDS

Electrode running buffer (5X)

125mM Tris Base

960mM Glycine

0.5% (v/v) 10% SDS

pH 8.3

Anode buffer I

0.3M Tris-HCl pH10.4

10% methanol

Anode buffer II

25mM Tris-HCl (pH10.4)

10% methanol

Cathode buffer

25mM Tris-HCl (pH9.4)

40mM glycine

10% Methanol

Tris-HCl solution

0.5M Tris Base

Final pH 6.8

Wash solution: PBS-Tween 20

100mM sodium chloride

80mM sodium phosphate dibasic

20mM sodium phosphate monobasic

0.05% Tween 20

TBS-Tween 20

20mM Tris-HCl (pH7.5)

150mM sodium chloride

0.05% Tween-20

Bromophenol blue stock solution

10X bromophenol blue

Laemmli sample buffer (2X)

Distilled water 27.5%

0.0625M Tris-HCl pH6.8

Glycerol 20%

10% SDS 20%

 β -mercaptoethanol 5%

Bromophenol blue (10X) 15%

Lysis buffer: NP-40

50mM Tris

150 mM NaCl

1% NP-40

10% (v/v) protease inhibitors were added fresh to the buffer before use

Chemiluminescent solution

Immobilon Western Blot Chemiluminescent substrate (Millipore):

500µl of peroxide solution

500µl of luminol solution

1ml total was required per blot. The solutions were mixed, upon reaching room temperature, as per manufacturer's guidelines.

Strip solution

1 in 10 dilution of Re-Blot Plus Strong strip solution was used per blot

Blocking buffer:

5% bovine serum albumin (BSA) in TBS-Tween 20

5% non-fat dried milk powder in TBS-Tween 20

Antibody solutions:

Antibodies were prepared in a solution of 1% BSA in TBS-T or 1% non-fat dried milk powder in TBS-T

2.1.8 General laboratory wares

2.1.8.1 Reagents and chemicals

Acetic Acid	(Sigma Aldrich, Ireland)
Albumin from bovine serum	(Sigma Aldrich, Ireland)
Ammonium persulphate	(Fisher Scientific, UK)
Ethanol: High Performance Liquid Chromatography (HPLC) grade	(Merck, USA)
Ethylene glycol	(Acros Organics, UK)
Glycerol	(Acros Organics, UK)
Glycine	(Fisher Scientific, Ireland)
Hydrogen peroxide	(Sigma Aldrich, Ireland)
Methanol	(Sigma Aldrich, Ireland)

Molecular grade (HPLC) water	(Sigma Aldrich, Ireland)
N,N,N',N'Tetramethylethylene-diamine (TEMED)	(Sigma Aldrich, Ireland)
Paraformaldehyde	(Sigma Aldrich, Ireland)
pH meter buffer solution pH 4 pH 7 pH 10	(Reagecon, Shannon, Ireland)
Potassium chloride (pH meter)	(Sigma Aldrich, Ireland)
Protein assay solution	Biorad Laboratories Inc., USA)
Protogel	(National Diagnostics, USA)
Protogel 4X resolving buffer	(National Diagnostics, USA)
Sodium azide	(Sigma Aldrich, Ireland)
Sodium chloride	(Sigma Aldrich, Ireland)
Sodium hydroxide	(Sigma Aldrich, Ireland)
Sodium phosphate dibasic	(Sigma Aldrich, Ireland)
Sodium phosphate monobasic	(Sigma Aldrich, Ireland)
Sucrose	(Sigma Aldrich, Ireland)
Tris hydrochloride (Tris HCl)	(Fisher Scientific, Ireland)
Triton X-100	(Sigma Aldrich, Ireland)
Tween 20	(Fisher Scientific, Ireland)
Virkon	(Antec International Ltd., USA)

2.1.8.2 Equipment

1.5ml microfuge tubes	(Sarstedt, Germany)
15ml disposable centrifuge tubes	(Fisherbrand, Fisher Scientific, UK)
50ml disposable centrifuge tubes	(Fisherbrand, Fisher Scientific, UK)
5ml Pasteur pipettes	(Fisher Scientific, UK)
Assorted glassware: beakers, conical flasks, cylinders:50ml, 100ml, 200ml, 600ml, 800ml, 1L, 2L	(Fisher Scientific, UK)
Disposable scalpels	(Swann-Morton, Sheffield, England)
Disposable serological pipettes 25ml	(Fisher Scientific, UK)
Disposable single edge blades	(Fisher Scientific, UK)
EDTA K ₂ 15ml tubes	(Sarstedt, Germany)
Forceps	Fisher Scientific, UK

Heraeus multifuge 3SR+ centrifuge	(Thermo Scientific, UK)
Hermle Z 233 M-2 centrifuge	(Hermle, Germany)
Hotplate magnetic stirrer	(Fisher Scientific, UK)
Magnetic stirrers and rod	(Fisher Scientific, Ireland)
Microwave	(NordMende, UK)
Mini see-saw rocker SSM4	(Stuart, Bibby Scientific Ltd., UK)
Mini-centrifuge	(Sprout, USA)
Needles Microlance	
325Gx5/8" 0.5mmx16mm	(BD Science, UK)
Parafilm "M"	(Pechiney Packaging Plastics, USA)

2.1.8.2.1 Pipettes

Finnpipette: 0.1-10µl; 10-100µl, 100-1,000µl; 2-10ml	(Thermo Scientific, UK)
Fisherbrand: 0.1-2.5µl	(Fisher Scientific, UK)
Eppendorf research pro electronic pipettes: 0.5-10µl, 5-100µl	(Eppendorf, USA)
Tipor-M ⁺ : 50-300µl 8-channel pipette	(Orange Scientific, Belgium)

2.1.8.2.2 Pipette tips

TipOne 10µl graduated filter tip (Sterile)	(StarLab, Ireland)
TipOne 100µl bevelled filter tip (Sterile)	(StarLab, Ireland)
TipOne 1000µl graduated filter tip (Sterile)	(StarLab, Ireland)
0.1-10µl crystal-G pipette tips	(Fisherbrand, Fisher Scientific, UK)
100-1,000µl SureOne non-filter pipette tips	(Fisherbrand, Fisher Scientific, UK)
1-200µl Yellow Gilson 1 pipette tips	(Fisherbrand, Fisher Scientific, UK)
2-10ml Finntip pipette tips	(Thermo Electron Corporation, USA)

Powerpac (Basic)	(Biorad Laboratories Inc., USA)
Powerpac (High Capacity)	(Biorad Laboratories Inc., USA)

Spatula	(Fisher Scientific, UK)
Stericup and steritop vacuum driven filters	(Millipore, UK)
Syringe plastipak 5ml disposable	(BD Science, UK)
Ultrabasic pH meter UB-5	(Denver Instruments, U.S.A)
Vortex IKA MS3 basic	(IKA Works Inc, NC, USA)
Weighboats:8cmx8cmx2.5cm; 13.5cmx13.5cmx2.5cm	(Fisher Scientific, UK)

Table 2-1 Recipe for resolving gel buffer and stacking gel buffer

Resolving Buffer		8%	10%	12%	Stacking Buffer		4%
	Protogel	26.6%	33.25%	40%		Protogel	13%
	4X Protogel Resolving Buffer	25%	25%	25%		Protogel Stacking Buffer	25%
	Water	51.7%	41.6%	35%		Water	62%
	APS (10% w/v)	0.10	0.10%	0.10%		APS (10% w/v)	0.05%
	TEMED	0.01%	0.01%	0.01%		TEMED	0.01%

Equation 1 Formula used to calculate different percentages of SDS-PAGE gels using Protogel components

$$V_p = X.V_t/30$$

V_p = volume of protogel

V_t = total volume of gel solution required

X = Percentage gel required

30 (% of Protogel neat solution)

2.2 Methods

2.2.1 Animal housing

Adult, male Sprague-Dawley rats (Harlan, UK) weighing 150g-200g at intake were used in all experiments. The experiments were conducted in accordance with EU council directive 86/806/EEC and guidelines of the BioResources Animal Ethics Committee, Trinity College Dublin. The animals were randomised so that both treatment and control animals were represented in each cage. The animals had free access to filtered tap water and standard laboratory diet (Red Mills, UK). The rats were maintained under 12 hour light-dark cycle conditions. The animals housing rooms were kept under ambient temperature and humidity conditions ($22\pm 2^{\circ}\text{C}$; 35-40% humidity).

2.2.2 Electroconvulsive stimulation (ECS) treatment

The animals were handled for one week prior to the start of treatment. They were habituated to the ear clip electrodes; the electrodes were clipped to the animals' ears each day during the week of handling. ECS was administered as follows:

The animal to be treated was removed from the home cage to a transport cage and taken to the treatment room. Each animal was weighed before treatment. The ECT unit (Figure 2-1) was charged. Ear clip electrodes, dampened in 0.9% saline solution, were applied to the animal's ears. The animal was placed in a tissue-lined treatment box. The ear clip electrodes were connected to the ECT unit (ECT Unit 57800, Ugo Basile, Italy). When the electrodes were appropriately applied, the charge was administered immediately by pressing the red delivery button. The animal was instantly rendered unconscious, entering the tonic phase of the seizure. The durations of the tonic phase and the clonic phase of the seizure were recorded. When the seizure behaviour ended, the unconscious animal was removed from the treatment cage to a heated recovery cage. The recovery period was monitored and the duration of recovery was recorded. Fully recovered animals were transported back to their home cages.

The standard ECS treatment was administered at 100 pulses per second for 0.7 seconds at a charge of 75 milliamps and a pulse width of 0.5 milliseconds or 0.7 milliseconds (Ito et al. 2010; Warner-Schmidt et al. 2008a; Banerjee et al. 2005; Newton et al. 2003). Control animals were handled identically to treated animals. They were placed in the treatment box for thirty seconds with the electrodes on their ears, but no charge was applied.



Figure 2-1 ECT unit used to administer electroconvulsive stimulation

The panel where the seizure parameters were set and the button that administered the shock are in the foreground of the image.

2.2.3 BrdU administration

The animals were administered BrdU (Sigma Aldrich) two to four hours after the fourth to the tenth, inclusive, treatments with ECS (seven BrdU injections in total). The animals were weighed each day prior to treatment. The BrdU was prepared according to the recipe in section 2.1.3.4. 40mg/ml BrdU was administered at 100mg/kg. The solution was allowed to cool before being administered intraperitoneally to the animals (Wojtowicz et al. 2006).

2.2.4 Sample collection for immunohistochemistry

The animals were sacrificed twenty four hours after the final ECS treatment. Animals were housed in a separate room until they were anaesthetised to minimise stress exposure for the animals. The animals were anaesthetised with urethane (Sigma-Aldrich) (3g/10ml of saline administered at a dose of 0.56ml/100g). The urethane was administered intraperitoneally. When animals were unconscious (non-responsive to stimuli: toe pinch) approximately twenty minutes after urethane administration, they were perfused. The animal was placed in a supine position on a drained, supporting surface.

The fur on the chest was cut away using a scissors starting at the base of the rib cage. The tissue over the abdominal cavity was gently snipped to reveal the base of the rib

cage. The rib cage was cut on the left and right side, allowing the rib cage to be lifted upwards. The diaphragm was gently cut so the heart was not damaged. The base of the sternum was clamped and pulled towards the head, allowing access to the chest cavity. The pericardium was cut, freeing the heart. The heart was held in position, pulled slightly downwards. A cut was made at the base of the heart to the right of the midline. The pump needle was inserted into the hole, through the heart and into the aorta. The needle was clamped into position. The pump was switched on to allow saline (0.9%) to flow slowly through. Immediately, a deep cut was made in the right side of the heart to allow blood flow out of the body. To ensure the needle was placed in the correct vessel (aorta), the colour of the heart and liver were checked to ensure they paled in colour. When the blood ran clear after approximately 250ml of saline had been pumped through, the pump was switched off and the needle was moved to a 4% paraformaldehyde (Sigma-Aldrich) solution. The pump was switched on. Approximately 200ml of paraformaldehyde were required. When fixation was complete, the animal's paws were checked for hardening, the pump was switched off. The animal was decapitated and its brain dissected out with care. The brain was stored in 4% paraformaldehyde solution for no longer than forty-eight hours (to prevent over-fixing) at 4°C. The brains were transferred to a 30% cryoprotectant sucrose solution for two weeks before being stored at -80°C.

2.2.5 Tissue preparation and sectioning for immunohistochemistry procedure

Perfused brains for sectioning were manually trimmed, with reference to the “The Rat Brain in Stereotaxic Coordinates” (Paxinos et al, 2007) atlas so that the hippocampus could be obtained with minimal trimming on the cryostat. This resulted in a piece of tissue 1-2cm² to be sectioned. The bulb end of a plastic Pasteur pipette (Fisher Scientific) was cut so that the tissue, immersed in OCT (TissueTek Inc.) could be contained within it (Figure 2-2). The tissue was placed in this mould and covered in OCT. The mould was not overfilled to avoid creating large blocks of OCT and difficulties when sectioning. The tissue was oriented so that the anterior coronal tissue face was in place for cutting. A container was filled with dry ice pellets. Isopentane (Sigma Aldrich) was poured over the dry ice, in a fume hood. The mould was placed into this mix until the OCT solidified. No isopentane was allowed to enter the mould. The moulds were wrapped in foil and stored at -80°C until sectioning.

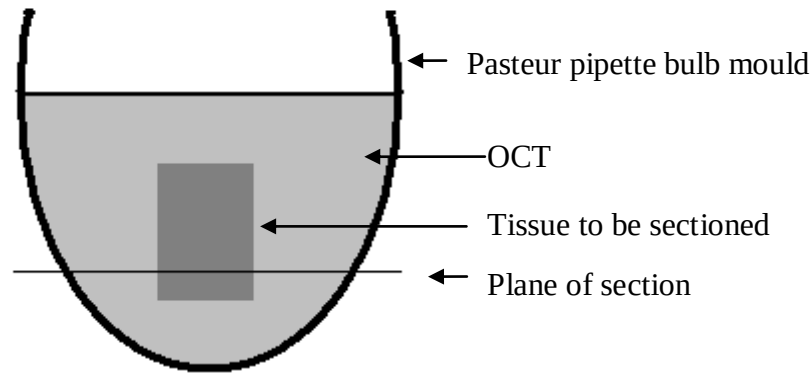


Figure 2-2 Tissue mould to support and orient tissue for sectioning

Cryosectioning and immunohistochemical processing was conducted according to the following protocol:

The right hemisphere of each brain was sectioned coronally. The brains were sectioned serially so that each slide contained every sixth section of tissue that was cut (Figure 2-3) (Malberg et al. 2000). Three sections were placed on each slide.

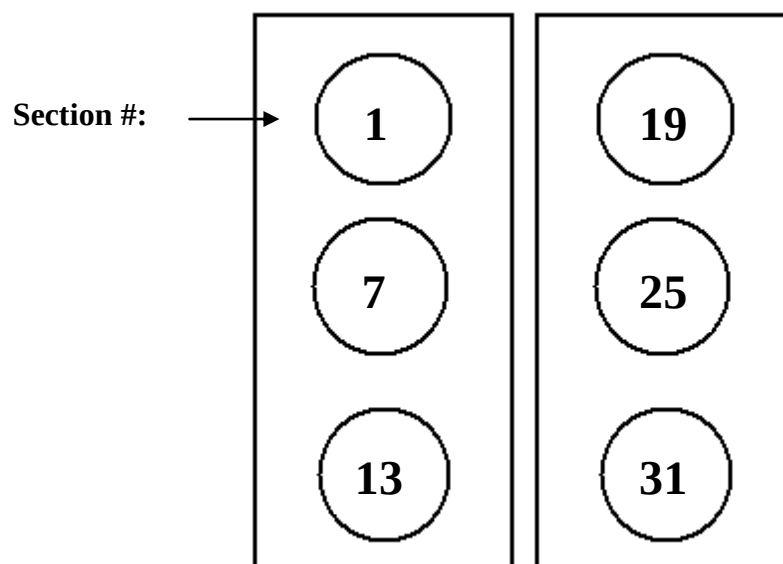


Figure 2-3 Tissue section order for staining procedure

Tissue sections were 10µm thick. Twenty-four sections were collected from each brain throughout the width of the hippocampus.

2.2.6 Immunohistochemistry staining procedure for BrdU-labelled cells

Slides were coded with brain number and section number (e.g. 3, 15 =brain number three, section number fifteen) to allow for blind counting at a later time (Warner-Schmidt et al. 2008a). Slides were placed in staining racks. OCT was dissolved in TBS (recipe, section 2.1.3.4) for ten minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (Fisher Scientific). The hydrogen peroxide solution was washed for ten minutes in running tap water. The slides were microwaved on full power for thirteen minutes in a solution of citric acid buffer (recipe, section 2.1.3.4). The slides were cooled on ice, and then washed twice with TBS for ten minutes each time. 150µl of avidin (Vector Laboratories) was added to each slide and incubated at room temperature for ten minutes. The slides were washed twice with TBS for ten minutes. 150µl of biotin (Vector Laboratories) was added to each slide and incubated for ten minutes at room temperature. The slides were washed twice with TBS for ten minutes. 150µl of 10% normal rabbit serum (Dako) in TBS was added to each slide for five minutes at room temperature. Incubation with avidin, biotin and normal rabbit serum acted to block non-specific binding.

The rabbit serum was not washed off before the primary antibody was applied. 150µl of rat anti-BrdU (Abcam) monoclonal primary antibody 1:10,000 dilution (100 ng/ml concentration) was incubated on each slide for one hour at room temperature. 150µl of rat anti-ECF-L (R&D Systems) at a 1:5,000 dilution (100ng/ml concentration) was applied to control sections acting as negative controls and incubated for one hour at room temperature. The slides were washed three times with TBS for ten minutes. 150µl of biotinylated rabbit-anti-rat secondary antibody (1:50 dilution in TBS, final concentration 10µg/ml; Vector Laboratories) was applied to each slide and incubated for thirty minutes at room temperature. The StrepAB/HRP complex (1 drop A: 1 drop B: 2.5ml TBS; Vector Laboratories) was prepared, thirty minutes before use. The slides were washed twice with TBS for ten minutes. 150µl of the StrepAB/HRP complex was added to each slide and incubated for thirty minutes at room temperature. The slides were washed once with TBS and twice with distilled water for ten minutes.

300µl of DAB was applied to each section (SigmaFAST DAB tablets, prepared according to manufacturer's instructions, section 2.1.3.4) and incubated for fifteen minutes at room temperature. The slides were washed with distilled water. The slides were placed in a staining rack of distilled water. In a fume hood, the racked slides were counterstained with haematoxylin for six seconds. The slides were placed under running tap water for one minute. The racks of slides were transferred to a staining jar containing 70% ethanol (Fisher Scientific) for one minute.

The slide racks were transferred to a staining jar containing absolute ethanol for one minute. The slide rack was transferred to a staining jar containing xylene 4 (Fisher scientific) for one minute. The slide rack was transferred to a staining jar containing xylene 3 for one minute. In a fume hood, the slides were mounted in DPX (Fluka) using No. 1.5 (0.17mm thick) coverslips.

2.2.7 Cell counting

BrdU-labelled cells were counted using an Olympus BX51 light microscope (DP72 camera attachment, Olympus). The granule cell layer and the subgranular zone of the dentate gyrus were counted (Figure 2-4). If cells were within two cell diameters of the subgranular zone, they were counted (Warner-Schmidt et al. 2008a). Dark brown stained rounded shaped cells were counted. If stained cells were fibrillar in appearance they were not counted. The cells were counted under 20X magnification. CellB (Soft Imaging Systems, Olympus) software was used to capture live and still images when cell counting.

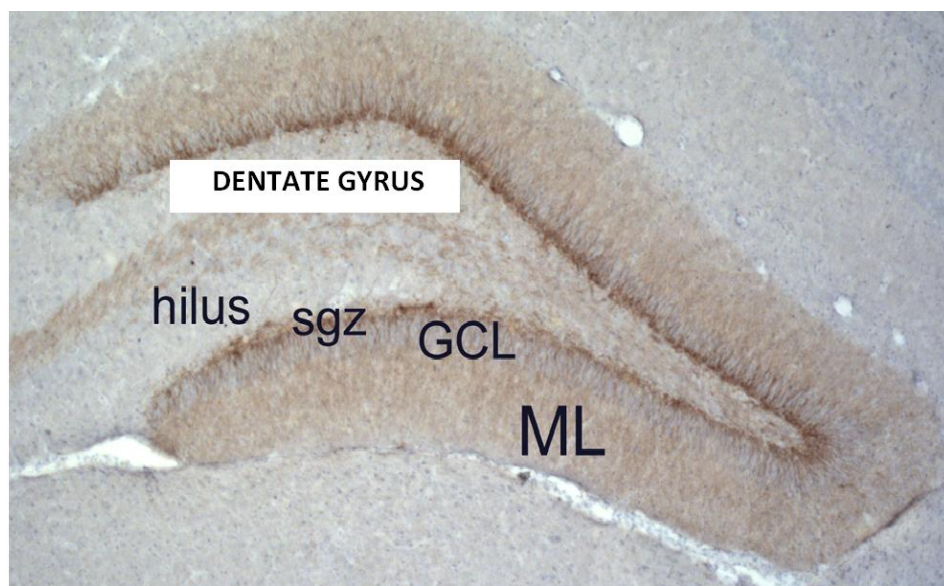


Figure 2-4 The cell layers of the hippocampal dentate gyrus

ML, molecular layer; GCL, granule cell layer; SGZ, subgranular zone

Proliferating cells in the subgranular zone and the granule cell layer of the dentate gyrus were counted. The subgranular zone and granule cell layer are the inner cell layers of the distinctively shaped dentate gyrus. They are surrounded by a thicker outer layer of cells (the molecular layer) and the inner region called the hilus; Image modified from Oomen, wikipedia.com, 2011.

2.2.8 Behaviour experiments

2.2.8.1 The water plus maze

Following the final ECS treatment, all animals were moved to a different housing area. They were habituated to this area for seven days. Their behaviour was monitored during this time to ensure the movement led to minimal disruption. Animal caging conditions did not differ between ECS treatment and behavioural experiments.

The water plus maze, a hybrid water maze and radial arm maze (Diamond et al. 1999; Alamed et al. 2008) was adapted to measure spatial memory.

The water maze arena and cues were set up as in Figure 2-5. The water plus maze consists of four arms, 53.5cm high and 9.5cm wide filled with water to a depth of 45cm. A hidden platform, black in colour and indistinguishable from the maze, was placed in one arm. The platform was 2.5cm below the level of the water offering an escape for the animals. The centre of the maze was designated as the decision zone. The maze was drained and cleaned each evening after use and refilled in preparation for the next morning. Surface faecal matter was removed as necessary after each animal.

The four-day procedure consisted of three training days and a fourth day of probe trials. Animals were removed from their home cage and brought into the experimental area, giving a clear view of the arena and the wall cue. The wall cue was a large black and white circle as in Figure 2-5. Each animal was placed into a preselected right or left entry arm for all of its trials on day 1-3. Entry arm selection was varied to prevent right/left arm bias. A trial was 60 seconds long. The animal was removed before the end of the trial if it swam into the wrong arm or found the platform. Each animal completed 10 trials with a 30s interval between each trial. The animal was kept in the holding cage in view of the visual cues in the arena during the inter-trial intervals.

Upon completion of the ten trials, animals were removed, dried and placed in a heated recovery cage before being returned to their home cage. This procedure was repeated for three days. On the final day, day 4, animals underwent 30 trials. The platform position did not change across trials; however, animal entry into arms was pseudorandomised across the 30 trials so that each animal started 10 trials in a different arm (A, B, C). The frequency of platform acquisition was taken as a measure of reference memory. Platform acquisition was manually scored. The velocity of the animals and the time spent in the decision zone were recorded by Ethovision vers 3.1 (Noldus Information Technology).

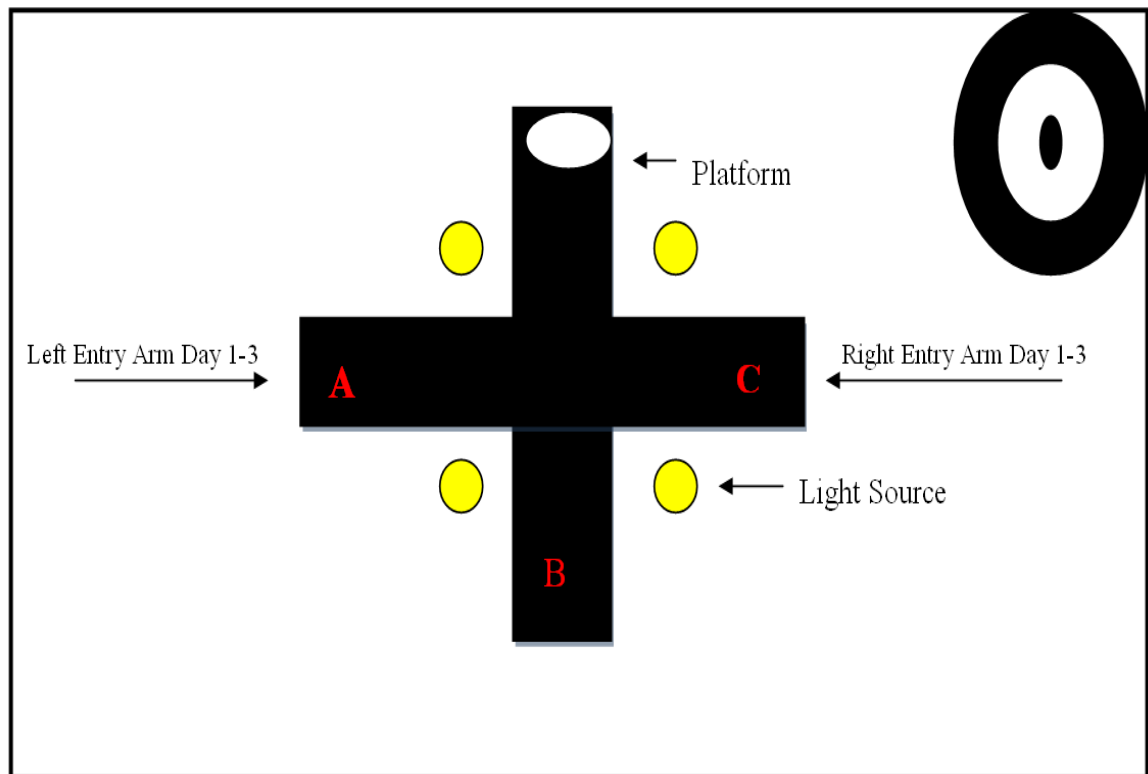


Figure 2-5 Water plus maze

Animals started in the left or right start arms on training days 1-3. On day 4, animal entry was randomised to arm A, B or C. The platform was located at the end of the fourth arm. A visual cue was placed on the wall above the arena in view of the animals in the maze.

2.2.8.2 The forced swim test

The forced swim test was carried out in two sessions; a pre-test followed by a test session 24 hours later. The pool used was a 50cm high clear plexiglass cylinder. The cylinder was filled to 35cm from the base with tap water. The water temperature was maintained at $25 \pm 1^\circ\text{C}$ (Porsolt et al. 1977; Porsolt et al. 2001). The animals were removed from their home cage and carried individually to the treatment room in a transport cage. Each animal was placed gently into the water so that all four limbs entered the water at the same time. The animals were allowed to swim for fifteen minutes on the first day. Their behaviour was monitored and behaviour durations were recorded for the first five minutes of the test session. The behaviours recorded for the first five included the length of time the animals spent immobile, swimming, climbing and the number of dives the animals made (Detke et al. 1995; Gersner et al. 2005).

Immobility behaviour was recorded when the animals were only making those movements required to keep their heads above water (Porsolt et al. 1977).

When the fifteen-minute pre-test was complete, the animals were removed from the cylinder, dried and caged near a heat source. All animals underwent the same procedure. The water in the cylinder was replaced after each cage of animals had been tested. The same procedure was repeated twenty-four hours later on the test day but animals were allowed to swim for five minutes only. The same behaviours were recorded during this five minute session as in the pre-test session.

2.2.9 Sample collection for ELISA

The animals were sacrificed by decapitation to collect tissue samples for ELISA. All surfaces were wiped clean with water or 1% Virkon between animals to reduce animal exposure to blood, which could cause stress. Briefly, following decapitation, the hippocampus and frontal cortex were dissected out on ice. The right hemisphere of each tissue was snap frozen in liquid nitrogen and stored at -80°C.

2.2.10 Tissue preparation for ELISA

Frontal cortex and hippocampus samples were prepared for analysis by ELISA (Altar et al. 2003; Griffin et al. 2009). The tissue samples were homogenised on ice in 10% w/v homogenisation buffer using a handheld motorised homogeniser and disposable pestles (Sigma Aldrich). The homogenisation buffer was prepared according to the manufacturer's instructions (section 2.1.4.3). The homogenates were centrifuged at 14,000g for thirty minutes. The resulting supernatants were removed and used in the assay.

2.2.11 Protein assay

Sample protein concentration was measured using a modified Bradford protein assay (Ramagali et al. 1985). This method is semi-quantitative as the protein content is obtained from a standard curve generated using bovine serum albumin (BSA) (Sigma-Aldrich).

BSA was prepared at a concentration of 5mg/ml. The following BSA standards were prepared in triplicate: 0µg, 5 µg, 10 µg, 20 µg, 40 µg, 50 µg and pipetted onto a 96-well plate (Sterilin). A corresponding volume of distilled water was added to a final volume of 10µl (Table 2-2). The Bradford assay has an overall sensitivity range of approximately 1µg protein/ml to 1,400µg protein/ml although a narrower range, 0µg -50µg protein range, was used in this study.

5.0 mg/ml BSA μl	Water μl	BSA μg
0	10	0
1	9	5
2	8	10
4	6	20
8	2	40
10	0	50

Table 2-2 Bradford protein assay BSA dilutions

The tissue samples were diluted 1 in 10 and run in triplicate. Protein assay solution (Biorad) was diluted 1 in 5 with distilled water. 200μl of diluted solution were added to each well. Air bubbles were removed and the Bradford reaction was allowed to occur for approximately fifteen minutes. The Bradford assay is a colorimetric assay that is based on the principle that proteins binding to Coomassie brilliant blue G-250 cause a shift in the absorbance of the dye from the red form ($A_{\max}$ 465nm) to the blue form ($A_{\max}$ 595) (Bradford, 1976). Therefore, samples and standards were read at 595nm using a plate reader (Biotek) within forty-five minutes of the addition of the protein assay reagent reaction starting.

Following the protein assay, all samples to be analysed by ELISA were normalised to 40μg/μl.

2.2.12 BDNF ELISA

A Chemikine Brain Derived Neurotrophic Factor (BDNF) Sandwich ELISA Kit (Millipore) was used to determine the amount of BDNF present in the samples. The kit supplied:

Chemikine BDNF ELISA Plate;
Wash Buffer concentrate (10X) Standard/Sample Diluent,
BDNF Standard (Recombinant Human),
Biotinylated Mouse anti-Human BDNF Monoclonal Antibody,
Streptavidin-Enzyme conjugate,
TMB/E Solution,
Stop Solution.

2.2.12.1 Hippocampal and frontal cortex sample dilution

Prior to running the assay, a trial ELISA was run to ensure that the tissue sample BDNF concentration would fall on the standard curve at the dilutions recommended by the manufacturer (0-500pg/ml). Excess hippocampal tissue and frontal cortex tissue from an animal chronically treated with ECS was used. The hippocampal supernatant was diluted 1:2, 1:10, 1:20, 1:50, 1:100, 1:200. The frontal cortex supernatant was diluted 1:2, 1:10, 1:20 and 1:50. Neat, undiluted hippocampal and frontal cortex supernatant was also run on the ELISA plate.

An optimal dilution of 1:10 for frontal cortex and 1:20 for hippocampus tissue was determined based on the assay results.

2.2.12.2 Standard preparation

The BDNF standards were prepared according to the manufacturer's directions. Briefly, lyophilised BDNF standard was reconstituted with 1.5ml of standard/sample diluent to give a stock relative BDNF concentration of 10,000pg/ml. 50µl of stock solution was added to 950µl of standard/sample diluent and vortexed, giving a 500pg/ml concentration (#1). 500µl of standard/sample diluent was added to seven other tubes (#2-8). 50µl of dilution #1 was added to the second tube containing 500µl of standard/sample diluent and vortexed giving a concentration of 250pg/ml - dilution #2. 50µl of dilution #2 was added to the next tube and vortexed to form dilution #3-125pg/ml. A 1:2 serial dilution was continued until a final dilution of 7.8pg/ml was formed. A "zero" dilution, containing no BDNF standard was also included (Table 2-3).

Standard #	1	2	3	4	5	6	7	8
Initial volume of Standard/Sample Diluent (µl)	950	500	500	500	500	500	500	500
Concentration (pg/ml)	500	250	125	62.5	31.25	15.63	7.82	0

Table 2-3 BDNF Standard concentrations following serial dilutions

2.2.12.3 BDNF assay

An appropriate number of BDNF well strips were placed in the well plate holder. All standards and samples were assayed in duplicate. 50µl of each standard or diluted hippocampal or frontal cortex sample was added to each well. The plate was sealed and incubated overnight at 4°C on a rocker.

The wash buffer (10X) was prepared (see recipe section 2.1.4.3). The plate sealer was gently removed. The plate was washed by discarding the fluid in the wells by flicking the liquid out over a sink and blotting on paper towels until no excess liquid remained. 200µl of wash buffer was pipetted into each well using a multichannel pipette (Orange Scientific). The wash buffer was also removed by flicking over the sink and blotting the plate. This was repeated four times for each wash cycle.

The biotinylated mouse anti-human BDNF monoclonal antibody was prepared by diluting 1:1000 with standard/sample diluent. 50µl of the antibody was added to each well. The plate was covered with a plate sealer and incubated for three hours at room temperature on a shaker. The plate was washed four times as described. The streptavidin-enzyme conjugate was prepared immediately before use by diluting the HRP conjugate 1:1000 with standard/sample diluent. 50µl of the solution was added to each well. The plate was covered and incubated at room temperature for one hour on a shaker. The plate was washed four times as described. The TMB/E solution was warmed to room temperature. 50µl of TMB/E substrate was added to each well and incubated at room temperature for 15-30 minutes, until the 500pg/ml standard was a deep blue colour. The reaction was stopped by adding 50µl of stop solution to each well, turning the blue colour to yellow. The plate was read on a plate reader (BioTek Instruments Inc.) at 450nm.

A standard curve was created by plotting the known concentration of the BDNF standards against the corresponding optical density (OD) using the graph function in Microsoft Excel. Unknown BDNF concentrations were then “read” off the curve based on their absorbance values.

2.2.13 Tissue collection for proteomic analysis

Animals were sacrificed by decapitation after the final ECS treatment. The animals were housed in a separate area until they were ready to be sacrificed. All surfaces and equipment were wiped down with 1% Virkon or water between animals. Following decapitation, the brain was carefully removed from the skull and placed on a chilled surface. Excess vasculature was removed. The frontal cortex and hippocampus were dissected out.

Each piece of tissue was immediately wrapped in foil, placed in a screw-top cryovial and frozen in liquid nitrogen. The time interval between each animal's death and freezing the tissue sample, as well as the tissue weight, was recorded. Dissection time was five minutes or less.

2.2.14 Sample preparation

Approximately 100mg of tissue was homogenised in 1ml of DiGE compatible lysis buffer (9.5M urea, 2% CHAPS, 20mM Tris, pH8.0). The tissue was sonicated (Misonix) in short bursts, on ice. The probe was cleaned between samples. Samples were centrifuged at 1500 rpm for ten minutes at 17°C. Samples were aliquoted into smaller volumes and stored at -80°C.

2.2.15 2 dimensional-difference in gel electrophoresis (2D-DiGE)

All proteomic experiments were conducted in the Separations Laboratory in the Conway Institute, University College Dublin. The following methods were adapted from established laboratory protocols.

2D DiGE is a well established technique that is used to effectively separate proteins according to their pH and molecular weight (Gorg et al. 2000; Swatton 2004). It allowed for the effective identification of proteins that are differentially expressed following treatment with ECS.

Differences in spot reproducibility and intensity between 2D gels limit the number of spots that can be reliably matched and statistically analysed in a study. Also, the same protein can run in different positions and have different spot intensities, even when the same amount is loaded, on different gels (Voss et al. 2000). The 2D-DiGE method of protein separation was used in this study as it overcomes some of these obstacles.

DiGE involves differentially labelling samples and an internal standard, a pool of all experimental samples, with fluorescent dyes. The inclusion of an internal standard reduces study variation and improves the reliability of gel statistical analysis compared to standard 2D electrophoresis techniques (Alban 2003).

2.2.15.1 Internal standard

The internal standard consists of a pool of all samples to be run in a study and therefore contains all proteins that could potentially be resolved on a gel. It is run on all gels and differentially labelled with a CyDye (Cy3). The internal standard is used to normalise protein expression levels across multiple gels in the experiment (Figure 2-6).

Each sample that is run on a gel is co-detected with the internal standard and all sample spots are compared in-gel to the same internal standard spot map, separating experimental variation from biological variation in spot expression levels (Alban et al. 2003).

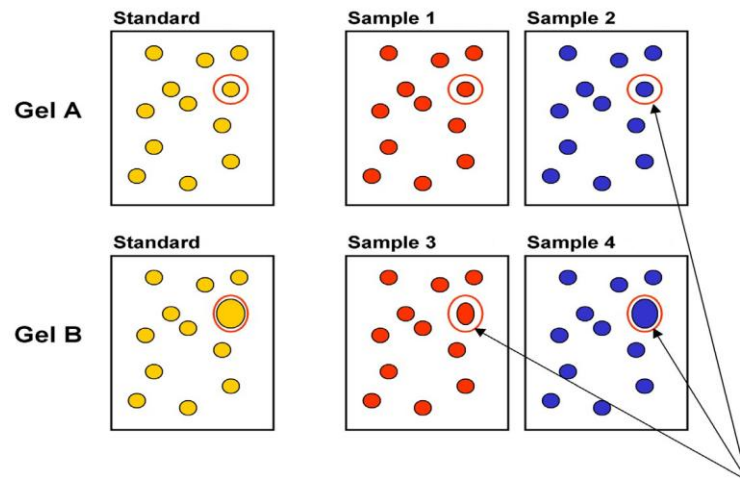


Figure 2-6 Demonstration of internal standard efficacy in detecting gel-gel variation (Amersham Biosciences, 2002)

The advantages of including an internal standard in the study design are illustrated in Figure 2-6. An apparent increase in the circled spots volumes can be seen on gel B in samples 3 and 4 compared to the same spot in sample 1 and 2. However, the internal standard shows that due to gel-to-gel variation, the circled spot on gel B has an increased volume compared to the same spot on gel A. Therefore, the spot in sample 3 has a lower relative volume compared to the same spot on gel A. In the absence of an internal standard, it would be assumed that the spot in sample 4 had a significantly larger volume and the spot in sample 3 a moderately larger volume than in sample 1 and 2, which is not the case. The internal standard exposed the gel-to-gel variation and allowed for the detection of the possible biological variation in sample 3 compared to the other samples (Amersham Biosciences, 2002).

2.2.15.2 Sensitivity

Another advantage to using fluorescent dyes is that they are more sensitive than other detection methods including silver staining and Coomassie blue staining (Gharbi et al. 2002). The sensitivity of Coomassie blue staining ranges from a detection limit of 8ng to 100ng (Patton 2002; Poland et al. 2005; Sitek et al. 2002).

Silver staining ranges from 1ng to 5ng (Patton, 2002) while CyDye minimal labelling has a detection limit of 0.1ng to 2.0ng (Patton 2002; Sitek et al. 2002). While all of the above staining techniques were used throughout the project, the initial protein spot detection was conducted using CyDye fluorescent labelling.

2.2.15.3 DiGE workflow

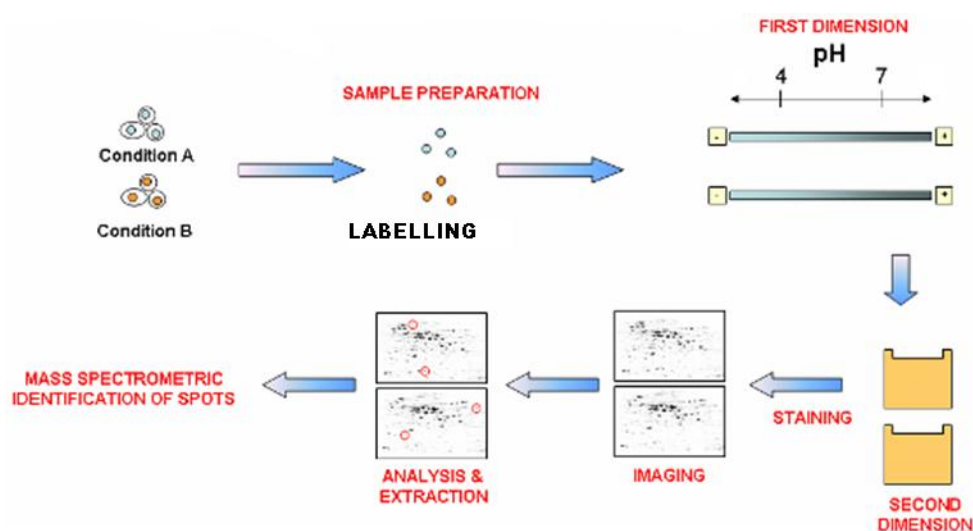


Figure 2-7 2D DiGE experiment workflow

Adapted from Expression Proteomics: Tools for Protein Separation and Analysis, derived from Principles of Proteomics, in presentation by Dr. LinVasc Biorad, S.C.

The workflow of a standard DiGE experiment is outlined in Figure 2-7. Briefly, samples are prepared in lysis buffer, protein content was calculated by protein assay and all samples are normalised. The protein samples are labelled with Cy 3 or Cy 5 fluorescent Cydyes. Each sample is labelled with Cy 5 while an internal standard consisting of a pool of all samples is labelled with Cy 3. The samples are loaded onto immobilised pH gradient (IPG) strips by overnight rehydration to evenly distribute the samples onto the strip. They are then focused, to separate the proteins according to their isoelectric point.

The focused strips are equilibrated in preparation for separation on SDS-PAGE gels. Proteins are then separated for a second time but this time according to their molecular weight. To visualise the fluorescently labelled proteins, the 2D gels are scanned and uploaded into an analysis software package. Protein spots whose abundances are significantly different following treatment with ECS are identified. These protein spots are then excised from preparative gels, i.e. 2D gels run with higher protein loads and visualised with mass spectrometry compatible stains.

2.2.15.4 Protein assay and sample normalisation

Sample protein concentration was measured using a Bradford assay similar to that in section 1.2.11, with minor modifications (Ramagli et al. 1985). All standards and samples were assayed in triplicate.

BSA was prepared at a concentration of 5µg/µl. The following BSA standards were prepared in duplicate: 0µg, 5 µg, 10 µg, 20 µg, 40 µg, 50 µg. 10µl of DiGE lysis buffer, 10µl 1M HCl and a corresponding volume of distilled water were added to disposable cuvettes (Sarstedt) to a final volume of 100µl (Table 2-4).

Protein assay reagent (Biorad) was diluted one in five with distilled water. 2.5ml of diluted solution were added to each cuvette. Tissue samples were diluted. Hippocampal samples were diluted 1 in 10 and frontal cortex samples were diluted 1 in 5 to a final volume of 10µl. Samples were added to a cuvette with 10 µl of DiGE lysis buffer, 10µl of 1M HCl and 80µl of distilled water. 2.5ml of protein assay solution, diluted 1 in 5, was added to the cuvettes.

Cuvettes were inverted several times, to ensure thorough mixing of sample or standard and reagent, and Bradford reaction was allowed to occur for approximately fifteen minutes. Samples and standards were read at 595nm using a spectrophotometer (Biorad) within forty-five minutes of the addition of the protein assay reagent reaction starting.

Protein samples were normalised using DiGE lysis buffer. Hippocampal samples were normalised to 10µg/µl. Frontal cortex samples were normalised to 5µg/µl.

5.0 mg/ml BSA μl	Lysis Buffer μl	Water μl	0.1M-HCl μl	BSA μg
0	10	80	10	0
1	10	79	10	5
2	10	78	10	10
4	10	76	10	20
8	10	72	10	40
10	10	70	10	50

Table 2-4 Bradford Protein assay BSA dilutions

2.2.15.5 Sample labelling

CyDyes (GE Healthcare) were used to minimally label 1-2% of lysine residues on each protein so each labelled protein has only one dye molecule and appears as a single spot on a gel. CyDyes contain an N-hydroxysuccinimide ester group that binds to the lysine group of a protein by an amide linkage (Amersham Biosciences, 2002; Figure 2-8).

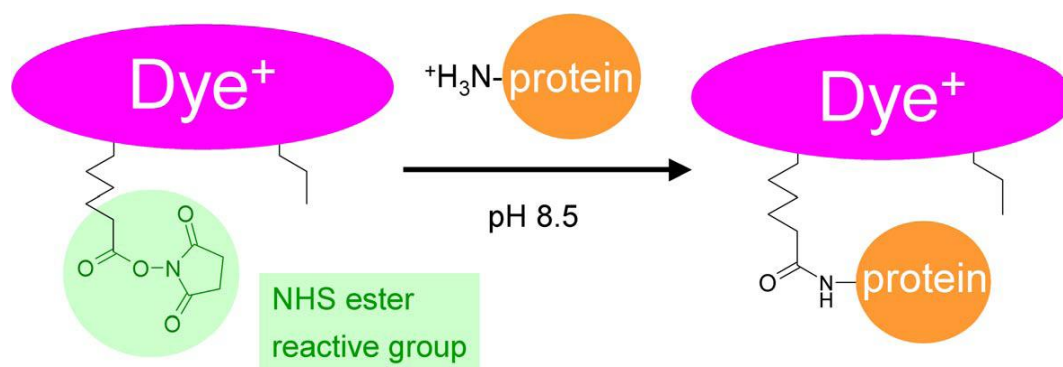


Figure 2-8 CyDye minimal labelling reaction

Adapted from Ettan DiGE System User manual, Amersham Biosciences.

NHS, N-hydroxysuccinimide.

Sample pH was measured using pH 6.0-9.0 strips (Biorad). A pH of ~8.5 was required for successful labelling. Minimal labelling using CyDyes Cy3 to label the internal standard and Cy5 (GE Healthcare) to label the samples was conducted. The labelling procedure was conducted on ice with no direct or strong lighting in the area.

CyDye preparation: CyDyes were removed from storage at -20°C and allowed to warm to room temperature before being opened to prevent condensation that could interfere with labelling.

A stock solution of CyDye was made by adding 5µl of anhydrous dimethylformide (DMF) (Sigma Aldrich) to 5nmol of CyDye. The CyDye tube was vortexed, to ensure total dye resuspension and centrifuged for one minute at 11,000rpm.

A working dilution was prepared by adding 1.5 volumes of anhydrous DMF to 1 volume of CyDye stock solution. The final concentration of the working dilution was 400pg/µl. Samples to be labelled were removed from storage at -80°C. 50µg of individual samples were labelled with 400pg of Cy5 working dilution. 1µl of CyDye labelled 50µg of protein.

A pool of all samples was created and labelled with 400pg of Cy3 per 50µg of protein. This pool of samples, the internal standard, was of sufficient volume to provide 50µg of protein to be run in combination with each individual sample. After adding the working dilution of CyDye, each sample was vortexed, spun briefly and left in darkness for thirty minutes on ice. 1µl of 10mM lysine (Sigma) was then added to each sample to stop the reaction. The samples were vortexed, spun and left on ice for ten minutes. The labelled samples were stored at -80°C until required.

2.2.15.6 IPG strip rehydration

Equal volumes of internal standard and sample were pooled together. 2X dilution buffer was added in a ratio of 1:1:2 (sample: internal standard: 2X dilution buffer). Rehydration buffer was added to a final volume of 450µl.

The prepared samples were pipetted directly along the length of the level, twelve-channel rehydration trays (Amersham Biosciences, UK), ensuring even distribution of the sample. Air bubbles were removed to prevent uneven strip rehydration of the 24cm pH 4-7 (linear) Immobilised pH Gradient (IPG) strips (GE Healthcare). The strips were covered with 2ml of PlusOne strip cover fluid (GE Healthcare) and the rehydration tray lid to prevent evaporation. The strips were rehydrated in darkness, overnight.

2.2.15.7 Isoelectric Focusing

Isoelectric focusing separates proteins on the basis of their isoelectric points (pIs). The net charge of a protein is determined by the pH of its environment. Proteins are positively charged in solutions at pHs below their isoelectric point and negatively charged in solutions above their isoelectric point (Garfin, 2003). The focusing process is described in Figure 2-9.

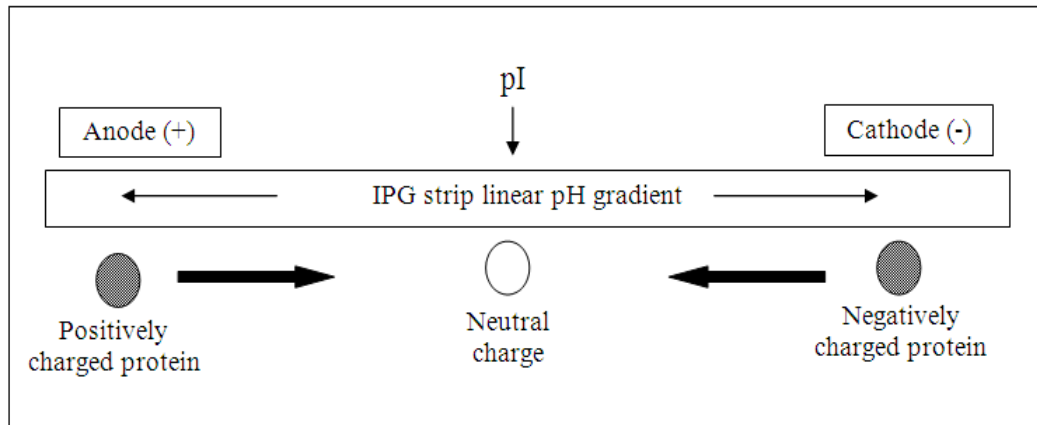


Figure 2-9 The principle of IPG focusing

The proteins are placed on an IPG strip which contains a linear pH gradient. When the proteins are subjected to an electric field they move towards the electrode (anode or cathode) with the opposite charge. As proteins migrate along the pH gradient they pick up or lose protons, decreasing net charge and slowing their migration. As the protein arrives at the pH that equals its pI (neutral charge), it stops migrating. At this point the proteins focus at their characteristic isoelectric points.

2.2.15.8 IPG focusing method

The IPGphor bed and ceramic manifold (IPGphorII, Amersham; Figure 2-10) were cleaned and dried. The ceramic manifold was placed on the IPGphor and adjusted so that it provided a level surface.



Figure 2-10 IPGPhor unit used to focus IPG strips

The focusing parameters were set using the keypad at the front of the machine.

From 2D electrophoresis and first dimension IEF user manual, Amersham Biosciences.

Immobiline DryStrip Cover Fluid (GE Healthcare) was poured into all channels where strips were run and the surrounding unused channels. The rehydrated IPG strips were placed gel side up on the ceramic tray. The strips were aligned so that the anodic (+) end was positioned on the appropriate marking on ceramic manifold, ensuring the gel of the strips covered both electrodes on the IPGphor. Paper wicks were soaked in distilled water and the excess water drained off. The wicks were placed at either end of the IPG strips covering the end of the gel of the strip and the electrode. The electrode assembly was positioned to overlap the paper wicks, the end of the strip and the electrodes on the base of the IPGphor. The electrode assembly was secured by swivelling the cams into the closed position. The level of cover fluid in the channels was topped up so the paper wicks were also covered with fluid. The lid of the IPGphor was closed and covered to ensure focusing was carried out in the dark. The IPG strips were focused using the parameters described in Table 2-5. The current was limited to 50 μ A per strip. The strips were focused overnight. Focused strips were stored at -80°C in sealed gel bags.

Step number	Step type	Voltage	Time
Step 1	Step	3,500V	75,000 Volt Hours
Step 2	Gradient	8,000V	10 Minutes
Step 3	Step	8,000V	1 Hour
Step 4	Holding Step	100V	72 Hours

Table 2-5 Focusing parameters

2.2.15.9 Gel casting for 2DiGE gels

All strips were run on 12% SDS-PAGE gels, 27.5cm wide x 22cm long x 1mm thick (Figure 2-11).

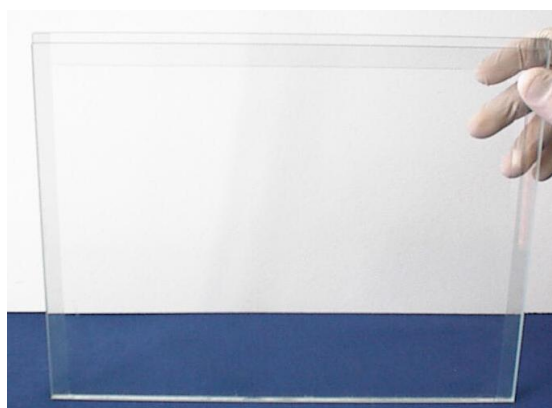


Figure 2-11 Glass plate cassettes used to cast large 2D gels

Adapted from www.precastgels.com.

Low fluorescence glass plates were used for DiGE studies containing fluorescently labelled samples. All glass plates were washed, dried and wiped down with ethanol and lint-free tissue before use. The backing plate and short plate were aligned; electrical tape was used to seal them together. Up to six gels were cast at a time using an Ettan DALT 6-gel caster (Amersham). The caster was assembled. A separator sheet was placed against the back wall followed by a glass gel cassette. Separator sheets and glass gel cassettes were alternated until all six glass gel cassettes had been inserted, finishing with separator sheets. The short plate of the glass gel cassettes faced the pouring channel of the caster.

The gasket of the caster faceplate was inserted into its channel and sealed with a light coating of GelSeal (GE Healthcare). The faceplate was placed carefully onto the caster to ensure it was correctly positioned, screwed into position along the base and clamped at the sides using the six required clamps, to prevent leakage.

To cast six 1mm 12% SDS PAGE gels a minimum total volume of 600ml was required. The gels were cast according to the gel recipe (Table 2-1). The gel solution was poured evenly into the channel at the back of the caster until the solution was approximately 0.5cm below the level of the short glass plate, leaving sufficient room for IPG strip following gel setting.

A layer of water-saturated butanol (Sigma-Aldrich) was pipetted on top of the gel to ensure an even top layer and to prevent air bubble formation and evaporation.

Water-saturated butanol was washed off after one hour with water. The gels were covered in moistened paper towels and cling-film and allowed to polymerise overnight at 4°C.

2.2.15.10 Equilibration

IPG strips were equilibrated to reduce (DTT), alkylate (IAA) and allow for detergent exchange (SDS buffer). The strips were equilibrated in 2ml of Equilibration Buffer I per strip (recipe section 2.1.6.3). The buffer was prepared by adding 1% w/v final concentration of DTT to a stock SDS equilibration buffer.

Equilibration Buffer II was prepared by adding 2.5% w/v iodoacetamide to the stock SDS equilibration buffer. The strips were equilibrated in Equilibration Buffer II for fifteen minutes. All equilibration was conducted on a shaker.

Agarose sealing solution was melted in a microwave and allowed to cool slightly before use.

The IPG strips were rinsed with 1X SDS electrophoresis buffer. The space between the glass plates was filled with 1X SDS Electrophoresis Buffer to allow the IPG strips to be positioned more easily. The strips were placed across the top of the gel between the glass plates with the gel side of the strip facing the short plate. The positive (+) end of the strip was placed to the left of the gel plate. The excess electrophoresis buffer was drained off. The IPG strips were sealed in place with molten, cooled, agarose sealing solution, ensuring no air bubbles were trapped between the strip and the gel. The gels were transferred to the gel tank when the agarose had set.

2.2.15.11 2nd dimension separation

The gel tank (separation unit, Figure 2-12) was half-filled with 1X SDS electrophoresis buffer (~12 litres). The MultiTemp cooling unit (GE Healthcare) was set to 15°C and switched on. The water pump was switched on to ensure the buffer circulated throughout the tank. The gaskets were placed in the tank with the rubber fringe facing outwards. The first gasket was placed in the outermost ridge; the second was placed in the innermost ridge. All gels were lubricated with electrophoresis buffer and inserted into the gaskets. The gels were oriented so that the IPG strip was on the right hand side of the tank. Blank plastic cassettes were run as substitutes for gels or to fill the tank to capacity if required.

The remaining buffer was added to the tank to the start of the glass spacer so that all of the gel plate was submerged fully under buffer.

The lid was placed on the tank and the power leads were attached to the high capacity power pack (Biorad). The gels were run at 0.2W per gel for one hour. This was increased to 1W per gel overnight. The gel run was complete when the bromophenol blue dye front from the agarose sealing solution reached the end of the gel. The tank was covered so that second dimension separation was conducted in darkness.

The gels were removed from the separation unit when the run was complete. Excess agarose was removed and the glass plates were washed and dried well to remove buffer residue. All plates were wiped down with ethanol and lint-free tissue wipes to remove any dust particles or streaking that could fluoresce and interfere with the scanning process.

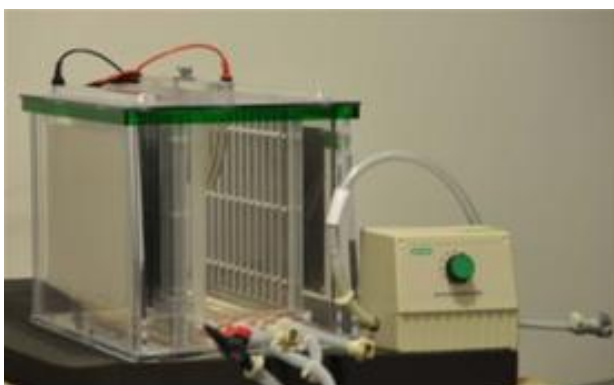


Figure 2-12 The PROTEAN tank and the pump used to separate proteins on 2D gels according to molecular weight Adapted from www.labx.com

2.2.16 Gel scanning

All gels were fluorescently scanned using the Typhoon 9410 scanner (Amersham Biosciences). The scanner bed was cleaned and wiped with ethanol and lint free tissue to remove any dirt that could fluoresce during scanning. Each gel cassette was placed on the scanner between a long and a short bumper to position and hold them in place. The gel cassettes were oriented so that the IPG strip was on the left hand side of the scanner.

Image analysis was conducted using ImageQuant TL software (GE Healthcare). The settings for analysing DiGE gels were:

- Acquisition mode: fluorescence
- Tray: DiGE Ettan DALT
- Focal plane: +3mm
- Image Analysis: ImageQuant
- DiGE file naming format was selected

Each initial scan was carried out at 500 μ m (pixelation). The photomultiplier tube (PMT) value was set at 600V. Cy3 and Cy5 saturation intensities were adjusted individually and the gel was rescanned as required until acceptable PMT values were achieved. The maximum accepted saturation values were between 60,000 and 80,000. Cy3 and Cy5 saturation intensity values were also within 10,000 of each other for each gel. The final scan was conducted at 100 μ m (pixel) to produce a higher quality image for further analysis.

Gel images were cropped and prepared for export using ImageQuant software. Image files were opened in .ds format using ImageQuant Tools. The images were maximised and the grey/colour adjust function was inverted to more easily visualise the gel boundaries. The crop tool was selected and the image was cropped at the gel-strip and glass-plate cassette boundaries.

2.2.17 Spot analysis

Progenesis SameSpot (Nonlinear Dynamics) software was used to analyse and compare the gels. The cropped gel images were uploaded into the software and quality control processing was conducted. This ensured the uploaded files were uncorrupted and the images were of a high standard and could be used for analysis. No gel images were excluded following quality control.

The “DiGE with Internal Standards” experiment format was selected in the next step “DiGE Setup”. Each image was arranged so that the internal standard and sample

(Cy3 and Cy5) images were paired together, grouping them into the gels they originated from. The software automatically identified the internal standard (Cy3) of each pairing.

A reference image for the hippocampus and frontal cortex was selected manually based on the clarity and quality of the gel images.

This image contained no streaking, warping or other disfigurement. The reference gel acted as the model to which all other gel images were aligned. Therefore, the best representative gel was chosen.

Image alignment was conducted to correct positional variation between spots and allow for 100% spot matching, ensuring accurate statistical analysis. Each internal standard gel (Cy3) was matched to the same reference gel and each sample gel (Cy5) was aligned to its internal standard. Five to ten vectors were manually selected for all gels before automatic alignment was applied.

Manual vector selection involved identifying strongly expressed spots from different areas of the Cy5 image and dragging them directly over the corresponding spot in the gel Cy3 image. This helped to guide automatic alignment of Cy5 spots to the Cy3 spots. The automatically applied vectors were manually checked and corrected as required to ensure accurate mapping of individual gel images to the reference image (Figure 2-13).

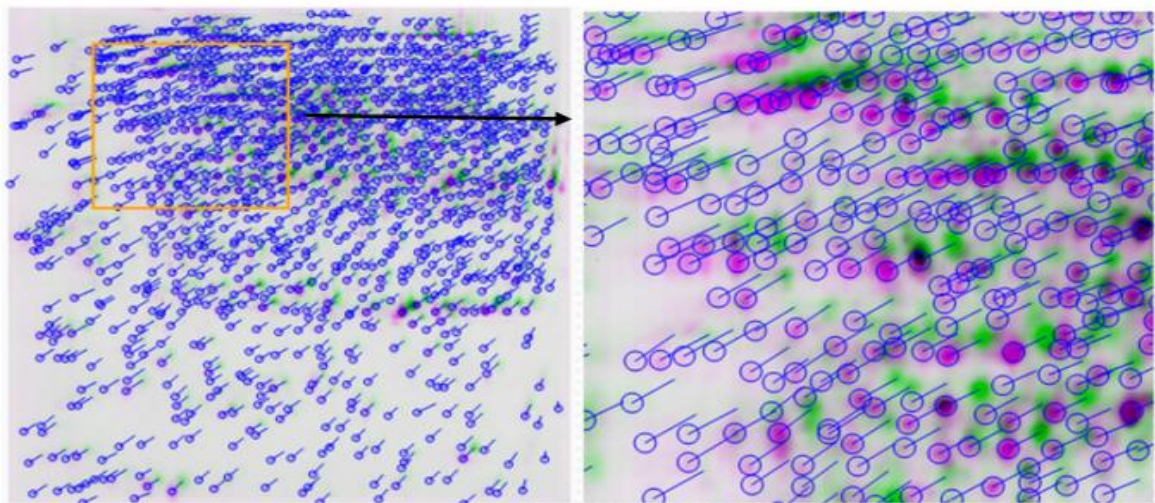


Figure 2-13 Spot alignment tool Progenesis Samespot Adapted from nonlineardynamics.com

The blue circles indicate the sample spots (pink) that are being aligned. The blue lines indicate the direction of the automatically applied alignment that will move the sample spots and overlay them onto their corresponding internal standard spots (green), which has been matched, with all other internal standards, to a single reference gel.

Experiment design set-up arranged the gels into groups for comparison. The “between-subject” design was used to compare control and treated animals. The effects of ECS on hippocampal and frontal cortex tissue were analysed as separate experiments (Table 2-6).

Tissue	ECS Treatment	Control
Hippocampus	Acute	Acute
	Chronic	Chronic
	Chronic + 4	Chronic + 4
Frontal Cortex	Acute	Acute
	Chronic	Chronic
	Chronic + 4	Chronic + 4

Table 2-6 2D DiGE gel Progenesis SameSpot (NonLinear Dynamics) group setup

Acute: animals received 1 ECS treatment; chronic: animals received 10 ECS treatment; chronic + 4: animals received 10 ECS treatments and were sacrificed 4 weeks later.

The protein spots were normalised to correct for experimental variation that results when running 2D gels. The internal standard acted as the fixed reference point and the sample was calibrated to it. Therefore, the normalised spot volume was compared to determine differences in protein expression in treated and control samples.

A one way ANOVA was used to compare treated and control groups. All spots were manually validated and reviewed. Non-spots (dirt, smears) on the gel were tagged and removed from analysis. This was done using the 2D montage and 3D topography tools in Progenesis (Figure 2-14). False discovery rate values (Q values), were calculated from one way ANOVA results to allow for the multiple comparison error (false discovery rate) associated with the large-scale protein spot comparisons.

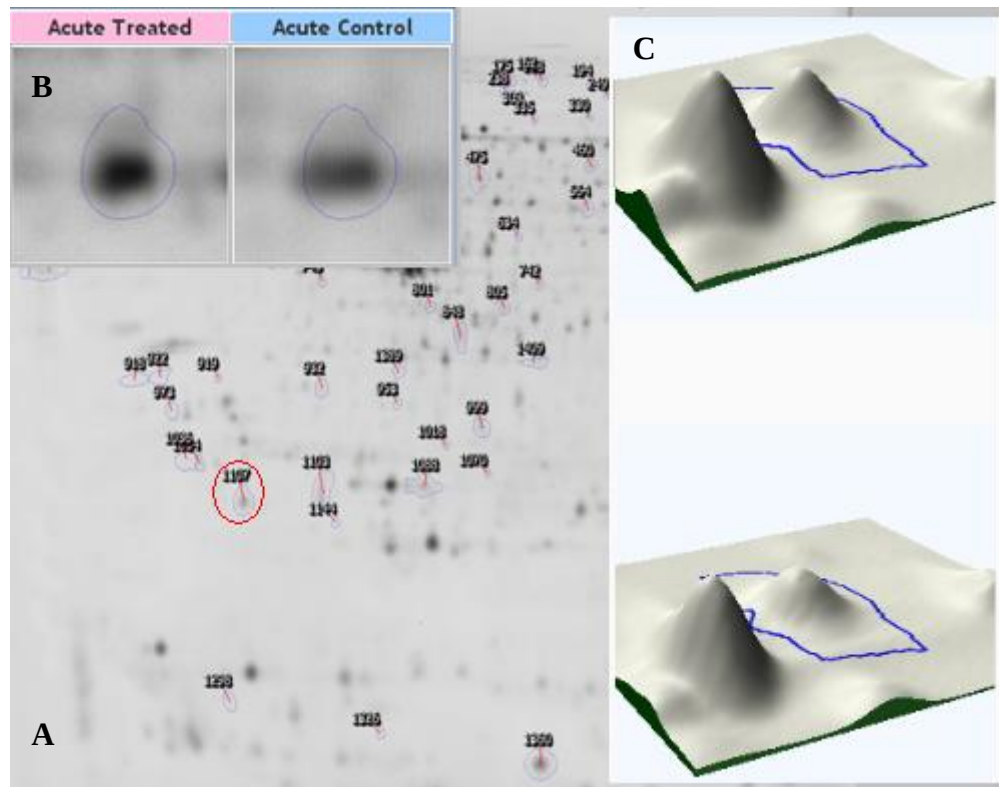


Figure 2-14 Diagnostic tools available in Progenesis SameSpot software (Nonlinear Dynamics) used to review protein spots Adapted from ProgenesisSamespot.com

A spot identified on the gel spot map (e.g. circled spot; **(A)**) can be compared in treated and control gels (2D montage; **(B)**) or in the 3D topography tool **(C)** to determine whether a genuine difference exists in the spots or if the statistically detected difference is due to a gel artefact.

All significant spots ($p < 0.05$) were tagged and carried forward for multivariate statistical analysis. Multivariate analysis consists of statistical methods that are used to analyse data from more than one variable (Rencher, 2002). Principal component analysis, an exploratory data analysis technique that separates gel samples according to expression variation, was conducted to detect outlying gels in the study (Majek et al. 2011). It also detected whether samples clustered according to their experimental treatment.

The average normalised spot volumes were imported into Unscrambler X (CAMO Software, Sweden) statistical software for multivariate analysis. The regression technique partial least squares discriminant analysis (PLS-DA) was used to explain variance in the data. Each gel was assigned a category variable, +1 for treated and -1 for control samples (Y variables). PLS-DA correlates variation in a dataset with class membership. Each spot

was identified as an X-variable. The data was mean centered and weighted (X weight: 1/standard deviation, Y weight: 1/standard deviation).

Full cross validation was selected and the analysis was run to build a first model of the data. All studies were analysed using the NIPALS algorithm. Using the diagnostic tools in the software, outliers were removed and a new, more robust, model drawn. The significance of weighted regression beta (β)-coefficients is calculated for each variable. This identifies the X-variables that are most important in the regression model. Spots with a beta-coefficient value of $p < 0.05$ were considered to significantly contribute to the variation seen in data and were identified as protein spots for potential identification by mass spectrometry.

2.2.18 Preparative gels for mass spectrometry analysis

Spots for mass spectrometric analysis were picked from preparative gels. Preparative gels were run using the internal standard, which was focused on pH4-7 IPG strips as previously described. 12% SDS-PAGE gels were cast according to Table 2-1 recipes. The glass plates used had a wider spacer, creating 1.5mm thick gels. Internal standard concentration ranging from 200 μ g to 800 μ g was run on gels to obtain higher spot-protein concentrations by less sensitive staining techniques. Preparative gels were stained using silver staining and Coomassie blue staining techniques that were compatible with mass spectrometry analyses.

2.2.18.1 Silver staining

2D gels were carefully removed from glass plate cassettes to avoid breaking the gels. The gels were stained using a PlusOne silver staining kit (GE Healthcare). A volume of 250ml was required for each step for each gel. All steps were carried out on a shaker-rocker. The gels were fixed in fixing solution for thirty minutes. The solution was changed and the gels were fixed for fifteen minutes. This was repeated once more and the gels were fixed for a final fifteen minutes (one hour total fixing time). A sensitising solution, and all following solutions, was prepared as per the manufacturer's guidelines. The gels were placed in sensitising solution for thirty minutes. The gels were washed in distilled water three times for five minutes. The gels were stained in silver solution for twenty minutes. The gels were washed twice for one minute in distilled water.

The gels were developed in developing solution until protein spots were clearly visible on the gel. A stop solution was added to stop the development reaction. The gels

were washed in distilled water three times for five minutes per wash. Stained gels were sealed in plastic gel bags and stored at 4°C.

2.2.18.2 Coomassie blue staining

250ml volume of each solution was required for every gel. All steps were conducted on a shaker rocker. The gels were fixed for one hour in fixing solution. The gels were washed for two hours in sensitising solution. The Coomassie blue stain was prepared and the gels were agitated in staining solution for at least four hours or overnight. The gels were destained for one hour in destaining solution I. The gels were destained in destaining solution II until a clear background was obtained. Gels were stored sealed in plastic gel bags in 5% v/v acetic acid until required.

2.2.19 Protein spot preparation

2.2.19.1 Spot cutting

Spot cutting was carried out in a laminar flow hood to reduce keratin contamination. Gels were cut on a light box to aid visualisation. All surfaces were wiped down with ethanol. The spots to be cut were identified. Pipette tips were cut short and the blunt end was used to manually pick out spots by stabbing vertically into the gel. The spots came away in the tip. Larger spots were cut using a blade. While as much of each spot as possible was cut out, the area surrounding the spots was avoided. The cut spots were placed in individual microfuge tubes and labelled according to spot location.

2.2.19.2 Spot destaining

An equal volume of 30mM potassium ferricyanide (Sigma Aldrich) and 100mM sodium thiosulphate were freshly mixed just before use. 50µl of this solution, sufficient to cover the gel spot, was added to each tube. The gel spots were incubated at room temperature on a thermomixer (Eppendorf) at 300rpm until the brown colour disappeared. The supernatant was discarded and the gel spots were washed with distilled water until the remaining yellow colour had disappeared. Gel spots were incubated in 100µl of 200mM ammonium bicarbonate for twenty minutes at room temperature on a thermomixer at 300rpm.

2.2.19.3 Dehydrating gel spots

A solution of 200mM ammonium bicarbonate: Acetonitrile (2:3) was prepared. The gel spots were incubated at 37°C on a thermomixer at 300rpm for ten minutes. The tubes were spun briefly. The supernatant was replaced with 70µl of 50mM ammonium

bicarbonate and incubated at 37°C on a thermomixer at 300rpm for ten minutes. The tubes were spun briefly. The supernatant was replaced with 70 µl of acetonitrile (Fisher Scientific) and the tubes were incubated at 37°C on a thermomixer at 300rpm for ten minutes. The tubes were spun briefly and the supernatant removed. The gel pieces were dried at 37°C on a speedivac (Eppendorf). The dried gel pieces were stored until required for trypsin digest.

Gel pieces were stored overnight at 37°C on a shaking incubator in 50 µl of trypsin solution (20ng/µl trypsin in 25mM ammonium bicarbonate) to digest the protein in the dried gel spots into peptides. The tubes were spun down and the supernatant was removed and saved in new, labelled microfuge tubes. The tryptic peptides were extracted three times by incubating for five minutes each time on a thermomixer in 50 µl of extraction solution (70% acetonitrile, 5% formic acid). The extracts were dried in a speedivac, and then stored at -20°C until resuspension.

Tryptic peptides were resuspended in 2µl of 0.1% formic acid that was pipetted up and down several times to wash the walls of the tube near the base, ensuring all peptides were resuspended for mass spectrometry. The tubes were vortexed for thirty seconds and spun briefly to ensure the entire sample gathered at the bottom of the tube.

2.2.20 Mass spectrometry analysis

2.2.20.1 Introduction

Peptic digests were handled and analysed by technical staff in the Mass Spectrometry Resource unit in the Conway Institute of Biomolecular and Biomedical Science, University College Dublin.

Briefly, mass spectrometry resolves the mass of a molecule by measuring the mass to charge (m/z) ratio of its ions. Mass spectrometers consist of three features, an ion source, mass analyser and a detector (Figure 2-15). Measurements are conducted in the gas phase on ionised peptides. The mass analyser measures the mass to charge (m/z) ratio of the ionised peptides and the detector measures the number of ions at each m/z value. Ionisation sources are coupled to ion traps that generate fragment ion (collision induced) spectra from selected precursor ions (Aebersold et al. 2003; Yates et al. 2009; ThermoFisher Scientific, 2008). MS/MS spectra are searched against sequence databases which can be species specific, in order to identify the peptides and proteins present in the sample.

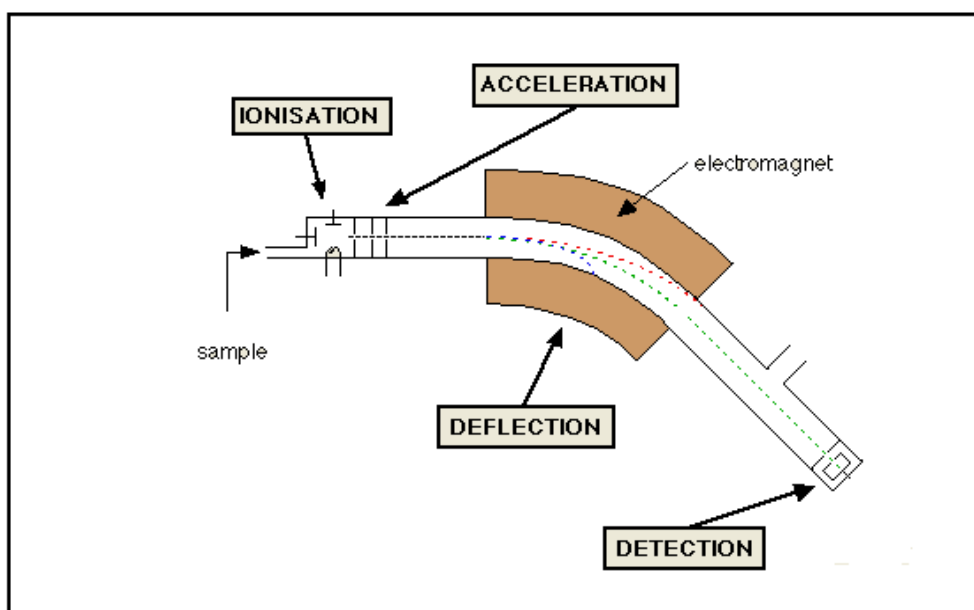


Figure 2-15 The basic principle of mass spectrometry

Samples are ionised, manipulated in the mass analyser by electromagnetic fields and directed to the detector. Figure adapted from chemguide.co.uk.

The type of mass spectrometry used in this project was a linear trap quadrupole (LTQ) Orbitrap mass spectrometer. Samples are separated by liquid chromatography prior to ionisation. In this machine electrospray ionisation is coupled to a hybrid linear ion trap and Orbitrap mass analyser (Figure 2-16). The ions are captured in the long, cylindrical linear ion trap which can store, isolate and fragment ions for analysis or in preparation for further detection in the Orbitrap. Ions move from the linear ion trap through the curved linear trap (C-trap), which allows for stable ion injection into the Orbitrap. The Orbitrap uses electrical fields to trap ions. Stable ions rotate around an axial central electrode (Figure 2-16 (B)). The instrument uses the axial oscillations to obtain the m/z ratio of the ions (ThermoFisher Scientific, 2008). The Orbitrap acquires full MS data while the LTQ acquires MSMS peptide scans data through collision induced dissociation (CID) (ThermoFisher Scientific, 2008).

(A)



(B)

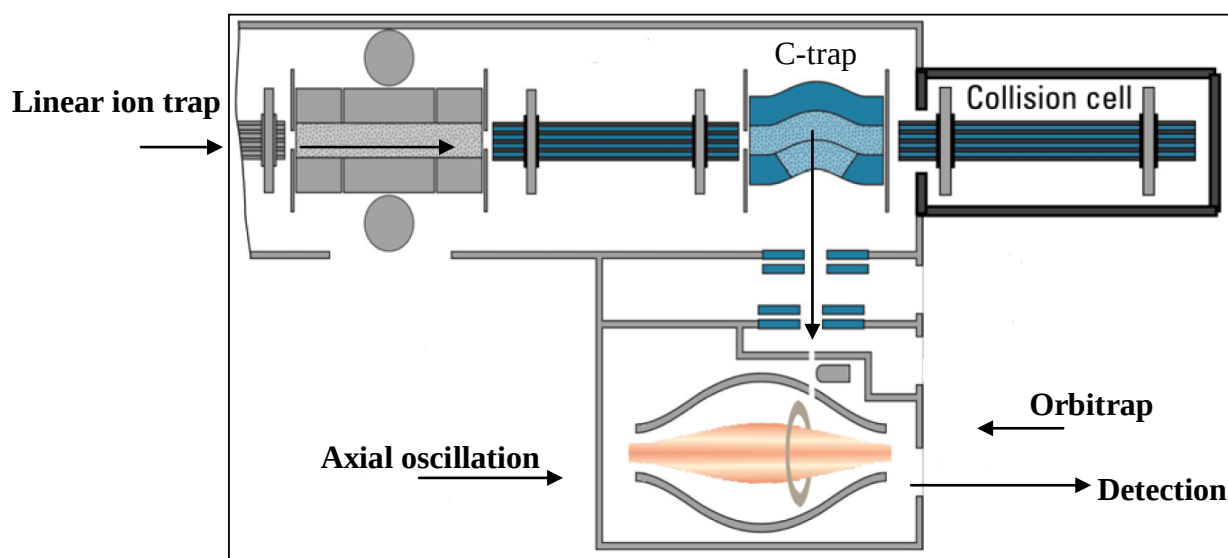


Figure 2-16 The LTQ Orbitrap XL mass spectrometer and linear ion trap (A) with a more detailed schematic of the ion trap, collision cell and Orbitrap mass analyser (B)

Adapted from LTQ Orbitrap XL Hardware manual (ThermoFisher Scientific, 2008).

2.2.20.2 Mass spectrometry methods

The samples were run on a Thermo Scientific LTQ ORBITRAP XL mass spectrometer (Figure 2-16 (A)) connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Each sample was loaded onto Biobasic C18 PicofritTM column (100 mm length, 75 mm ID) and was separated by an increasing acetonitrile gradient, using a 30 min reverse phase gradient at a flow rate of 300nL/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 200 °C, a capillary voltage of 45V, a tube lens voltage of 100V and with a potential of 1900V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution MS scan (300-2000 Dalton) was performed using the Orbitrap to select the 7 most intense ions prior to MS/MS analysis using the ion trap.

For peptide data analysis MS/MS data was searched using SEQUEST (BioWorks 3.3.1, Thermo Finnigan, UK) software against the Uniprot/Swiss-Prot rat database (September 2010 release version 74; 513948 total database entries). The following criteria were used to search for protein identification: precursor mass ion tolerance (instrumental error in determining mass of ions before fragmentation) 50ppm (1.5 atomic mass units (AMU)), fragment ion tolerance 1 AMU, methionine oxidation (e.g. if a peptide contains 3 methiones, the software will search for peptides with 0, 1, 2, or 3 oxidised methionine residues) and carboxyamidomethylation modifications. Trypsin was specified as the proteolytic enzyme and up to two missing cleavages (sites that were not tryptically digested) were allowed. The peptides were filtered according to the following parameters: Xcorr above 1.90 (+1), 2.50 (+2) and 3.20 (+3) (Xcorr is the correlation between the observed and theoretical peptide mass fragments, set for charge states of +1, 2, 3), peptide probability of $p \leq 0.001$.

Uniprot accession numbers of identified proteins were uploaded into the Database for Annotation, Visualisation and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov>). Protein accession numbers were converted to gene identifiers allowing biological meaning to be extracted from the lists of proteins detected following mass spectrometry (Dennis et al. 2003; Huang et al. 2009). DAVID was used to search the data, using tools including the gene function classification tool and the functional annotation tool, in the Gene Ontology (GO) database (Gene Ontology Consortium, 2001; Huang et al. 2009; Malik et al. 2010). The GO terms biological process (BP) FAT, molecular function (MF) FAT and cellular component (CC) FAT were selected. The FAT search criteria filtered out the broadest classification terms. Gene functional classification (genes classified into gene families), functional annotation charts (biological

terms that share similar biological meaning) and functional annotation clustering charts (terms from the functional annotation charts are clustered) under medium classification stringency were examined for each study. Functional annotation chart results with a significance level of $p < 0.001$ and an FDR of less than 5 were collated. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway database was also searched, through DAVID, to determine which established pathways the genes/proteins were involved in.

Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA, www.ingenuity.com) was used to compile a map of protein interactions based on the protein accession information for each study. The protein accession numbers and corresponding fold change values for each protein were uploaded. The accession numbers were converted to its gene identifier and mapped to the corresponding gene object in the IPA knowledge base (Levin et al. 2010; Mallei et al. 2011). The genes (focus genes) were overlaid onto a global molecular network developed from literature-based information in the IPA knowledge base. Networks of focus genes were then algorithmically generated based on their connectivity. Each gene is a node in the pathway and the potential relationships between nodes was drawn as an edge (line). All edges are supported by at least for reference from literature, textbook or canonical information stored in the IPA knowledge base (Mallei et al. 2011; Levin et al. 2010; Piubelli et al. 2011c). Networks were ranked according to a score. The score takes into consideration the number of network eligible molecules and the total number of molecules in the IPA knowledge base. Fishers's exact test was used to calculate p-values. The score is the negative log of this p-value (Levin et al. 2011). Only the highest ranked network is included in the results. The network components were assigned to cellular locations.

The experimental molecular weights and isoelectric points of protein spots were calculated based on the location of the spot in the gel. As the proteins were separated on linear pH 4-7 IPG strips, the pI of each spot was read directly from the gel images. The molecular weight of protein spots was determined based on the relative mobility of protein bands in the gel (R_f). To calculate the molecular weight:

$$\text{Molecular weight} = 10^y = 10^{(m(x) + c)}$$

Equation 2 Determining the molecular weight from a standard curve of mobility of protein bands

m = slope of the line; c = the y-intercept; $x = \log R_f$ (distance migrated by a protein/distance migrated by the dye front).

2.2.21 Semi-quantitative immunoblotting

Mass spectrometry can identify several proteins in any given spot. The abundance of differentially expressed candidate proteins of interest were analysed, in a second set of rats treated the same as the original sample, by semi-quantitative immunoblotting techniques (Southern, 1975; Towbin et al. 1979; Renart et al. 1979). Briefly, semi-quantitative immunoblotting involves running protein samples on an SDS-PAGE gel. The proteins are then transferred from the gel to a PVDF membrane (blotting). Antibodies, specific to proteins of interest, are used to target protein bands. These bands are then detected using chemiluminescent methods. The densities of the visualised bands are relative to the amount of protein present in those bands.

2.2.22 Sample preparation

Frontal cortex and hippocampus tissue was weighed and homogenised in 10% w/v lysis buffer (NP-40 Buffer, with fresh protease inhibitor (Sigma Aldrich) by sonication. The samples were centrifuged at 12,000 rpm at 4°C for twenty minutes. The supernatant was collected and used in the immunoblotting procedure. The sample protein concentration was calculated by the protein assay method described in 2.2.11. Tissue samples were normalised to 10µg/µl of protein.

2.2.23 Mini-gel casting and loading

Mini-gels were cast according to the formula described in section 2.1.9. Briefly, 10% or 12% resolving gels and 4% stacking gels were cast. 10-spacer combs were used to create wells for sample loading. 50µg of protein (5ul) was mixed with an equal volume of 2X Laemmli buffer (Laemmli, 1970). The sample was vortexed and heated to 95°C for five minutes. Protein molecular weight markers (ThermoScientific) and samples were loaded onto each mini-gel. The outer lanes were not loaded with sample to avoid protein band distortion. Control and treated samples were loaded onto each gel. The gels were run at 50mA per gel until the blue dye front reached the end of the gel.

2.2.24 Semi-dry transfer

PVDF membrane (Immobilon-P, Millipore) was cut to the same size as the gel. Six pieces of filter paper (Fisher Scientific) were cut to the same size as the membrane. The membrane was soaked in methanol for thirty seconds then transferred to distilled water for two minutes. The opaque membrane was then transferred to anode buffer II for fifteen minutes.

Two pieces of filter paper were soaked in anode buffer I, one piece was soaked in anode buffer II and three pieces were soaked in cathode buffer for fifteen minutes. The filter paper, membrane and gel were placed together in the following order (Figure 2-17) on the blotting apparatus (Transblotter SD, Biorad).

- Two pieces of filter paper soaked in Anode Buffer I
- One piece of filter paper soaked in Anode Buffer II
- The membrane
- The gel
- Three pieces of filter paper soaked in Cathode Buffer

Air bubbles were removed from between each layer. The cathode plate and the lid were placed onto the transblotter. The electrodes were attached to the power pack. The gels were transferred at 0.23A per gel for one hour and forty five minutes.

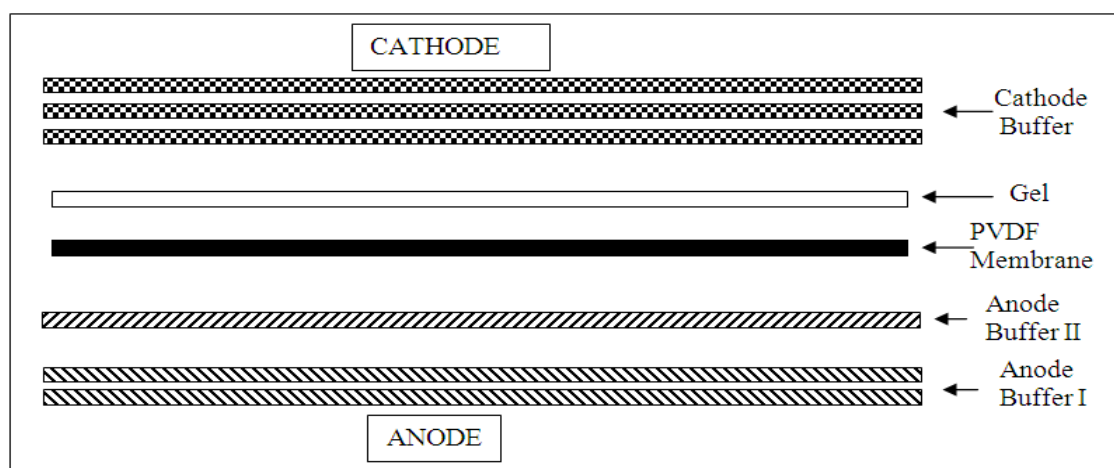


Figure 2-17 Immunoblot filter paper, membrane and SDS-PAGE gel sandwich

2.2.25 Immunoblotting and protein visualisation

The transferred membrane was rinsed briefly in distilled water to remove any traces of gel. The membrane was then incubated for one hour in blocking buffer (5% BSA in TBS-T or 5% dried milk in TBS-T) at room temperature on a shaker. The membrane was washed three times for ten minutes in PBS-T and then incubated overnight at 4°C in primary antibody solution. The following morning, the membrane was washed three times for ten minutes and then incubated in secondary antibody at room temperature for one hour.

The membrane was removed from the antibody, washed three times for ten minutes in PBS-T and then incubated in chemiluminescent solution (Millipore) (prepared as per manufacturer's guidelines, section 2.1.7.4) for five minutes. The blot was drained of chemiluminescent solution and visualised in a darkbox imager (LAS 3000, Fujifilm). The membrane exposure times were adjusted to optimise the quality of the captured image.

Following imaging, the membrane was washed three times in PBS-T for ten minutes. It was then incubated with strip solution (section 2.1.7.4) for fifteen minutes at room temperature. The membrane was washed twice in PBS-T for fifteen minutes. The membrane was then blocked and reprobed with a primary antibody as above if required.

Antibody	Supplier	Dilution	Concentration
GAPDH	Abcam	1 in 5,000	200ng/ml
Neurogranin	Millipore	1 in 1,000	-
Spectrin	Millipore	1 in 1,000	100ng/1ml
CamKII	Abcam	1 in 1,000	1µg/ml
Tropomodulin 2	Abcam	1 in 1,000	1µg/ml
GFAP	Dako	1 in 1,000	2.9µg/ml
B-Actin	Sigma	1 in 5,000	400ng/ml
BASP-1	Millipore	1 in 2,500	-
Calponin 3	Santa Cruz	1 in 1,000	200ng/ml
Rabbit anti-mouse polyclonal antibody HRP	Millipore	1in10,000	100ng/ml
Goat anti-rabbit polyclonal antibody HRP	Millipore	1in10,000	100ng/ml

Table 2-7 Antibody dilutions used in immunoblotting

2.2.26 Semi-quantitative immunoblotting: analysis

Immunoblot bands were analysed using ImageJ (Image Processing and Analysis in Java) software. ImageJ is free, open access software that was downloaded for use from (Rasband, 1997-2005) www.rebwen.nih.com.

Immunoblot image files were opened in 8-bit format in ImageJ software. The rectangular selections tool was chosen to select the protein bands of interest on the blot image. After all bands were selected, they were plotted as profiles. The darkest bands were considered to have the highest density and they formed the highest peaks in the profile plots (Figure 2-18).

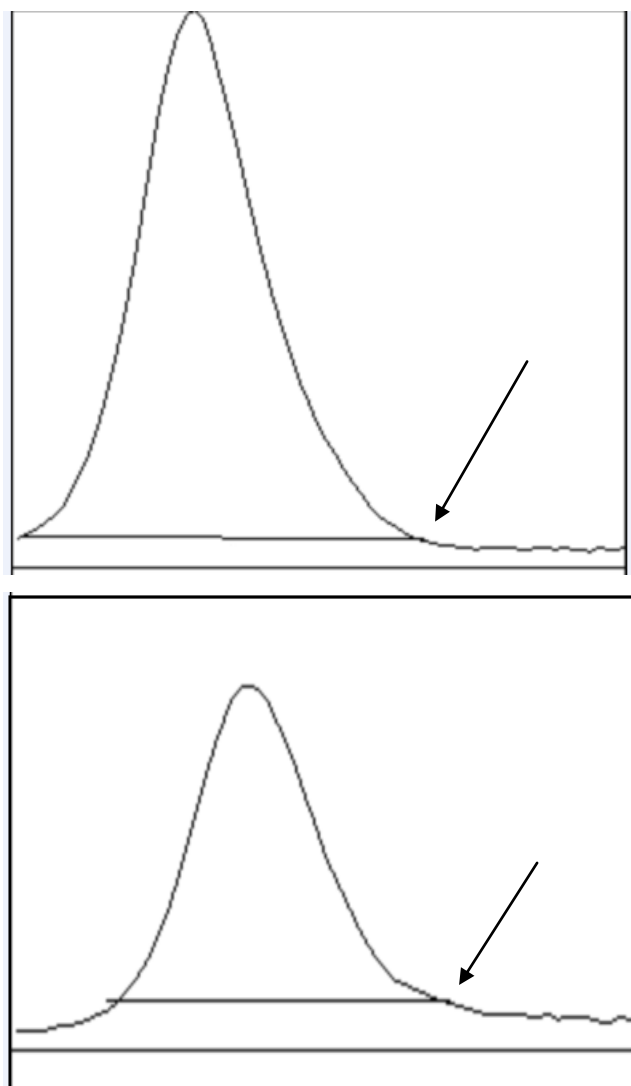


Figure 2-18 profile peaks obtained in ImageJ software

Higher peaks represent denser bands. The arrows point to the line drawn to enclose the base of the peaks.

Wider peaks represented bands that covered a wider size on the gel. The straight line selection tool was used to close the base of the peaks. As background noise is present in all Immunoblot gels, the peaks never reach the baseline and were enclosed to allow for further analysis. The wand tool was used to select the peak area only from the profile plot. The area of the peak was calculated based on the size of the selected, enclosed peak. The percent value was also calculated. All values obtained were relative. Relative band densities were used to calculate the differences in protein expression levels in ECS treated and control animals. All normalised band density values were tested using Graphpad Prism's Grubb's Test (<http://graphpad.com/quickcalcs/Grubbs1.cfm>) to determine if any of the values were outliers. Outlying values were excluded from further immunoblot analysis.

2.2.27 Statistics

Data was analysed using the statistical analysis package Graphpad Prism (Graphpad Inc.) or Microsoft Excel (Microsoft Inc.). The data was compared statistically using a Student's t-test, one or two way analysis of variance or non-parametric statistical tests as specified in the results. If significant changes were detected following ANOVA the data was further analysed using a Student Newman-Keuls or Bonferroni post hoc test as appropriate. A p value of less than 0.05 was considered statistically significant. Results are expressed as means with standard error of the mean (SEM) or mean percentage control with SEM.

Proteomic data was analysed using Progenesis SameSpot software (NonLinear Dynamics). Multivariate proteomic analysis was conducted using UnscramblerX (Camo Inc.) as detailed in the relevant results chapter. Data were expressed as normalised spot volumes, a p value of less than 0.05 was considered statistically significant. Immunoblot images were analysed using ImageJ software. Student's t-test or Mann-Whitney test for non-normal data was used to compare the effects of ECS treatment compared to control on selected antibodies of interest.

The effects of brief and ultra brief electroconvulsive stimulation

3.1 Introduction

ECT is recognised as the most effective treatment available for depression (The UK ECT Review Group, 2003). However, ECT is associated with cognitive side effects that, although mainly short-lived (Semkovska, 2011; Semkovska and McLoughlin, 2010), limit its use for depressed patients. Since it was first developed as a therapy in the 1930's, ECT has been modified to improve the adverse effects experienced by patients. The level of impairment induced by ECT varies with the use of different technical parameters including waveform and electrode placement (Lisanby et al. 2000; Ingram, 2000).

Modifications of the parameters by which ECT is administered can improve the treatment. For example, changing pulse wave form from sine-wave to square wave preserved the efficacy of ECT treatment (Fox et al. 1989; Kho et al. 2003) and reduced the associated cognitive side-effects such as attention and executive function tasks (Fujita et al. 2006). ECT electrodes are traditionally placed bitemporally and such placement is still standard practice today (The UK ECT Review Group, 2003). Right unilateral electrode placement, first described over forty years ago (d'Elia, 1970; Sackeim, 2000) was introduced as an alternative to bitemporal placement, to reduce patient confusion and cognitive deficits after ECT treatments (Abrams, 2002). However not all patients respond to right unilateral ECT (Rosa et al. 2006). In order to treat the symptoms of depression as effectively as bilateral ECT, unilateral ECT is administered at higher doses above seizure threshold (McCall et al. 2000; Semkovska, 2011). Higher dosage may lead to an increase in associated cognitive side effects (Kellner et al. 2010).

Reducing pulse width from the standard brief pulse duration, which ranges from 0.5ms - 1.5ms duration (Lukoyanov et al. 2004), may also reduce the cognitive side effects of ECT. The standard pulse-width is physiologically inefficient as neuronal depolarisation takes an estimated 0.1 ms-0.2 ms (Ranck, 1975; Sackeim, 2004). A reduction of pulse width to an ultra brief pulse width of 0.3 ms (Loo et al. 2008; Sienaert et al. 2010) may provide an improvement in the side effects associated with ECT while maintaining antidepressant efficacy.

To date, reports of the effectiveness of ultra-brief ECT have been somewhat mixed. Reports of remission rates as high as 70% with few side effects following ultra brief pulse ECT administration (Sackeim et al. 2008) have not been replicated (Quante et al. 2011; Sienart et al. 2009).

The effects of ultra brief pulse ECT have yet to be examined in ECS, an animal model of ECT. While molecular, cellular and behavioural effects of brief pulse ECS have been elucidated, there is currently no preclinical data available for ultra brief pulse ECS. If ultra brief pulse ECS is as effective therapeutically as brief pulse ECS, similar increases in cell proliferation (Madsen et al. 2000; Hellsten et al. 2002; Malberg et al. 2000), neurotrophin expression (Altar et al. 2003) and changes in behaviour (Porsolt, et al. 1977) would be expected in animals treated with either form of ECS.

In order to determine whether ultra brief pulse ECT may provide a form of therapy that has equivalent efficacy to current treatments but improves upon their side effects, a greater understanding of it is required. This study shall attempt to elucidate the preclinical effects of ultra brief pulse ECS in an animal model by comparing it to its well established predecessor, brief pulse ECS.

3.1.1 Aims of the study

The purpose of this study was to compare the behavioural, cellular and molecular effects of ultra brief pulse and brief pulse ECS. Firstly, the seizures induced by brief pulse ECS and ultra brief pulse ECS were compared. Behaviourally, the cognitive effects of brief pulse and ultra brief pulse ECS were examined in the water plus maze and the antidepressant efficacy of brief pulse and ultra brief pulse ECS were tested in the forced swim test (FST). The dentate gyrus was examined for increased cell proliferation in ECS treated animals as measured by an increase in BrdU labelled cells. The effect of brief pulse and ultra brief pulse ECS on protein expression levels of the neurotrophin BDNF in the hippocampus and frontal cortex were measured by ELISA.

3.2 Methods

3.2.1 Ultra brief pulse study: study design

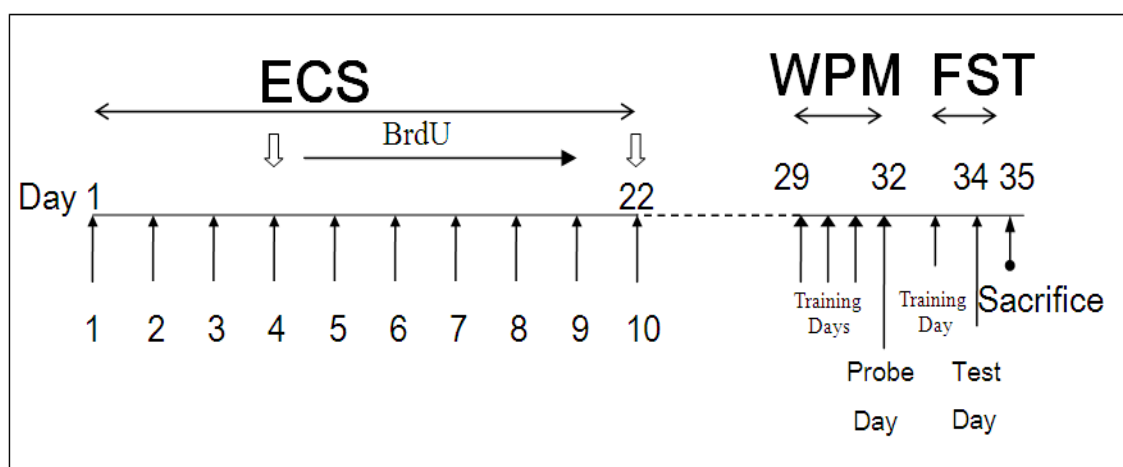


Figure 3-1 Ultra brief pulse study design

The ECS treatment number (1-10) is listed below the timeline. Experiment day (1-35) is denoted above the timeline.

BrdU, bromodeoxyuridine; ECS, electroconvulsive stimulation; WPM, water plus maze; FST, forced swim test

The effects of brief pulse and ultra brief pulse ECS were compared in the ultra brief pulse study (Figure 3-1). The behavioural and molecular effects of ECS treatment were examined using a subset of animals, $n=6/\text{group}$. One animal was excluded from the water plus maze study due to barbering and housing isolation. The final group number of animals in each group for this behavioural protocol was therefore: control group $n=6$, ultra brief pulse group $n=6$, brief pulse group $n=5$.

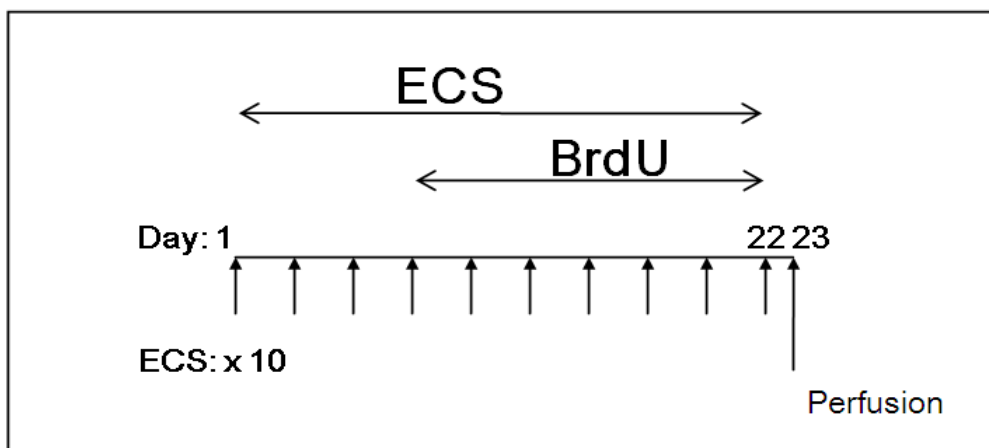


Figure 3-2 ECS and BrdU administration study design

ECS treatments (1-10) are denoted by arrows placed below the timeline. Experiment day (1-23) is denoted above the timeline.

ECS, electroconvulsive stimulation; BrdU, bromodeoxyuridine.

A subset of animals $n=4/\text{group}$ were treated with BrdU in order to examine the effects of different ECS pulse widths on cell proliferation in the brain (Figure 3-2). BrdU was administered two to four hours after ECS from treatment days 4 to 7 (section 2.2.3).

3.2.2 Ultra brief pulse study: animal ECS treatment

The animals were administered a chronic course of ECS consisting of ten treatments. Animals were housed and treated as described in section 2.2.1. Control animals underwent the same handling as treated animals but no charge was delivered. The animals were treated every Monday, Wednesday and Friday morning using the parameters described in Table 3-1.

Treatment	Pulses/ second	Pulse Width (msec)	Shock Duration (seconds)	Current (mAmp)
Brief Pulse	100	0.5	0.7	75
Ultra Brief	100	0.3	0.7	75

Table 3-1 ECS treatment parameters

3.2.3 Experimental procedures

All procedures were conducted in line with protocols detailed in the Methods section 2.2 of Chapter 2. Following ECS treatment, animals were habituated to the behavioural area for one week prior to starting water plus maze training. The water plus maze consisted of three training days followed by a fourth day of probe trials (section 2.2.8.1). Twenty four hours after completing the water plus maze, the animals started the forced swim test protocol. This protocol was two days long. The first day was a training day (15 minute session) and the second day was a testing day (5 minute session). Animals were administered a two-day modified forced swim test (FST) protocol. As described (section 2.2.8.2), the training day consisted of a fifteen minute swim session followed 24 hours later on test day by a 5 minute swim session. Behaviour was monitored and recorded manually.

Briefly, tissue for molecular analysis was collected twenty four hours after the forced swim test (n=6 per group). Animals were sacrificed and the hippocampus and frontal cortex were dissected out. BDNF protein levels were measured using a sandwich ELISA kit according to the manufacturer's instructions (Chemikine, Chemicon (Millipore), USA). Hippocampal samples were homogenised in the recommended lysis buffer (10% w/v). Homogenates were centrifuged at 14,000g for thirty minutes and the supernatant was collected for use in the BDNF assay. The amount of BDNF in each sample was analysed in duplicate and determined from the regression line generated by BDNF standards (range 7.8pg/ml to 500pg/ml). Total protein concentrations were measured by Bradford protein assay. BDNF results are presented as pg BDNF per mg of total protein content in each sample.

To label proliferating cells in the dentate gyrus, BrdU treated animals were anaesthetised with urethane and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde. Hippocampal coronal sections (10µm) were mounted on slides for staining. Briefly, endogenous peroxidase activity was blocked using 3% hydrogen peroxide solution. Slides were heated in 10mM citric acid buffer, washed in TBS, incubated in avidin (Vector Laboratories, USA) for 10 minutes, washed and then incubated in biotin (Vector Laboratories, USA) for 10 minutes. Following washes and incubation in 10% normal rabbit serum (Dako, UK) for 5 minutes, sections were incubated with rat anti-BrdU (1:10,000; ab6326, Abcam, UK) for 1 hour. After this, slides were washed and incubated in biotinylated rabbit anti-rat secondary antibody (1:50; Vector Laboratories, USA) for 30 minutes.

Following washes, StrepAB/HRP complex (Vector Laboratories, USA) was incubated on each slide for 30 minutes. Slides were washed before a 15 minute DAB (SigmaFAST DAB tablets) incubation to visualise the reaction product. Sections were counterstained with Hematoxylin. Sections were imaged using an Olympus DP72 at 20x magnification. Numbers of BrdU-positive cells in dentate gyrus subgranular zone and granule cell layer were counted blind on coded slides from every sixth section over a range of 24 sections per animal.

Data analysis was conducted as described in section 2.2.27 of Chapter 2.

3.3 Results

3.3.1 Tonic-clonic seizure duration comparison

Ultra brief pulse ECS induced a visually identical seizure to that of brief pulse ECS. The duration of the tonic and clonic phases of seizure were recorded (Table 3-2).

Following comparison by Student's *t*-test, there was no significant difference in the total length of seizure ($p=0.984$) or the length of the tonic ($p=0.25$) or clonic phases ($p=0.66$) of seizure induced by either treatment.

	Brief pulse ECS	Ultra brief pulse ECS
Tonic seizure duration	18 ± 0.82 seconds	17 ± 0.60 seconds
Clonic seizure duration	23 ± 1.5 seconds	24 ± 2.5 seconds
Total seizure duration	41 ± 1.7 seconds	41 ± 2.8 seconds
Recovery duration	550 ± 17 seconds	420 ± 29 seconds

Table 3-2 Seizure and recovery duration following brief pulse and ultra brief pulse ECS administration

Tonic and clonic seizure duration did not differ significantly between treatment groups. Total average seizure duration did not differ between treatments. The average length of time it took animals to recover is also recorded in the table. Data are expressed as mean with standard error of the mean (SEM), $n=6$ per group.

3.3.2 Recovery times

Recovery time was significantly longer in brief pulse treated animals than ultra brief pulse treated animals (Figure 3-3) ($p=0.0025$) although the average recovery time was within ten minutes of seizure administration (Table 3-2). An animal was considered to have recovered when it was moving normally around the cage and no longer reacted with hypersensitivity to its environment (Altar et al. 2004). No reoccurrence of seizures were observed in the home cage following treatment.

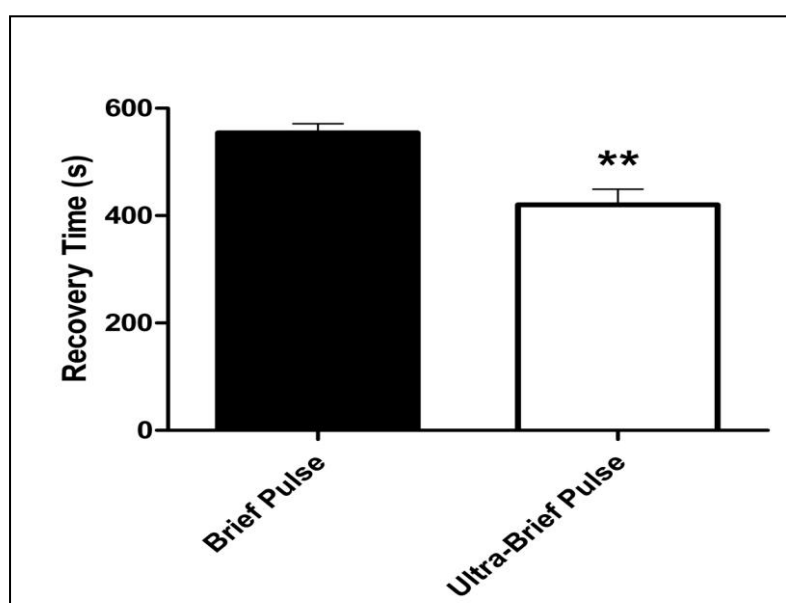


Figure 3-3 Recovery times for brief pulse and ultra brief pulse treated animals

The recovery time was significantly longer in brief pulse treated animals than in ultra brief pulse treated animals, Student's *t*-test $p<0.01$. Data was presented as mean with SEM, $n=6$ per group, ** $p<0.01$.

3.3.3 Animal weight

The average animal weight per treatment group is shown in Table 3-3.

Treatment	Weight (g) Treatment Day 1	Weight (g) Treatment Day 10
Brief pulse ECS	265±9.1	292±10
Ultra brief pulse ECS	278±5.7	304±7.6
Control	274±4.7	309±5.3

Table 3-3 The average weight (g) of control, brief pulse and ultra brief pulse ECS treated animals

Data is expressed as mean with SEM, n=6 per group.

Animal weights did not differ significantly between treatment groups on day 1 (Figure 3-4) and day 10 (Figure 3-5) of treatment. On day 1, analysis showed no significant difference in animal weight between groups before the start of treatment ($F_{(2,15)}=0.93$, $p=0.4176$). On the final ECS treatment day, day 10, one way ANOVA again showed no significant difference in animal weight between brief pulse, ultra brief pulse and control groups ($F_{(2,15)}=1.3$, $p=0.3082$).

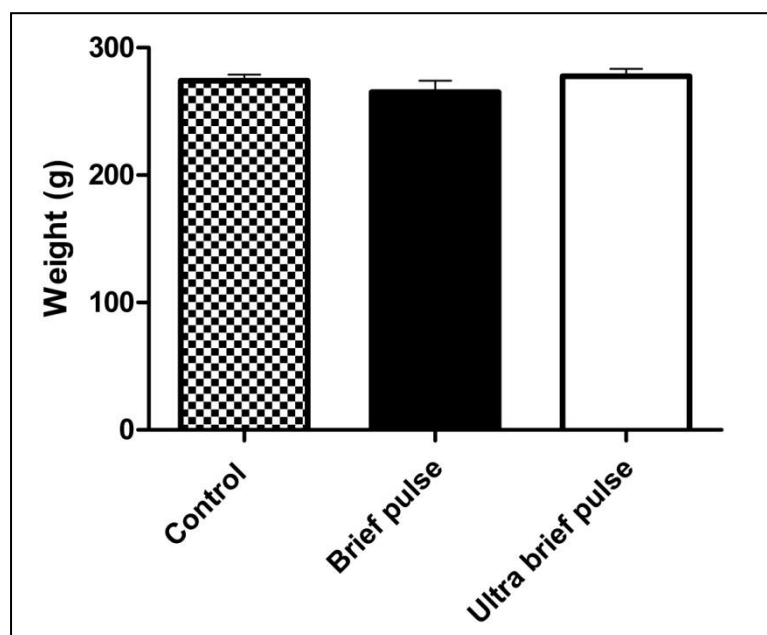


Figure 3-4 Animal weight on treatment day 1

There was no significant difference between brief pulse ECS, ultra brief pulse ECS and sham treated animal weight. Data are represented as mean $\pm$ SEM, $n=6$ per group, one way ANOVA.

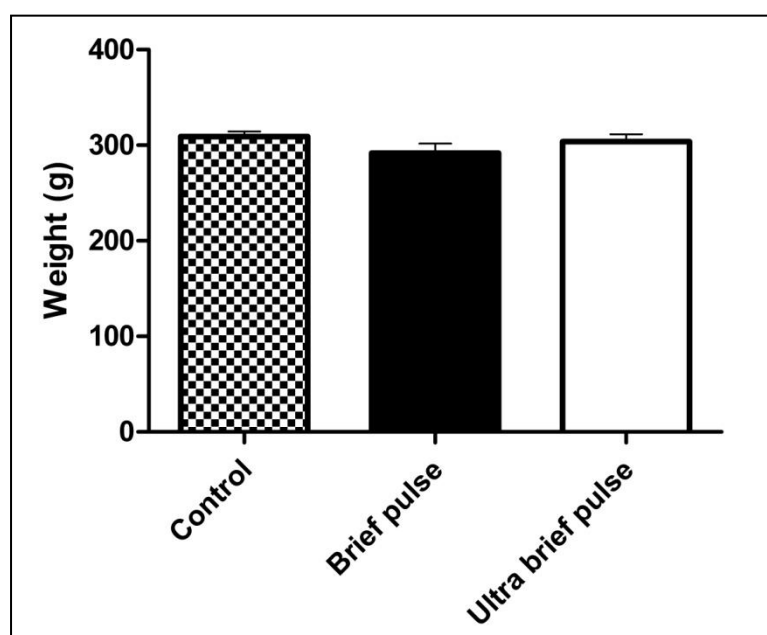


Figure 3-5 Animal weight on treatment day 10

There was no significant difference between brief pulse ECS, ultra brief pulse ECS and sham treated animal weight. Data are represented as mean $\pm$ SEM, $n=6$ per group, one way ANOVA.

Animal weight gain was monitored throughout the course of ECS treatment (Figure 3-6). Data are presented as animal weight as % of treatment day 1 animal weight. Two way (repeated measures) ANOVA showed that there was no difference between treatment groups ($F_{(2,135)}=1.16$, $p=0.3402$). There was a significant difference in animal weight between treatment days ($F_{(9,135)}=48.40$, $p<0.0001$). There was also no significant interaction effect ($F_{(18,135)}=1.64$ $p<0.0579$).

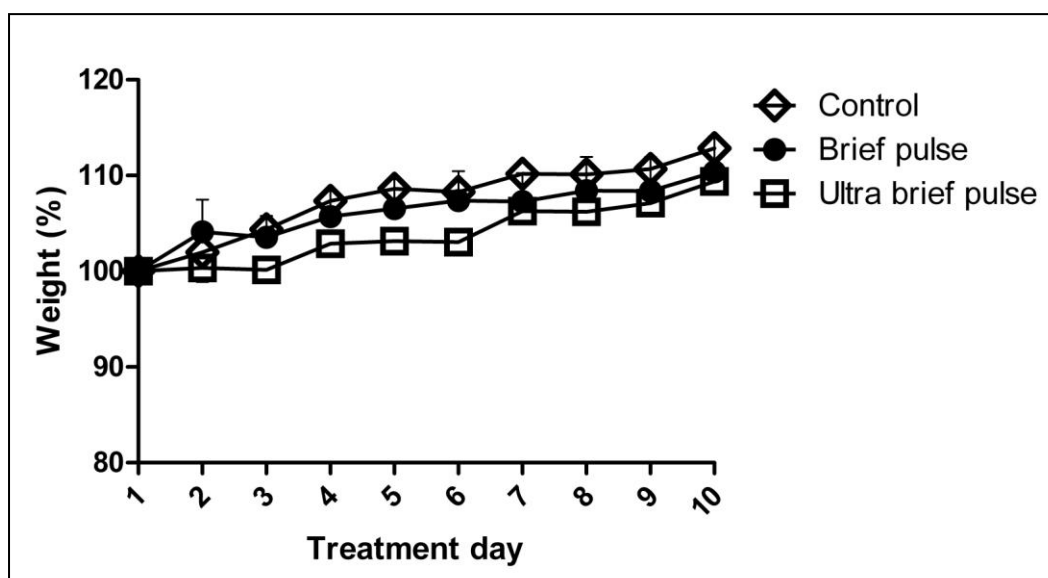


Figure 3-6 Animal weights treatment days 1-10

There was no significant difference between treatment groups on treatment days 1-10 as determined by Bonferroni post test. Data are mean $\pm$ SEM, $n=6$ per group, two way (repeated measures) ANOVA and Bonferroni post test.

3.3.4 Seizure duration comparisons

Clonic and tonic seizure duration was compared on ECS treatment days one, five and ten (Figure 3-7-3.8) to compare differences in seizure quality over the course of treatment.

3.3.4.1 Tonic seizure duration comparison days 1, 5, 10

Tonic seizure duration did not differ significantly between brief pulse and ultra brief pulse treated animals as demonstrated by repeated measures ANOVA ($F_{(1,20)} = 0.27$, $p=0.6157$; Figure 3-7). However, the seizure durations did increase significantly with time ($F_{(2,20)}=3.88$, $p=0.0378$). Post hoc analysis with Bonferroni post test did not detect significant differences between groups. There was no significant interaction effect between factors treatment and time ($F_{(2,20)}=0.01$ $p=0.9852$).

3.3.4.2 Clonic seizure duration comparison days 1, 5, 10

Following analysis by repeated measures ANOVA, treatment type had no effect on clonic seizure duration ($F_{(1,20)}=0.82$, $p=0.3861$; Figure 3-8). The day on which the seizure was administered was found to be a significant factor as clonic seizure duration increased significantly over time ($F_{(2,20)}=18.63$, $p<0.0001$). However, post hoc analysis with Bonferroni post hoc did not detect any significant differences. There was no significant interaction effect ($F_{(2,20)}=0.27$; $p=0.7663$).

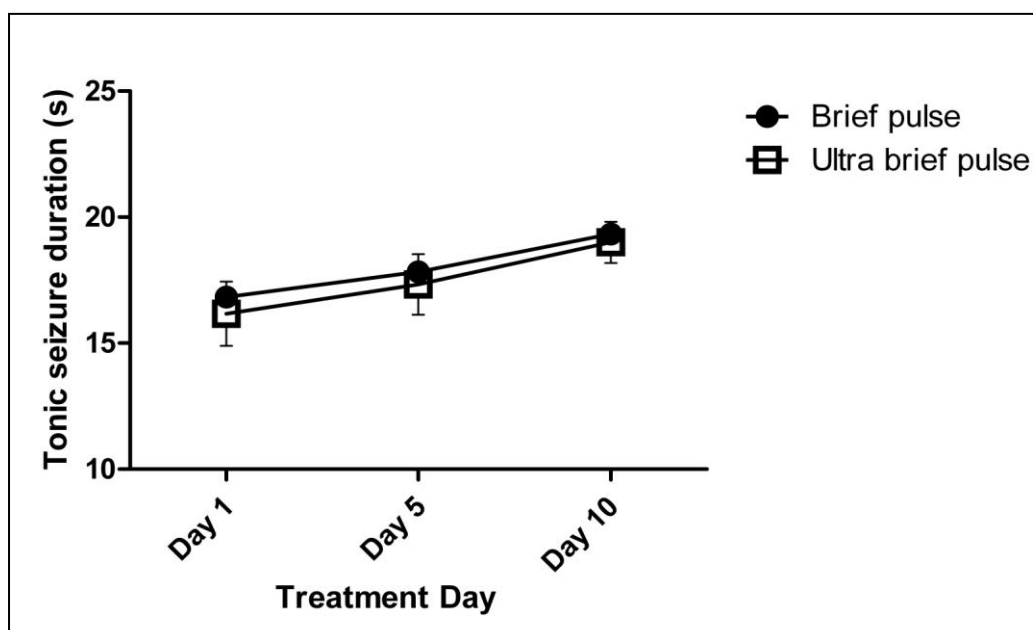


Figure 3-7 ECS-induced tonic seizure duration comparison

The tonic phase of the induced seizures was measured on treatment days 1, 5 and 10. Tonic seizure length did not differ between treatments but increased significantly over time. Data is presented as mean $\pm$ SEM, $n=6$ per group, repeated measures ANOVA.

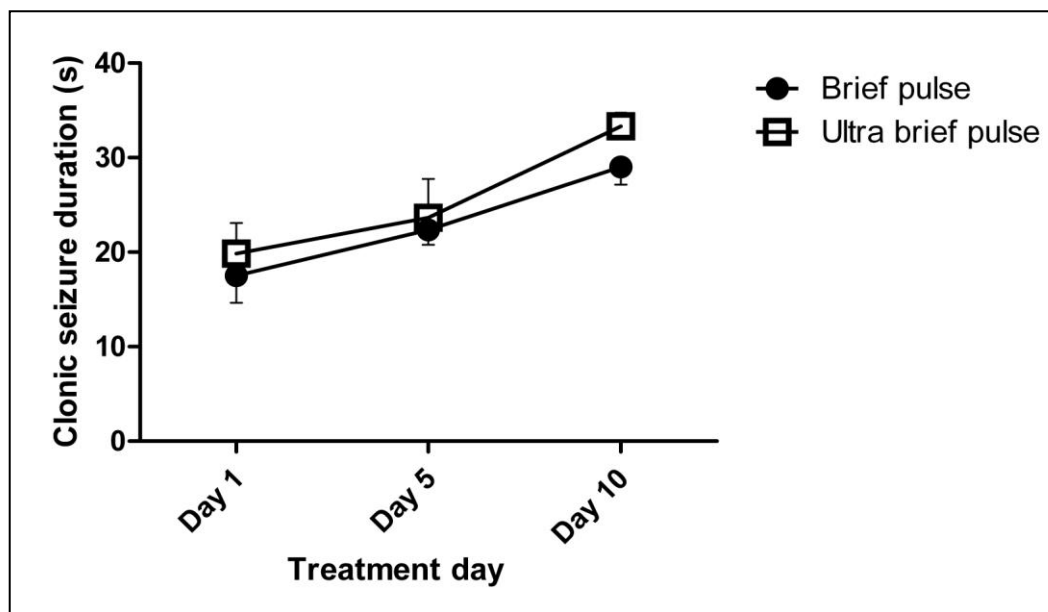


Figure 3-8 ECS induced clonic seizure duration comparison

The tonic and clonic phases of the induced seizures were measured on treatment days 1, 5 and 10. Treatment with brief pulse or ultra brief pulse ECS did not lead to significantly different clonic seizure durations following repeated measures ANOVA. However, seizure duration was significantly longer with treatment day as a factor. All values are means $\pm$ SEM, $n = 6$ per group.

3.3.5 Water plus maze

The water plus maze was conducted to test spatial learning, a test of hippocampus dependent memory. Animals were trained for three days to find a hidden platform in a four arm water maze. On the fourth day, a probe test consisting of thirty trials was carried out. The animal's ability to recall the location of the platform was measured.

3.3.5.1 Platform acquisitions: day 1-3

The number of times the platform was found by animals during the three training days increased significantly over time, following repeated measures ANOVA, indicating overall learning of the task occurred ($F_{(2,42)} = 4.09$, $p=0.0238$; Figure 3-9). There was no difference in platform acquisition between brief pulse ECS, ultra brief pulse ECS and sham ECS treated animals ($F_{(2,42)} = 0.77$, $p = 0.4705$). There was no significant interaction effect between treatment and time in this analysis ($F_{(4,42)} = 0.27$, $p = 0.8952$).

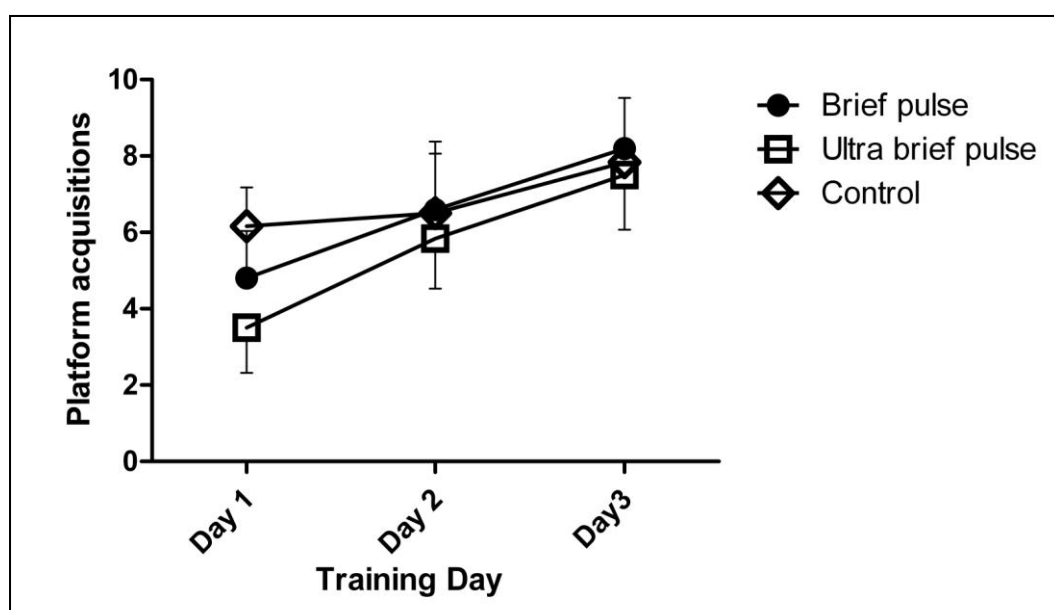


Figure 3-9 Platform acquisitions day 1-3

The number of platform acquisitions increased from training day 1 to training day 3, however, there was no treatment difference (between brief pulse ECS, ultra brief pulse ECS or sham treated control animals) in the average number of times the platform was acquired. Data was presented as mean $\pm$ SEM, $n=5/6$, repeated measures ANOVA.

3.3.5.2 Platform acquisitions: day 4

Brief pulse and ultra brief pulse treated animals obtained the platform less frequently on the water plus maze probe day than control animals (Figure 3-10). However, this difference did not reach statistical significance ($F_{(2,14)} = 1.8$, $p = 0.2091$). The average number of times the platform was acquired during the 30 trials carried out on the probe day was: control group 25 ± 3 ; brief pulse treated group 18 ± 2.6 ; ultra brief pulse treated group 17 ± 3.6 .

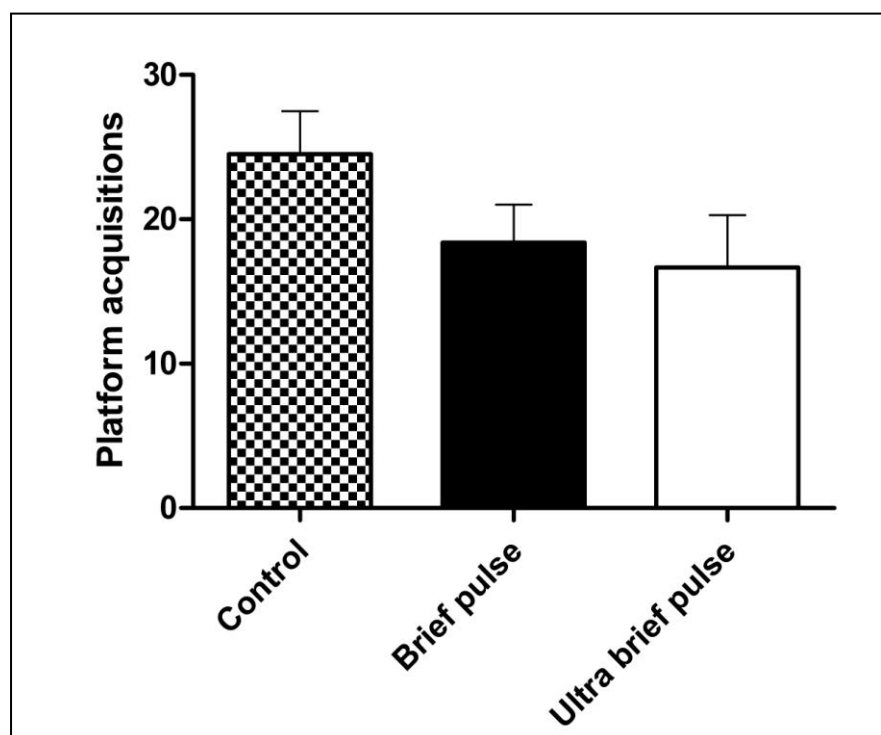


Figure 3-10 Probe day platform acquisitions

No significant differences were detected between ECS treatments on the number of times the platform was acquired in the probe trial. Data are presented as mean $\pm$ SEM, $n=5/6$ per group, one way ANOVA.

3.3.5.3 Velocity to platform

The velocity (cm/s) to the platform was also measured on training and probe days to detect whether there was a difference in the time it took control, brief pulse and ultra brief pulse treated animals to reach the platform.

3.3.5.3.1 Day 1-3

No significant difference in velocity to platform was found between brief pulse, ultra brief pulse and control groups following analysis by repeated measures ANOVA ($F_{(2,42)}=0.16$, $p=0.8496$) over the three training days (Figure 3-11). However, velocity to platform increased over time ($F_{(2,42)}=6.47$, $p=0.0035$).

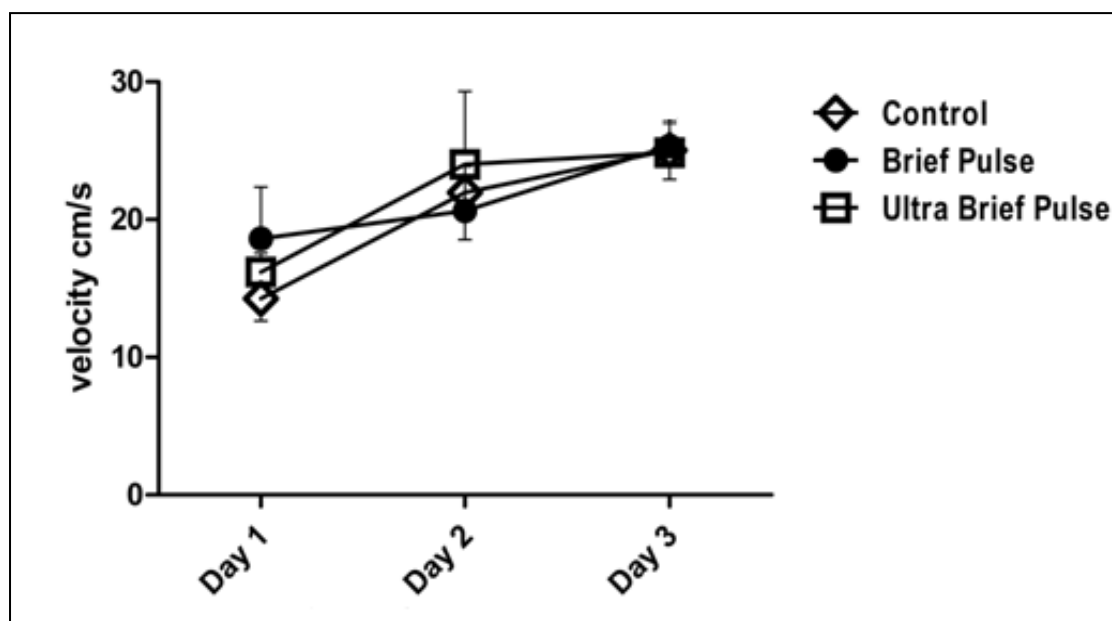


Figure 3-11 Velocity on training days 1-3 in the water plus maze

Velocity to platform increased over time but did not significantly differ between brief pulse, ultra brief pulse and control groups. Data was presented as mean $\pm$ SEM, $n=5/6$, repeated measures ANOVA.

3.3.5.3.2 Day 4

The velocity (cm/s) was also measured on the probe day by one way ANOVA (Figure 3-12). There was no significant difference between brief pulse, ultra brief pulse and sham ECS treated animals ($F_{(2,14)}=0.18$, $p=0.8331$). The velocity to the platform for control animals was 21 ± 2.7 cm/s, for brief pulse animals was 21 ± 2.4 cm/s and for ultra brief pulse treated animals was 23 ± 2.9 cm/s.

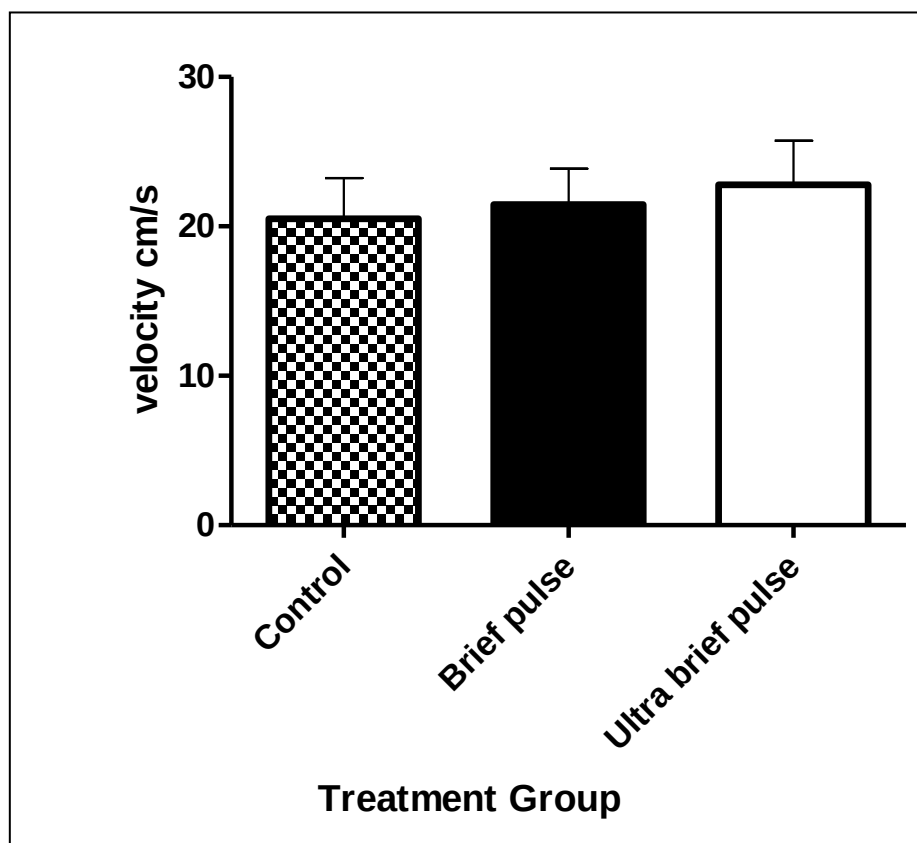


Figure 3-129 Velocity to platform probe day

The velocity to the platform on the water plus maze probe day did not differ significantly between animals treated with brief pulse, ultra brief pulse or sham ECS. Data expressed as mean $\pm$ SEM, $n=5/6$ per group, one way ANOVA.

3.3.5.4 Time spent in decision zone

The decision zone is the central area of the water plus maze at the junction between the four arms of the maze (Figure 3-13). An animal is not considered to have entered the platform or wrong arms of the maze until it has passed through, and out of, the decision zone. While in the decision zone, an animal can turn into one arm yet change direction and choose another arm without the first arm being scored as the acquired arm.

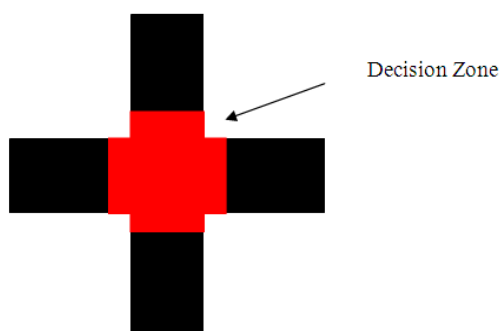


Figure 3-13 Decision zone of water plus maze

3.3.5.4.1 Training day 1-3

Repeated measures ANOVA of time spent in the decision zone showed that there was no significant difference between the length of time brief pulse, ultra brief pulse and sham ECS treated animals spent in this zone on treatment days 1-3 ($F_{(2,28)}=0.37$, $p=0.6986$). With time as a factor, there was a significant difference over the three training days ($F_{(2,28)}= 9.3$, $p=0.0008$), however post hoc analysis with Bonferroni post test did not reveal a significant difference. There was no significant interaction effect between treatment and time ($F_{(4,28)}=0.92$, $p=0.4669$; Figure 3-14).

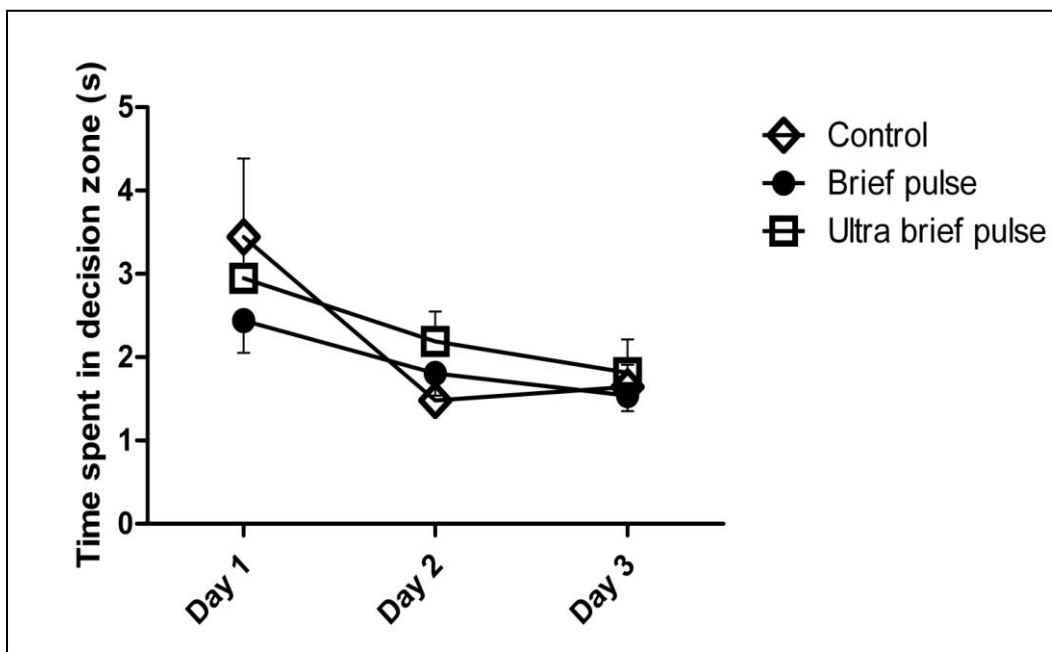


Figure 3-14 Time spent in decision zone

The time animals spent in the decision zone did not differ significantly between treatments on training days 1 to 3. The amount of time animals spent in the decision zone differed significantly over training days. Data expressed as mean $\pm$ SEM, $n=5/6$ per group, repeated measures ANOVA.

3.3.5.4.2 Probe Day 4

Following analysis by one way ANOVA, no significant difference in the length of time animals spent in the decision zone was detected ($F_{(2,14)}=0.4$, $p=0.6771$). The average length of time animals spent in the decision zone was: control 1.7 ± 0.16 s, brief pulse ECS treated 2.3 ± 0.7 s, ultra brief pulse treated 2.3 ± 0.74 s (Figure 3-15).

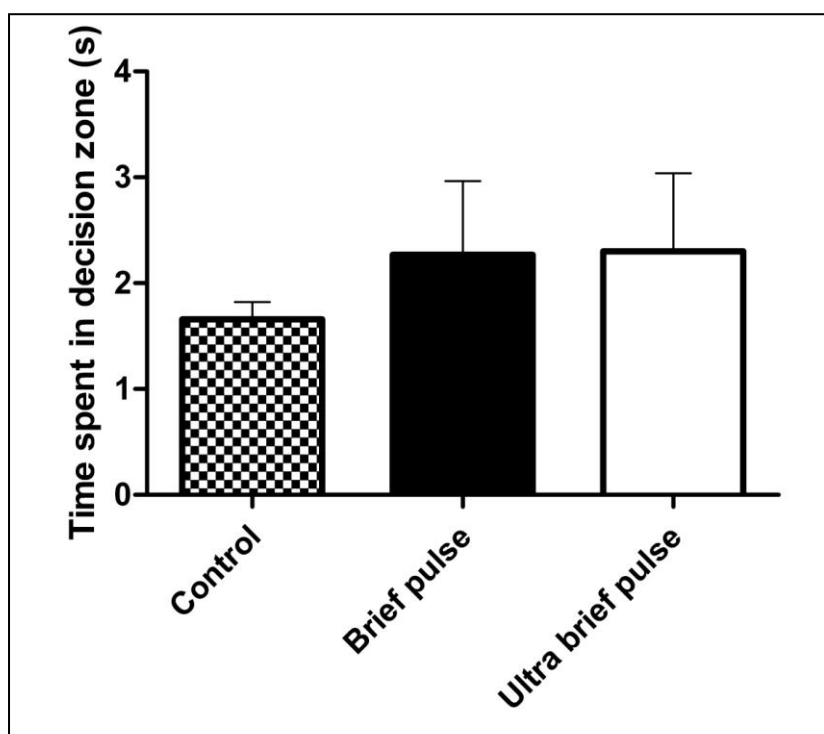


Figure 3-15 Time spent in the decision zone on probe day

The time animals spent in the decision zone did not differ significantly between groups on the water plus maze probe day. However, brief pulse and ultra brief pulse ECS treated animals appear to spend more time in the decision zone than sham treated control animals. Data expressed as mean $\pm$ SEM, $n=5/6$ per group, one way ANOVA.

3.3.6 Forced Swim Test

3.3.6.1 Training day

The forced swim test was administered over two days, the first day consisting of a fifteen minute trial. The first five minutes were monitored and animal behaviour was recorded (Detke et al. 1995). Immobility time, when animals made only those motions required to stay afloat, did not differ significantly between treatment groups and control (Figure 3-16; one way ANOVA ($F_{(2,15)} = 2.2$, $p = 0.1483$). However, ECS treated animals spent less time immobile than control animals; brief pulse immobility time, 97 ± 7.7 s; ultra brief pulse immobility time, 83 ± 18 ; control immobility time, 119 ± 9.6 s.

Other escape behaviours, including climbing (when the rat was making active movements with its forepaws in and out of the water, directed against the walls), swimming (when the animal was making active movements with its forepaws and was moving around the swim tank) and the number of dives (when the animal was fully submerged) were also recorded. No significant difference was detected between treatments in any of these behaviours (Table 3-4).

3.3.6.2 Test day

The test day consisted of a five minute session in the swim tank. ECS treatment resulted in a significant decrease in immobility time in the forced swim test when compared to control animals ($F_{(2,15)} = 3.8$, $p = 0.0465$; Figure 3-17). Post hoc analysis (Newman-Keuls test) showed that brief pulse ECS only, had a significantly reduced immobility time in this test ($p < 0.05$). A non-significant decrease in immobility time was seen in the ultra brief pulse group.

There was no difference in the amount of time animals spent climbing, swimming or in the number of dives the animals made (Table 3-5).

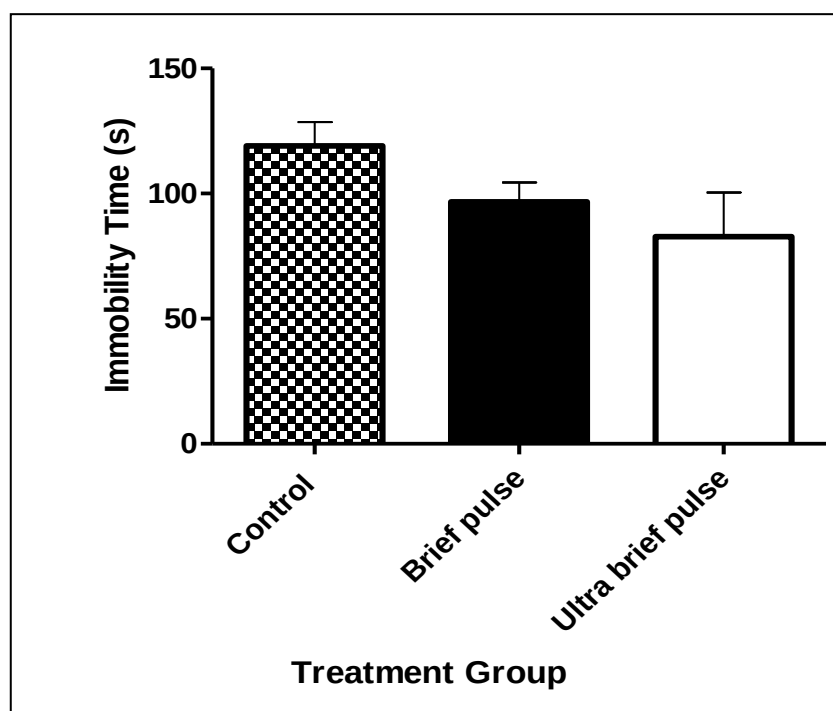


Figure 3-1016 Training day immobility times in the FST

There was no significant difference in immobility times between groups following the first five minutes of observation of the training day swim test session. Data was presented as mean immobility time (s) $\pm$ SEM, n=6 per group, one way ANOVA.

	Control	Brief pulse ECS	Ultra brief pulse ECS	P value	
Climbing (s)	48 $\pm$ 9.8	53 $\pm$ 9.8	58 $\pm$ 3.8	P=0.7072	$F_{(2,15)}=0.35$
Swimming (s)	128 $\pm$ 10	133 $\pm$ 14	155 $\pm$ 19	P=0.4167	$F_{(2,15)}=0.93$
No. Dives	0.33 $\pm$ 0.2	0.67 $\pm$ 0.67	1 $\pm$ 1	P=0.802	$F_{(2,15)}=0.22$

Table 3-4 Training session behaviour times and analysis

The amount of time (s) animals spent engaged in climbing and swimming behaviour, as well as the number of dives animals made, is detailed in Table 3-5. Following analysis by one way ANOVA, no significant differences were detected in these behaviours following brief pulse, ultra brief pulse and sham ECS treatment. Data is presented as mean $\pm$ SEM, n=6 per group.

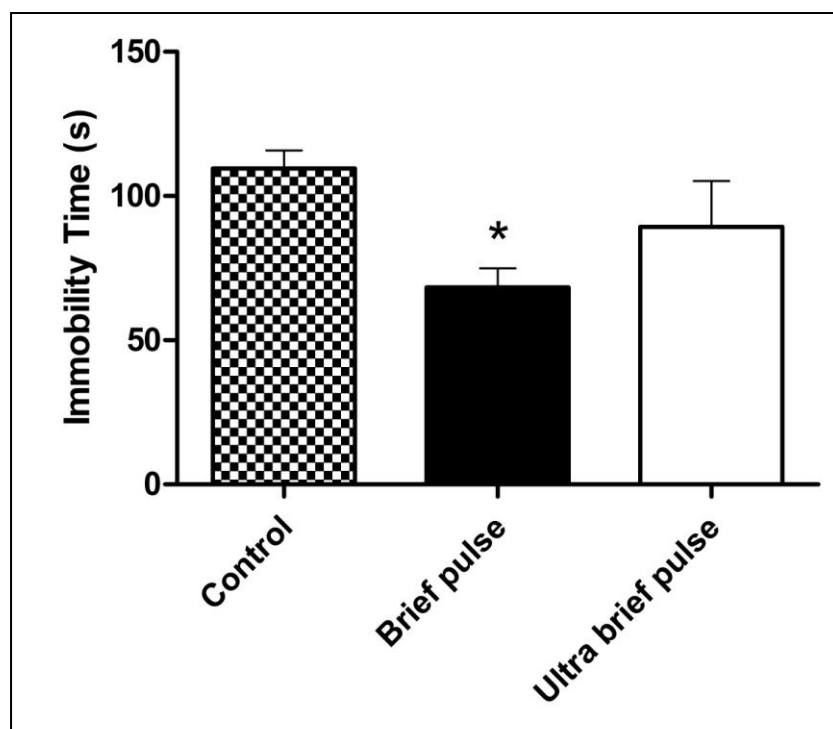


Figure 3-17 Forced swim test: day of testing immobility times

Brief pulse treated animals spend significantly less time immobile (s) during the forced swim test than ultra brief pulse or control animals ($p < 0.05$). Ultra brief pulse treated animals showed a non-significant decrease in immobility time compared to control animals. There was no significant difference between the immobility times of brief pulse and ultra brief pulse animals. All values are means $\pm$ SEM, $n = 6$, * $p < 0.05$ compared to control group following one way ANOVA and Newman Keuls post hoc test.

	Control	Brief pulse ECS	Ultra brief pulse ECS	P-value	ANOVA
Climbing (s)	43 $\pm$ 12	47 $\pm$ 4.4	56 $\pm$ 3.9	P=0.4781	F _(2,15) =0.78
Swimming (s)	141 $\pm$ 12	175 $\pm$ 13	155 $\pm$ 13	P=0.1996	F _(2,17) =1.8
No. Dives	0.17 $\pm$ 0.2	1.8 $\pm$ 0.91	0 $\pm$ 0	P=0.0527	F _(2,15) =3.6

Table 3-5 Forced swim test: day of testing behaviour results and analysis

There was no difference between climbing and swimming times between treatment groups. There was a trend towards significance in the number of dives undertaken by animals (Table 3-5). Data is presented as mean $\pm$ SEM, $n=6$, one way ANOVA.

3.3.7 Effect of ECS on cell proliferation

ECS is a robust inducer of cell proliferation in the dentate gyrus (Scott et al. 2000; Malberg et al. 2000). To determine the efficacy of the ECS protocol applied in these studies, a measure of cell proliferation was taken following chronic ECS administration.

Cell proliferation in the dentate gyrus was measured by counting the average number of BrdU labelled cells per slide. There was a significant increase in the number of BrdU labelled cells in the dentate gyrus following ECS administration as determined by Kruskal-Wallis non-parametric test ($p=0.0264$). Post hoc analysis revealed that levels of proliferation were higher in brief pulse treated animals compared to control animals. This increase was almost 3-fold compared to sham ECS treated animals. An increase in cell proliferation was also seen following ultra brief pulse ECS, however this did not reach statistical significance when compared to control.

The percentage of BrdU labelled cells in each study is listed in Table 3-6. The results are presented as percentage of control counts (Figure 3-18). Figure 3-19 shows images of BrdU labelled proliferating cells from the dentate gyrus control, brief pulse and ultra brief pulse treated animals.

Treatment	% Expression
Brief pulse ECS	279 $\pm$ 29
Ultra brief pulse ECS	139 $\pm$ 31
Control	100 $\pm$ 7.6

Table 3-6 % BrdU labelled cells following brief pulse, ultra brief pulse and sham-ECS treatment

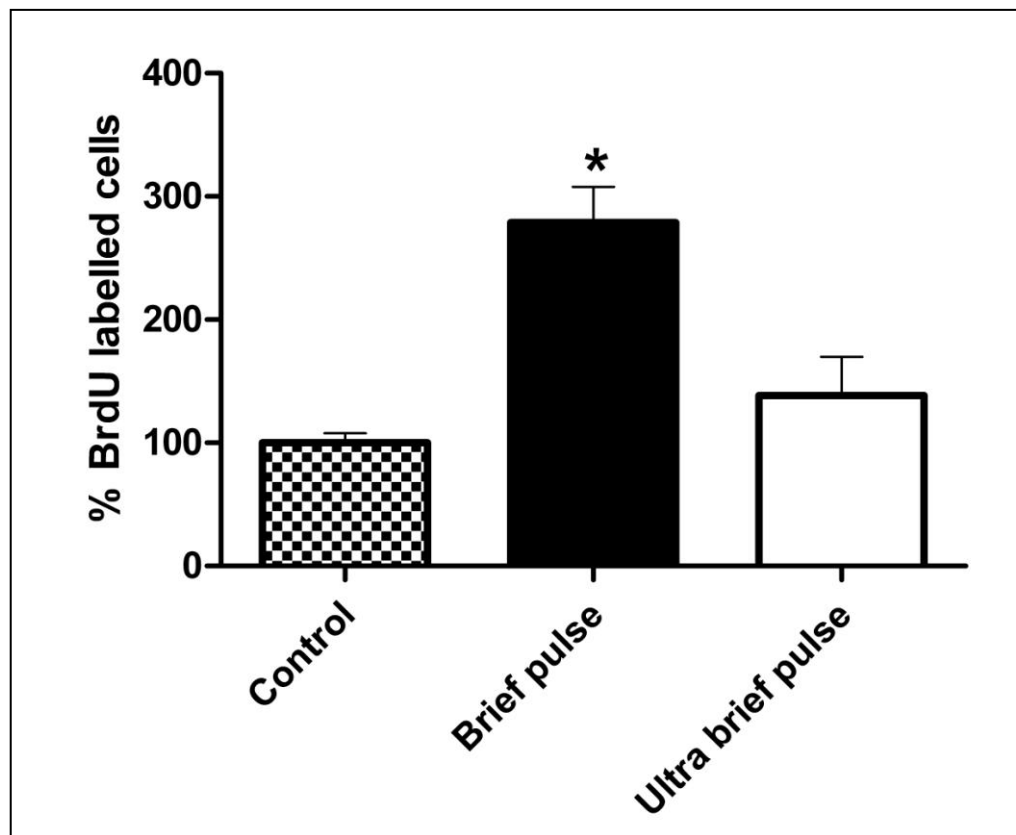


Figure 3-18 BrdU labelled cell counts in the dentate gyrus

Brief pulse treatment significantly increased the number of BrdU labelled cells (Dunns multiple comparison post hoc test, $p < 0.05$) in the dentate gyrus compared to sham treated control animals (percentage of control). The increase in BrdU labelled cells was not significantly higher in ultra brief pulse treated animals than in control animals. All values are means $\pm$ SEM, $n = 4$ per group, one way ANOVA, * $p < 0.05$

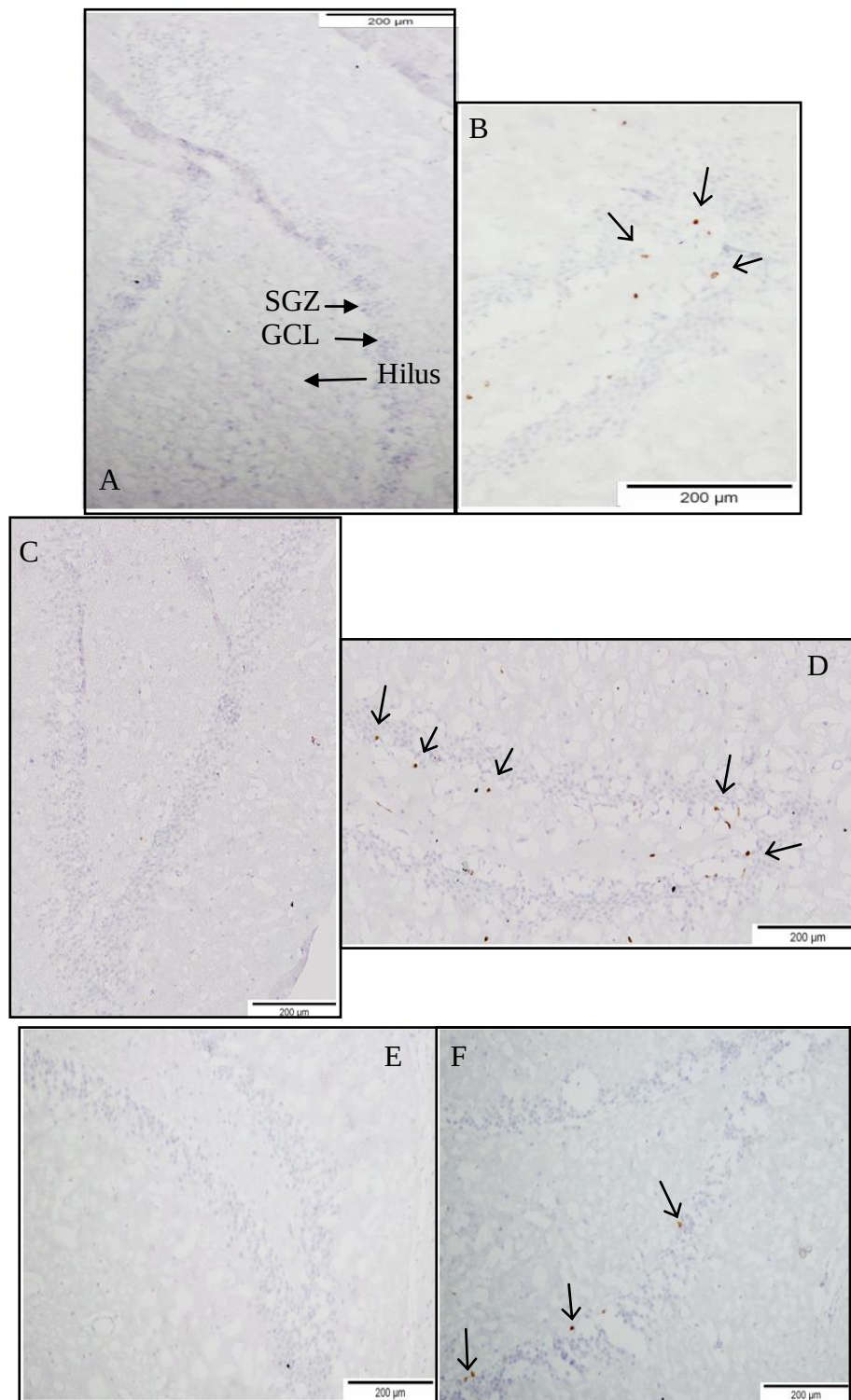


Figure 3-19 Cell proliferation in the dentate gyrus

Figure **(A)** Sham treated samples negative control ECF-L tissue; Figure **(B)** Sham treated sample tissue stained for BrdU labelling; Figure **(C)** Brief pulse treated samples negative control ECF-L tissue; Figure **(D)** Brief pulse treated sample tissue stained for BrdU labelling; Figure **(E)** Ultra brief pulse treated samples negative control ECF-L tissue; Figure **(F)** Ultra brief pulse treated sample tissue stained for BrdU labelling. Arrows indicate BrdU labelled (proliferating) cell. Unless otherwise stated, images were captured at 10X magnification; 200μm scale bar. GCL, granule cell layer; SGZ, subgranular zone.

3.3.8 BDNF ELISA

The level of BDNF protein expression was measured in the hippocampus and frontal cortex using a sandwich ELISA kit (section 2.1.5; 2.2.12).

3.3.8.1 Standards

The BDNF standard provided in the ELISA kit (Chemikine, Millipore) was diluted from 0ng to 500ng for use as a standard. The absorbance values of BDNF at multiple dilutions (section 2.2.12.2) of known concentration were used to create a standard curve (Figure 3-20). The BDNF content of samples was calculated using the formula for the slope of the line, $y = 0.0014x + 0.016$. As the BDNF standards on the graph are closely fitted to a straight line ($R^2 = 0.9955$), the results based on the slope of this line will give more accurate sample results.

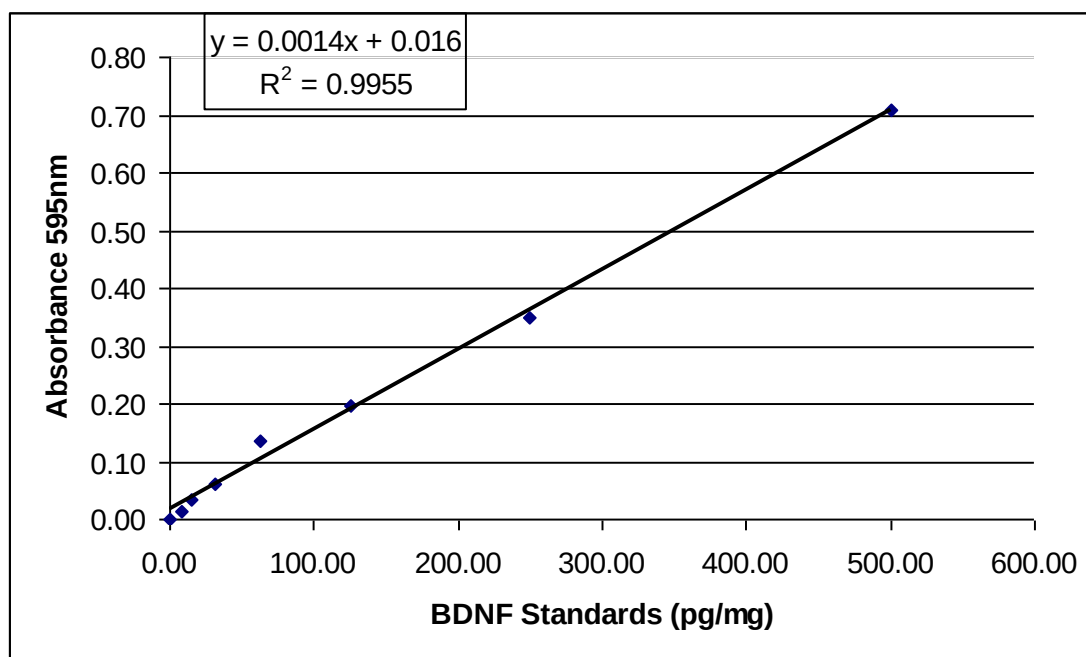


Figure 3-20 BDNF standard included in the ELISA kit

3.3.8.2 Hippocampus BDNF ELISA

One way ANOVA detected a significant increase in BDNF protein levels in the hippocampus ($F_{(2,15)}=6.3$ $p=0.01$). Post hoc analysis found that brief pulse ECS induced higher levels of BDNF protein expression than sham treatment ($p<0.01$) and ultra brief pulse ECS treatment ($p<0.05$) (Figure 3-21).

The average level of BDNF protein in control animals was 55.14 ± 12.09 pg/mg total protein; 137.3 ± 22.8 pg/mg total protein in brief pulse treated animals and 82.73 ± 12.69 pg/mg total protein in ultra brief pulse treated animals.

3.3.8.3 Frontal cortex BDNF ELISA

BDNF protein expression levels did not differ significantly between brief pulse, ultra brief pulse and control animals in the frontal cortex following analysis by one way ANOVA ($F_{(2,15)}=1.3$, $p=0.3027$; Figure 3-22).

The average level of frontal cortex BDNF in control animals was 69 ± 14 pg/mg total protein, in brief pulse treated animals was 57 ± 8.3 pg/mg total protein and in ultra brief pulse treated animals was 46 ± 8 pg/mg total protein.

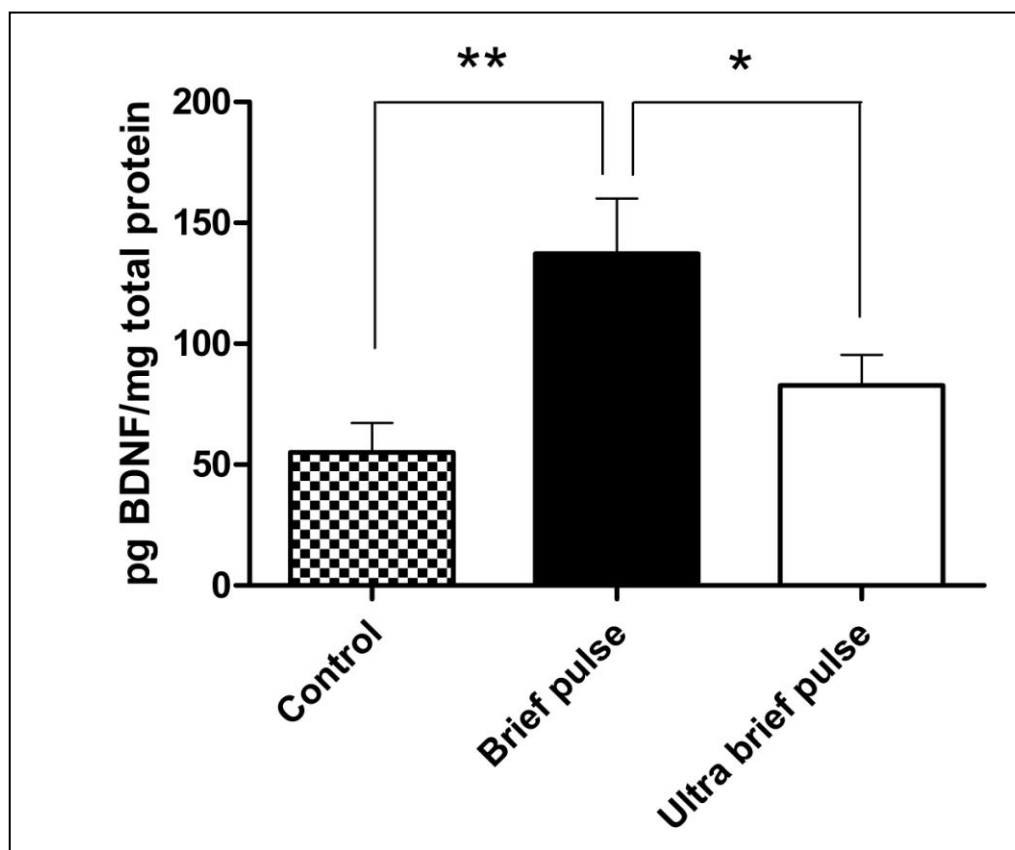


Figure 3-21 Hippocampal BDNF protein expression

BDNF protein expression was significantly increased in the brief pulse group compared to both control and the ultra brief pulse groups following analysis by one way ANOVA. Data presented as mean pg BDNF per mg total protein $\pm$ SEM, $n=6$ per group. ** $p<0.01$ compared to control and * $p<0.05$ ultra brief pulse groups.

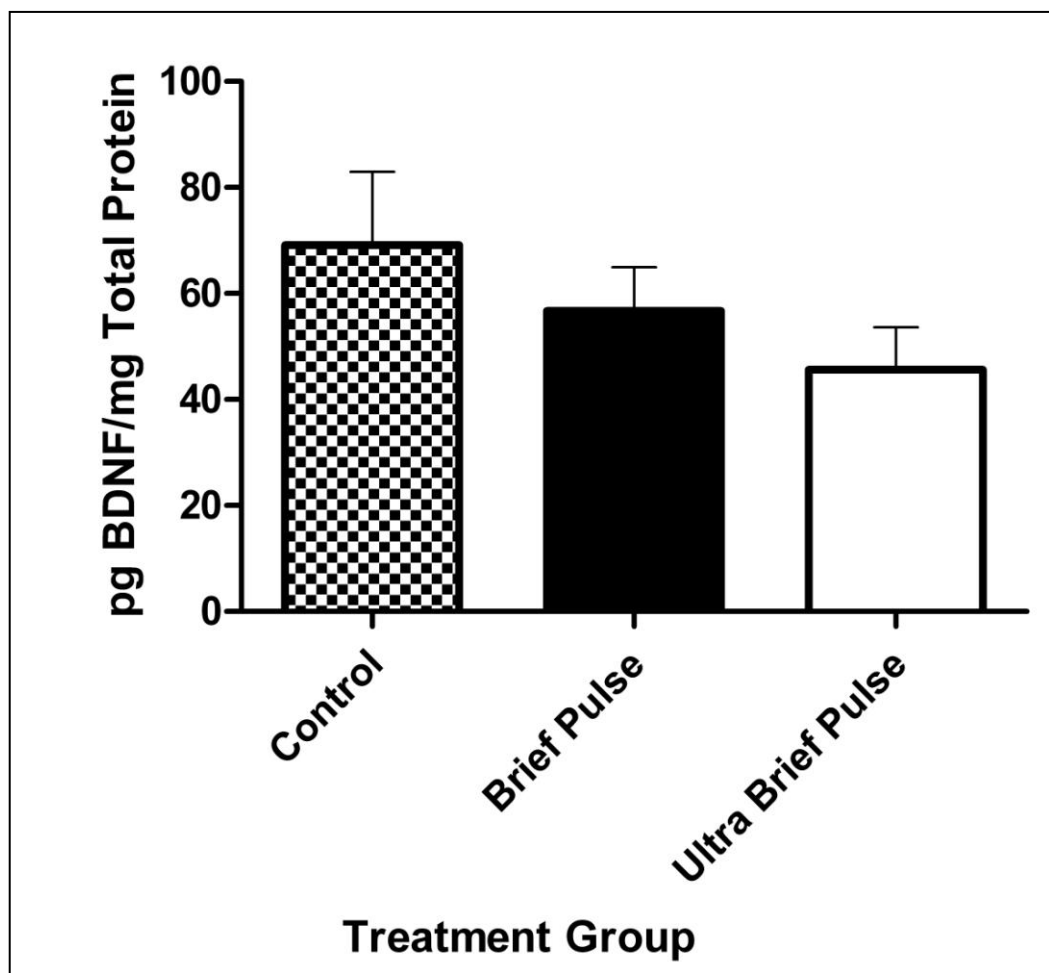


Figure 3-22 BDNF expression levels in the frontal cortex

There was no significant difference in BDNF protein levels in the frontal cortex following analysis by one way ANOVA. Data was presented as mean pg/mg total protein $\pm$ SEM, $n=6$ per group.

3.4 Discussion

3.4.1 Introduction

While the effects of ultra brief pulse ECT have been investigated in humans, there are several advantages to conducting preclinical studies into the impact of adjusting the parameters of ECT seizure induction (Fochtman, 1994). Clinical studies are expensive and labour intensive. A large number of variables must be controlled for due to the heterogenous nature of human populations compared to animals. There are also a finite number of patients who receive ECT and qualify for inclusion in such clinical studies. To date, while the effects of ultra brief pulse ECT have been investigated by several authors (Roepke et al. 2011; Loo et al. 2008), few of these studies are fully randomised, masked, control trials (Sackeim et al. 2008; Sienaert, et al. 2009; Quante, et al. 2011). The promising results initially obtained (remission rates 73% following unilateral ultra brief pulse treatment; Sackeim et al. 2008) were not replicated (44% remission rate following unilateral ultra brief pulse treatment; Sienaert et al. 2009). Conversely, animal studies, using homogenous animal populations, can control for a greater number of variables. Conducting pilot studies in animals can therefore inform clinical studies, allowing for more productive study design and optimal knowledge gain from such experiments (Fochtman, 1994).

3.4.2 Seizure duration, recovery and weight

The qualities of brief and ultra brief pulse ECS seizures were compared, to determine whether modifying its existing parameters might improve the administration of ECT. Total seizure duration did not significantly vary between treatments, nor did the duration of the tonic or clonic phases of seizure when studied individually. When the tonic and clonic phases of seizures were compared, the average seizure duration did not differ between treatments. The length of seizure on days one, five and ten of treatment were also compared to determine if they changed over time. The treatment day affected the duration of the tonic and clonic phases, which lasted significantly longer on day ten than day one. This result is similar to those previously reported which found a significant increase in the duration of tonic seizure length over ten ECS treatments (Jansson et al. 2009). However, there was no treatment effect, that is, no difference between brief pulse and ultra brief pulse ECS. Overall, ultra brief pulse ECS parameters induced a seizure that did not differ from that induced by brief pulse ECS treatment.

While the seizures induced by both treatments did not differ, the length of time it took animals to recover was significantly longer following brief pulse ECS. Although the

average animal recovery time was within ten minutes of treatment, ultra brief pulse treated animals' regained consciousness and were returned to their home cages more quickly. This may be due to the overall lower charge that these animals received following treatment (1.575 millicoulombs (mC)) compared to brief pulse treated animals (2.625mC) (Sackeim et al. 2000).

Animal weight was monitored over the course of treatment. Animal weight can be indicative of the health of an animal, with excessive weight loss or weight gain denoting health problems (Metz et al. 2005). The average animal weight did not differ significantly between control and ECS treated animals on day one or the final day of ECS treatment. Nor was there a significant difference in the percentage increase in animal weight between treatment groups over the course of treatment. Over the course of ten treatments, all animals gained weight, as has previously seen in ECS treated animals (Ito et al. 2010;). Therefore, neither brief pulse nor ultra brief pulse ECS administration impacts negatively on animal weight when compared to sham treated control animal weight gain. Equally, ultra brief pulse and brief pulse ECS treated animal weight did not respond differently to ECS administration.

Therefore, ultra brief pulse ECS induces seizures as effectively as brief pulse ECS. The seizures and the animal response to the seizures induced by either pulse form are almost indistinguishable.

3.4.3 Cell Proliferation

ECS treatment is also associated with an increase in proliferating cells in the hippocampus of treated animals (Madsen et al. 2000; Segi-Nishida et al. 2008). A significant and similar increase in BrdU labelled cells, denoting newly formed cells, was seen in this study following brief pulse treatment. A similar increase was not observed in animals treated with ultra brief pulse ECS. The number of BrdU labelled cells visualised following ultra brief pulse ECS was also not significantly different from the control group.

Previous studies have repeatedly determined that the majority of proliferating cells in the dentate gyrus mature into new neurons [approximately 70-90% (Malberg et al. 2000; Scott et al. 2000)]. While it has been reported by some authors that hippocampal neurogenesis is required for the behavioural effects of antidepressants (Santarelli et al. 2003), an increase in cell genesis is not necessarily associated with the therapeutic effects of antidepressant treatments (Holick, 2008; Bessa et al. 2009). However, in combination with the behavioural and molecular results obtained in this study, the greater number of

proliferating cells found following brief pulse ECS treatment suggests it is a more effective antidepressant treatment than ultra brief pulse ECS.

A limitation of this study is the small sample size. A greater number of samples may have revealed the increase in cell proliferation seen following ultra brief pulse treatment to be significant, although it was not found to be so in this study. However, the number of samples in this study was sufficient to detect robust increases in cell proliferation following brief pulse ECS. Although ultra brief pulse ECS administration induces a seizure similar to brief pulse ECS, it does not have the same effect on hippocampal cell proliferation.

3.4.4 BDNF

Neurotrophins, in particular BDNF are implicated in the mechanisms of action of antidepressant treatments. The role of neurotrophins in depression and antidepressant action has been reviewed in detail (Schmidt et al. 2007; Castren, et al. 2009). ECS has repeatedly been shown to increase the expression levels of BDNF protein in the hippocampus (Nibuya et al. 1995; Altar et al. 2003).

In this study, brief pulse ECS also induced a significant change in the level of BDNF protein being expressed in the hippocampus. This robust increase was not seen following ultra brief pulse ECS administration. Significantly increased levels of hippocampal BDNF have been found in the hippocampus up to seven days after ECS treatment compared to sham treatment (Li et al. 2007). However, a similar increase in frontal cortex expression of BDNF protein was not found. ECS has previously increased BDNF protein levels in the frontal cortex (Balu et al. 2008; Jacobsen et al. 2004) up to three days after the final ECS administration (Altar et al. 2003). The time delay between the end of ECS treatment and animal sacrifice may explain why no increase was seen in frontal cortex BDNF protein levels in ECS treated animals.

However, in both the hippocampus and frontal cortex, ultra brief pulse ECS treated animals expressed lower levels of BDNF protein than brief pulse treated animals. As increases in BDNF expression following antidepressant treatment are associated with the efficacy of treatment (Balu et al. 2008) this suggests that brief pulse ECS is a more effective antidepressant treatment.

3.4.5 Forced swim test

The forced swim test, developed by Porsolt in 1977, is a robust method to test the efficacy of antidepressant treatments. The animals try and escape from the swim tank on the training day but learn that this is not possible, before giving up and becoming immobile. Immobility behaviour is thought to reflect despair (Fedorova et al. 2006). On the test day, 24 hours later, this behaviour is more pronounced. Antidepressant treatment generally reduces the time spent immobile. The test is relatively selective in identifying clinically effective antidepressant drug therapies and ECS (Porsolt et al. 1977).

Brief pulse width ECS has been repeatedly shown to reduce the immobility time of treated animals compared to sham treated controls (Porsolt et al. 1977; Tsutsumi et al. 2002; Maayan et al. 2005; Li, 2007). In this study, we saw a similar result. Once again, brief pulse ECS has an antidepressant effect on treated animals, with these animals spending significantly less time immobile than control animals. Ultra brief pulse ECS administration does not appear to act as an effective antidepressant treatment. There is no significant decrease in animal immobility time when compared to sham or brief pulse treated animals.

While immobility is the traditional measure of antidepressant efficacy in the forced swim test, active behaviours such as swimming, climbing and diving can also be informative. Dive behaviour was recorded but because it occurred so rarely in the test it was difficult to interpret reliably. Active behaviours have previously been used to detect nuances between the effects of different antidepressant drug classes in the forced swim test (Detke et al. 1995; Cryan et al. 2002). However, there is no statistical difference in these behaviours following treatment with brief pulse and ultra brief pulse ECS. Instead, brief pulse ECS has greater efficacy than ultra brief pulse ECS as determined by immobility times.

3.4.6 Water plus maze

The hippocampus is involved in memory formation and consolidation and is particularly implicated in depression as reviewed (Campbell et al. 2004a). The water plus maze was used to measure hippocampal dependent spatial memory in the rat (Metz et al. 2005). This maze hybridises the effective learning of the Morris water maze with the more complex spatial learning strategies associated with the radial maze (Diamond et al. 1999). The water maze exploits the natural swimming ability of the rat. Also, the animal is motivated by immersion in the water, removing the need to deprive them of food or water. Integration of the four-arm maze with the water maze gives a more accurate means of

measuring memory by allowing errors to be scored, leading to fewer options when interpreting the results (Diamond et al. 1999; Alamed et al. 2006). Substantially different results may be obtained following testing in the hybrid maze than in the radial maze or Morris water maze alone as discussed by Conrad (2010) following a review of the literature on spatial learning and memory testing in a chronic stress model. A similar strategy of combining these maze tests have been successfully used by other authors (Diamond et al. 1999; Morgan et al. 2000).

The water plus maze tests the animal's ability to learn the location of the maze's platform and form a spatial reference memory for the task. Reference memory is defined here as memory for features that do not vary over multiple trials. A probe trial was conducted twenty-four hours after the last training session to ensure reference memory and not memory from the last training session was measured.

Previous studies have demonstrated that ECS treatment can have a significant impact on memory. Studies investigating the effects of ECS on memory have previously detected deficits in spatial learning in the water maze (Khan et al. 1994; Dong et al. 2010) and the radial arm maze (Beatty et al. 1985; Maki et al. 1985) following treatment with ECS.

In this study, the animals treated with brief pulse and ultra brief pulse ECS acquired the platform less often than the control animals. This suggests some impairment in their ability to recall the platform location following ECS treatment. The difference in platform acquisition levels between control and treated animals was not statistically significant. This may be due to the delay between ECS treatment and behavioural testing. Testing of the animals' deficit in spatial learning began one week after treatment. However, deficits in spatial learning up to two months after ECS treatment have been observed previously (Lukoyanov et al. 2004).

Although animals treated with ultra brief pulse ECS receive a smaller overall charge than that administered to brief pulse treated animals, it is sufficient to impair memory to almost the same extent as brief pulse ECS (brief pulse treated animals obtained the platform on average 18 times out of 30 trials, ultra brief pulse animals 17 out of 30 trials). There was no statistically significant difference between platform acquisitions by brief pulse and ultra brief pulse treated animals. Therefore, while a chronic course of ECS impacts negatively on the animals' ability to navigate reference memory tasks compared to sham treatment (but not significantly), ultra brief pulse ECS does not appear to offer an improvement over brief pulse ECS.

The velocity of the animals in the water plus maze was also measured on the training and probe days. As expected, velocity increased over time from training day 1 to training day 3 as the animals' familiarity with the maze increased. There was no significant difference in velocity between treated and control animals. ECS treatment did not impair the animals' ability to reach the platform or navigate the maze in comparison to sham treated animals. There was also no difference between the velocity of brief pulse and ultra brief pulse treated animals. A similar result was seen on the probe day with velocity measures for control and ECS treated animals indistinguishable between groups.

The length of time animals spent in the decision zone (Figure 3-20) decreased from training day 1 to training day 3 as the animals learned the location of the platform which allowed them to escape from the water. The amount of time control, brief pulse and ultra brief pulse treated animals spent in the decision zone did not differ. On the probe day, animal times in the decision zone did not significantly differ. However, ECS treated animals did spend more time in the zone than control animals. This suggests that it took the ECS treated animals longer to determine which arm contained the platform, although not substantially so. There was no difference between brief pulse and ultra brief pulse ECS in this measure.

3.4.6.1 Water plus maze: summary

At all measures taken during the water plus maze, the effects of brief pulse and ultra brief pulse closely mimic each other. However, it is also important to note that there is no statistically significant difference between ECS treatment and sham treatment on the probe day with regards to platform acquisition, velocity to platform and time spent in the decision zone. Despite this, there were interesting patterns in the results. For example, platform acquisition by ECS treated animals was lower than for control animals, as would be expected if ECS treatment induced cognitive impairment. Equally, ECS treated animals spent more time in the decision zone, indicating a slower response in determining which arm the platform lay in, although these responses were not statistically significant compared to controls. There may be several explanations for this. A larger number of animals may be required to conclusively determine the effects of brief pulse and ultra brief pulse ECS treatment on hippocampal dependent memory in the water plus maze. Also, the time delay between the end of ECS treatment and the start of training may have allowed for sufficient recovery of ECS induced cognitive deficits so that any deficiencies were not detected as being significant by the water plus maze. There appears to be no improvement

to the cognitive side effects associated with ECS by treating animals with ultra brief pulse rather than brief pulse parameters.

3.4.7 Conclusion

Brief pulse and ultra brief pulse ECS induce seizures that are similar in both duration and quality. However, their effects differ quite significantly. Brief pulse ECS is a powerful inducer of cell proliferation and BDNF protein expression in the hippocampus. Behaviourally, brief pulse ECS treatment results in a solid antidepressant response in the forced swim test. Ultra brief pulse ECS does not appear to be as effective an antidepressant treatment. Ultra brief pulse ECT was proposed to be therapeutically comparable to brief pulse ECT but with less cognitive side effects (Loo et al. 2008). Behavioural studies revealed no difference in hippocampal dependent memory deficits between brief and ultra brief pulse treated animals, although all ECS treated animals showed impairment compared to control animals.

A limitation of this study is the sample size, which may cloak the antidepressant effects and changes induced by ultra brief pulse ECS. However, as brief pulse ECS was demonstrated to induce a robust antidepressant effect, ultra brief ECS seems to be a less effective antidepressant treatment. This may be due to the lower levels of energy delivered during ultra brief pulse administration compared to brief pulse ECS. A single parameter was changed in this study to determine whether reducing pulse width would prove beneficial in reducing the cognitive side effects of ECS, while maintaining its therapeutic efficacy. Future work investigating the effects of ultra brief pulse width, in combination with changes to other ECS parameters (duration of seizure, amplitude, pulses delivered per second) are required, to determine if the potential cognitive benefits of ultra brief pulse ECS outweigh its current lack of antidepressant efficacy.

Analysis of the hippocampal proteome following electroconvulsive stimulation

4.1 Introduction

Neuropsychiatric disorders are frequently investigated using proteomic techniques (Martins-de-Souza et al. 2010). A study of the proteome, the pool of proteins expressed by an organism, provides insight into the wide range of protein changes occurring in the brain at a given time. The effect of a treatment or disease state on the expression of proteins involved in such divergent activities as metabolism, cellular processes and signalling can be quantified. Therefore, proteomic analysis of antidepressant-related effects may be particularly informative due to the multi-faceted nature of these treatments and the underlying disorder (Taurines et al. 2011; Sullivan et al. 2000).

4.1.1 Proteomic studies of depressed patients

Proteomic analysis has revealed changes in the expression of a number of proteins in human frontal cortex samples from patients with psychiatric disorders (Johnston-Wilson et al. 2000). Protein changes that were unique to depressed patients or shared by patients with depression, bipolar disorder and schizophrenia were found. These included proteins involved in energy metabolism, cell differentiation and nervous system development. The other brain region that has been investigated in depressed patients is the anterior cingulate cortex (Beasley et al. 2006). Certain proteins were common to both the frontal cortex and anterior cingulate cortex, although some were regulated in different directions. For example, dihydropyrimidinase related protein 2, a member of a family of proteins involved in axonal guidance and neuronal differentiation (Quinn et al. 1999) was upregulated in the anterior cingulate cortex and downregulated in the frontal cortex. Recently, changes in the cerebrospinal fluid proteome of patients with depression were investigated (Ditzen et al. 2011). Identified proteins were involved in neuronal development and protection (e.g. neurofilament heavy polypeptide and neuronal cell adhesion molecule) and sleep regulation (e.g. prostaglandin-D2 synthase) which may have potential roles in the pathogenesis of depression. Overall, few proteomic studies have looked at the effects of depression or antidepressant treatment in the human brain. Due to the complex nature of depression and the influence of external factors, such as drug treatment, the study of the effects of depression and antidepressants on the rodent brain proteome (Martins-de-Souza et al. 2010).

4.1.2 Proteomic studies of the effects of antidepressant treatments

The effects of antidepressant treatments on the rodent hippocampal proteome have been thoroughly studied. Well-established antidepressants including fluoxetine (Carboni et al. 2006b; Guest et al. 2004; Khawaja et al. 2004), venlafaxine (Khawaja et al. 2004) and paroxetine (McHugh et al. 2010) as well as novel compounds with potential antidepressant properties including CRF1 and NK1 receptor antagonists (Carboni et al. 2006b) and the substance P antagonist L-000760735 (Guest et al. 2004) have been investigated. The herbal antidepressant treatments St. Johns Wort and xiao-yao-san are clinically effective (Kasper et al. 2006; Zhang et al. 2007) and their effects on the proteome of hippocampal cells has been studied (Pennington et al. 2009).

The effect of exercise on the rat hippocampal proteome (Chen et al. 2007; Kirchner et al. 2008) has also been studied as exercise is an effective treatment for mild or moderate depression (Rethorst et al. 2009; Martinsen et al. 1985). Similar to studies of antidepressant pharmacotherapies, a wide range of proteins were found to be differentially expressed following exercise.

The most commonly detected proteins of interest following antidepressant treatment were involved in metabolism (e.g. alpha enolase, which underwent a 1.1 to 1.9 fold change in expression; Pennington et al. 2009) neural plasticity and the cytoskeleton (e.g. vimentin, which underwent a 1.4-2.86 fold change in expression; alpha synuclein, a 0.63 expression change (Pennington et al. 2009; McHugh et al. 2010). The consistent changes in expression of proteins with plasticity and metabolic functions highlight the potential role of these networks in the action of different classes of antidepressant in the hippocampus. However, few of the changes in protein expression found following proteomic analysis have been confirmed and validated.

4.1.3 Proteomic studies of animal models of depression

The effects of animal models of depression on protein expression in the hippocampus have also been investigated. Chronic stress (Mu et al. 2007), restraint stress (Kim et al. 2007), psychosocial stress (Carboni et al. 2006a) and chronic corticosterone treatment (Skynner et al. 2006) protocols were employed to determine the effects of models of depression on the hippocampal proteome.

A role for neurogenesis and changes in pathways regulating cell morphology and cellular assembly and organisation were also suggested by these studies, implicating, for example, actin binding proteins gelsolin and fascin (fold changes of 1.17 and 1.18 to 1.28; Skynner et al. 2006) in the mechanisms of depression.

4.1.4 Proteomic studies of antidepressant treatments with animal models of depression

Studies looking at antidepressant treatment in models of depression were also conducted. A range of depression models were employed including the Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) and maternal separation administered with escitalopram (Carboni et al 2010; Piubelli et al. 2011 a, b) or nortriptyline (Piubelli et al. 2011c) and maternal separation administered with escitalopram (Marais et al. 2009). Carbohydrate and lipid metabolism, immunity proteins and proteins involved in neuronal remodelling were the most frequently modulated following antidepressant administration in animal models of depression. Again, plasticity and metabolic proteins were the most strongly implicated in antidepressant action in the proteome. The cytoskeletal proteins tubulin and actin were frequently identified, with fold changes in expression ranging from 1.3 to 2.4 and 1.6 to 1.9 respectively (Piubelli et al. 2011a; Piubelli et al. 2011c). Overall, though, few of the identified proteins have been validated.

4.1.5 Other proteomic studies of the hippocampus

While most research has focused on whole brain regions, studies looking at the proteome of hippocampal slices (McNair et al. 2006), hippocampal neurons (Piccoli et al. 2007; Dahl et al. 2003; Pennington et al. 2009) and the synaptosome proteome (Mallei et al. 2011) again implicated proteins associated with energy metabolism (e.g. pyruvate dehydrogenase underwent a 1.2 fold change in expression; Mallei et al. 2011), cellular remodelling and synaptic and neural plasticity (dihydropyrimidinase related protein 2, a 1.18 to 2.04 fold change in expression; Pennington et al. 2009).

Proteomic studies to date, therefore, strongly support a function for proteins that are involved in neural plasticity in both depression and the response of antidepressant treatments. Although hundreds of proteins have been identified as potentially being involved in the mechanisms of antidepressant action following proteomic analysis, the roles of few of these proteins have been confirmed or validated.

4.1.6 ECT, ECS and the proteome

The effects of different classes of antidepressant drug treatments and different forms of antidepressant treatment have been studied, under a range of conditions, on the hippocampal proteome. However, the proteomic effects of electroconvulsive therapy (ECT), the most effective treatment currently available for severe depression (UK ECT Review Group, 2003) have not yet been extensively investigated.

A single targeted study looked at the effect of anaesthetic administration on a range of proteins in serum in patients receiving ECT (Stelzhammer et al. 2011). It found that changes in several proteins that had been associated with ECT administration (e.g. S100 calcium binding protein B) were instead an effect of the anaesthetic that was administered prior to ECT.

A single study has applied the animal model of ECT, electroconvulsive stimulation (ECS), to the study of the proteome (Lee et al. 2009). However, this study looked at the entire rat brain proteome using silver staining techniques. Significant, whole brain protein changes following ECS may not be informative of the mechanisms of ECS. ECS treatment is known to induce cellular and molecular changes in specific brain regions such as the hippocampus and frontal cortex (Scott et al. 2000; Jacobsen et al. 2004). Subtle, low level protein changes induced by ECS in these brain regions of interest may not be detected in whole-brain proteomic analysis. Also, silver staining techniques are not as sensitive as fluorescent minimal labelling methods (Gharbi et al. 2002).

4.1.7 Commonly expressed proteins found in proteomic studies

However, it must be noted that certain individual proteins and families of proteins are repeatedly identified in human and rodent proteomic studies regardless of the tissue type or experiment (Petrak et al. 2008). These include proteins such as dihydropyrimidinase-like protein-2 which was identified in 17% of all proteomic studies measured (Petrak et al. 2008) as well as in many of the antidepressant and depression related studies discussed above (Mallei et al. 2011; Pennington et al. 2009; Piubelli et al. 2011a; Johnston-Wilson et al. 2000). None the less, dihydropyrimidinase-like protein-2 is implicated in neuronal maturation and plasticity (Crews et al. 2011; Hensley et al. 2011) and is therefore a valid protein of interest in depression and antidepressant related proteomic studies.

4.1.8 Aims of the study

Therefore, the purpose of this study was to analyse the effects of ECS on the hippocampal proteome using 2-dimensional difference in gel electrophoresis (DiGE) to detect changes in protein expression. In line with studies conducted to examine the effects of antidepressant pharmacotherapy, a comprehensive study of the protein changes induced by ECS in the hippocampus shall be undertaken.

4.2 Methods

4.2.1 Study design and animal treatment

Male Sprague-Dawley rats (150g-200g) were housed four per cage under a 12hr light-dark cycle (lights on 08:00) with food and water available ad libitum throughout the experiment. The experiments were conducted in accordance with EU council directive 86/806/EEC and guidelines of the Bioresources Animal Ethics Committee, Trinity College Dublin. Animal housing was randomised so that treated and sham treated animals were housed in each cage. Animals were divided into different groups in order to identify changes induced by either a single (Acute) or repeated course (10 sessions; Chronic) of ECS as well as any longer-lasting changes apparent four weeks after completing a course of ECS (Chronic + 4) (Figure 4-1).

Chronic, but not acute ECS administration is required to induce many of the changes associated with treatment (Malberg et al. 2000). Therefore, the proteomic changes induced following chronic ECS treatment will be of particular interest. Although an acute course of ECS is not as effective, insight into the proteins that are induced following a single ECS may be informative of the initial effects of ECS and the proteins that are subsequently induced chronically. Equally, the proteins that are expressed four weeks after the final ECS treatment may be of relevance in explaining the long-term effects of ECS. For these reasons, the Acute, Chronic and Chronic + 4 week effects of ECS on the hippocampal proteome were studied.

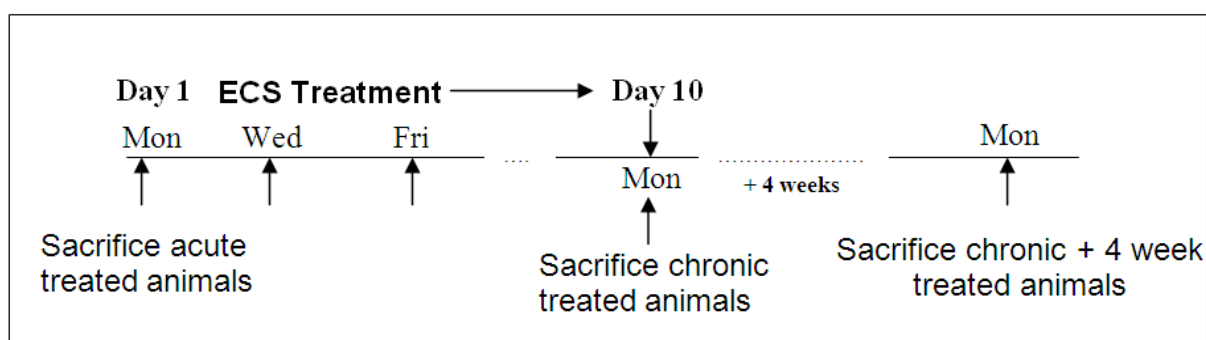


Figure 4-1 ECS treatment course timeline for proteomic studies

All animals were administered ECS under the following parameters:

100 pulses per second; 0.5ms pulse width, 0.7s duration; 75mA. Each study contained two groups of animals; seven animals received ECS treatment, seven animals were sham treated.

Acute ECS treatment: animals were treated with one session of ECS (Figure 4-1) at the same time as animals following chronic and chronic + 4 weeks of ECS treatment, that is, on the first day of ECS treatment for these animals. The animals were sacrificed four hours after ECS treatment.

Chronic CS treatment: animals were treated with 10 sessions of brief pulse ECS (Figure 4-1). The animals were treated every Monday, Wednesday and Friday morning. The animals were sacrificed four hours after the last ECS treatment.

Chronic + 4 weeks of ECS treatment: These animals received 10 sessions of ECS treatment. The animals were treated every Monday, Wednesday and Friday morning. Following the final ECS treatment, the animals were housed for a further four weeks under the same conditions (Figure 4-1). No other treatments were administered. The animals were sacrificed four weeks after the last ECS treatment.

4.2.2 Experimental procedures

Animals were sacrificed four hours after ECS for the Acute and Chronic studies and four weeks after ECS for the Chronic +4 week study. The hippocampus was dissected out and prepared for proteomic analysis as described in section 2.2.13-2.2.16.

Briefly, hippocampus samples were homogenised in 1ml of DiGE compatible lysis buffer (9.5M urea, 2% CHAPS, 20mM Tris, pH8.0). Each sample was vortexed and centrifuged for 10 min at 1500 rpm. The protein concentration was determined using the Bradford protein assay.

A pool of all samples, the internal standard, was labelled with the fluorescent Cy3 labelling reagent while all hippocampal samples were labelled with fluorescent Cy 5 Cydye (GE Healthcare, Ireland). 50µg of protein was labelled with 400 pmol of CyDye for 30 min at 4°C. The reaction was stopped with 1µl of 10mM lysine for 10 min at 4°C. Each sample and the pooled sample were combined with dilution buffer (9.5M urea, 2% CHAPS, 2% DTT, 1.6% Pharmalyte) in a ratio of 1:1:2. This solution was brought to a final volume of 450µl in rehydration buffer (8M urea, 0.5% CHAPS, 0.2% DTT, 0.2% Pharmalyte, bromophenol blue) and used to rehydrate immobilised pH 4-7 gradient (IPG) strips (GE Healthcare, Ireland) overnight at room temperature.

IPG strips underwent isoelectric focusing on an Ettan IPGphor II (GE Healthcare, Ireland) for 75000 Vh at 50 μ A per strip. Following focusing, the IPG strips were equilibrated for 15 min with equilibration buffer (6M urea, 30% glycerol, 2% SDS, 0.05M Tris-HCl) containing 1% DTT, then in equilibration buffer with 2.5% iodoacetamide.

Proteins were separated in the second dimension by SDS-PAGE using 1mm, 12% gels overnight at 1W per gel (Ettan Dalt 12; GE Healthcare, Ireland). The gels were scanned using a Typhoon 9400 scanner (GE Healthcare, Ireland) and analysed using Progenesis SameSpots software (Nonlinear Dynamics). Univariate (one-way ANOVA) analysis and principal component analysis (PCA) were conducted using Progenesis SameSpot software (NonLinear Dynamics). Multivariate analysis (partial least squares-discriminant analysis) was conducted using Unscrambler X software (CAMO, Inc.).

To identify the protein constituents of target spots where expression levels were found to be significantly altered by ECS, 200-800 μ g of the pooled protein samples (internal standard) was separated on 1.5mm thick preparative gels that were run as described above. The gels were stained using mass spectrometry compatible silver stain (PlusOne, GE Healthcare, Ireland) or Coomassie blue stain (Sigma Aldrich, Ireland). Selected target spots were destained (30mM potassium ferricyanide, 100mM sodium thiosulphate) and trypsin digested overnight at 37°C. Samples were dried under centrifuge vacuum. The samples were resuspended in 2 μ l of 0.1% formic acid for analysis by mass spectrometry.

The samples were run on a Thermo Scientific LTQ Orbitrap XL mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system, as detailed in the methods section 2.2.20.2. For peptide data analysis MS/MS data was searched using SEQUEST (BioWorks 3.3.1, Thermo Finnigan, UK) software against the Uniprot/Swiss-Prot rat database (September 2010 release version 74).

4.2.3 Data analysis

Figure 4-2 summarises the statistical methods used to determine the protein spots whose expressions are significantly altered by ECS. Univariate and multivariate statistical techniques were carried out to gain a comprehensive understanding of the expression changes seen following treatment. Both forms of analysis are frequently used to evaluate proteomic datasets in an effort to fully mine the data (Karp et al. 2005b; Pedreschi et al. 2009).

Univariate analysis was applied to the proteomic data to determine differences in protein spot expression levels in ECS treated samples compared to controls. A one-way ANOVA was applied to the data, by Progenesis SameSpot software (NonLinear Dynamics), to detect these expression changes. Progenesis SameSpot software (NonLinear Dynamics) addressed the multiple testing problem by calculating the false discovery rate (FDR), the probability of a result being a false positive.

However, comparing expression changes in a single protein spot following ECS to a sham treated control does not give a complete analysis given the complex interactions that may be found among proteins in the dataset. Therefore, a multivariate analysis technique, partial least squares-discriminant analysis (PLS-DA) was also applied to the data. PLS-DA allows for data exploration using a series of statistical plots (Figure 4-2). Principal component analysis (PCA), a non-supervised technique, was also applied by Progenesis SameSpots software (NonLinear Dynamics) to allow the detection of outliers in the data as well as the visualisation of ECS treated sample and control gel clustering. The statistical methods used in this study are described in greater detail below.

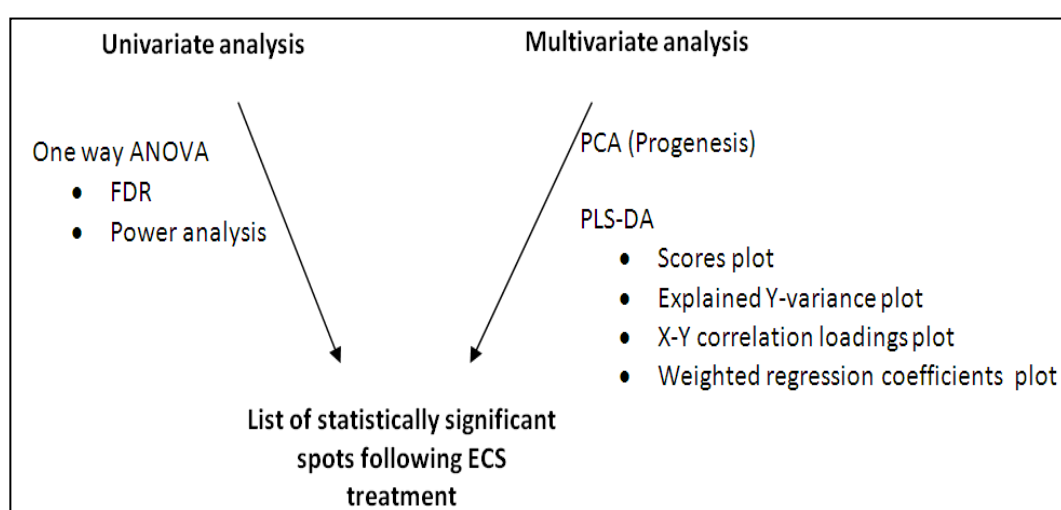


Figure 4-2 Analysis of proteomic data FDR false discovery rate; PCA principal component analysis; PLS-DA partial least squares discriminant analysis; ECS electroconvulsive stimulation

4.2.4 Univariate analysis of protein spots

4.2.4.1 One way ANOVA

To determine whether a protein spot's expression was significantly different, a one-way ANOVA was conducted by Progenesis SameSpot software (NonLinear Dynamics), ECS treated and control normalised expression values were compared for each spot. Spots with a value $p \leq 0.05$ were selected as being statistically significant (Pedreschi et al. 2007; Di Carli et al. 2011). Other statistical measures were provided by Progenesis SameSpot (NonLinear Dynamics), including the FDR and the study power in accordance with the software manual guidelines (NonLinear Dynamics I, 2011; NonLinear Dynamics II, 2011).

4.2.4.2 Multiple testing problem

Due to the large number of spots being tested in each study, the multiple comparisons testing problem was allowed for (Karp et al. 2005b; Pedreschi et al. 2009). As the p-value was set at 0.05, there was a 5% chance that the significant difference in spot volume for any given spot was a false positive. A 5% chance of a result being a false positive may lead to an unsatisfactory number of false positives when multiple tests are conducted.

To correct for this, the false discovery rate (FDR) method was applied. Adjusted p-values were calculated for each test (i.e. treated-control spot comparison) so that the numbers of false discoveries in the tests that resulted in significance were controlled. Therefore, a FDR adjusted p-value (q-value) of 0.05 meant a 5% chance that a significant result may be a false positive (Table 4-1) (Karp et al. 2005b; NonLinear Dynamics I, 2011).

4.2.4.3 Power analysis

Power analysis was also calculated for each spot (Karp et al. 2005b). This value was a measure of the probability of finding a real difference if it exists. The power result showed the probability of finding a spot to be significant if the difference between group means was actually a true difference. The number of samples present in a study and the ANOVA value of the spot contributed to the power of the study (Karp et al. 2005b; NonLinear Dynamics II, 2011).

4.2.5 Multivariate analysis of hippocampal spots

4.2.5.1 Principal component analysis (PCA)

All gels were analysed using PCA (Marengo et al. 2006), applied by Progenesis Samespot software (as detailed in NonLinear Dynamics III, 2011). Spot expression levels were used to determine the principal axes of expression variation (NonLinear Dynamics III, 2011). The expression data were plotted in principal component space. Principal components are based on axes drawn through the data that represent the direction of maximum variation in the data.

Thus, gel samples were separated according to expression variation. This allowed outlying gels to be identified. All gels were represented in two-dimensional space so that spots that did not show much variation appeared closer together on the PCA plot and spots that showed greater variation were further apart on the plot. Each gel in a study was plotted based on expression variance as determined by spot expression values across the gels (Jolliffe, 2002; NonLinear Dynamics III, 2011).

4.2.5.2 Partial least squares-discriminant analysis (PLS-DA)

PLS-DA was the multivariate method employed to analyse the data using Unscrambler X (CAMO) software (Marengo et al. 2008; Karp et al. 2005b; Kjaersgard et al. 2006; Norden et al. 2005). PLS-DA is a form of regression; therefore, its basic principal is to find out how predictor “X” variables explain variation in response “Y” variables. Partial least squares models the X and Y datasets simultaneously to find the latent (hidden) variables in X that will best predict the latent variables in Y (see Figure 4-3).

PLS-DA is the use of PLS for classification. It uses one response variable (Y) which codes for class membership (+1 for treated and -1 for control). The “X” variables are the individual protein spot values (section 2.2.17). All analysis was conducted according to the manufacturer’s guidelines that are detailed in a series of Unscrambler X manuals (CAMO I, 2011; CAMO II, 2011; CAMO III, 2011; CAMO IV, 2011).

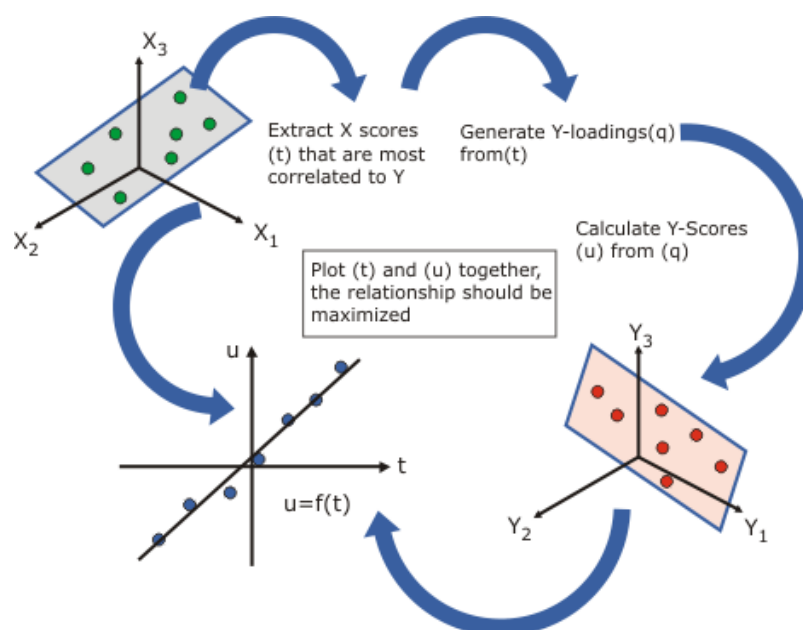


Figure 4-3 Description of theory of PLS (CAMO I, 2011)

t-scores are coordinates of data-points in the X-space. They capture the part of the structure in X that best predicts Y. u-scores are part of the structure in Y which is best explained by X. The relationship between t- and u-scores summarises the relationship between X and Y. q-loadings express the direct relationship between the Y-variables and the t-scores. Scores and loadings are used to express how samples and variables are projected along model components (CAMO I, 2009).

PLS-DA is initially conducted using all variables (X-variables) to create a model of the data. Variables that contributed to non-structured variation (noise) were removed. This was done by selecting variables that were identified as being significant and creating a new model. The Unscrambler X software (CAMO) conducts PLS-DA based on algorithms derived from the Martens' test (Martens and Martens, 2000) to automatically mark non-significant variables.

Therefore, the number of spots present in each study was reduced until the best model to explain variation in the data was drawn (CAMO I, 2011). PLS-DA gives a model with an equation expressed by the regression coefficients (β -coefficients). Regression coefficients showed the relative importance of variables. Significant spots were identified based on a value of $p \leq 0.05$ assigned to the β -coefficients.

The PLS-DA models were investigated and diagnosed using a series of plots that allowed for accurate interpretations of the models. The different plots demonstrate different aspects of the model (explained in detail in: CAMO II, 2011; CAMO III, 2011; CAMO IV, 2011). The plots used included: a scores plot, explained Y-variance plot, X-Y correlation loadings plot and a weighted regressions coefficients plot. The plots allow the visualisation of the data and the detection of outlying factors or variables that do not contribute significantly to explaining variation in the data following ECS treatment. These variables can be removed and the data remodelled so that significant variables can be identified. The scores plot shows which factors (ECS or sham treated gels) are outliers and can be removed. The X-Y correlation loadings plot and weighted regressions coefficients plot highlight the individual X-variables that contribute significantly to variation in the data. The explained Y-variance plot shows the impact of removing non-significant variables on the level of explained variance in the response variable Y.

4.2.5.2.1 Scores plot

A scores plot is a two-dimensional scatter plot of scores for two factors, representing the treated and control gels. The scores plot shows patterns in the factors. The scores plot summarises variation in the data and is used to interpret differences and similarities in the data. Factors (ECS or sham treated gels) clustered together on the plot are similar while factors at greater distances apart are less similar (Figure 4-10).

The Hotelling T^2 ellipse is derived from the Hotelling T^2 statistic which is the multivariate analogue of the univariate t-distribution (Sauers, 1997). The Hotelling T^2 ellipse is displayed for models (particularly the scores plot) as a way to detect outliers. Factors that fall outside the ellipse are considered to be outliers (CAMO II, 2009).

4.2.5.2.2 Explained Y-variance plot

This plot shows how much variation is described by each component in the PLS-DA model (Figure 4-11). In this plot, the calibration and validation data are compared. The calibration dataset contains all the data used to compute the model. The validation dataset is computed from full cross validation, where one object is left out and the value of the left out object is predicted. This is repeated until all objects are individually left out and their predicted values computed. The validation variance is calculated from a combination of the prediction residuals.

The closer the explained calibration variance and the explained validation variance curves are, the more representative the model is of the data. Models with a high percentage of total explained variance explain most of the variation in Y.

4.2.5.2.3 X-Y correlation loadings plot

This plot is most useful for interpreting the relations between variables as the percentage of explained variance, as indicated by ellipses, can be seen in the plot (Figure 4-12). The Hotelling T^2 outer ellipse indicates 100% explained variance. The Hotelling T^2 inner ellipse indicates 50% explained variance. Variables close to each other have a high positive correlation. Variables in diagonal quadrants tend to be negatively correlated. The variables in the centre are poorly explained and cannot be readily interpreted. The X-Y correlation loadings plot is useful for interpreting patterns and structure in X and Y.

4.2.5.2.4 Weighted regression coefficients

This plot is used in combination with the correlation loadings plot to determine which variables influence the regression coefficient profile the most (Figure 4-13). The weighted regression coefficients provide information about the importance of the X-variables. Variables with a large regression coefficient have a larger role in the regression model.

4.3 Results

4.3.1 Cydye labelled gels

Following protein separation, all gels were scanned using a fluorescent scanner (Typhoon 9400, GE Healthcare, Ireland) and the images were uploaded and manipulated as discussed in section 2.2.16. High quality protein sample labelling and gel running were required for accurate analysis of the gels by Progenesis SameSpot software (NonLinear Dynamics) for DiGE analysis.

Representative raw images of samples labelled with Cy5 (Figure 4-4) and the internal standard labelled with Cy3 (Figure 4-5) are shown. The scanned images of the Cy3 and Cy5 fluorescently labelled samples were overlaid (Figure 4-6) and converted to black/white images (Figure 4-7) for analysis using Progenesis SameSpot software (NonLinear Dynamics).

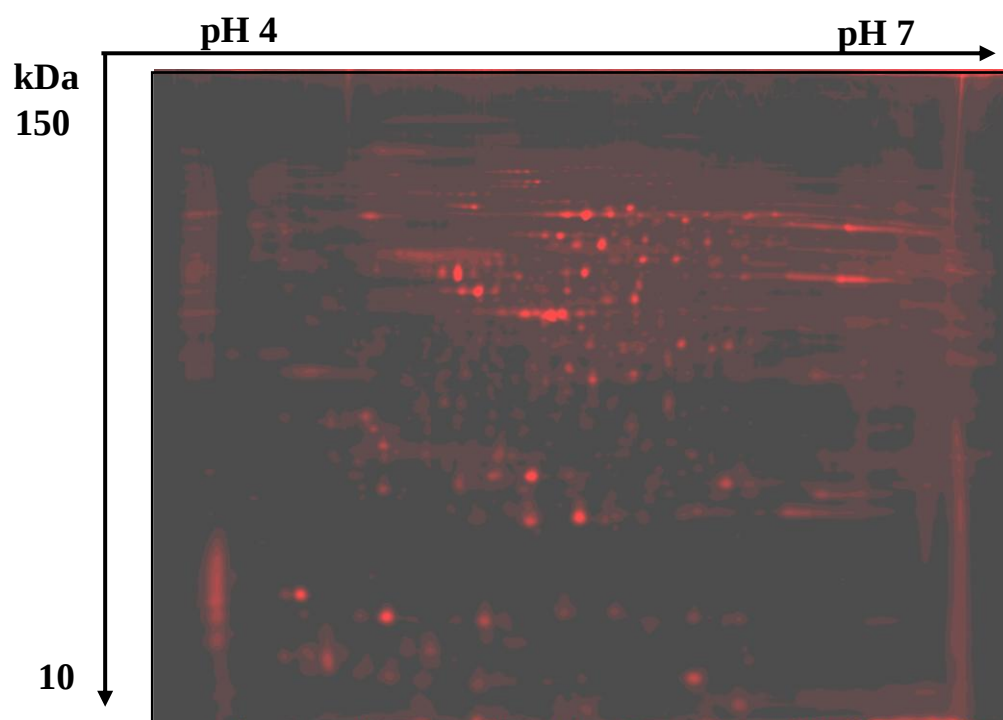


Figure 4-4 Representative fluorescent scan of Cy5 labelled hippocampal sample

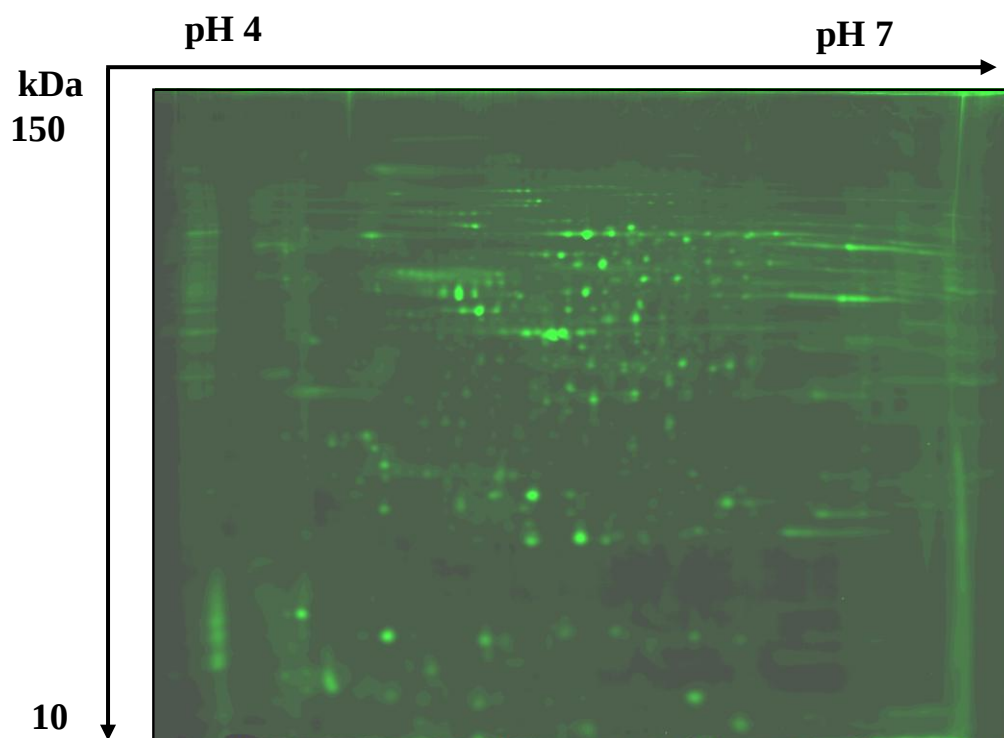


Figure 4-5 Representative fluorescent scan of Cy3 labelled internal standard

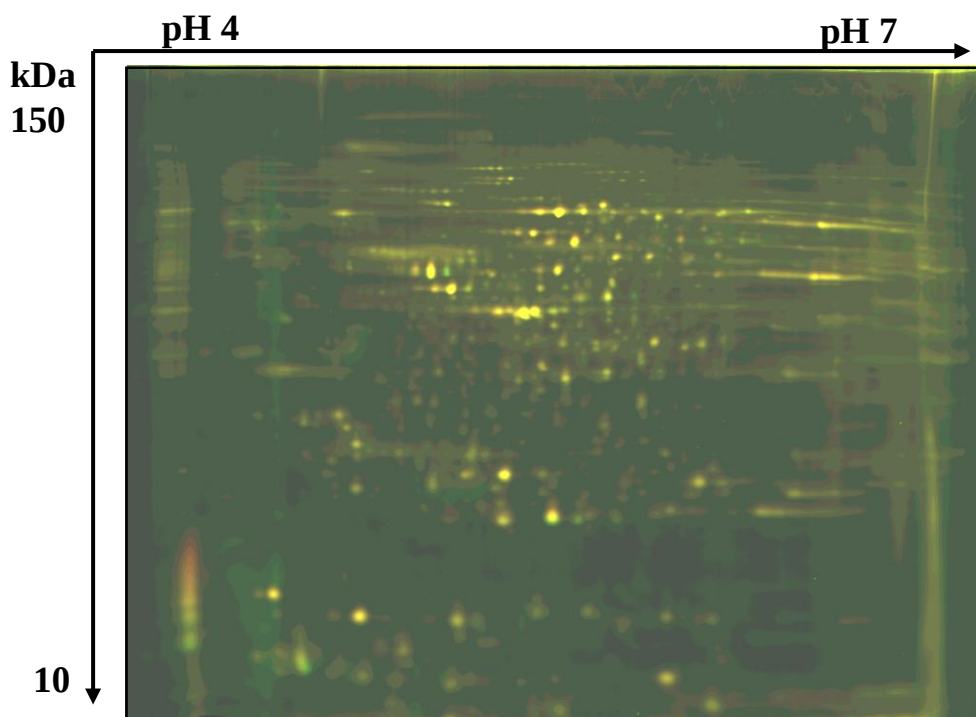


Figure 4-6 Overlay of Cy3 and Cy5 fluorescently scanned gels (representative)

The internal standard containing a pool of all samples visualised with the hippocampal sample that is run concurrently on the gel.

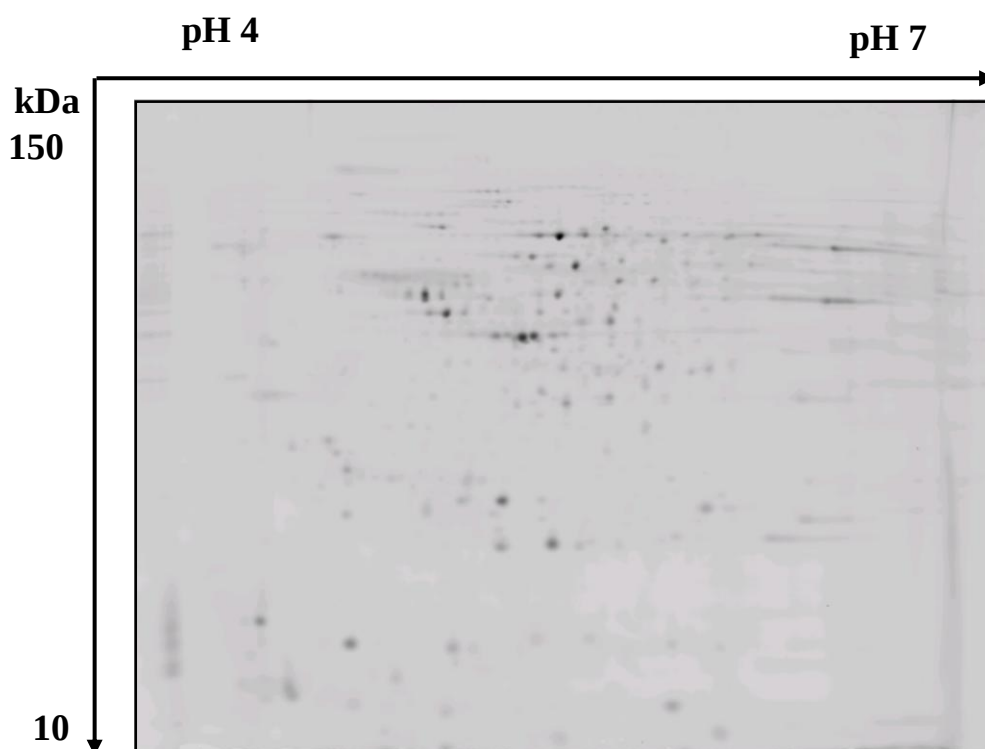


Figure 4-7 Black/white conversion of fluorescently labelled gels (representative)

The fluorescent images are converted to black/white images which are analysed by Progenesis SameSpots software (NonLinear Dynamics).

4.3.2 Hippocampus: Analysis of the effects of acute ECS treatment

4.3.2.1 Univariate analysis

The univariate results table (Table 4-1) includes the p-value associated with the one way ANOVA comparison of treated and control ECS samples. The average normalised volume, the fold change between spots, the q-value, which controls for the FDR and the power analysis value, are also included in Table 4-1.

Figure 4-8 shows the location, on a representative acute gel, of the significant spots detected by univariate analysis in Progenesis SameSpot software (NonLinear Dynamics).

The PCA conducted in Progenesis SameSpot software (NonLinear Dynamics) was used to detect outlying samples and patterns of clustering in the data. Although it is a multivariate technique, it was applied by Progenesis SameSpot software (NonLinear Dynamics) and was used to detect outlying gels that could skew the data. The PCA plot, as discussed above, is shown (Figure 4-9).

Table 4-1 List of significantly differentially expressed spots in the hippocampus following treatment with acute ECS, detected by univariate analysis

The table lists the standard spot numbers, ranked according to p-value, based on ANOVA results. The fold change, calculated from the average of the normalised volumes of each spot from treated and control samples are included in the table. The calculated power value and the q-value, a measure of FDR, are also listed in the table.

Hippocampus: acute treatment result					Average normalised volumes	
Spot #	Anova (p)	q Value	Power	Fold change	Acute treated	Acute control
947	0.000549	0.302094	0.989399	1.4	0.74	1.064
460	0.002106	0.328589	0.946721	1.2	1.179	0.961
516	0.002834	0.328589	0.928588	1.3	0.998	0.761
287	0.003795	0.328589	0.906621	1.2	1.05	1.262
918	0.004255	0.328589	0.896808	1.3	1.011	1.267
193	0.004383	0.328589	0.894158	1.3	1.096	0.868
61	0.006645	0.328589	0.851758	1.2	0.948	0.771
801	0.007244	0.328589	0.841731	1.1	1.025	0.909
575	0.008509	0.328589	0.821885	1.3	1.024	0.818
953	0.008869	0.328589	0.816524	1.2	0.964	0.783
669	0.011862	0.328589	0.776243	1.3	1.061	1.415
1298	0.013381	0.328589	0.758151	1.5	0.853	1.305
475	0.014271	0.328589	0.748171	1.1	1.061	0.927
564	0.015299	0.328589	0.737138	1.2	1.066	0.864
848	0.016184	0.328589	0.728037	1.2	1.054	0.882
468	0.016234	0.328589	0.727531	1.3	1.102	0.833
1088	0.017967	0.328589	0.710686	1.3	1.136	0.9
479	0.018044	0.328589	0.709965	1.2	1.075	0.878
194	0.018238	0.328589	0.708161	1.1	1.071	0.94
691	0.019524	0.328589	0.696516	1.2	1.067	1.307

238	0.020987	0.328589	0.683928	1.2	1.055	0.848
647	0.021124	0.328589	0.682779	1.4	1.396	0.988
330	0.022717	0.328589	0.669849	1.3	1.025	0.796
223	0.023173	0.328589	0.666278	1.4	1.161	0.824
175	0.024046	0.328589	0.65958	1.4	0.956	0.675
360	0.025417	0.328589	0.649439	1.7	2.197	3.705
309	0.025926	0.328589	0.645781	1.2	1.047	0.876
168	0.027222	0.328589	0.636717	1.3	1.025	0.805
361	0.027684	0.328589	0.633565	1.4	1.313	1.872
285	0.028000	0.328589	0.629116	1.2	0.905	1.051
1165	0.028348	0.328589	0.629116	1.6	1.001	1.578
973	0.028646	0.328589	0.627144	1.2	1.067	1.33
162	0.030829	0.328589	0.61319	1.2	0.966	1.167
335	0.031017	0.328589	0.612023	1.2	0.996	0.801
1018	0.031632	0.328589	0.608255	1.5	0.871	1.285
1326	0.031908	0.328589	0.606586	1.6	0.855	1.332
932	0.033373	0.328589	0.597914	1.2	1.115	1.307
478	0.033683	0.328589	0.596123	1.2	1.121	0.939
1054	0.03371	0.328589	0.595967	1.4	1.128	1.532
1070	0.033754	0.328589	0.595711	1.4	0.911	1.26
634	0.033913	0.328589	0.594797	1.5	1.111	0.737
1389	0.033937	0.328589	0.594661	1.2	0.991	0.819
805	0.03406	0.328589	0.59396	1.1	1.055	0.925
780	0.03419	0.328589	0.593218	1.2	1.098	0.927
664	0.035209	0.328589	0.587484	1.2	1.232	1.5
578	0.035556	0.328589	0.585568	1.3	1.243	1.605
351	0.036478	0.328589	0.580543	1.2	1.036	0.868
999	0.037795	0.328589	0.573553	1.2	0.978	1.147
919	0.03958	0.328589	0.564408	1.2	1.056	1.315
1360	0.040169	0.328589	0.561469	1.3	1.202	1.51
249	0.040177	0.328589	0.561431	1.2	1.005	0.84
1144	0.040417	0.328589	0.560245	1.5	1.048	1.543

1036	0.040829	0.328589	0.558223	1.2	1.137	1.355
740	0.040879	0.328589	0.557978	1.2	1.058	1.302
1107	0.041312	0.328589	0.555875	1.1	1.105	1.019
1239	0.043613	0.328589	0.545011	1.9	0.608	1.14
742	0.04386	0.328589	0.543874	1.3	1.113	0.886
687	0.043942	0.328589	0.543497	1.1	1.084	1.197
332	0.044073	0.328589	0.542898	1.3	1.094	1.37
1005	0.044489	0.328589	0.541006	1.3	1.078	1.437
922	0.045141	0.328589	0.538076	1.2	0.974	1.207
1409	0.045736	0.328589	0.535432	1.3	0.954	0.733
1103	0.046713	0.328589	0.531156	1.1	1.169	1.302
65	0.050082	0.328589	0.517015	1.3	0.672	0.857
366	0.050264	0.328589	0.516273	1.5	1.105	1.632

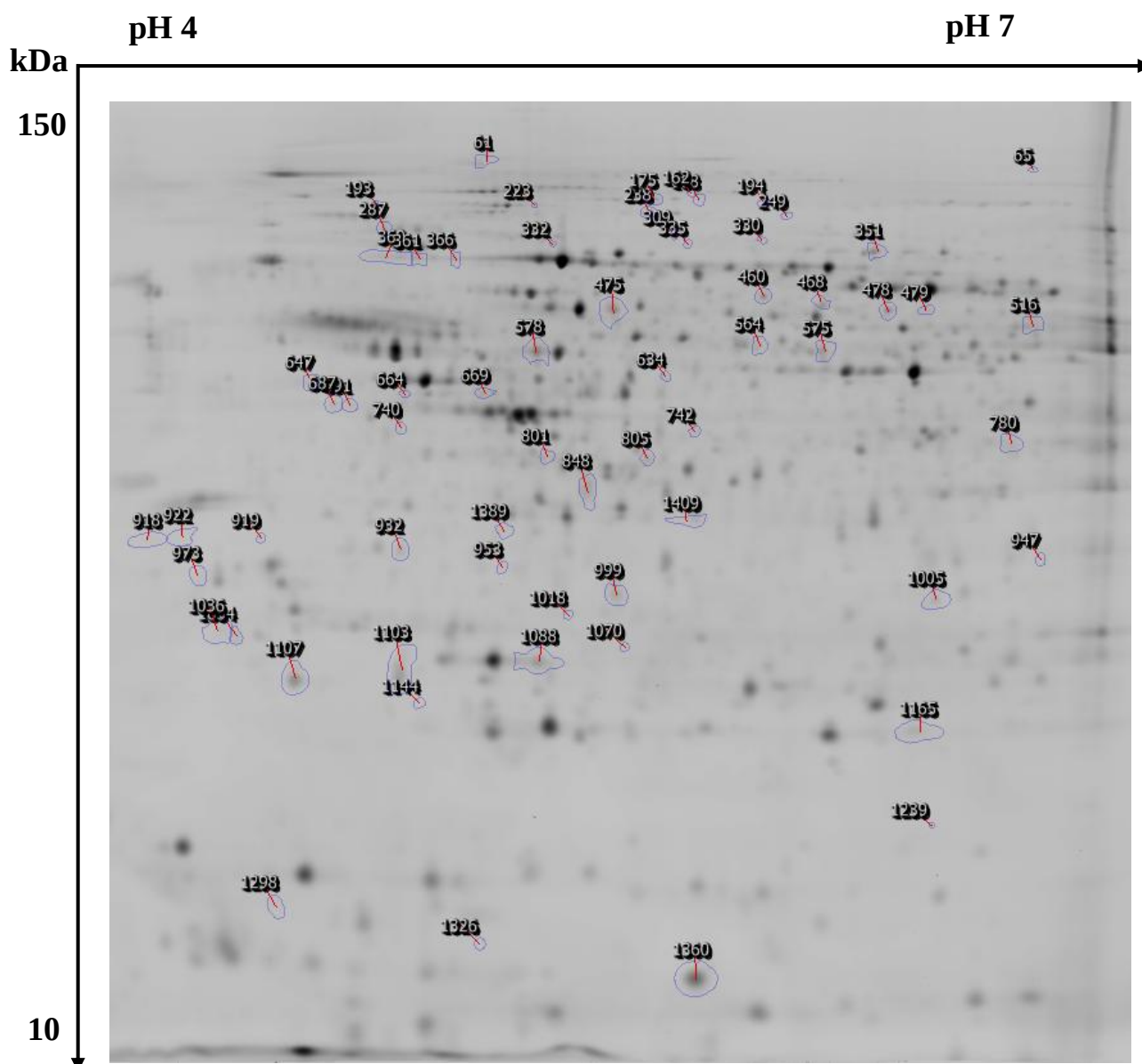


Figure 4-8 Hippocampus: Location of significant spots following univariate analysis and acute ECS treatment

Following analysis by Progenesis SameSpot software (NonLinear Dynamics) the spots highlighted on the gel were identified as being significantly up- or down-regulated ($p \leq 0.05$, one way ANOVA).

4.3.2.1.1 Principal component analysis

Principal component analysis (PCA), conducted by Progenesis SameSpot software (NonLinear Dynamics) for the detection of outliers, was applied to the data (Figure 4-9).

Treated samples cluster together and control samples cluster together. A single control gel (circled) appears to be a potential outlying sample and may require further investigation in combination with the PLS-DA scores plot.

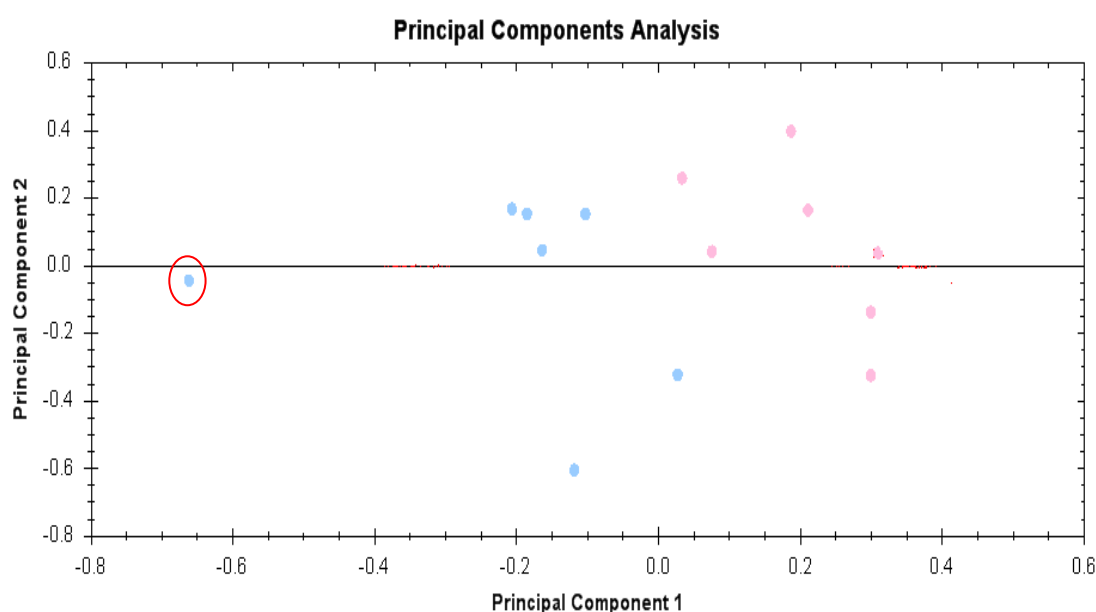


Figure 4-9 PCA plot of treated and control hippocampal samples following acute ECS

Treated gels are represented by pink spots and control gels are represented by blue spots. The circled blue spot, representing a control gel, is of particular interest as a potentially outlying gel.

4.3.2.2 Multivariate analysis

The spots whose expression contributed to variation in the data following multivariate analysis are listed according to p-value below (Table 4-2). Those spots found to be significant at $p \leq 0.05$ are listed in the adjoining columns. Only spots significant at this level were considered for further analysis.

Variables that contribute to variation in the hippocampus		Significant variables $p \leq 0.05$	
Spot number	P-value	Spot #	P-value
102	0.26009	66	0.01576
175	0.67241	460	0.04321
209	0.2811	947	0.01497
193	0.1547		
287	0.19966		
330	0.54781		
360	0.51046		
475	0.27266		
479	0.81695		
516	0.06205		
529	0.99027		
534	0.84531		
547	0.78124		
537	0.98932		
575	0.29297		
669	0.59495		
848	0.25224		
893	0.68578		
918	0.2358		
919	0.79014		
1088	0.7494		
1165	0.67693		
1280	0.97688		
1298	0.66294		

Table 4-2 Protein spots identified as statistically significant following multivariate analysis and acute ECS treatment All spots that were determined to be significant following PLS-DA and significant spots with a p-value less than 0.05 are listed in Table 4-2. The list was compiled from the p-values of β coefficients after outliers were removed and the model redrawn based on the diagnostic plots in Figure 4-10 - 4-13.

4.3.2.2.1 PLS-DA diagnostic plots

The different plots used to analyse and detect the sources of variation in each study are shown. Representative plots for the models drawn with all data are included for the X-Y correlation loadings and weighted regression coefficients. They demonstrate the effect of removing non-significant variables in order to detect significant variables in the data. The scores plots and explained variance plots that contained all the variables (Figure 4-10 (A), 4-11 (A)) and the redrawn models containing significant variables only (Figure 4-10 (B), 4-11(B)) are shown below. The “before” and “after” plots demonstrate the improvement in explained variance (Figure 4-11) when models were drawn using the significant data only, as well as detecting sample clustering patterns and outliers (Figure 4-10).

Figure 4-10: Following analysis of this plot and the PCA plot of this data drawn in Progenesis SameSpot software (NonLinear Dynamics), a single control gel was removed from analysis (circled in red, Figure 4-10 (A)). Although this gel did not fall outside the Hotelling T^2 ellipse (indicating outlying variable), in combination with the result obtained from PCA analysis (Figure 4-8), this gel was removed from further models of the data. Removal of this variable improved the variance that could be explained by the remaining data and improved clustering of the gels into treated or control groups.

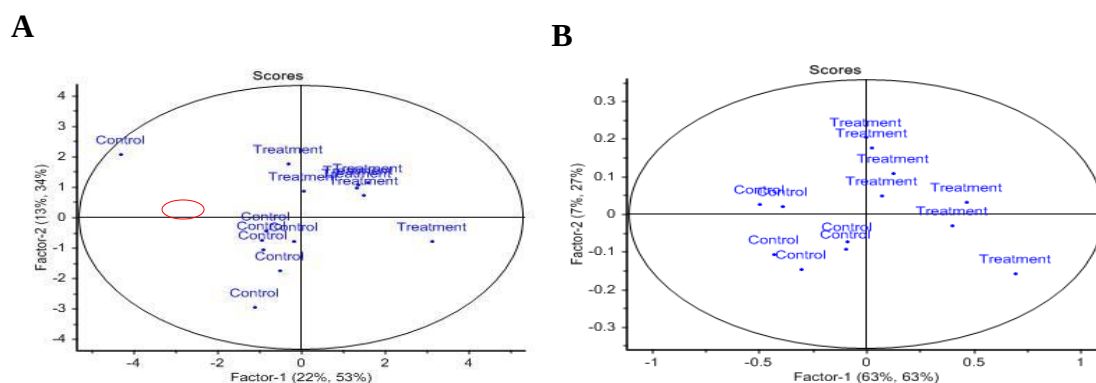
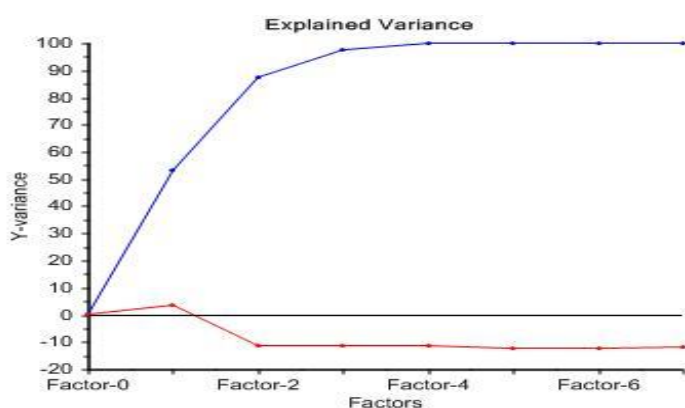


Figure 4-10 Scores plots following acute ECS treatment of the hippocampus

(A) Scores plot drawn from initial model with all data included; **(B)** Scores plot drawn following removal of all variables marked as non-significant in initial model.

This plot (Figure 4-11) allows comparison of how well the validation curve (red) matches the calibration curve (blue). The model indicated two factors were the optimal model dimension by full cross validation. Cross validation created 27 submodels, where 1 sample was left out in each. The validation curve showed 60% explained variance for two factors, after which it reached a plateau. Two factors explain 90% of the variance in Y on the calibration curve.

A



B

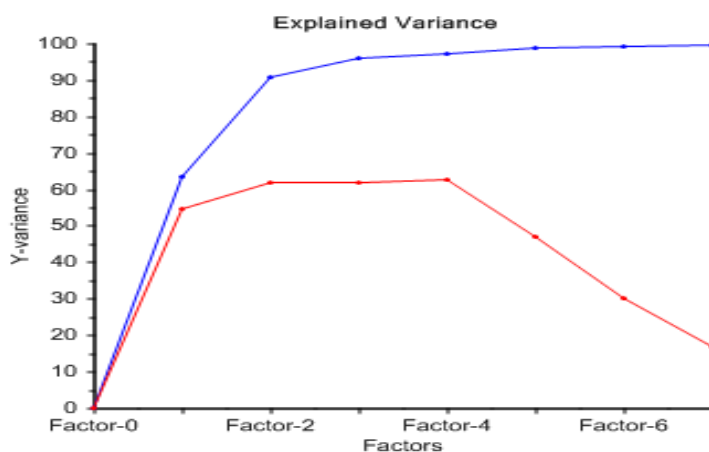
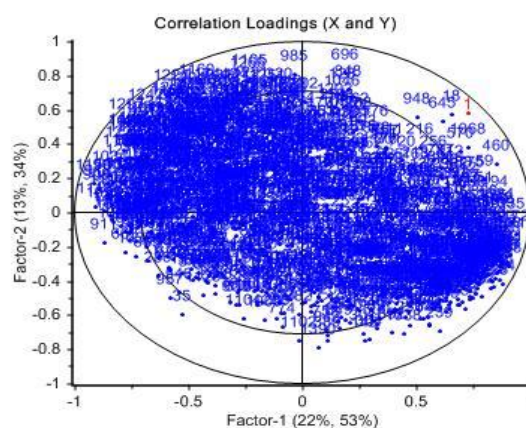


Figure 4-11 Plots of explained variance following acute ECS treatment of the hippocampus

(A) The variance explained by all the data; **(B)** The explained variance plot drawn from significant variables

Figure 4-12 (A) is difficult to interpret due to the presence of so many variables. Variables that fall in the centre of the plot cannot be interpreted and do not contribute to explaining overall variance in the data. X-variables selected as non-significant were removed and the plot was redrawn (Figure 4-12 (B)) with the remaining variables. These variables contributed significantly to the variance found in the model. The importance of these variables is interpreted by weighted regression plots (Figure 4-13). Circled variables were found to be significant at $p \leq 0.05$.

A



B

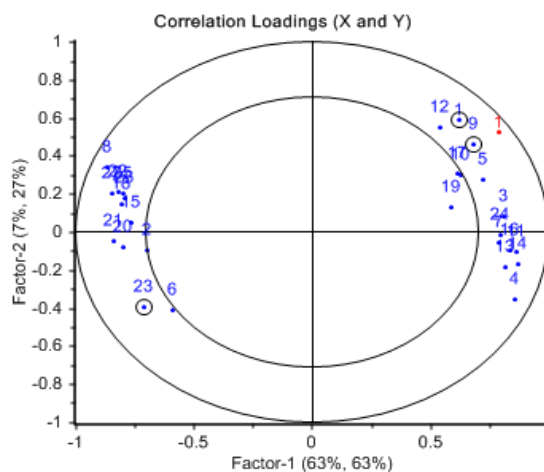
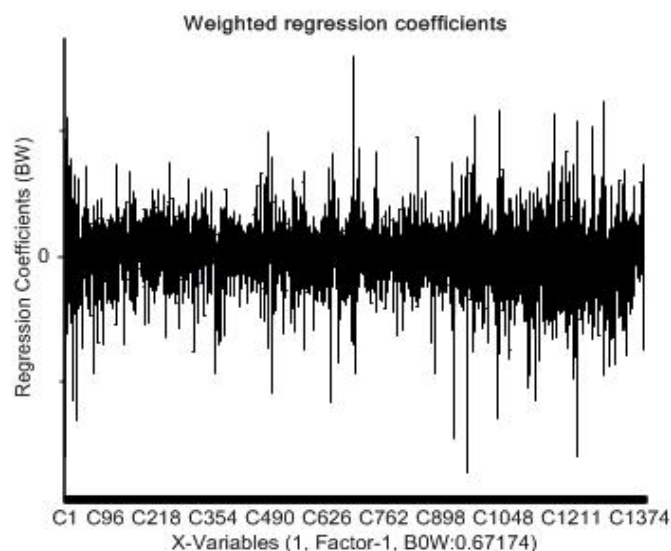


Figure 4-12 X-Y correlation loadings following acute ECS treatment of the hippocampus

(A) Representative X-Y correlation loadings of all X variables included in the initial model;
 (B) X-Y correlation loadings plot of X-variables marked significant. Y (treated/control) is represented by the number 1, in red, in each plot.

All variables (Figure 4-13 **(A)**) are difficult to interpret. In Figure 4-13 **(B)** the significant variables only are plotted. The importance of individual variables is more easily visualised in terms of the weighted regression coefficients than in the previous plot (Figure 4-12). A 95% confidence interval estimates the uncertainty limits. The variables significant at $p \leq 0.05$ are circled.

A



B

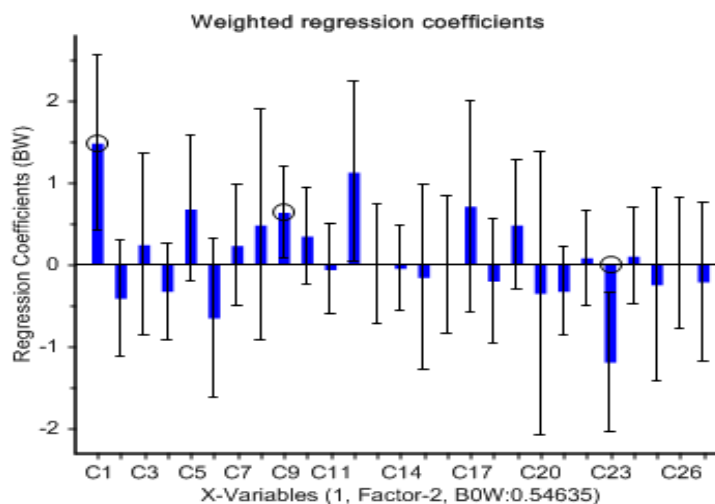


Figure 4-13 Plot of weighted regression coefficients

(A) Representative weighted regression coefficients of all data; **(B)** weighted regression coefficients plot containing significant variables only.

4.3.2.3 Comparison of univariate and multivariate significant spot findings

A collection of the spots found to be statistically different following both univariate and multivariate analysis in the hippocampus following acute ECS is also included. A Venn diagram describes the number of spots common to both univariate and multivariate analysis as well as unique to each analysis (Figure 4-14). The significant spot numbers from each analysis are listed in Table 4-3.

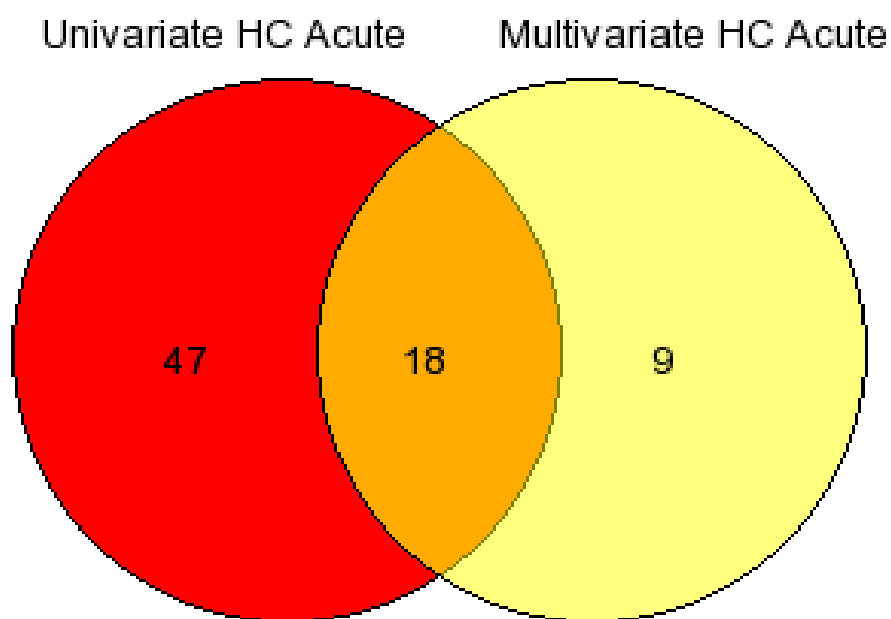


Figure 4-14 Venn diagram of significant spot distribution following univariate and multivariate analysis in the hippocampus following acute ECS treatment

HC, hippocampus. Drawn in www.genevenn.com.

Significant spot number following univariate analysis		Significant spot numbers common to both	Significant spot numbers following multivariate analysis
61	740	175	66
65	742	193	102
162	780	287	209
168	801	330	529
194	805	360	534
223	922	460	537
238	932	475	547
249	953	479	893
285	973	516	1280
309	999	575	
332	1005	669	
335	1018	848	
351	1036	918	
361	1054	919	
366	1070	947	
468	1103	1088	
478	1107	1165	
564	1144	1298	
578	1239		
634	1326		
647	1360		
664	1389		
687	1409		
691			

Table 4-3 List of significant spots following univariate and multivariate analysis of the hippocampus following acute ECS treatment

4.3.3 Protein spots picked for mass spectrometry

A number of significant spots from the hippocampus were picked from preparatory gels for identification by mass spectrometry. The location of the silver-stained picked spots is highlighted in Figure 4-15 on representative preparative gels. These spots, their fold change, p-value and the statistical method by which they were detected are listed in Table 4-4.

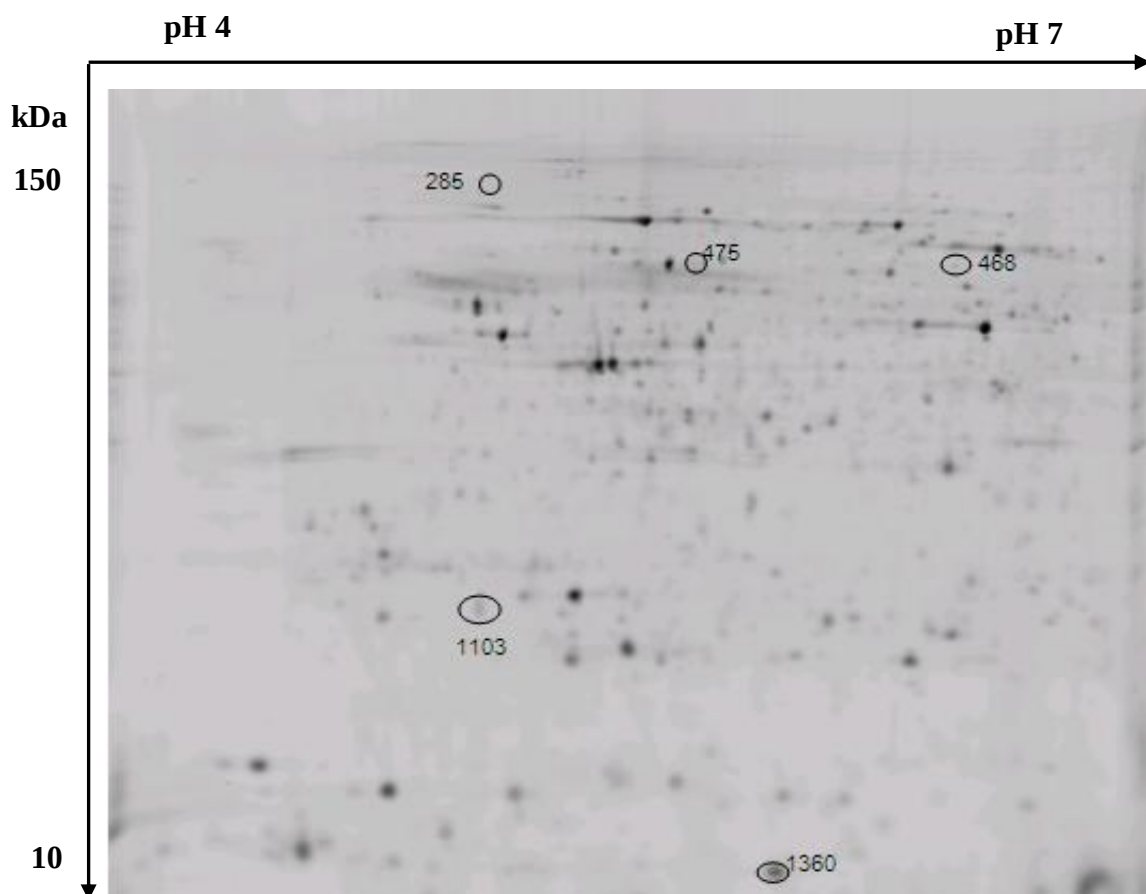


Figure 4-15 Preparative gel indicating significant spots that were picked for identification from the hippocampus following acute ECS treatment

Hippocampus	Standard spot no.	Fold change	Up / down regulated	p-value	Analysis
Acute	468	1.3	Up	$P \leq 0.017$	UVA
	1103	1.1	Down	$P \leq 0.046$	UVA
	1360	1.3	Down	$P \leq 0.04$	UVA
	285	1.2	Down	$P \leq 0.028$	UVA
	475	1.1	Up	$P \leq 0.014$	MVA/UVA

Table 4-4 Protein spots picked for identification

UVA, univariate analysis; MVA, multivariate analysis

4.3.4 Hippocampus: Analysis of the chronic effects of ECS treatment

4.3.4.1 Univariate analysis

The univariate results table following chronic ECS treatment includes the p-value associated with the one way ANOVA comparison of treated and control ECS samples, The average normalised volume, the fold change between spots, the q-value, which controls for the FDR and the power analysis value (Table 4-5).

Figure 4-16 shows the location, on a representative chronic gel, of the significant spots detected by univariate analysis in Progenesis SameSpot software (NonLinear Dynamics). PCA in Progenesis SameSpot software (NonLinear Dynamics) was used to detect outlying samples and patterns of clustering in the data. The PCA plot is shown in Figure 4-17.

Table 4-5 List of significantly differentially expressed spots in the hippocampus following chronic ECS treatment, detected by univariate analysis

Hippocampus, chronic ECS					Average normalised volumes	
Spot #	Anova (p)	q Value	Power	Fold change	Chronic treated	Chronic control
450	0.000117	0.098461	0.999184	1.1	1.103	1.25
1230	0.000166	0.098461	0.998414	1.7	1.006	0.602
580	0.001855	0.403064	0.953291	1.2	1.328	1.1
1216	0.005797	0.403064	0.866743	1.2	0.889	1.096
570	0.006172	0.403064	0.859989	1.2	1.211	0.994
1198	0.006796	0.403064	0.849177	1.1	0.953	0.838
494	0.006894	0.403064	0.847524	1.2	1.037	0.892
743	0.007395	0.403064	0.839269	1.2	0.874	1.058
872	0.009	0.403064	0.814618	1.1	1.044	1.178
875	0.009568	0.403064	0.806486	1.5	1.252	0.85
497	0.009774	0.403064	0.8036	1.2	1.088	0.9
1019	0.013132	0.403064	0.76103	1.2	0.888	1.07
1120	0.013221	0.403064	0.759993	1.2	0.865	1.031
1151	0.013257	0.403064	0.75958	1.6	1.015	0.62
726	0.01702	0.403064	0.719747	1.1	0.969	1.067
523	0.019142	0.403064	0.699912	1.2	1.026	1.196
1148	0.020585	0.403064	0.687316	1.2	0.939	1.093
1256	0.020586	0.403064	0.68731	1.2	1.107	1.311
1149	0.021245	0.403064	0.681774	1.6	0.889	0.562
1082	0.021736	0.403064	0.677726	1.1	0.977	1.098

1316	0.021949	0.403064	0.675998	1.2	1.104	0.908
333	0.024238	0.403064	0.658133	1.1	0.95	1.072
786	0.025116	0.403064	0.65163	1.2	1.184	1.008
1334	0.026546	0.403064	0.6414	1.2	0.869	1.075
1222	0.028681	0.403064	0.626911	1.4	0.919	0.659
1142	0.028743	0.403064	0.626503	1.2	0.839	1.034
1275	0.029551	0.403064	0.621256	1.2	0.971	1.171
1242	0.031058	0.403064	0.611771	2	0.952	0.48
758	0.0318	0.403064	0.60724	1.1	1.029	1.142
1365	0.032164	0.403064	0.605047	1.3	0.647	0.828
1280	0.034276	0.403064	0.592727	1.2	0.889	1.047
192	0.034315	0.403064	0.592506	1.3	1.202	0.929
1279	0.034406	0.403064	0.591991	1.2	1.007	1.165
257	0.034541	0.403064	0.591229	1.2	1.109	0.924
568	0.037159	0.403064	0.576904	1.1	1.158	1.042
573	0.037451	0.403064	0.575362	1.3	0.6	0.783
194	0.038081	0.403064	0.572064	1.3	1.176	0.911
964	0.039556	0.403064	0.564531	1.4	0.973	0.712
932	0.042193	0.403064	0.551653	1.1	1.287	1.148
1249	0.043487	0.403064	0.545592	1.3	1.132	0.865
1015	0.043977	0.403064	0.543337	1.1	1.005	1.099
85	0.045806	0.403064	0.535123	1.3	0.857	1.11
693	0.046078	0.403064	0.533925	1.2	1.012	0.842
870	0.050051	0.403064	0.517139	1.1	1.153	1.037

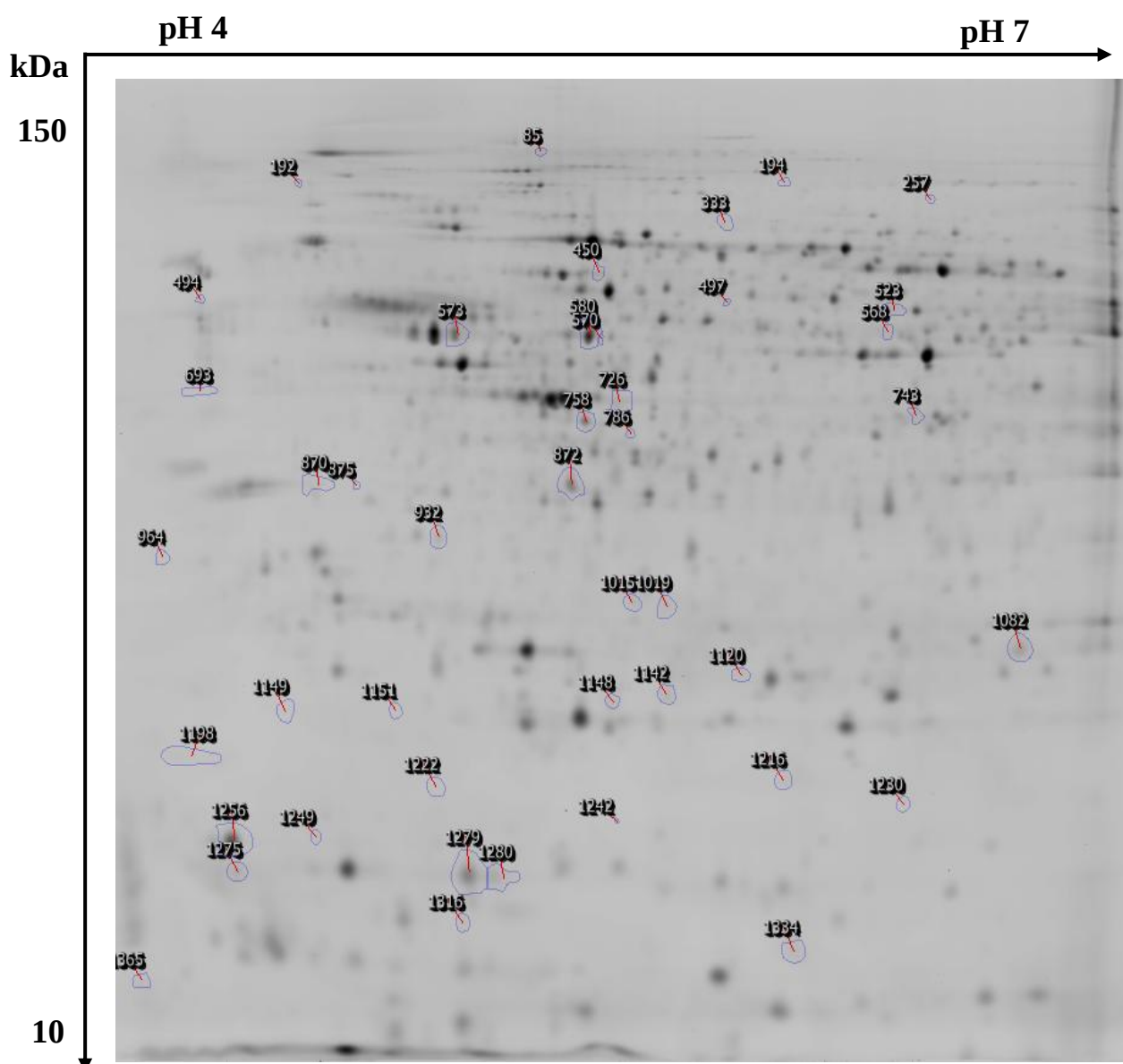


Figure 4-16 Hippocampus: Location of significant spots following univariate analysis and chronic ECS treatment

Following analysis by Progenesis SameSpot software (NonLinear Dynamics) the spots highlighted on the gel were identified as being significantly up-or down-regulated following univariate analysis ($p \leq 0.05$, one way ANOVA).

4.3.4.2 Principal component analysis

Principal component analysis (PCA) was conducted by Progenesis SameSpot software (NonLinear Dynamics) to detect outliers in the the data. ECS treated gels cluster strongly together as do control gels, suggesting a strong ECS treatment effect (Figure 4-17). There were no outlying gels following chronic ECS treatment.

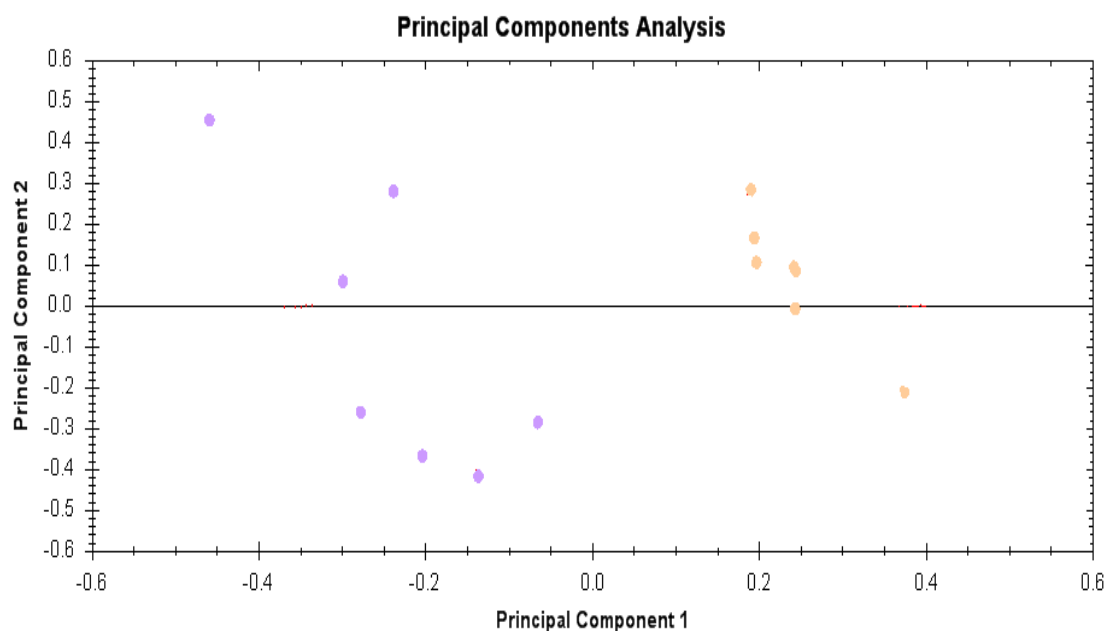


Figure 4-17 PCA plot of treated and control hippocampal samples following chronic ECS

Control sample gels are represented by purple spots, the ECS treated gels are represented by the peach coloured spots.

4.3.4.3 Multivariate analysis

The spots that were found to contribute to variation following multivariate analysis are listed according to p-value below (Table 4-6). Those spots found to be significant at $p \leq 0.05$ are listed in the adjoining columns. Only those spots detected at this level of significance were considered for further analysis.

Variables that contribute to variation in the hippocampus after chronic ECS		Significant variables $p \leq 0.05$	
Spot number	P-value	Spot number	P-value
1	0.59682	1151	0.05742
14	0.85027	1230	0.01218
46	0.44449		
208	0.80436		
312	0.59488		
435	0.18137		
450	0.23062		
500	0.98497		
525	0.69904		
523	0.4812		
746	0.2373		
743	0.56372		
758	0.93264		
767	0.85749		
778	0.69427		
872	0.97275		
1082	0.44898		
1142	0.74552		
1148	0.57873		
1149	0.57365		
1152	0.6671		
1151	0.05742		
1166	0.90334		
1174	0.25542		
1216	0.19478		
1212	0.80438		
1222	0.71204		
1242	0.97555		
1256	0.72964		
1279	0.71587		
1315	0.50349		

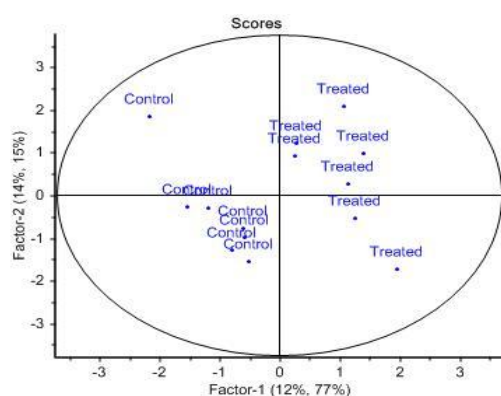
Table 4-6 Protein spots identified as being statistically significant following multivariate analysis and chronic ECS List is based on p-values of β -coefficients after outliers have been removed. Spots determined to be significant at $p \leq 0.05$ are listed in the right column.

4.3.4.3.1 PLS-DA diagnostic plots

The different plots used to analyse and detect the sources of variation in each study include representative plots for the X-Y correlation loadings and weighted regression coefficients following the removal of non-significant spots. The scores plots and explained variance plots that contain all the variables (Figure 4-18 (A), 4-19 (A)) and the redrawn models containing significant variables only (Figure 4-18 (B), 4-19 B) are shown below. The before and after plots demonstrate the improvement in explained variance (Figure 4-19) using the significant data only as well as detecting sample clustering patterns and outliers (Figure 4-18).

In Figure 4-18, Scores plot **A** and **B** there is evidence of control samples clustering together and treated samples clustering together. No factors fall outside the Hotelling T^2 ellipse therefore, no samples are excluded as outliers in this study.

A



B

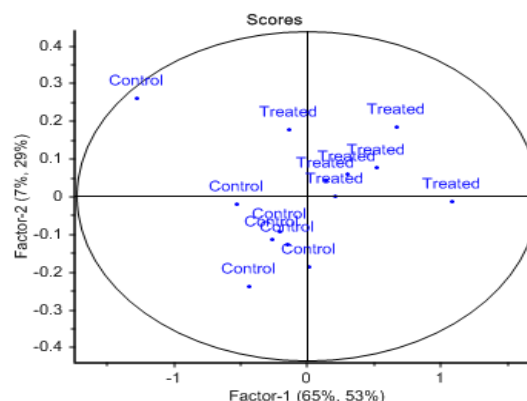
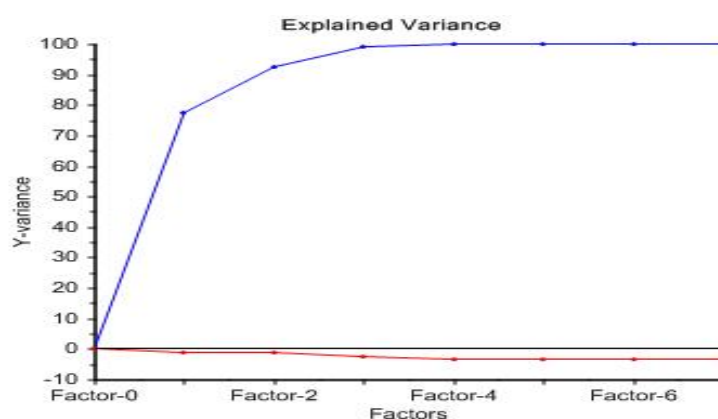


Figure 4-18 Scores plot following chronic ECS treatment of the hippocampus

A Scores plot drawn in initial model using all data; **B** Scores plot of all factors following removal of non-significant variables.

This plot (Figure 4-19) allows comparison of how well the validation curve (red) matches the calibration curve (blue). The model indicated four factors were the optimal model dimension by full cross validation (Figure 4-19 (B)). Cross validation created 32 submodels, where 1 sample was left out in each. The validation curve showed 55% explained variance for four factors, then reached a plateau. Two factors explain 80% of the variance in Y on the calibration curve.

A



B

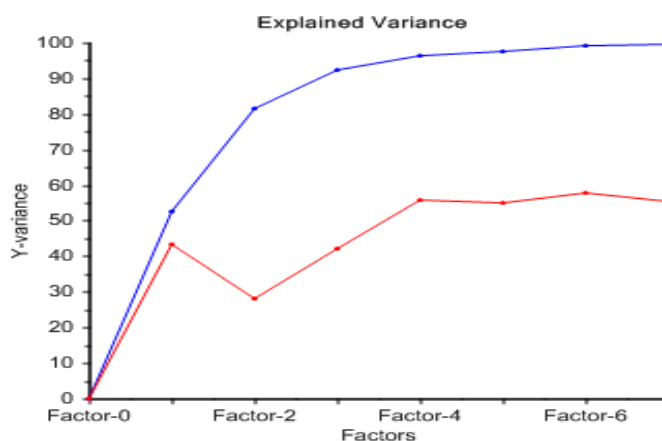


Figure 4-19 Plots of explained variance following chronic ECS treatment of the hippocampus

A Variance explained by model containing all data; **B** Variance explained by model drawn with significant variables.

Variables identified as being non-significant were removed and the plot redrawn (Figure 4-20). The significant variables explain more variation in the data. The variables in the top right quadrant are important for explaining variation in Y (“1”). Each number represents a gel spot. Circled variables were found to be significant at a value of $p \leq 0.05$.

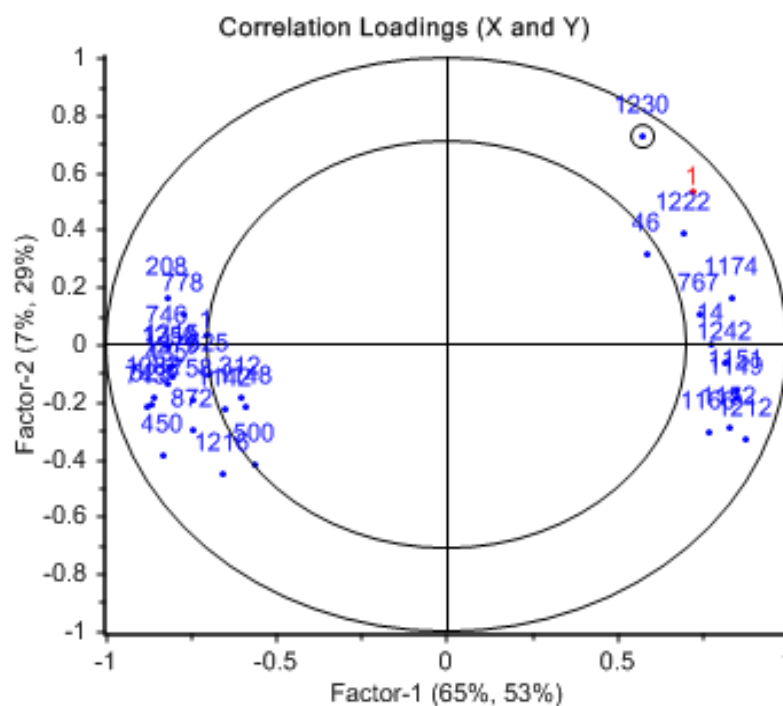


Figure 4-20 X-Y correlation loadings following chronic ECS treatment of the hippocampus

X-Y correlation loadings of variables marked significant only. Y is represented by “1” in red in each plot.

Figure 4-21 shows the importance of the variables is more easily visualised in terms of the regression coefficients than the X-Y plot. They are more relevant to interpretation of the importance of individual variables for modelling. A 95% confidence interval estimates the uncertainty limits.

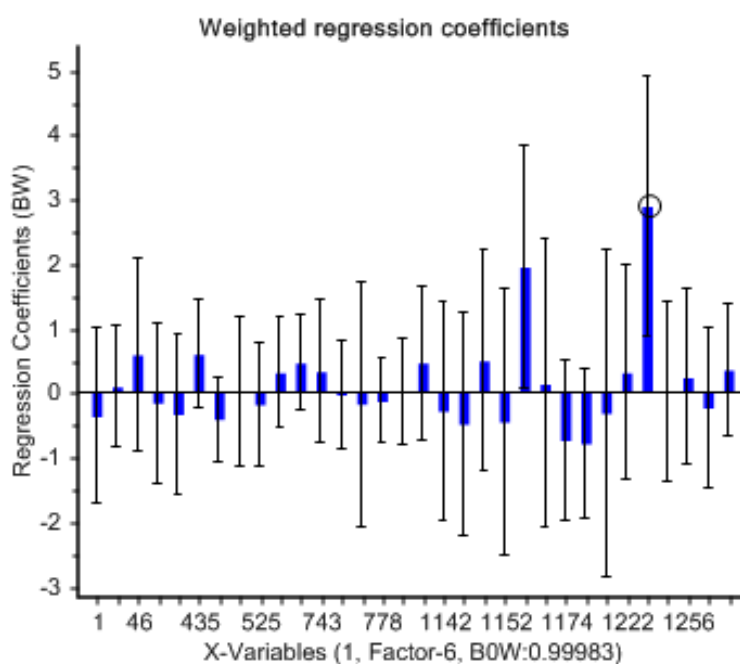


Figure 4-21 Plot of weighted regression coefficients

The weighted regression coefficient plot of significant variables in the data.

BW weighted regression coefficient. The circled variable is significant at $p \leq 0.05$.

4.3.4.4 Comparison of univariate and multivariate significant spot findings

Proteins spots found to be statistically different following both univariate and multivariate analysis in the hippocampus following chronic ECS are presented below. A Venn diagram describes the number of spots common to both univariate and multivariate analysis as well as unique to each analysis (Figure 4-22). The significant spot numbers from each analysis are listed in Table 4-7.

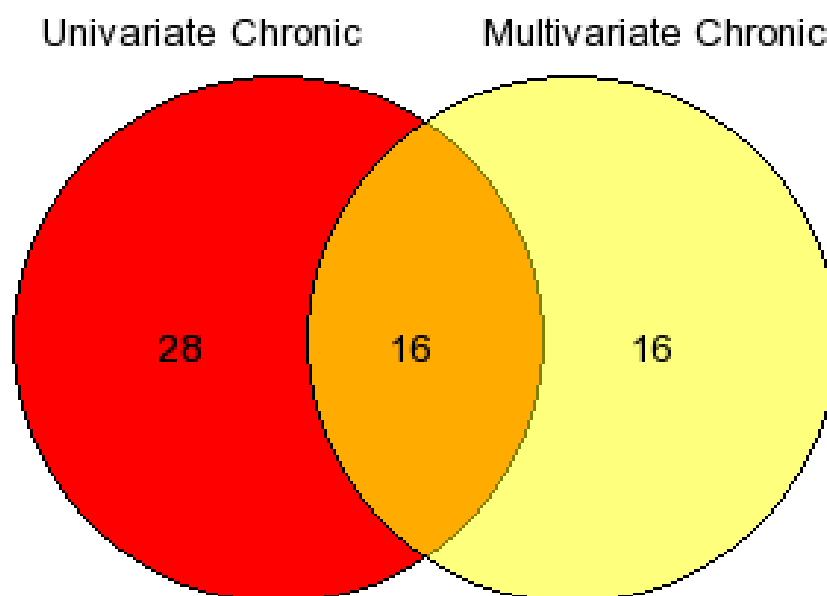


Figure 4-22 Venn diagram of significant spot distribution found following univariate and multivariate analysis in the hippocampus following chronic ECS treatment

Drawn in www.genevenn.com

Significant spots common to univariate analysis	Significant spots common to both analyses	Significant spots common to multivariate analysis
85	450	1
192	523	14
194	743	46
257	758	208
333	872	312
494	1082	435
497	1142	500
568	1148	525
570	1149	746
573	1151	767
580	1216	778
693	1222	1152
726	1230	1166
786	1242	1174
870	1256	1212
875	1279	1315
932		
964		
1015		
1019		
1120		
1198		
1249		
1275		
1280		
1316		
1334		
1365		

Table 4-7 List of significant spots following univariate and multivariate statistical analysis of the hippocampus following chronic ECS treatment

4.3.5 Protein spots picked for mass spectrometry analysis

A number of significant spots from the hippocampus following chronic ECS treatment were picked from the densitometer scanned silver-stained preparatory gels for identification by mass spectrometry. The location of the picked spots is highlighted in Figure 4-23. These spots, their fold change, p-value and the statistical method by which they were detected are listed in Table 4-8.

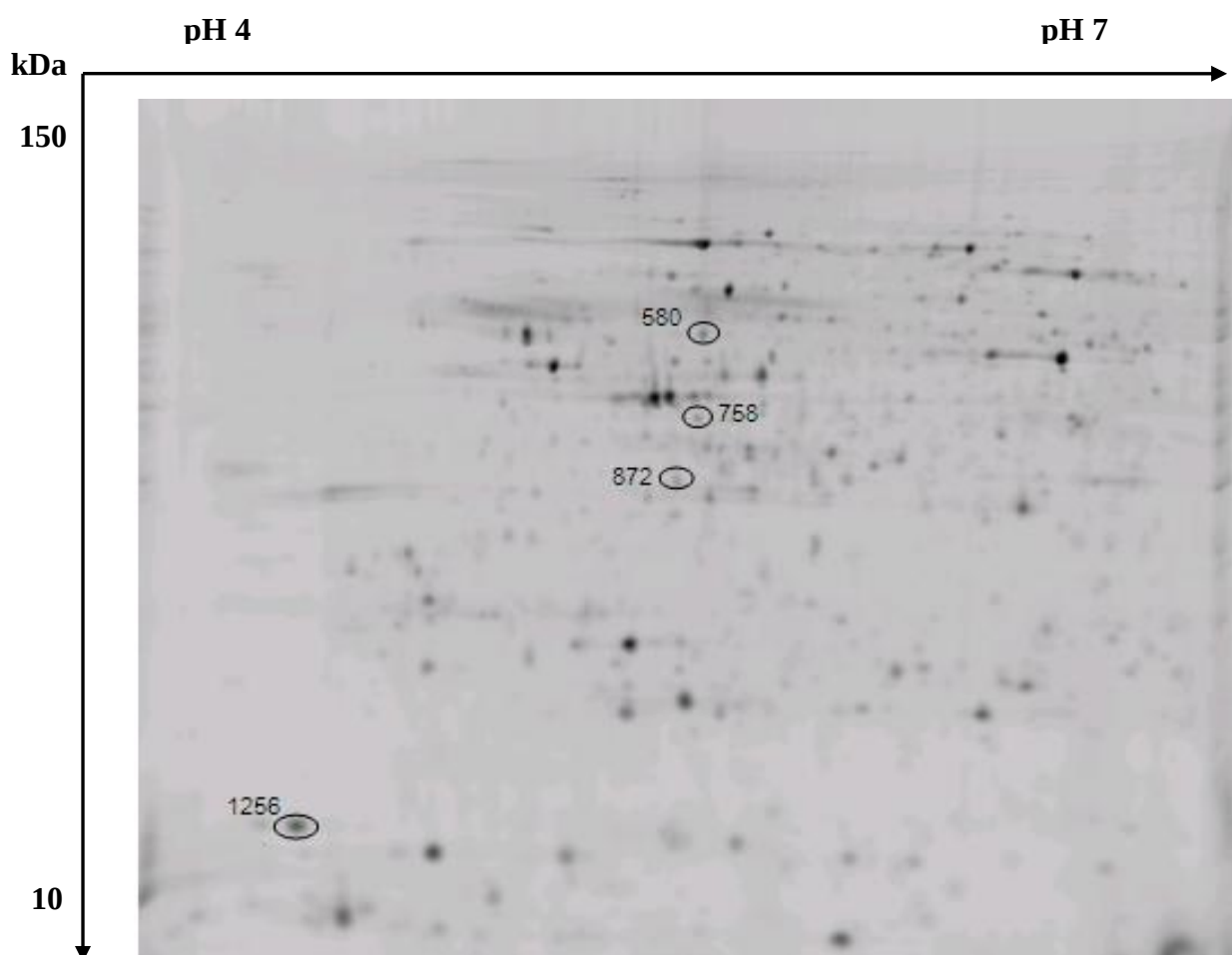


Figure 4-23 Preparative gel indicating significant spots that were picked for identification from the hippocampus following chronic ECS treatment

Hippocampus	Standard spot no.	Fold change	Up / down regulated	P-value	MVA/UVA
Chronic	1256	1.2	Down	P=0.02	MVA/UVA
	580	1.2	Up	P=0.001	UVA
	872	1.1	Down	P=0.009	MVA/UVA
	758	1.1	Down	P=0.031	MVA/UVA

Table 4-8 Spots picked for identification by mass spectrometry

MVA multivariate analysis; UVA univariate analysis

4.3.6 Hippocampus: Analysis of the effects of chronic + 4 weeks of ECS treatment

4.3.6.1 Univariate analysis

Following chronic + 4 weeks of ECS treatment, the univariate results table includes the p-value associated with the one way ANOVA comparison of treated and control ECS samples. The average normalised volume, the fold change between spots, the q-value that controls for the FDR and the power analysis value, is also included (Table 4-9) for each study.

Figure 4-24 is a representative gel that identifies the location of the significant spots detected by univariate analysis, following chronic + 4 weeks of ECS, in Progenesis SameSpot software (NonLinear Dynamics). Principal component analysis (PCA) in Progenesis SameSpot software (NonLinear Dynamics) was used to detect outlying samples and patterns of clustering in the data. The PCA plot is shown in Figure 4-25

Table 4-9 List of significantly differentially expressed spots in the hippocampus following chronic + 4 weeks of ECS, detected by univariate analysis

Hippocampus chronic + 4					Average normalised volumes	
Spot #	Anova (p)	q Value	Power	Fold change	Chronic + 4 treated	Chronic + 4 control
235	0.000173	0.081932	0.998302	1.1	1.09	0.958
1270	0.000192	0.081932	0.997942	1.6	0.559	0.922
913	0.000437	0.103797	0.988064	1.2	1.184	0.974
234	0.000598	0.103797	0.988064	1.1	1.161	1.048
686	0.000666	0.103797	0.986198	1.2	0.938	1.137
316	0.000679	0.103797	0.985823	1.2	1.108	0.961
227	0.001096	0.107062	0.974099	1.1	1.025	0.904
517	0.001114	0.107062	0.9736	1.9	1.272	0.668
751	0.001135	0.107062	0.973013	1.2	1.087	0.918
824	0.001353	0.115161	0.966933	1.1	1.228	1.13
637	0.002307	0.162486	0.941569	1.1	1.117	1.014
1257	0.002834	0.171943	0.928579	1.3	1.448	1.822
221	0.003696	0.171943	0.908777	1.1	1.169	1.263
414	0.003787	0.171943	0.906801	1.2	1.15	0.985
872	0.004163	0.171943	0.898749	1.1	1.185	1.258
1281	0.004419	0.171943	0.893412	1.2	0.875	1.067
415	0.004496	0.171943	0.891837	1.3	0.951	1.21
741	0.004643	0.171943	0.888864	1.2	0.968	0.829
1286	0.004959	0.171943	0.88259	1.5	0.707	1.037
472	0.005004	0.171943	0.881702	1.3	1.183	0.934
303	0.005058	0.171943	0.880652	1.1	1.185	1.049
158	0.005166	0.171943	0.878562	1.2	1.042	0.87
327	0.005699	0.171943	0.868535	1.3	1.037	0.791
320	0.005978	0.171943	0.863461	1.2	1.087	0.911
402	0.006253	0.171943	0.858567	1.1	1.191	1.071

470	0.006275	0.171943	0.858176	1.1	1.082	0.975
241	0.006342	0.171943	0.857002	1.3	1.011	0.793
281	0.006766	0.171943	0.849693	1.2	1.108	0.945
214	0.006893	0.171943	0.847542	1.2	0.99	0.815
1032	0.007072	0.171943	0.844556	1.1	1.034	1.135
307	0.007394	0.171943	0.839274	1.2	0.978	0.85
1283	0.00743	0.171943	0.838703	1.2	0.832	1.012
863	0.007892	0.171943	0.831355	1.2	1.082	1.256
168	0.007938	0.171943	0.830624	1.2	1.016	0.815
483	0.008011	0.171943	0.829495	1.5	1.105	0.75
152	0.008061	0.171943	0.828711	1.2	1.126	0.968
408	0.008342	0.171943	0.824404	1.1	1.014	0.945
801	0.008507	0.171943	0.82191	1.1	1.042	0.924
864	0.0088	0.174601	0.817542	1.1	0.984	1.039
834	0.008909	0.174601	0.815951	1.1	1.071	0.998
790	0.009337	0.177276	0.809758	1.1	1.106	1.012
515	0.011588	0.212915	0.779656	1.2	1.139	0.972
407	0.012027	0.215262	0.774205	1.1	1.063	0.964
1380	0.012119	0.215262	0.773075	1.2	0.849	1.059
662	0.012561	0.215409	0.767747	1.3	1.002	0.782
113	0.013161	0.217647	0.760687	1.2	0.832	1.008
917	.013	0.218126	0.75951	1.1	1.026	0.901
306	0.013263	0.218126	0.75951	1.2	1.091	0.9
236	0.013849	0.218126	0.752855	1.1	0.995	0.89
537	0.013863	0.218126	0.752701	1.3	1.126	0.887
1279	0.014245	0.218126	0.748451	1.1	1.083	1.189
498	0.015399	0.22365	0.736088	1.3	1.184	1.556
1022	0.015568	0.22365	0.734332	1.2	0.854	0.99
660	0.015581	0.22365	0.734195	1.2	0.898	1.039
317	0.015876	0.22365	0.731159	1.1	1.081	0.984
576	0.016279	0.226936	0.727073	1.1	1.076	1.225
862	0.016518	0.226936	0.72469	1.1	1.219	1.393

339	0.017297	0.226936	0.717058	1	1.059	1.01
1388	0.018458	0.226936	0.706131	1.2	1.13	0.927
1315	0.018517	0.226936	0.705581	1.1	1.119	1.238
771	0.018695	0.226936	0.703956	1.3	0.833	1.055
312	0.01874	0.226936	0.703543	1.1	1.188	1.055
340	0.019017	0.226936	0.701035	1.2	1.056	0.893
479	0.019163	0.226936	0.69973	1.1	0.895	0.782
324	0.019677	0.226936	0.695165	1.5	1.53	2.366
136	0.019894	0.226936	0.693265	1.2	0.996	0.838
153	0.019992	0.226936	0.692413	1.2	0.972	0.819
422	0.020045	0.226936	0.691951	1.2	0.907	0.732
784	0.02034	0.226936	0.689413	1.2	1.145	0.952
304	0.020652	0.226936	0.686747	1.1	1.235	1.108
1313	0.021125	0.226936	0.682771	1.6	0.614	0.955
481	0.021193	0.226936	0.682203	2.1	1.382	0.646
289	0.021196	0.226936	0.682181	1.2	1.008	0.865
1244	0.021419	0.226936	0.680333	1.2	0.886	1.066
423	0.022065	0.226936	0.675061	1.2	0.994	0.847
1284	0.022541	0.226936	0.671243	1.2	0.918	1.062
325	0.023248	0.226936	0.665696	1.1	1.079	0.994
1371	0.023273	0.226936	0.665505	1.2	0.957	1.104
676	0.023784	0.226936	0.661571	1.2	0.98	0.848
196	0.023911	0.226936	0.660609	1.1	1.094	1
937	0.02394	0.226936	0.660385	1.2	0.916	0.758
859	0.024546	0.226936	0.655834	1.1	1.061	0.995
566	0.024701	0.226936	0.65468	1.1	1.078	0.962
326	0.025253	0.227779	0.650628	1.1	1.052	0.917
534	0.025423	0.227779	0.649396	1.1	0.898	0.822
1004	0.025858	0.227779	0.646267	1.2	1.002	1.167
495	0.026212	0.227779	0.643749	1.1	1.175	1.323
177	0.026564	0.227779	0.641274	1.2	0.994	0.851
888	0.027669	0.235196	0.633671	1.1	1.136	1.215

554	0.028081	0.235196	0.630895	1.1	1.112	1.057
703	0.029202	0.235196	0.623507	1.2	1.049	0.866
842	0.029431	0.235196	0.62203	1.1	1.116	1.236
1073	0.030055	0.235196	0.618043	1.1	0.944	1.014
977	0.030127	0.235196	0.617584	1.2	0.964	1.204
725	0.030246	0.235196	0.616837	1.2	1.054	0.903
526	.031	0.235196	0.608694	1.1	1.088	0.982
63	0.03156	0.235196	0.608694	1.3	0.727	0.957
1366	0.031696	0.235196	0.607867	1.2	0.896	1.084
1143	0.031718	0.235196	0.607738	1.1	1.189	1.317
253	0.032163	0.235196	0.605054	1.2	1.088	0.891
1085	0.032381	0.235196	0.603753	1.1	1.103	1.22
956	0.033074	0.235196	0.599659	1.2	0.808	0.95
1330	0.0331	0.235196	0.599506	1.2	0.96	1.162
724	0.034137	0.235196	0.593517	1.1	1.096	1.011
946	0.034194	0.235196	0.593193	1.1	1.051	0.958
1194	0.035542	0.235196	0.585644	1.1	1.098	1.234
774	0.036342	0.235196	0.581279	1.1	1.067	0.974
556	0.03727	0.235196	0.576316	1.3	1.103	0.867
1365	0.037285	0.235196	0.576236	1.3	0.887	1.128
172	0.037784	0.235196	0.57361	1.2	1.131	0.946
990	0.038073	0.235196	0.572104	1.2	0.958	0.779
770	0.038708	0.235196	0.568831	1.3	0.766	0.986
943	0.038784	0.235196	0.568444	1.3	0.988	1.241
518	0.039127	0.235196	0.566695	1.1	1.057	0.937
1252	0.03921	0.235196	0.566274	1.2	0.882	1.059
1147	0.040121	0.235196	0.561709	1.2	0.974	1.159
965	0.040147	0.235196	0.561582	1.1	0.966	1.096
690	0.040154	0.235196	0.561547	1.3	0.878	1.137
484	0.040741	0.235196	0.558654	1.8	1.214	0.659
381	0.040978	0.235196	0.557497	1.1	1.029	1.102
238	0.042235	0.235196	0.551454	1.1	0.966	0.867

973	0.043363	0.235196	0.546167	1.2	0.978	1.125
1170	0.044931	0.235196	0.539013	1.2	0.862	1.069
89	0.047504	0.235196	0.527753	1.1	0.937	1.022
1379	0.047572	0.235196	0.527464	1.1	0.898	1.017
697	0.048046	0.235196	0.525453	1.2	0.832	0.98
421	0.048301	0.235196	0.524378	1.2	0.978	0.844
175	0.05011	0.235196	0.516901	1.2	0.971	0.801

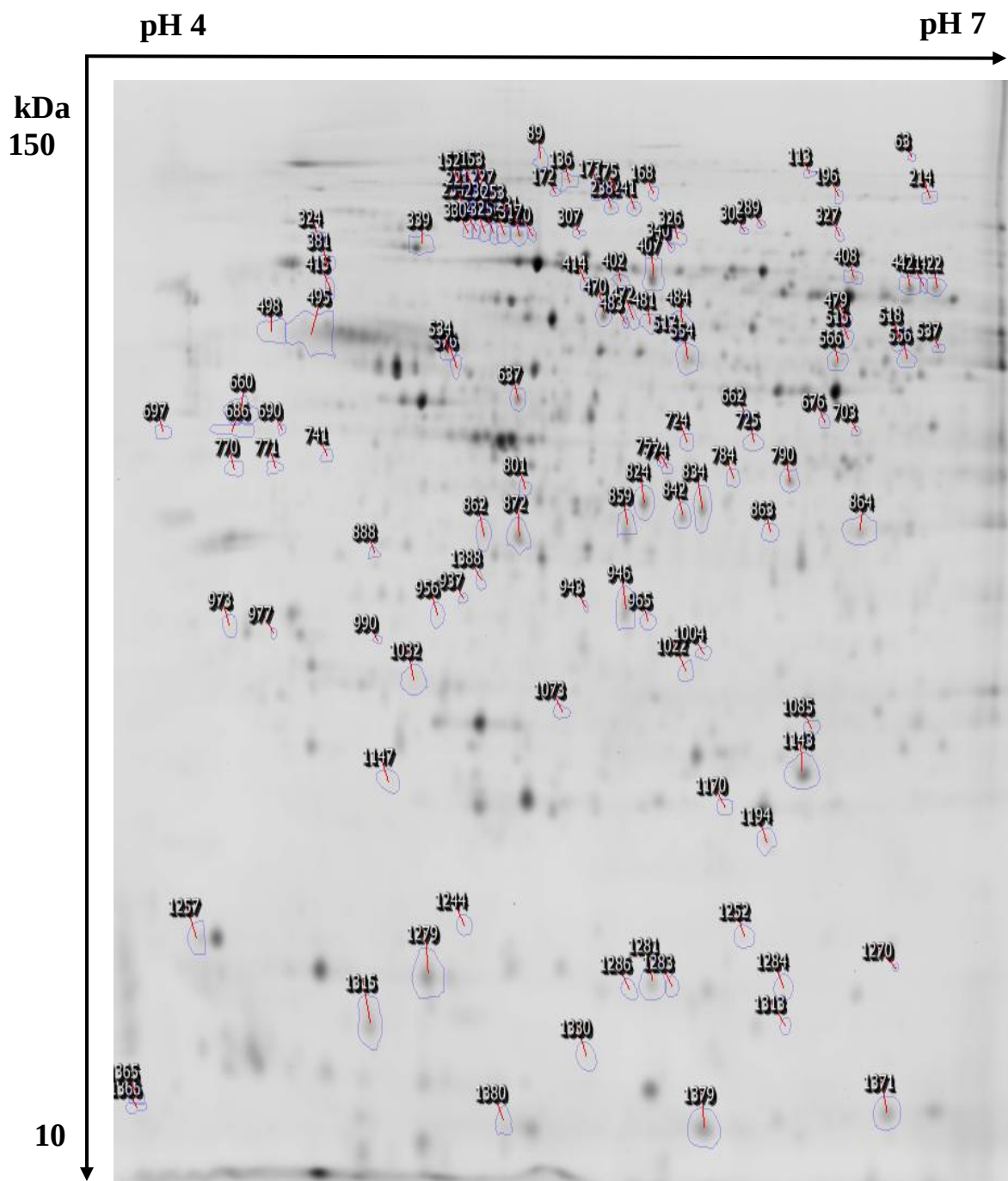


Figure 4-24 Hippocampus: Location of significant spots following univariate analysis and chronic + 4 weeks of ECS treatment

Following analysis by Progenesis SameSpot software (NonLinear Dynamics) the spots highlighted on the gel were identified as being significantly up- or down-regulated ($p \leq 0.05$ one-way ANOVA). The linear pI scale and molecular weights (kDa) are marked at the gel.

4.3.6.1.1 Principal component analysis

Principal component analysis (PCA), conducted by Progenesis SameSpot software (NonLinear Dynamics) for the detection of outliers, was applied to the data. The ECS treated gels and the control treated gels cluster well according to treatment (Figure 4-25). There was no interaction between the two groups, suggesting a robust treatment effect. There were no outlying gels following chronic + 4 weeks of ECS.

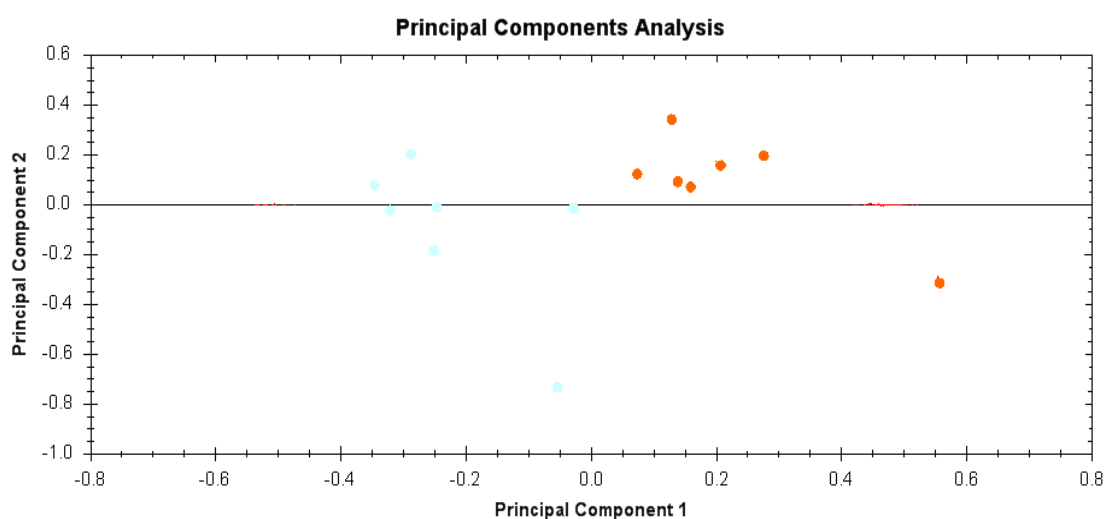


Figure 4-25 PCA plot of treated and control hippocampal samples following chronic + 4 weeks of ECS treatment

Control sample gels are represented by blue spots, the ECS treated gels are represented by the orange coloured spots.

4.3.6.2 Multivariate analysis

The spots that contributed to variation following chronic + 4 weeks of ECS treatment after multivariate analysis are listed according to p-value below (Table 4-10). Those spots found to be significant at $p \leq 0.05$ are listed in the adjoining columns. Only the spots that are significant at this level were considered for further analysis.

Table 4-10 Protein spots identified as being statistically significant following multivariate analysis and chronic + 4 weeks of ECS treatment

List is based on p-values of β coefficients determined to be significant after outliers have been removed. $p \leq 0.05$ values listed in column on right.

Variables that contribute to variation in the hippocampus chronic+4 study				Significant variables $p \leq 0.05$	
Spot number	P-value	Spot number	P-value	Spot number	P-value
55	0.74431	842	0.52144	1270	0.01321
64	0.23794	859	0.89316	316	0.01572
113	0.30095	864	0.14713		
134	0.17366	872	0.14018		
131	0.45303	862	0.68045		
136	0.82225	863	0.31695		
132	0.18513	888	0.3722		
158	0.9155	905	0.55432		
153	0.26082	913	0.38822		
133	0.13516	917	0.85474		
152	0.97136	946	0.70842		
135	0.11413	945	0.615		
170	0.96737	937	0.52766		
177	0.29715	950	0.57988		
176	0.27009	982	0.85531		
175	0.13782	1005	0.18751		
174	0.28793	1004	0.91918		
168	0.44492	1022	0.31109		
172	0.62457	1032	0.14951		
184	0.1768	1055	0.86224		
196	0.34153	1044	0.70856		
227	0.17555	1082	0.53845		
185	0.26752	1080	0.41982		
207	0.8736	1085	0.89605		
214	0.54585	1111	0.92825		
221	0.09191	1143	0.78229		
181	0.76466	1127	0.92216		
238	0.96652	1147	0.68482		
241	0.10648	1157	0.5887		
234	0.23333	1172	0.63581		
235	0.1166	1170	0.94949		

236	0.6126	1186	0.9013		
253	0.85064	1194	0.75453		
281	0.41424	1202	0.33058		
289	0.75211	1268	0.31815		
311	0.84374	1244	0.22653		
325	0.74182	1257	0.25483		
312	0.55205	1252	0.89703		
303	0.83277	1279	0.24718		
304	0.88549	1281	0.49802		
317	0.31551	1274	0.23866		
306	0.45176	1284	0.51908		
320	0.35022	1283	0.06204		
327	0.22968	1286	0.07439		
307	0.44528	1294	0.44188		
326	0.92374	1315	0.64743		
339	0.14698	1313	0.43749		
329	0.57536	1330	0.66463		
407	0.31388	1360	0.92353		
402	0.49793	1365	0.33907		
423	0.78802	1371	0.30959		
414	0.35603	1366	0.45535		
421	0.68007	1380	0.30618		
422	0.81242	1379	0.50822		
415	0.10164	1374	0.38577		
418	0.56859	1381	0.06471		
450	0.45207	660	0.31704		
470	0.80657	1388	0.70223		
472	0.45506	1391	0.34145		
495	0.97027	1397	0.52753		
484	0.97676	1408	0.83394		
481	0.93423	1409	0.5816		
485	0.10223				
483	0.46271				
498	0.52016				
524	0.80245				
518	0.87741				
515	0.68368				
517	0.13154				
534	0.30602				
547	0.92353				
537	0.46046				

555	0.6784				
566	0.36101				
557	0.68866				
576	0.27803				
581	0.71799				
592	0.44958				
629	0.33287				
637	0.11906				
717	0.60498				
676	0.52342				
695	0.64489				
712	0.68572				
686	0.17811				
690	0.38484				
703	0.902				
725	0.38541				
748	0.40534				
751	0.10527				
771	0.45378				
770	0.56179				
768	0.09059				
774	0.53924				
790	0.39327				
789	0.74568				
834	0.32243				
824	0.1791				
814	0.92243				

4.3.6.2.1 PLS-DA diagnostic plots

A number of different plots were used to analyse and detect the sources of variation in each study during multivariate analysis. These include representative plots for the X-Y correlation loadings and weighted regression coefficients following the removal of non-significant spots. The scores plots and explained variance plots that contain all the variables (Figure 4-26 (A), 4-27 (A)) and the redrawn models containing significant variables only (Figure 4-26 (B), 4-27 (B)) are shown below. The before and after plots demonstrate the improvement in explained variance (Figure 4-27) using the significant data only as well as detecting sample clustering patterns and outliers (Figure 4-26).

The Scores plot (Figure 4-26) shows control samples clustered together and treated samples clustered together in both plots (A, B). There are no outliers present as all factors fall inside the Hotelling T^2 ellipse.

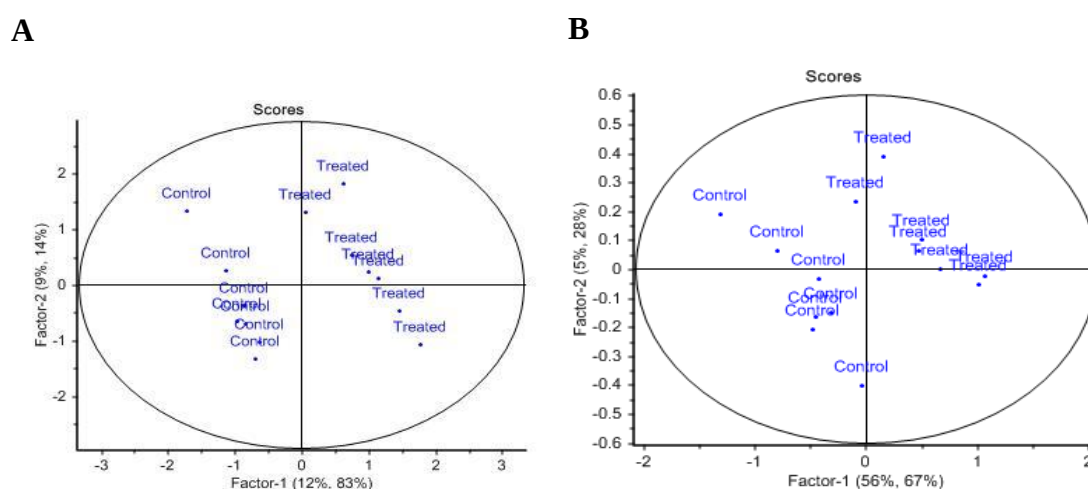
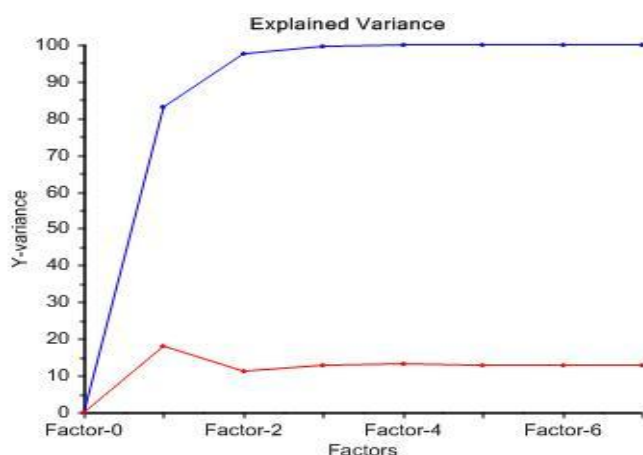


Figure 4-26 Scores plot following chronic +4 weeks of ECS treatment of the hippocampus

(A) Scores plot containing all data; **(B)** Scores plot following removal of non-significant data.

Little variance is explained in plot (A) (Figure 4-27); approximately 20% following one factor which dropped to approximately 10% and then reached a plateau. The model (B) (Figure 4-27) indicated three factors were the optimal model dimension by full cross validation. Cross validation created 163 submodels, where 1 sample was left out in each. The validation curve showed 65% explained variance for three factors. Two factors explain 95% of the variance in Y on the calibration curve.

A



B

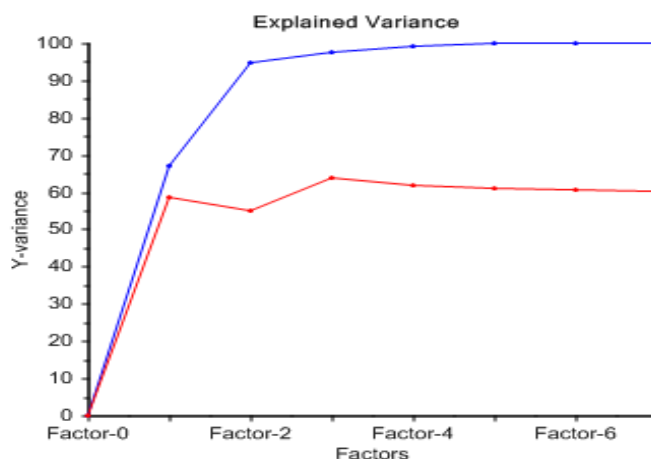


Figure 4-27 Plots of explained variance following chronic + 4 weeks of ECS treatment of the hippocampus

(A) Explained variance curve based on all variables; **(B)** Explained variance plot based on significant variables only.

The variables that are most important for explaining variance in the data (Figure 4-28) can be seen in Figure 4-29, the weighted regression coefficient plot. Circled variables are significant at $p \leq 0.05$.

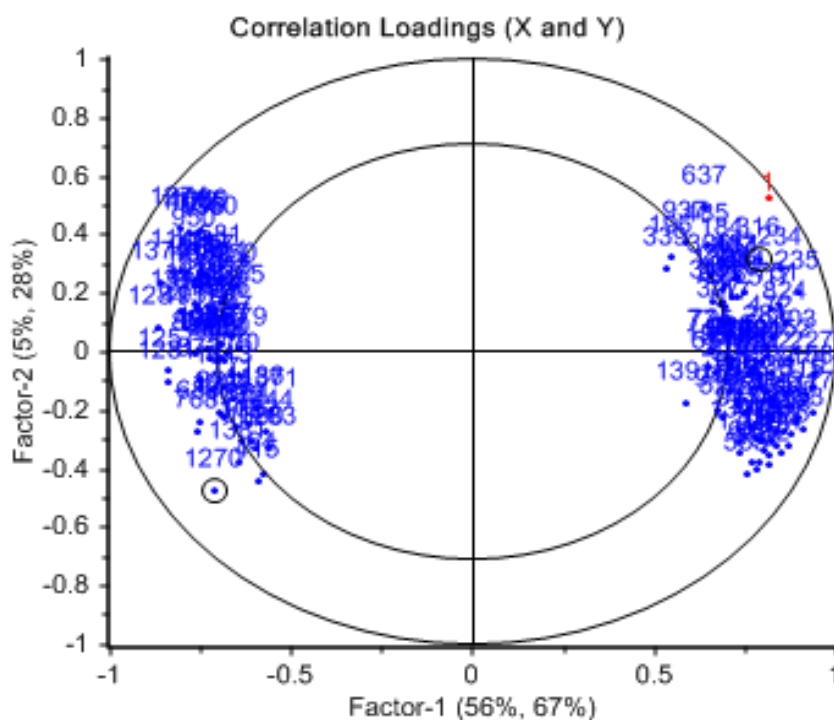


Figure 4-28 X-Y correlation loadings following chronic + 4 weeks ECS treatment of the hippocampus

X-Y correlation loadings plot of data marked as significant only. The Y variable is represented by "1" (red) in each plot.

Figure 4-29 contains the weighted regression coefficients of the significant variables only. The importance of individual variables can be seen in this plot. A 95% confidence interval estimates the uncertainty limits. Variables with uncertainty limits that cross zero are not significant at $p \leq 0.05$. The variables significant at this level are circled.

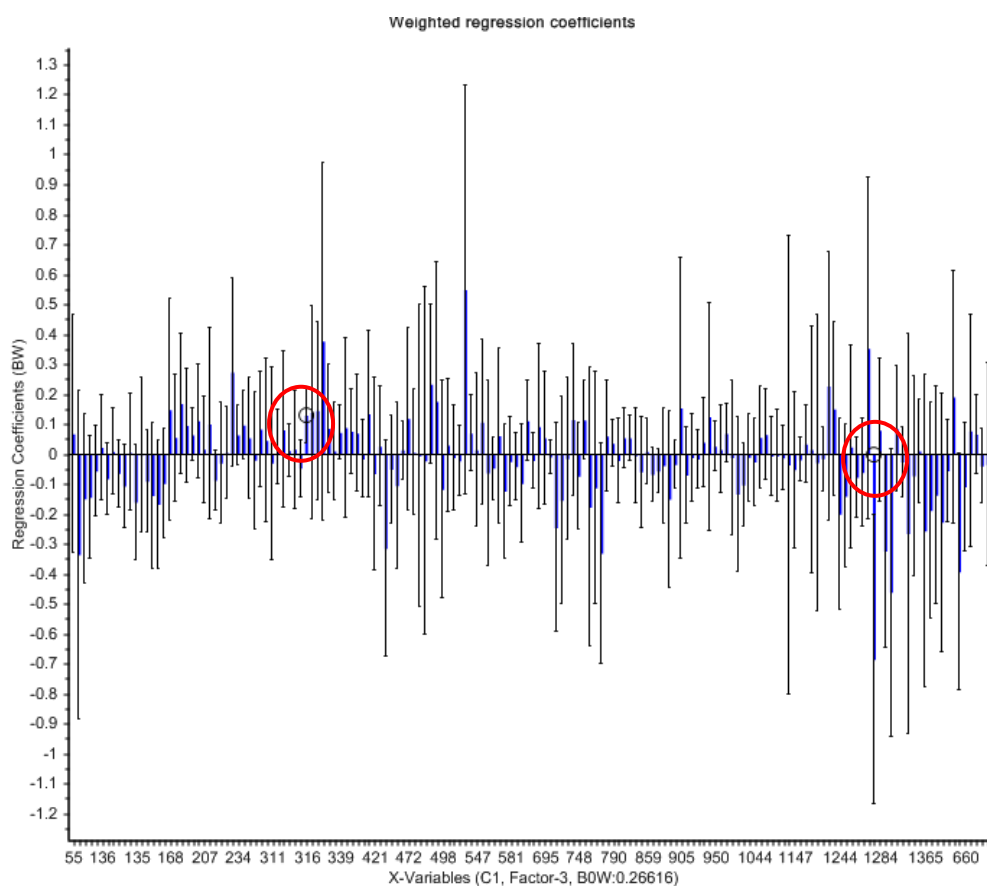


Figure 4-29 Plot of weighted regression coefficients

BW, weighted B-coefficient.

4.3.7 Comparison of univariate and multivariate significant spot findings

A Venn diagram (Figure 4-30) describes the number of spots common to both univariate and multivariate analysis as well as unique to each analysis. The significant spot numbers from each type of analysis are listed in Table 4-11.

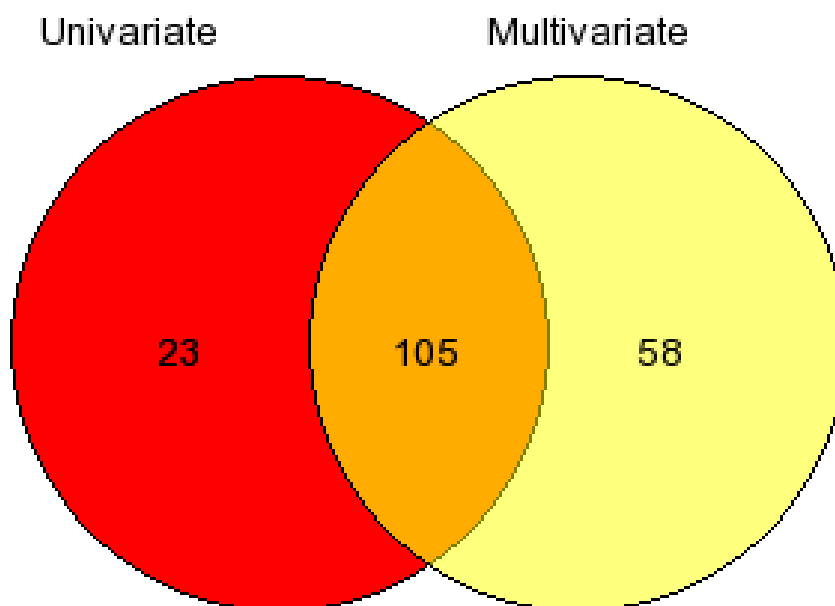


Figure 4-30 Venn diagram of significant spot distribution following univariate and multivariate analysis in the hippocampus following chronic + 4 weeks of ECS treatment

Drawn in www.genevenn.com.

Table 4-11 List of significant spots following univariate and multivariate analysis of the hippocampus following chronic + 4 weeks of ECS treatment

Significant spots unique to univariate analysis	Significant spots common to both		Significant spots unique to multivariate analysis
63	113	690	55
89	136	703	64
324	152	725	131
340	153	751	132
381	158	770	133
408	168	771	134
479	172	774	135
526	175	790	170
554	177	824	174
556	196	834	176
662	214	842	181
697	221	859	184
724	227	862	185
741	234	863	207
784	235	864	311
801	236	872	329
943	238	888	418
956	241	913	450
965	253	917	485
973	281	937	524
977	289	946	547
990	303	1004	555
1073	304	1022	557
	306	1032	581
	307	1085	592
	312	1143	629

	316	1147	695
	317	1170	712
	320	1194	717
	325	1244	748
	326	1252	768
	327	1257	789
	339	1270	814
	402	1279	905
	407	1281	945
	414	1283	950
	415	1284	982
	421	1286	1005
	422	1313	1044
	423	1315	1055
	470	1330	1080
	472	1365	1082
	481	1366	1111
	483	1371	1127
	484	1379	1157
	495	1380	1172
	498	1388	1186
	515		1202
	517		1268
	518		1274
	534		1294
	537		1360
	566		1374
	576		1381
	637		1391
	660		1397
	676		1408
	686		1409

4.3.8 Spots picked for mass spectrometry

The location of the significant protein spots picked from the hippocampus following chronic + 4 weeks of ECS is highlighted in Figure 4-31 on representative preparative gels. These spots, their fold change, p-value and the statistical method by which they were detected are listed in Table 4-12.

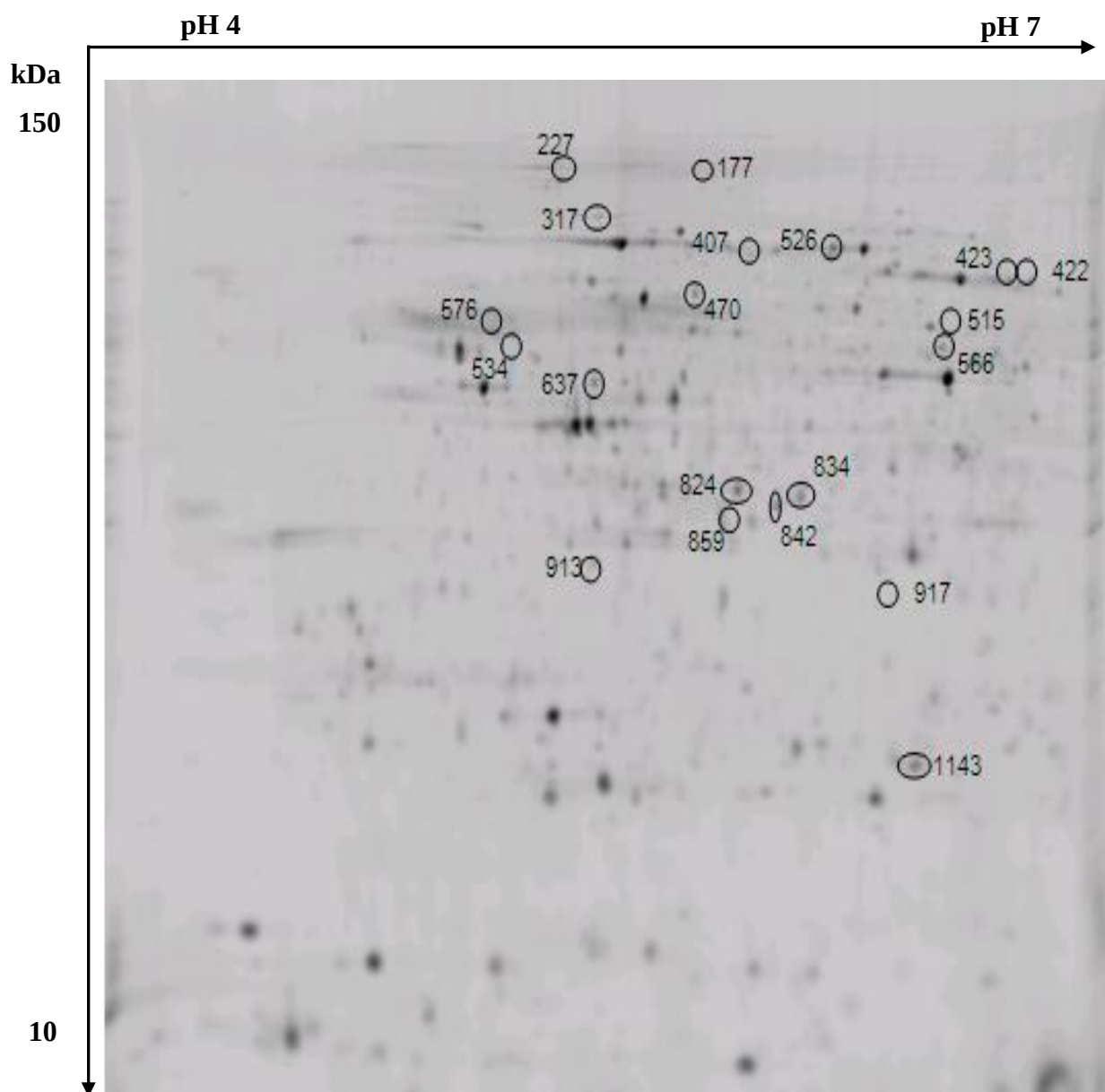


Figure 4-31 Preparative gel indicating significant spots that were picked for identification from the hippocampus following chronic + 4 weeks ECS treatment

Hippocampus	Standard spot no.	Fold change	Up / down regulated	p-value	MVA/UVA
Chronic + 4	534	1.1	Up	P=0.025	MVA/UVA
	422	1.2	Up	P=0.02	MVA/UVA
	637	1.1	Up	P=0.002	MVA/UVA
	407	1.1	Up	P=0.012	MVA/UVA
	317	1.1	Up	P=0.015	MVA/UVA
	566	1.1	Up	P=0.024	MVA/UVA
	423	1.2	Up	P=0.022	MVA/UVA
	470	1.1	Up	P=-0.006	MVA/UVA
	859	1.1	Up	P=0.024	MVA/UVA
	824	1.1	Up	P=0.001	MVA/UVA
	842	1.1	Down	P=0.029	MVA/UVA
	227	1.1	Up	P=0.001	MVA/UVA
	834	1.1	Up	P=0.008	MVA/UVA
	576	1.1	Down	P=0.016	MVA/UVA
	917	1.1	Up	P=0.013	MVA/UVA
	177	1.2	Up	P=0.026	MVA/UVA
	515	1.2	Up	P=0.011	MVA/UVA
	1143	1.1	Down	p=0.031	MVA/UVA
	913	1.2	Up	P=0.0004	MVA/UVA
	526	1.1	Up	P=0.031	UVA

Table 4-12 Spots picked for identification by mass spectrometry

UVA univariate analysis; MVA multivariate analysis; MVA/UVA identified by both multivariate and univariate analysis.

4.4 Discussion

2D-DiGE gels were run to separate out proteins that may be involved in the response to ECS in the hippocampus and frontal cortex. The effects of ECS at different time points were investigated, including after a single treatment or ten treatments, and the longer term effects when animals were sacrificed four weeks after chronic treatment.

The effects of ECS on the rat brain have previously been investigated (Lee et al. 2009); however these authors looked at the whole brain and used silver staining and Coomassie staining techniques to visualise protein changes. As a result, fewer proteins (<1,000 proteins) were resolved on the gels. Twenty-four differentially expressed spots were found and six proteins in total were identified.

2D-DiGE was chosen as the method to separate proteins as it is a highly sensitive technique (section 2.2.15). It allows for the use of an internal standard which reduces variation across gels.

Protein separation was conducted on pH 4-7 IPG strips. These strips give a detailed protein spread over a shorter pH range of interest. Protein pI values usually fall within the pH range 3-12 with the pIs of many proteins between pH 4-7 (Garfin, 2003). The pH 4-7 range of IPG strips has frequently been used in studies investigating the effects of antidepressant pharmacotherapy on the rodent brain (Khawaja et al. 2004; Lee et al. 2009; McHugh et al. 2010).

4.4.1 Univariate analysis

Univariate analysis directly compares the volume of a protein spot from a treated sample to the same spot from a control sample to determine if ECS treatment affects the level of protein expression in that spot. One way ANOVA was used to carry out these comparisons and significance was set at $p \leq 0.05$. A number of spots from each study were found to be significantly different (Table 4-13).

Study	Number of significant spots ($p \leq 0.05$)
acute ECS	65
chronic ECS	44
chronic+4 weeks ECS	128

Table 4-13 The number of significantly different spots determined by univariate analysis

4.4.1.1 p-value & q-value

The significant spots were listed according to p-value but also by q-value. As the p-value was increased, the q-value increased and the power of the study decreased.

Therefore, the spots that are most likely to be truly significant are those with the lowest p-value and q-value but the highest power (NonLinear Dynamics IV, 2011). These spots were particularly targeted for identification by mass spectrometry.

4.4.1.2 Fold change

The fold change in protein expression, as well as the p-value, can be used to identify proteins of interest although it was not used as a mark of significance alone in this study. Large fold changes may be more easily visualised on gels, however most fold changes in protein expression in the hippocampus following ECS treatment were small. A small fold change in protein expression may be statistically significant (Carboni et al. 2010) and could lead to a biological effect (Cecconi et al. 2007). The location of the significant spots detected by univariate analysis can be seen in e.g. Figure 4-24.

4.4.1.3 Across study comparisons

Although it is possible to compare the effects of acute, chronic and chronic+4 week ECS treated animals across gels due to the presence of an internal standard on each gel, this comparison was not made directly. This was due to the different age and weight of animals on the day they were sacrificed. Although a statistical study of a number of spots that were common to more than one study was not made, a non-statistical comparative approach may be informative. Spots found in the acute and chronic+4 week study include 168, 175, 238, 479, 534, 537, 547, 801, 973, 1005 and 1360. Two spots, 932 and 1280, were found following both acute and chronic ECS treatment. These proteins could respond acutely to ECS and therefore may not represent long term changes in expression. Spots found following chronic and chronic + 4 weeks of ECS treatment may respond to ECS chronically with persistent changes in expression. These spots include 194, 312, 450, 572, 1082, 1279, 1315, 1365 and 1409.

4.4.2 Multivariate analysis

Multivariate statistical methods were also applied as it uses information from many variables simultaneously to more fully understand the data. It is likely that many variables contribute to the response to ECS in treated compared to control animals. Applying multivariate statistics can find patterns and relationships between variables. This can be useful, as a change in one variable can lead to changes in other variables that may not be detected by univariate analysis, which looks at a single variable at a time (CAMO V, 2011).

Multivariate analysis is also a graphical technique. Many plots were drawn and used to visualise the data and the variables that contribute to variation in the data in these studies. It was necessary to remove outliers and make adjustments to optimise analysis. Multivariate analysis was initially conducted with all the data (average normalised volumes). Therefore the initial plots were first drawn with all the X-variables included.

4.4.2.1 The effects of acute ECS on the hippocampus

The scores plot and PCA plot were used to detect outlying gels. There were no outlying gels present in the studies. However, one gel was excluded based on its placement in both plots in the hippocampus following acute ECS. If included, the presence of the gel skewed the results and the models did not explain as much variance. Excluding the gel led to better results and more variance explained.

The final explained variance plot showed that over 60% of data is explained by two factors, indicating a robust model. A relatively high level of variation in the data can be explained by a small number of factors (Figure 4-11) implying that the model is representative of the data.

The X-Y correlation plot (Figure 4-12) looked at X-variable (individual spots) expression and the influence they had on the Y variable. This plot was difficult to interpret when first drawn with all the variables as most variables contribute to noise and the role of individual variables was difficult to visualise. The plots were redrawn with the significant spots only. Spots 1, 5, 9, 12, 10 and 17 are strongly positively correlated with Y. Spots 6 and 23 are negatively correlated with Y. The circled spots are significant at $p \leq 0.05$. Variables in the outer ellipse of this plot are defined as having contributed to up to 100% of the variation. While this plot is useful, the weighted regression plot is used to identify the individual contribution each variable makes to variation.

The importance of removing non-significant variables and redrawing the model to visualise the significant variables can be seen in the weighted regression plot. While the

plots of the original data are difficult to interpret, the plots of the significant variables easily highlight the variables that contribute to variation. The variables with a p-value less than 0.05 are denoted in each figure with a circle. All the spots in the weighted regression plot are significant but those with certainty limits (95% confidence intervals) that cross zero are not significant at the 5% level. The corresponding p-values for β -coefficients were included in Table 4-2. Therefore, variables 1 and 9 are significantly positively correlated to variation in Y and variable 23 is significantly negatively correlated to variation in Y. Variables 1, 9 and 23 correspond to spots 66, 460 and 947 respectively. The location of these spots can be visualised in Figure 4-15.

Spot 66 is unique to multivariate analysis as being statistically significant; however spots 460 and 947 were found to be significant following univariate and multivariate analysis. In total 74 spots were found to be significantly changed in the hippocampus of animals treated acutely with ECS. Only those with significance levels of $p \leq 0.05$ shall be highlighted for potential identification by mass spectrometry.

Due to technical difficulties in comparing preparative gels to DiGE gels, spots can be difficult to reliably isolate and identify. This is due to the different protein loadings and staining techniques being compared. Therefore, although precedence was given to the most significant spots detected by statistical analysis, only those spots that could be accurately cut from the preparative gels were chosen. Five spots in total were picked from the hippocampus following acute ECS. One of these was identified by univariate and multivariate analysis (475) while the other four were detected by univariate analysis only.

4.4.2.2 The effects of chronic ECS on the hippocampus

The scores plot and PCA plot were used to detect outlying gels. No gels were excluded as outliers from this study. A single control gel (Figure 4-18) did not cluster as tightly with the other control gels and following removal of the non-significant variables it was found close to the Hotelling T^2 ellipse. However, as it did not fall outside the ellipse it was not excluded from the study.

The explained variance plot showed that approximately 55% of the variation in the data is explained by four factors. This is a relatively high number of factors required to explain a moderate level of variance. However, four factors were required to explain 95% variance in the fitted, validation curve in this plot. Therefore the model for the hippocampus following chronic ECS is not as robust as that for the other hippocampus studies.

The X-Y correlation plot looked at X-variables and found a single circled ($p \leq 0.05$) variable that was strongly positively correlated to Y, variable 1230. While several other variables significantly affected the variance in the hippocampus following chronic ECS (Figure 4-20), they were not determined to be significant at the cut-off level of $p \leq 0.05$. The individual contribution each variable makes to variation was examined in the weighted regression plot.

Variable 1230 is the only variable with certainty limits (95% confidence intervals) that do not cross zero and is therefore significant at the 5% level. Variable 1151 is almost significant at this level ($p = 0.057$) and this can be seen by its corresponding certainty limits (Figure 4-21).

Variable 1230, corresponding to spot 1230, is common to multivariate analysis and univariate analysis as being statistically significant at $p \leq 0.05$ and was therefore selected as being of particular interest for mass spectrometry identification. The location of the spot can be visualised in Figure 4-23. Significant spots detected by univariate analysis (44 in total) shall also be highlighted for potential identification by mass spectrometry.

Overall, the hippocampus chronic multivariate analysis is of lower quality than in the other studies. A greater number of factors were required to explain moderate levels of variance in the data. A single variable was found to contribute to the model at the designated level of statistical significance.

While the model can describe variation seen in the data, it is less robust than other hippocampus models. This may be due to the presence of outliers which can cause large residual variance. There may be an insufficient number of factors to explain the hippocampus chronic data set. Fewer significant spot volume changes were also detected by univariate analysis than in the acute and chronic + 4 week studies (44 compared to 65 and 128, respectively).

Despite the potential limitations of the analysis of this study, spots identified as being statistically significant were selected for further analysis.

A small number of spots were picked for identification from this study. One spot was identified by univariate analysis alone (580), the others were common to both analyses but not at $p \leq 0.05$ following multivariate analysis. Although the effect of chronic antidepressant treatment in the hippocampus is particularly interesting (Kedracka-Krok et al. 2010; Khawaja et al. 2004), the low number of significant spots picked for identification may be justified by the weaker results obtained following statistical analysis.

4.4.2.3 The effects of chronic + 4 weeks of ECS on the hippocampus

The scores plot and PCA plot were used to detect outlying gels. There were no outlying gels present in this study as determined by both plots (Figure 4-25 and 4-26). The gels cluster together well based on treatment.

The explained variance plot showed that three factors explain approximately 65% of the variation in Y (Figure 4-27). The significant variables explain a substantial amount of the variation in the data. Fewer factors are required to explain the variation than seen in following chronic ECS, suggesting this model is more robust. However, a stronger model would explain a greater amount of variance in the data. While only two spots were significant at $p \leq 0.05$ following multivariate analysis, many other spots (161 in total) were found to be significant.

The X-Y correlation plot looked at X-variable (individual spots) expression and the influence they had on the Y variable. Following the removal of the non-significant spots, a large number of significant spots remained in this plot. Two variables, 1270 and 316, were circled ($p \leq 0.05$). Variable 1270 is negatively correlated to Y whereas variable 316 is strongly positively correlated to Y.

This result was more clearly visualised in the weighted regression coefficient plot (Figure 4-29) which clearly identified spots 1270 and 316 as being statistically significant ($p \leq 0.05$; uncertainty limits did not cross zero). All significant variables as well as variables 1270 and 316 are listed according to their p-values in Table 4-11. A large number of spots (105 spots) were found to be significant following both univariate and multivariate analysis including variable 316 (spot 316). Variable 1270 was only found to be significant following multivariate analysis. However, only those spots deemed statistically significant ($p \leq 0.05$) will be considered for selection for identification by mass spectrometry.

A large number of spots were picked following chronic + 4 weeks of ECS treatment for identification (Table 4-12). All but one of these spots (526) was identified by both univariate and multivariate analyses. However, these multivariate spots were not significant at $p \leq 0.05$. The picked spots represent a range of proteins of different molecular weights and isoelectric points that may be informative about the long-term effects of ECS treatment on the hippocampus.

4.4.3 Summary

ECS induces a wide range of changes in the brain. The proteome contains a dynamic range of proteins that can number in the hundreds of thousands (Van Eyck et al. 2008). Proteomic analysis gives a snapshot of the protein expression in the hippocampus at a given time. Therefore, in order to best mine the data available from a single study, which has reliably detected on average 1500 proteins, multiple approaches must be taken to examine the data. Univariate analysis alone does not give a complete picture of the data but used in combination with multivariate analysis can be used to detect variables that may make a significant contribution to the variation seen in the data (Pedreschi et al. 2009). Therefore, spots that were found to be statistically significant ($p \leq 0.05$) by both analyses were highlighted for picking and identification by mass spectrometry. Although multivariate analysis identified many other spots as being significant in each study, they were not chosen for further analysis based solely on their p-value. Many significant spots that were also identified by univariate analysis were picked for mass spectrometric identification.

Difficulties in picking spots from preparative gels meant that many of the most significant spots were not selected for identification. However, a range of spots from throughout the gel were reliably picked for identification. The proteins expressed in these spots may help to explain the antidepressant effects of ECS.

The protein spots that were picked for identification by mass spectrometry resulted in the identification of dozens of proteins, with multiple proteins found in each spot. Protein identification and confirmation is discussed in chapter 5.

Identification and confirmation of hippocampal proteins following electroconvulsive stimulation

5.1 Introduction

Although the changes in protein expression in the hippocampus following antidepressant pharmacotherapy have been studied, the effects of ECS treatment have not been thoroughly investigated. Therefore in Chapter 4, the acute, chronic and longer term effects of ECS on hippocampal proteins were determined, using 2DiGE to separate proteins according to their molecular weight and isoelectric point. Univariate and multivariate statistical methods were applied to find the protein spots which were differentially expressed following ECS treatment. From this selection, several protein spots were picked to identify, by means of mass spectrometry, the proteins that underwent changes in expression following ECS.

5.1.1 Aims of the experiment

The purpose of this study was to identify hippocampal proteins that are differentially expressed following ECS treatment using mass spectrometry. These hippocampal proteins were then classified using the gene ontology (GO) database (Gene Ontology Consortium, 2001), according to molecular function of the proteins, the biological processes they are involved in and the cellular components they are found in. GO analysis was conducted in order to understand the potential role of the proteins in the hippocampus following ECS treatment.

To determine the interactions and the pathways between the proteins the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Ogata et al. 1999) and Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, www.ingenuity.com) were employed. Finally, to confirm the changes in protein expression levels detected in the proteomics study (Chapter 4) semi-quantitative immunoblotting of a number of proteins of particular interest was conducted.

A number of protein spots that underwent significant differential expression in the hippocampus following ECS were identified by mass spectrometry. This resulted in a list of proteins that may contribute to the effects of ECS treatment. The identified proteins are similar in nature to those detected in previous proteomic studies of antidepressant therapy, as discussed in Chapter 4 (section 4.1). Cytoskeletal and metabolic-related protein expression was particularly prevalent. Gene ontology (GO) analysis also highlighted the

cytoskeleton and metabolism as biological processes in which the identified proteins were involved.

While a large number of hippocampal proteins were identified, confirmation of a number of selected proteins was conducted to verify the changes in expression of particular proteins of interest. Protein confirmation is often overlooked in proteomic studies (Ji et al. 2009; Mallei et al. 2011; Marais et al. 2009; McHugh et al. 2010) but is important to fully understand the nature of the protein changes detected following ECS treatment.

The proteins identified by mass spectrometry, the analysis conducted to classify the proteins and visualise the potential interactions between them and the results of studies to confirm the expression of a number of these proteins is discussed below.

5.2 Methods

5.2.1 Study design and animal treatment

A study design similar to that used in Chapter 4 (section 4.2.1) was repeated for confirmation studies of selected proteins. Male Sprague-Dawley rats (150g-200g) were housed four per cage under a 12hr light-dark cycle (lights on 08:00) with food and water available ad libitum throughout the experiment. Control animals were handled identically to treated animals but no charge was delivered (section 2.2.1). ECS was administered using the following parameters: 100 pulses per second; 0.5ms pulse width; 0.7s duration; 75mAmp current.

Animals (n=7-10 per group) were administered an acute or chronic course of ECS according to the timeline in Figure 5-1. A subset of chronically treated animals was sacrificed four weeks after the final ECS treatment.

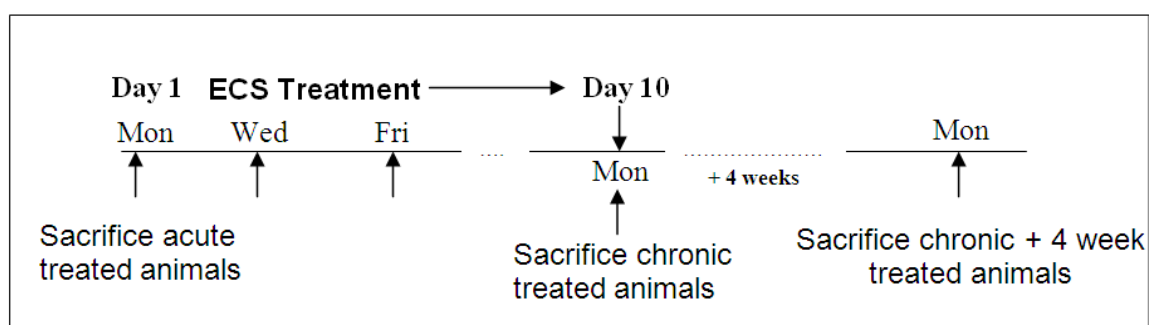


Figure 5-1 ECS treatment course timeline for proteomic studies

5.2.2 Experimental procedure

5.2.2.1 Mass spectrometry and bioinformatics

Proteins were identified using LTQ Orbitrap mass spectrometry. MS/MS results were searched against the Uniprot/Swiss-Prot rat database (section 2.2.20) using SEQUEST (Bioworks, ThermoFinnigan).

Bioinformatic analysis was conducted using the Database for Annotation, Visualisation and Integrated Discovery (DAVID) (Dennis et al. 2003). The protein accession identifications from each study were uploaded. FatGO terms ($p < 0.001$, FDR < 5; Chitteti et al. 2011) were listed for gene ontology (GO) categories: (i) molecular function, (ii) biological process and (iii) cellular component. The KEGG pathway database was also searched through DAVID for each study. Ingenuity pathway analysis (IPA) software (Ingenuity Systems, www.ingenuity.com) was used to draw maps of protein interactions based on the accession numbers of proteins identified by mass spectrometry in each study. The KEGG and IPA software was applied to the protein data as described in detail in section 2.2.20.2.

5.2.2.2 Database for Annotation, Visualisation and Integrated Discovery (DAVID) and Gene Ontology

The gene ontology (GO) database (Ashburner et al. 2000) is a multi species database that provides three structured ontologies of terms to describe the biological processes, molecular functions and cellular components of gene products (Dimmer et al. 2008). GO overcomes previous issues that arose when classifying proteins. A lack of consistency in the vocabulary used to describe proteins and their functions impeded automatic classification of similar proteins (Malik et al. 2010). Each protein can be described by a finite vocabulary (GO term) at different levels. Each GO term belongs to the biological process, molecular function or cellular component category. However, a protein can be involved in multiple biological processes, have several molecular functions and be active in more than one cellular component (Malik et al. 2010). GO is organised in a hierarchy and consists of parent and child terms so that proteins can be described at several levels. However, inclusion of all levels of the hierarchy can lead to an unnecessary level of detail in the set of results. For example, a protein that is involved in regulating the biological process “cholesterol transport” would be described as follows:

Biological process: biological regulation → regulation of biological process → regulation of localisation → regulation of transport → regulation of lipid transport → regulation of sterol transport → regulation of cholesterol transport (From Dimmer et al. 2008).

The broadest GO annotation terms were filtered out using the GO meta-tool DAVID (Dennis et al. 2003) which can search and integrate information in GO (Malik et al. 2010). Only FatGO terms from the DAVID search were included in the results as it filters out the broadest classification terms. Functional annotation results following DAVID search was conducted for the protein identifiers uploaded for each study (section 2.2.20.2). Due to the large number of results for each study, stringent statistical cut-off points for inclusion were used. Only terms with a significance level of $p < 0.001$ and a false discovery rate (FDR) of ≤ 5 were included.

5.2.2.3 Immunoblotting

Following ECS treatment the animals were sacrificed as previously described (section 2.2.13). Hippocampus tissue was collected for analysis by semi-quantitative immunoblotting as in section 2.2.21. Briefly, 50µg of protein were diluted 2X with Laemmli buffer (0.0625M Tris-HCl pH6.8, glycerol 20%, SDS 20%, β-mercaptoethanol 5%, Bromophenol blue (10X) 15%) and incubated at 95°C for 5 minutes. The proteins were separated on 8-12% SDS-PAGE gels in electrode running buffer (125mM Tris Base, 960mM glycine, 0.5% (v/v), 10% SDS). The proteins were blotted onto PVDF membrane (Millipore, Ireland) at 0.23A per blot for 1 hour 45 minutes. The membrane was blocked in 5% BSA (Sigma Aldrich, Ireland)/ dried milk powder in TBS-T for one hour at room temperature. The membrane was washed in PBS-T for 3 x 10 minutes and then incubated in primary antibody overnight at 4°C (antibodies and dilutions listed in Table 2-7). The primary antibody was washed off (3 x 10 minutes PBS-T) the next morning, and then incubated in secondary antibody for one hour at room temperature (antibodies and dilutions listed in Table 2-7). Protein bands were detected following five minute incubation in chemiluminescent solution (Millipore, Ireland) and membrane visualisation (Las 3000, Kodak, UK). The protein bands were compared by densitometry using ImageJ software. The relative % density of ECS treated samples as a percentage of control samples were presented.

5.3 Results

5.3.1 Hippocampus: protein identification and classification following acute ECS

The proteins identified by mass spectrometry in the hippocampus are detailed below (Tables 5-1 and 5-2). Table 5-1 lists the protein name, Uniprot (www.uniprot.org) protein accession number, the number of the 2-D gel protein spot in which the protein was found (e.g. Figure 4-8) and the theoretical and experimental molecular weights and isoelectric points of each protein. The proteins are loosely classified based on the function of the protein as described in the Uniprot Knowledgebase (UniprotKB) protein homepage.

A more detailed classification of the proteins, by the gene ontology (GO) categories biological process and molecular function that are listed in Uniprot, is listed in Table 5-2. Mass spectrometry information including the protein cross correlation (XCorr) score, the number of peptides, and percentage coverage, the mass spectrometry p-value, the fold change in protein expression and the one way ANOVA (univariate analysis) p-value for that spot is included in Table 5-2. XCorr is cross correlation score between the observed peptide mass fragment spectrum and the predicted one (ThermoScientific, 2007). The percentage coverage is based on the amino acid sequence of the protein (ThermoScientific, 2007).

Protein Name	Accession number	Standard spot no.	pI Experimental	pI Theoretical	MW (kDa) experimental	MW (kDa) Theoretical
CYTOSKELETON						
Actin, cytoplasmic 1	P60711	468	6.2	5.29	64176.3481	41737
		1103	5		26827.9507	
		1360	5.9		13197.9658	
Actin, aortic smooth muscle	P62738	468	6.2	5.24	64176.3481	42009
		1103	5	5.24	26827.9507	42009
Spectrin alpha chain, brain	P16086	285	5.1	5.2	77300.624	284637.5
Glial fibrillary acidic protein	P47819	285	5.1	5.35	77300.624	49957.09
F-actin-capping protein subunit alpha-1	B2GUZ5	468	6.2	5.44	64176.3481	32778.58
F-actin-capping protein subunit alpha-2	Q3T1K5	468	6.2	5.58	64176.3481	32835.87
TRANSCRIPTION REGULATION						
Histone H4	P62804	468	6.2	11.36	64176.3481	11236.15
Thioredoxin	P11232	468	6.2	4.8	64176.3481	11542.28
Abhydrolase domain-containing protein 14B	Q6DGG1	1103	5	5.65	26827.9507	22617.93
PROTEIN BIOSYNTHESIS						
Elongation factor 1-alpha 1	P62630	468	6.2	9.1	64176.3481	50113.84
Elongation factor 2	P05197	468	6.2	6.42	64176.3481	95152.82
CHAPERONE						
T-complex protein 1 subunit epsilon	Q68FQ0	475	5.6	5.51	63434.3509	59405.58
Protein DJ-1	O88767	468	6.2	6.32	64176.3481	19974.17
HEAT SHOCK FAMILY						
60 kDa heat shock protein, mitochondrial	P63039	475	5.6	5.35	63434.3509	57925.78
78 kDa glucose-regulated protein	P06761	285	5.1	5.01	77300.624	70474.59
METABOLIC PROCESSES						
L-lactate dehydrogenase B chain	P42123	468	6.2	5.7	64176.3481	36481.18
Pyruvate kinase isozymes M1/M2	P11980	468	6.2	6.69	64176.3481	57686.6
Lactoylglutathione lyase	Q6P7Q4	1103	5	5.12	26827.9507	20688.42
ATP synthase subunit gamma, mitochondrial	P35435	1103	5	8.87	26827.9507	30190.7
ANGIOGENESIS						
Rho-related GTP-binding protein RhoB	P62747	1103	5	5	26827.9507	21782.92
PROTEOLYSIS						
Proteasome subunit beta type-6	P28073	1103	5	4.98	26827.9507	21964.93
SYNAPTIC PLASTICITY						
Calcium/calmodulin-dependent protein kinase type II subunit beta	P08413	1360	5.9	6.73	13197.9658	60401.8
Neurogranin	Q04940	1360	5.9	6.53	13197.9658	7496.31
ENDOCYTOSIS/VESICLE TRANSPORT						
Myc box-dependent-interacting protein 1	O08839	285	5.1	4.95	77300.624	64533.21
Vesicle-fusing ATPase	Q9QUL6	285	5.1	6.55	77300.624	82652.49

Table 5-1 Hippocampus: protein identification following acute ECS

This table includes information on the protein identity, its theoretical and experimental weights and isoelectric points. MW, molecular weight; kDa, kilo Dalton; pI, isoelectric point.

Table 5-2 Hippocampus: mass spectrometry results following acute ECS

This table contains information from the mass spectrometry analysis of each protein and the protein classification according to biological process and molecular function as derived from GO in Uniprot database. The MS p-value is the significance value assigned to the protein following mass spectrometry identification. The STAT p-value refers to the one-way ANOVA p-value of the protein spot following univariate analysis (Chapter 4 section 4.2.4). MS, mass spectrometry; STAT, statistical (one-way ANOVA) value; XC, cross correlation (Xcorr).

Protein Name	Accession number	Standard spot no.	Protein Score (XC)	No. peptides	Coverage (%)	MS p-value	Fold change	STAT p-value	Gene Ontology (Biological Process)	Gene Ontology (Molecular Function)
CYTOSKELETON										
Actin, cytoplasmic 1	P60711	468	10.17	1	1.7	0.000911	1.3	0.018	axonogenesis	ATP binding; protein kinase binding
		1103	18.23	2	6.1	2.05E-05	1.1	0.032		
		1360	10.24	1	6.1	3.98E-06	1.2	0.033		
Actin, aortic smooth muscle	P62738	468	10.13	1	2.9	0.000103	1.3	0.018	muscle contraction	ATP binding
		1103	10.2	1	6.1	0.000257	1.1	0.032		
Spectrin alpha chain, brain	P16086	285	30.16	3	1.2	2.66E-05	1.2	0.028	actin cytoskeleton reorganisation; actin filament capping	actin binding; calcium ion binding; calmodulin binding; protein N-terminus binding; protein complex binding; syntaxin binding
Glial fibrillary acidic protein	P47819	285	10.13	1	2.6	0.000401	1.2	0.028	response to wounding	structural constituent of cytoskeleton
F-actin-capping protein subunit alpha-1	B2GUZ5	468	10.19	1	5.9	4.15E-05	1.3	0.018	actin cytoskeleton organisation; actin filament capping	actin binding
F-actin-capping protein subunit alpha-2	Q3T1K5	468	80.37	8	44.1	1.57E-10	1.3	0.018	actin cytoskeleton organisation; actin filament capping	actin binding
TRANSCRIPTION REGULATION										
Histone H4	P62804	468	20.16	2	19.4	2.89E-06	1.3	0.018	cell differentiation; multicellular organismal development; nucleosome assembly; ossification	DNA binding
Thioredoxin	P11232	468	10.13	1	8.6	0.000112	1.3	0.018	electron transport chain; positive	thioredoxin-disulfide reductase

									regulation of DNA binding; regulation of protein import into nucleus, translocation; response to radiation; transport	activity
Abhydrolase domain- containing protein 14B	Q6DGG1	1103	10.14	1	5.2	0.000627	1.1	0.032		hydrolase activity
PROTEIN BIOSYNTHESIS										
Elongation factor 1-alpha 1	P62630	468	10.17	1	1.7	0.000911	1.3	0.018	GTP catabolic process; translational elongation; translation	GTP binding; GTPase activity; translation elongation factor activity
Elongation factor 2	P05197	468	10.16	1	1	5.51E-05	1.3	0.018	translational elongation; GTP catabolic process	GTP binding; GTPase activity; translation elongation factor activity
CHAPERONE										
T-complex protein 1 subunit epsilon	Q68FQ0	475	10.14	1	1.8	6.19E-06	1.1	0.027	protein folding	ATP binding; unfolded protein binding
Protein DJ-1	O88767	468	10.19	1	10.1	2.56E-05	1.3	0.018	autophagy; cellular response to hydrogen peroxide; inflammatory response; mitochondrion organization; negative regulation of cell death; negative regulation of protein binding; Neuroprotection; positive regulation of oxidative phosphorylation uncoupler activity; protein stabilization; regulation of inflammatory response; response to drug; single fertilization	mRNA binding; peptidase activity; peroxidase activity; protein homodimerization activity
HEAT SHOCK FAMILY										
60 kDa heat shock protein, mitochondrial	P63039	475	20.15	2	3.7	7.19E-07	1.1	0.027	anti-apoptosis; chaperone mediated protein folding requiring cofactor; detection of misfolded protein; positive regulation of anti-apoptosis; positive regulation of inflammatory response; response to ATP; response to cocaine; response to drug; response to	ATP binding; insulin binding; misfolded protein binding; protease binding; protein complex binding; protein heterodimerization activity

									estrogen stimulus; response to heat; response to hydrogen peroxide; response to hypoxia; response to lipopolysaccharide	
78 kDa glucose-regulated protein	P06761	285	170.26	17	32.6	2.11E-10	1.2	0.028	anti-apoptosis; ER overload response; activation of signaling protein activity involved in unfolded protein response; cerebellum structural organisation; cerebellar Purkinje cell layer development	ATP binding; unfolded protein binding
METABOLIC PROCESSES										
L-lactate dehydrogenase B chain	P42123	468	10.18	1	4.8	7.87E-09	1.3	0.018	NAD metabolic process; glycolysis; lactate metabolic process; oxidation-reduction process	L-lactate dehydrogenase activity; NAD binding; identical protein binding
Pyruvate kinase isozymes M1/M2	P11980	468	50.19	5	11.1	6.94E-06	1.3	0.018	ATP biosynthetic process; Glycolysis; liver development; organ regeneration; pyruvate biosynthetic process; response to gravity; response to hypoxia; response to insulin stimulus; response to muscle inactivity; response to nutrient; skeletal muscle tissue regeneration	ADP binding; ATP binding; magnesium ion binding; potassium ion binding; protein binding; pyruvate kinase activity
Lactoylglutathione lyase	Q6P7Q4	1103	40.28	4	27.2	5.49E-11	1.1	0.032	anti-apoptosis; carbohydrate metabolic process; glutathione metabolic process; methylglyoxal metabolic process; lactoylglutathione lyase activity	metal ion binding
ATP synthase subunit gamma, mitochondrial	P35435	1103	10.15	1	4	0.000851	1.1	0.032	ADP biosynthetic process; ATP synthesis coupled proton transport; oxidative phosphorylation	hydrogen ion transporting ATP synthase activity, rotational mechanism; proton-transporting ATPase activity, rotational mechanism
ANGIOGENESIS										
Rho-related GTP-binding protein RhoB	P62747	1103	10.17	1	6.1	2.27E-07	1.1	0.032	Angiogenesis; cell adhesion; cell differentiation; endosome to lysosome transport; negative	GTP binding

									regulation of cell cycle; positive regulation of angiogenesis; protein transport; small GTPase mediated signal transduction; transformed cell apoptosis	
PROTEOLYSIS										
Proteasome subunit beta type-6	P28073	1103	20.18	2	8.8	2.99E-06	1.1	0.032	proteolysis involved in cellular protein catabolic process	threonine-type endopeptidase activity
SYNAPTIC PLASTICITY										
Calcium/calmodulin-dependent protein kinase type II subunit beta	P08413	1360	10.15	1	1.8	1.77E-05	1.2	0.033	long-term synaptic potentiation; protein phosphorylation	ATP binding; calmodulin binding; calmodulin-dependent protein kinase activity
Neurogranin	Q04940	1360	30.22	3	39.7	6.68E-05	1.2	0.033		calmodulin binding

5.3.2 Hippocampus: GO analysis following acute study

The GO terms found to be significant by DAVID following a search for FatGO terms for proteins in the (i) biological process, (ii) molecular function and (iii) cellular component ontologies are listed below in Tables 5-3 - 5-5. The number of proteins (count) and their identifier (accession number) as well as the p-value for each term following GO classification is also listed. The (i) biological process (ii) molecular function or (iii) cellular component each protein is involved in is listed under Term.

Table 5-3 Biological Processes

Term	Count	P-Value	Accession number
actin filament capping	3	1.72E-04	B2GUZ5, Q3T1K5, P16086
negative regulation of actin filament depolymerization	3	2.44E-04	B2GUZ5, Q3T1K5, P16086
negative regulation of actin filament polymerization	3	2.84E-04	B2GUZ5, Q3T1K5, P16086
regulation of actin filament depolymerization	3	3.27E-04	B2GUZ5, Q3T1K5, P16086
negative regulation of protein polymerization	3	4.75E-04	B2GUZ5, Q3T1K5, P16086
negative regulation of protein complex assembly	3	7.81E-04	B2GUZ5, Q3T1K5, P16086
negative regulation of protein complex disassembly	3	0.001423	B2GUZ5, Q3T1K5, P16086

Table 5-4 Molecular Function

Term	Count	P-Value	Accession number
purine ribonucleotide binding	11	3.09E-04	P62747, P63039, Q68FQ0, P60711, Q9QUL6, P06761, P05197, P62630, P62738, P08413, P11980
ribonucleotide binding	11	3.11E-04	P62747, P63039, Q68FQ0, P60711, Q9QUL6, P06761, P05197, P62630, P62738, P08413, P11980
nucleotide binding	12	4.29E-04	P62747, P63039, Q68FQ0, P42123, P60711, Q9QUL6, P06761, P05197, P62630, P62738, P08413, P11980
purine nucleotide binding	11	4.61E-04	P62747, P63039, Q68FQ0, P60711, Q9QUL6, P06761, P05197, P62630, P62738, P08413, P11980

Table 5-5 Cellular Component

Term	Count	P-Value	Accession number
soluble fraction	6	3.49E-04	Q6P7Q4, P62747, P42123, P60711, P62738, P11980
cytosol	9	0.001543	P11232, Q88767, P63039, Q68FQ0, P60711, Q9QUL6, P06761, P62630, P11980

5.3.3 Hippocampus: protein identification and classification following chronic ECS

Table 5-6 and 5-7 below are organised as described in section 5.4.1. Table 5-6 lists the protein name, accession number, the number of the spot in which the protein was found and the theoretical and experimental molecular weights and isoelectric points of each protein.

Proteins are assigned a more detailed classification, by biological process and molecular function in Table 5-7. Mass spectrometry information including the protein score (XCorr), the number of peptides, and percentage coverage, the mass spectrometry p-value, the fold change in protein expression and the one way ANOVA p-value for that spot are included.

Protein Name	Accession number	Standard spot No.	pI experimental	pI theoretical	MW (kDa) experimental	MW (kDa) theoretical
CYTOSKELETON						
Actin, cytoplasmic 1	P60711	1256	4.3	5.29	18066.35335	41737
		580	5.5		57799.02334	
		872	5.4		40776.08257	
		758	5.5		47430.9693	
Actin, aortic smooth muscle	P62738	1256	4.3	5.24	18066.35335	42009
		580	5.5		57799.02334	
		872	5.4		40776.08257	
Actin, alpha cardiac muscle 1	P68035	580	5.5	5.23	57799.02334	41784.64
Spectrin alpha chain, brain	P16086	580	5.5	5.2	57799.02334	284637.5
		872	5.4		40776.08257	
Tubulin beta-2A chain	P85108	580	5.5	4.78	57799.02334	49906.97
Tropomodulin-2	P70566	758	5.5	5.34	47430.9693	39491.83
Glial fibrillary acidic protein	P47819	758	5.5	5.35	47430.9693	49957.09
NEURONAL PLASTICITY						
Beta-synuclein	Q63754	580	5.5	4.48	57799.02334	14504.16
PROTEIN TRANSPORT						
Alpha-actinin-4	Q9QXQ0	580	5.5	5.27	57799.02334	104915.02
HEAT SHOCK FAMILY PROTEIN						
78 kDa glucose-regulated protein	P06761	758	5.5	5.01	47430.9693	70474.59

Table 5-6 Hippocampus: protein identification following chronic ECS

The table includes information on the protein identity, it's theoretical and experimental weights and isoelectric points. MW, molecular weight; kDa, kilo Dalton; pI, isoelectric point.

Table 5-7 Hippocampus: mass spectrometry results following chronic ECS

This table contains information from the mass spectrometry analysis of each protein and the protein classification according to biological process and molecular function as derived from GO in Uniprot database. XC, cross correlation; MS mass spectrometry; STAT, one way ANOVA value.

Protein Name	Accession Number	Standard Spot No.	Protein Score (XC)	Number Peptides	Coverage (%)	MS p-value	Fold change	STAT p-value	Gene Ontology (Biological Process)	Gene Ontology (Molecular Function)
CYTOSKELETON										
Actin, cytoplasmic 1	P60711	1256	20.26990891	2	8.8	1.49661E-09	1.2	0.021	axonogenesis	ATP binding; protein kinase binding
		580	64.28	7	26.7	5.83E-08	1.2	0.002		
		872	38.29	4	13.9	7.63E-10	1.1	0.009		
		758	10.22	1	6.1	0.00000978	1.1	0.032		
Actin, aortic smooth muscle	P62738	1256	18.18382263	2	8.8	1.88752E-05	1.2	0.021	muscle contraction	ATP binding
		580	8.23	1	6.1	0.000857	1.2	0.002		
		872	10.2	1	6.1	0.0000501	1.1	0.009		
Actin, alpha cardiac muscle 1	P68035	580	10.15	1	7.2	0.0000275	1.2	0.002	cardiac muscle contraction response to drug; response to ethanol	ATP binding
Spectrin alpha chain, brain	P16086	580	10.23	1	0.9	6.88E-13	1.2	0.002	actin cytoskeleton reorganisation; actin filament capping	actin binding; calcium ion binding; calmodulin binding; protein N-terminus binding; protein complex binding; syntaxin binding
		872	10.13	1	1	0.000143	1.1	0.009		
Tubulin beta-2A chain	P85108	580	10.19	1	2.2	0.000471	1.2	0.002	microtubule-based movement; protein polymerisation	GTP binding; GTPase activity; structural molecule activity
Tropomodulin-2	P70566	758	20.24	2	10.5	2.42E-11	1.1	0.032	actin filament organisation	actin binding; tropomyosin binding
Glial fibrillary acidic protein	P47819	758	10.16	1	2.6	0.0000132	1.1	0.032	response to wounding	structural constituent of cytoskeleton
NEURONAL PLASTICITY										

Beta-synuclein	Q63754	580	20.24	2	27.7	0.000000133	1.2	0.002	dopamine metabolic procedd; negative regulation of neuron apoptosis	calcium ion binding
PROTEIN TRANSPORT										
Alpha-actinin-4	Q9QXQ0	580	10.17	1	2.5	0.000153	1.2	0.002	protein transport; regulation of apoptosis; response to hypoxia	calcium ion binding; protein N-terminus binding; protein complex binding
HEAT SHOCK FAMILY PROTEIN										
78 kDa glucose- regulated protein	P06761	758	10.15	1	1.7	0.000819	1.1	0.032	anti-apoptosis; ER overload response; activation of signaling protein activity involved in unfolded protein response; cerebellum strucctural organisation;cerebellar Purkinje cell layer development	ATP binding; unfolded protein binding

5.3.4 Hippocampus: GO analysis following chronic ECS

The GO terms found to be significant by DAVID following a search for FatTGO terms for proteins in the biological process, molecular function and cellular component ontologies are listed below. The number of proteins (count) and their identifier (accession number) as well as the p-value for each term is also listed in Table 5-8 – 5-10.

Table 5-8 Biological Processes

Term	Count	P-Value	Accession number
actin cytoskeleton organization	5	1.58E-04	P68035, P70566, Q9QXQ0, P16086, P62738
actin filament-based process	5	1.81E-04	P68035, P70566, Q9QXQ0, P16086, P62738
actin filament organization	4	6.87E-04	P68035, P70566, Q9QXQ0, P62738
cytoskeleton organization	5	9.15E-04	P68035, P70566, Q9QXQ0, P16086, P62738

Table 5-9 Molecular Function

Term	Count	P-Value	Accession number
cytoskeletal protein binding	5	0.001099	P68035, P70566, Q9QXQ0, P16086, P62738

Table 5-10 Cellular Component

Term	Count	P-Value	Accession number
cytoskeleton	8	1.04E-05	P68035, P60711, P70566, P47819, Q9QXQ0, P16086, P85108, P62738
cortical cytoskeleton	3	4.29E-04	P60711, Q9QXQ0, P16086
intracellular non-membrane-bounded organelle	8	4.77E-04	P68035, P60711, P70566, P47819, Q9QXQ0, P16086, P85108, P62738
non-membrane-bounded organelle	8	4.77E-04	P68035, P60711, P70566, P47819, Q9QXQ0, P16086, P85108, P62738
cytoskeletal part	6	9.74E-04	P68035, P47819, Q9QXQ0, P16086, P85108, P62738
cell cortex part	3	0.001303	P60711, Q9QXQ0, P16086

5.3.5 Hippocampus: protein identification and classification following chronic + 4 weeks ECS

The proteins identified by mass spectrometry in the hippocampus are detailed below (Table 5-11, Table 5-12) as described in section 5.4. The protein name, accession number, the number of the spot in which the protein was found and the theoretical and experimental molecular weights and isoelectric points of each protein are found in Table 5-11.

Table 5-12 includes a more detailed classification of the proteins, by biological process and molecular function. Mass spectrometry information including the protein score (XCorr), the number of peptides, percentage coverage, the mass spectrometry p-value, the fold change in protein expression and the one way ANOVA p-value for that spot are listed in Table 5-12.

Table 5-11 Hippocampus: protein identification following chronic + 4 week ECS

This table includes information on the protein identity; it's theoretical and experimental weights and isoelectric points. MW, molecular weight; kDa, kilo Dalton; pI, isoelectric point.

Protein Name	Accession Number	Standard Spot No.	pI Experimental	pI Theoretical	MW (kDa) Experimental	MW (kDa) Theoretical
CYTOSKELETON						
Actin, cytoplasmic 1	P60711	534	5.3	5.29	57799.02334	41737
		422	6.8		68018.57043	
		637	5.6		53903.56638	
		407	6		68814.18965	
		317	5.5		75523.48116	
		566	6.5		57799.02334	
		423	6.8		68018.57043	
		859	5.7		42717.66445	
		824	5.8		43722.85373	
		842	5.9		42223.7693	
		834	6		43217.33673	
		515	6.5		61259.43684	
		1143	6.6		25019.83838	
		913	5.4		38027.91373	
Actin, aortic smooth muscle	P62738	637	5.6	5.24	53903.56638	42009
		317	5.5		75523.48116	
		859	5.7		42717.66445	
		824	5.8		43722.85373	
		842	5.9		42223.7693	
		834	6		43217.33673	
Tubulin alpha-1A chain	P68370	534	5.3	4.94	57799.02334	50135.63
		422	6.8		68018.57043	
		637	5.6		53903.56638	
Tubulin beta-2A chain	P85108	534	5.3	4.78	57799.02334	49906.97
		422	6.8		68018.57043	

		317	5.5		75523.48116	
		824	5.8		43722.85373	
		842	5.9		42223.7693	
		913	5.4		38027.91373	
Tubulin alpha-4A chain	Q5XIF6	534	5.3	4.95	57799.02334	49924.4
Tubulin beta-3 chain	Q4QRB4	534	5.3	4.82	57799.02334	50418.65
		317	5.5		57799.02334	
Tubulin alpha-1B chain	Q6P9V9	534	5.3	4.94	57799.02334	50151.63
Tubulin beta-2C chain	Q6P9T8	534	5.3	4.79	57799.02334	49800.98
		317	5.5		75523.48116	
Glial fibrillary acidic protein	P47819	534	5.3	5.35	57799.02334	49957.09
		637	5.6		53903.56638	
		317	5.5		75523.48116	
		566	6.5		57799.02334	
		470	5.8		64176.34805	
		859	5.7		42717.66445	
		227	5.4		80981.34769	
		917	6.3		38027.91373	
		177	5.7		82886.91963	
		515	6.5		61259.43684	
		526	6.6		60551.16453	
WD repeat-containing protein 1	Q5RKI0	422	6.8	6.15	68018.57043	66050.26
		637	5.6		53903.56638	
		423	6.8		68018.57043	
		470	5.8		64176.34805	
Dihydropyrimidinase-related protein 1	Q62950	526	6.1	6.64	70842.19352	62195.75
Dihydropyrimidinase-related protein 2	P47942	407	6	5.95	68814.18965	62277.57
		834	6		43217.33673	
		526	6.6		60551.16453	
Dihydropyrimidinase-related protein 5	Q9JHU0	423	6.8	6.6	68018.57043	61540.39
F-actin-capping protein subunit alpha-1	B2GUZ5	859	5.7	5.44	42717.66445	32778.58
		842	5.9		42223.7693	
F-actin-capping protein subunit alpha-2	Q3T1K5	859	5.7	5.58	42717.66445	32835.87
		842	5.9		42223.7693	
Alpha-soluble NSF attachment protein	P54921	913	5.4	5.3	38027.91373	33192.66
Beta-soluble NSF attachment protein	P85969	913	5.4	5.32	38027.91373	33469.87
Myelin basic protein S	P02688	1143	6.6	11.24	25019.83838	21371.02
Peripherin	P21807	534	5.3	5.37	57799.02334	53549.76
Microtubule-associated protein tau	P19332	526	6.1	5.95	70842.19352	78432.81
Septin-8	B0BNF1	534	5.3	5.75	57799.02334	51120.87
Microtubule-associated protein tau	P19332	423	6.8	5.95	68018.57043	78432.81
Desmin	P48675	534	5.3	5.21	57799.02334	53325.52
Abl interactor 1	Q9QZM5	407	6	6.61	68814.18965	51573.58
Myosin-Ic	Q63355	534	5.3	9.45	57799.02334	119811.42
Vimentin	P31000	534	5.3	5.06	57799.02334	53601.54
Neurofilament light polypeptide	P19527	913	5.4	4.63	38027.91373	61204.09
		526	6.6		60551.6	
VESICLE/ENDOCYTOSIS						
Protein kinase C and casein kinase substrate in neurons protein 1	Q9Z0W5	534	5.3	5.15	57799.02334	50499.14
		576	5.2		57799.02334	
NUCLEOSOME						
Histone H4	P62804	422	6.8	11.36	68018.57043	11236.15
		842	5.9		42223.7693	

Histone H1.2	P15865	422	6.8	11.10	68018.57043	21856.16
		842	5.9		42223.7693	
Histone H2B type 1	Q00715	422	6.8	10.37	68018.57043	13859.03
Histone H2B type 1	Q00715	842	5.9	10.77	42223.7693	10850.03
CELL ADHESION						
Junction plakoglobin	Q6P0K8	422	6.8	5.75	68018.57043	81800.75
RIBOSOME						
40S ribosomal protein S25	P62853	422	6.8	10.12	68018.57043	13742.13
60S ribosomal protein L8	P62919	422	6.8	11.04	68018.57043	27893.46
PROTEIN BIOSYNTHESIS						
Elongation factor 1-alpha 1	P62630	842	5.9	9.1	42223.7693	50113.84
Elongation factor 2	P05197	422	6.8	6.42	68018.57043	95152.82
		842	5.9		42223.7693	
PROTEOLASE/HYDROLASE/TRANSFERASE						
Cytosol aminopeptidase	Q68FS4	422	6.8	6.78	68018.57043	56150.14
Guanine deaminase	Q9WTT6	637	5.6	5.56	53903.56638	51016.1
Methionine aminopeptidase 2	P38062	407	6	5.72	68814.18965	52920.68
26S Protease Regulatory Subunit 6B	Q63570	227	5.4	5.09	80981.34769	47408.33
Leucine carboxyl methyltransferase 1	Q6P4Z6	317	5.5	5.73	75523.48116	38330.14
		824	5.8		43722.85373	
ANTIOXIDANT						
Peroxiredoxin-6	O35244	422	6.8	5.65	68018.57043	24687.4
METABOLISC PROCESSES						
Cytochrome b-c1 complex subunit 1	Q68FY0	637	5.6	5.22	53903.56638	49380.6
		576	5.2		57799.02334	
Gamma-enolase	P07323	637	5.6	5.03	53903.56638	47009.34
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	P08461	407	6	5.7	68814.18965	58734.05
		317	5.5		75523.48116	
		566	6.5		57799.02334	
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Q66HF1	317	5.5	5.28	75523.48116	76860.16
		859	5.7		42717.66445	
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Q99NA5	317	5.5	5.72	75523.48116	36682.24
		566	6.5		57799.02334	
		824	5.8		43722.85373	
		834	6		43217.33673	
		177	5.7		82886.91963	
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	Q561S0	470	5.8	5.96	64176.34805	36842.72
		842	5.9		42223.7693	
L-lactate dehydrogenase B chain	P42123	859	5.7	5.7	42717.66445	36481.18
		842	5.9		42223.7693	
L-lactate dehydrogenase A chain	P04642	859	5.7	8.47	42717.66445	36319.31
		842	5.9		42223.7693	
Pyruvate kinase isozymes M1/M2	P11980	859	5.7	6.69	42717.66445	57686.6
Lambda-crystallin homolog	Q811X6	917	6.3	5.95	38027.91373	35209.83
Transaldolase	Q9EQS0	842	5.9	6.57	42223.7693	37460.08
Phosphoglycerate kinase 1	P16617	227	5.4	8.02	80981.34769	44407.26
		834	6		43217.33673	
D-3-phosphoglycerate dehydrogenase	O08651	515	6.5	6.30	61259.43684	56362.23
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	P49432	913	5.4	5.29	38027.91373	35841.38

TRANSPORT						
Transitional endoplasmic reticulum ATPase	P46462	317	5.5	5.14	75523.48116	89217.64
		859	5.7		42717.66445	
		227	5.4		80981.34769	
Acyl-protein thioesterase 1	P70470	824	5.8	6.05	43722.85373	24708.72
Voltage-dependent anion-selective channel protein 1	Q9Z2L0	917	6.3	8.63	38027.91373	30624.31
Voltage-dependent anion-selective channel protein 2	P81155	917	6.3	7.44	38027.91373	31745.82
Phosphatidylinositol transfer protein alpha isoform	P16446	917	6.3	5.98	38027.91373	31776.23
HEAT SHOCK FAMILY						
Stress-induced-phosphoprotein 1	O35814	423	6.8	6.4	68018.57043	62570.1
Heat shock 70 kDa protein 1-like	P55063	859	5.7	5.91	42717.66445	70549.18
78 kDa glucose-regulated protein	P06761	859	5.7	5.01	42717.66445	70474.59
Heat shock-related 70 kDa protein 2	P14659	526	6.6	5.51	60551.6	69641.66
60 kDa heat shock protein, mitochondrial	P63039	470	5.8	5.35	64176.34805	57925.78
		1143	6.6		25019.83838	
CHAPERONE						
T-complex protein 1 subunit alpha	P28480	834	6	5.86	43217.33673	60359.65
T-complex protein 1 subunit epsilon	Q68FQ0	470	5.8	5.51	64176.34805	59405.58
		859	5.7		42717.66445	
		824	5.8		43722.85373	
		1143	6.6		25019.83838	
Protein DJ-1	O88767	824	5.8	6.32	43722.85373	19974.17
		1143	6.6		25019.83838	
GrpE protein homolog 1, mitochondrial	P97576	824	5.8	6.12	43722.85373	21292.33
NITRIC OXIDE GENERATION						
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	O08557	842	5.9	5.76	42223.7693	31294.82
		227	5.4		80981.34769	
		834	6		43217.33673	
ANTI COAGULANT						
Annexin A3	P14669	917	6.3	5.97	38027.91373	36232
SIGNALING MODULATOR						
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	P54313	913	5.4	5.6	38027.91373	37199.84
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	P54311	917	6.3	5.6	38027.91373	37245.77
		913	5.4		38027.91373	
APOPTOSIS/ TRANSCRIPTION						
Steroid receptor RNA activator 1	Q6QGW5	917	6.3		38027.91373	
DIFFERENTIATION/NEUROGENESIS						
Protein RUFY3	Q5FVJ0	1143	6.6	5.36	25019.83838	52906.84
Clusterin	P05371	1143	6.6	5.46	25019.83838	49076.38
Serum albumin	P02770	407	6	5.8	68814.18965	65916.36
		526	6.6		60551.6	
Serine racemase	Q76EQ0	842	5.9	5.68	42223.7693	35693.09
Mitochondrial inner membrane protein (Fragment)	Q3KR86	576	5.2	10.34	57799.02334	4747.61

Table 5-12 Hippocampus: mass spectrometry results following chronic + 4 weeks ECS

This table contains information from the mass spectrometry analysis of each protein and the protein classification according to biological process and molecular function as described for Table 5-2. MS, mass spectrometry; STAT, one-way ANOVA; XC, cross correlation (Xcorr).

Protein Name	Accession Number	Standard spot no.	Fold change	STAT p-value	Protein Score (XC)	Number peptides	Coverage (%)	MS p-value	Gene Ontology (Biological Process)	Gene Ontology (Molecular Function)
CYTOSKELETON										
Actin, cytoplasmic 1	P60711	534	1.1	0.012	20.3	2	6.1	2.57E-09	axonogenesis	ATP binding; protein kinase binding
		422	1.3	0.0000158	20.3	2	6.1	1.55E-7		
		637	1.1	0.0000515	20.28	2	6.1	1.54E-7		
		407	1.1	.012	20.27	2	6.1	0.000042		
		317	1.1	0.000618	20.3	2	6.1	2.16E-10		
		566	1.1	0.002	40.32	4	14.1	8.08E-12		
		423	1.2	0.002	10.19	1	6.1	9.96E-08		
		859	1.1	0.012	28.25	3	6.1	1.72E-08		
		824	1.1	0.00095	10.25	1	6.1	2.34E-09		
		842	1.1	.029	20.24	2	6.1	3.79E-6		
		834	1.1	0.003	20.26	2	9.1	2.87E-6		
		515	1.2	0.004	10.19	1	6.1	0.000387		
		1143	1.1	5.16E-4	10.17	1	6.1	0.000118		
		913	1.2	0.000437	10.25	1	6.13	1.69E-10		
Actin, aortic smooth muscle	P62738	637	1.1	0.0000515	20.2	2	5.6	0.0000371	muscle contraction	ATP binding
		317	1.1	0.000618	18.23	2	9	0.0000167		
		859	1.1	0.012	8.21	1	6.1	0.000545		
		824	1.1	0.00095	10.16	1	2.9	0.0000386		
		842	1.1	.029	10.18	1	2.9	0.0000455		
		834	1.1	0.003	10.18	1	2.9	0.0000825		
Tubulin alpha-1A chain	P68370	534	1.1	0.012	70.26	7	24.4	7.9E-09	microtubule-based	GTP binding; GTPase

		422	1.3	0.0000158	10.24	1	3.1	111E-7	movement; protein polymerisation	activity; protein domain specific binding; protein heterodimerisation activity; structural molecule activity
		637	1.1	0.0000515	10.14	1	3.1	736E-7		
Tubulin beta-2A chain	P85108	534	1.1	0.012	60.19	6	16	1.96E-08	microtubule-based movement; protein polymerisation	GTP binding; GTPase activity; structural molecule activity
		422	1.3	0.0000158	10.16	1	2.2	5.78E-7		
		317	1.1	0.000618	10.15	1	2.2	0.0000257		
		824	1.1	0.00095	10.17	1	2.7	0.000872		
		842	1.1	.029	10.16	1	2.2	0.000482		
		913	1.2	0.000437	20.15	2		0.000176		
Tubulin alpha-4A chain	Q5XIF6	534	1.1	0.012	10.16	1	3.3	5.13E-7	microtubule-based movement; protein polymerisation	GTP binding; GTPase activity; structural molecule activity
Tubulin beta-3 chain	Q4QRB4	534	1.1	0.012	10.17	1	2.7	0.0000186	axon guidance; microtubule-based movement; mitosis; protein polymerisation	GTP binding; GTPase activity; peptide binding; structural constituent of cytoskeleton
		317	1.1	0.000618	10.17	1	2.7	0.000123		
Tubulin alpha-1B chain	Q6P9V9	534	1.1	0.012	10.17	1	3.1	7.32E-7	microtubule-based movement; protein polymerisation	GTP binding; GTPase activity; structural molecule activity
Tubulin beta-2C chain	Q6P9T8	534	1.1	0.012	10.16	1	2	0.000301	microtubule-based movement; protein polymerisation	GTP binding; GTPase activity; structural molecule activity
		317	1.1	0.000618	10.15	1	2.7	0.000199		
Glial fibrillary acidic protein	P47819	534	1.1	0.012	10.16	1	2.6	1.008E-6	response to wounding	structural constituent of cytoskeleton
		637	1.1	0.0000515	10.15	1	2.6	6.75E-6		
		317	1.1	0.000618	10.16	1	2.6	3.16E-7		
		566	1.1	0.002	10.15	1	2.4	0.0000111		
		470	1.1	0.001	10.16	1	2.6	0.0000154		
		859	1.1	0.012	10.13	1	2.6	0.0000762		
		227	1.2	0.00000944	10.14	1	2.6	0.000254		
		917	1.1	0.013	10.15	1	2.56	0.000104		
		177	1.2	0.002	10.15	1	2.6	5.83E-6		
		515	1.2	0.004	10.16	1	2.6	1.07E-6		

		526								
WD repeat-containing protein 1	Q5RKIO	422	1.3	0.0000158	60.24	6	17.2	6.03E-12		actin binding
		637	1.1	0.0000515	10.2	1	3.6	0.000182		
		423	1.2	0.002	90.25	9	26.6	4.03E-11		
		470	1.1	0.001	10.19	1	3.5	1.97E-10		
Dihydropyrimidinase-related protein 1	Q62950	526	1.1	0.031	10.2	1	2.8	0.0000235	neuron development	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
Dihydropyrimidinase-related protein 2	P47942		1.1	.012					olfactory bulb development; positive regulation of glutamate secretion; regulation of axon extension; response to amphetamine; response to cocaine; response to drug; spinal cord development; synaptic vesicle transport	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds; protein binding
		407			20.14	2	3.8	0.0000773		
		834	1.1	0.003	30.24	3	7.3	1.38E-08		
			1.1	0.031						
		526			110.27	11	28.8	2.44E-10		
Dihydropyrimidinase-related protein 5	Q9JHU0	423	1.2	0.002	30.23	3	7.6	1.89E-5	neuron differentiation;	protein binding; hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
F-actin-capping protein subunit alpha-1	B2GUZ5	859	1.1	0.012	20.17	2	9.4	0.0000328	actin cytoskeleton organisation; actin filament capping	actin binding
		842	1.1	.029	10.16	1	5.9	6.51E-6		
F-actin-capping protein subunit alpha-2	Q3T1K5	859	1.1	0.012	80.35	8	44.1	4.39E-11	actin cytoskeleton organisation; actin filament capping	actin binding
		842	1.1	.029	10.2	1	5.6	0.000249		
Alpha-soluble NSF attachment protein	P54921	913	1.2	0.000437	20.17	2	7.12	0.0000616	cellular membrane fusion; vesicle-mediated transport	SNARE binding; syntaxin binding; protein binding
Beta-soluble NSF attachment protein	P85969	913	1.2	0.000437	90.25	9	38.4	1.59E-08	intracellular protein transport; vesicle-mediated transport	binding; syntaxin binding
Myelin basic protein S	P02688	1143	1.1	5.16E-4	10.14	1	5.6	0.000124	myelination; negative regulation of axonogenesis	protein binding; structural constituent of myelin sheath
Peripherin	P21807	534	1.1	0.012	10.14	1	2.1	0.000308	intermediate filament	structural molecule

									cytoskeleton organisation	activity
Microtubule-associated protein tau	P19332	526	1.1	0.031	10.14	1	1.3	0.0000329	apoptosis; microtubule cytoskeleton organization; negative regulation of microtubule depolymerization; neuron differentiation; positive regulation of axon extension; positive regulation of microtubule polymerization; response to nutrient; response to organic substance	microtubule binding; protein domain specific binding; protein kinase binding
Septin-8	B0BNF1	534	1.1	0.012	10.13	1	2	0.0000391	cell cycle	GTP binding
Microtubule-associated protein tau	P19332	423	1.2	0.002	20.14	2	2.9	0.0000563	Apoptosis; microtubule cytoskeleton organization; negative regulation of microtubule depolymerization; neuron differentiation; positive regulation of axon extension; positive regulation of microtubule polymerization; response to nutrient; response to organic substance	microtubule binding; protein domain specific binding; protein kinase binding
Desmin	P48675	534	1.1	0.012	10.13	1	1.9	0.00019		structural molecule activity
Abl interactor 1	Q9QZM5	407	1.1	.012	10.21	1	3.4	1.55E-6		
Myosin-Ic	Q63355	534	1.1	0.012	10.2	1	2.4	6.4E-8	protein transport; transmembrane transport	ATP binding; actin binding; calmodulin binding; motor activity
Vimentin	P31000	534	1.1	0.012	50.18	5	9.2	3.68E-7	intermediate filament-based process	protein kinase binding; structural constituent of cytoskeleton
Neurofilament light polypeptide	P19527	913	1.2	0.000437	10.15	1	1.85	6.69E-6	protein polymerisation; response to corticosterone stimulus; response to organic nitrogen; response to peptide hormone stimulus; response to toxin	phospholipase binding; protein domain specific binding; structural molecule activity
		526	1.1	0.031	10.14	1	1.8	0.0000861		

VESICLE/ENDOCYTOSIS										
Protein kinase C and casein kinase substrate in neurons protein 1	Q9Z0W5	534	1.1	0.012	50.28	5	13.6	5.2E-11	cytoskeleton organisation; endocytosis	cytoskeletal protein binding
		576	1.1	0.03	40.28	4	10.9	4.69E-08		
NUCLEOSOME										
Histone H4	P62804	422	1.3	0.0000158	20.16	2	19.4	3.92E-6	cell differentiation; multicellular organismal development; nucleosome assembly; ossification	DNA binding
		842	1.1	.029	20.16	2	19.4	2.23E-6		
Histone H1.2	P15865	422	1.3	0.0000158	10.14	1	5	0.000011	chromosome condensation; nucleosome assembly	DNA binding
		842			10.13	1	5	1.6E-7		
Histone H2B type 1	Q00715	422	1.3	0.0000158	10.17	1	12	9.9E-7	nucleosome assembly; defense response to bacterium	DNA binding
Histone H2B type 1	Q00715	842	1.1	.029	10.19	1	12	0.0000219	defense response to bacterium; nucleosome assembly	DNA binding
CELL ADHESION										
Junction plakoglobin	Q6P0K8	422	1.3	0.0000158	20.17	2	3.2	3.38E-6	cell-cell adhesion; protein heterooligomerisation	protein kinase binding; structural molecule activity
RIBOSOME										
40S ribosomal protein S25	P62853	422	1.3	0.0000158	10.14	1	7.2	0.000057	ribosomal small subunit assembly	structural constituent of ribosome
60S ribosomal protein L8	P62919	422	1.3	0.0000158	10.13	1	4.3	0.000569	translation	rRNA binding; structural constituent of ribosome
PROTEIN BIOSYNTHESIS										
Elongation factor 1-alpha 1	P62630	842	1.1	.029	10.15	1	2.4	0.000624	GTP catabolic process; translational elongation; translation	GTP binding; GTPase activity; translation elongation factor activity
Elongation factor 2	P05197	422	1.3	0.0000158	10.14	1	1	0.0000688	translational elongation; GTP catabolic process	GTP binding; GTPase activity; translation elongation factor activity
		842	1.1	.029	10.15	1	1	0.000122		
PROTEOLASE/HYDROLASE/TRANSFERASE										
Cytosol aminopeptidase	Q68FS4	422	1.3	0.0000158	10.17	1	2.3	0.000105	proteolysis	aminopeptidase activity; metal ion binding

Guanine deaminase	Q9WTT6	637	1.1	0.0000515	20.16	2	4.8	1.22E-08		guanine deaminase activity; zinc ion binding
Methionine aminopeptidase 2	P38062	407	1.1	.012	60.21	6	18.6	2.05E-09	N-terminal protein amino acid modification; peptidyl-methionine modification; protein processing; proteolysis	aminopeptidase activity; metal ion binding; metalloexopeptidase activity
26S Protease Regulatory Subunit 6B	Q63570	227	1.2	0.00000944	8.13	1	2.9	0.000104	protein catabolic process	ATP binding; ATPase activity
Leucine carboxyl methyltransferase 1	Q6P4Z6	317	1.1	0.000618	10.15	1	3	0.000645	C-terminal protein methylation; methylation	methyltransferase activity
		824	1.1	0.00095	10.16	1	3	0.000059		
ANTIOXIDANT										
Peroxiredoxin-6	O35244	422	1.3	0.0000158	10.13	1	4	0.00058	cell redox homeostasis; hydrogen peroxide catabolic process; oxidation-reduction process	glutathione peroxidase activity; peroxiredoxin activity; protein binding
METABOLIC PROCESSES										
Cytochrome b-c1 complex subunit 1	Q68FY0	637	1.1	0.0000515	50.22	5	10.4	1.55E-7	proteolysis; response to activity; response to alkaloid; transport	metalloendopeptidase activity; protein complex binding; zinc ion binding
		576	1.1	0.03	50.18	5	10.6	5.62E-08		
Gamma-enolase	P07323	637	1.1	0.0000515	10.16	1	2.3	0.000117	gluconeogenesis; glycolysis; response to drug; response to estradiol stimulus; response to organic cyclic compound	magnesium ion binding; phosphopyruvate hydratase activity; protein heterodimerisation activity; protein homodimerisation activity
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	P08461	407	1.1	.012	90.21	9	19.9	4.88E-14	acetyl-CoA biosynthetic process from pyruvate; glycolysis; sleep	dihydrolipolysine-residue acetyltransferase activity; nucleotide binding
		317	1.1	0.000618	20.24	2	5.9	1.71E-08		
		566	1.1	0.002	10.2	1	2.4	1.84E-7		
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Q66HF1	317	1.1	0.000618	120.24	12	19.9	7.27E-09	ATP metabolic process; ATP synthesis coupled electron transport; Apoptosis;	2 iron, 2 sulfur cluster binding; 4 iron, 4 sulfur cluster binding; NADH

		859	1.1	0.012	140.26	14	23.2	5.69E-10	regulation of mitochondrial membrane potential; transport	dehydrogenase (ubiquinone) activity; electron carrier activity; metal ion binding
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Q99NA5	317	1.1	0.000618	80.28	8	27.6	1.81E-08	2-oxoglutarate metabolic process; NADH metabolic process; isocitrate metabolic process; tricarboxylic acid cycle	NAD binding; isocitrate dehydrogenase (NAD+) activity; magnesium ion binding
		566	1.1	0.002	20.16	2	4.9	0.000237		
		824	1.1	0.00095	110.28	11	30.6	0.0000012		
		834	1.1	0.003	40.18	4	11.5	1.71E-08		
		177	1.2	0.002	60.17	6	16.1	0.0000038		
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	Q561S0	470	1.1	0.001	30.2	3	10.4	2.56E-7	electron transport chain; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; response to drug; transport	ATP binding; phosphotransferase activity, alcohol group as acceptor
		842	1.1	.029	30.2	1	10.7	6.02E-09		
L-lactate dehydrogenase B chain	P42123	859	1.1	0.012	40.2	4	16.2	4.05E-10	NAD metabolic process; glycolysis; lactate metabolic process; oxidation-reduction process	L-lactate dehydrogenase activity; NAD binding; identical protein binding
		842			60.29	6	22.5	1.58E-10		
L-lactate dehydrogenase A chain	P04642	859	1.1	0.012	18.2	2	8.7	1.58E-7	NAD metabolic process; glycolysis; lactate metabolic process; oxidation-reduction process; positive regulation of apoptosis; post-embryonic organ development; response to cAMP; response to drug; response to estrogen stimulus; response to glucose stimulus; response to hydrogen peroxide; response to hypoxia; response to nutrient; response to organic cyclic	L-lactate dehydrogenase activity; NAD binding; identical protein binding
		842	1.1	.029	18.2	2	8.7	5.96E-7		

									compound	
Pyruvate kinase isozymes M1/M2	P11980	859	1.1	0.012	10.14	1	1.9	0.0000192	ATP biosynthetic process; Glycolysis; liver development; organ regeneration; pyruvate biosynthetic process; response to gravity; response to hypoxia; response to insulin stimulus; response to muscle inactivity; response to nutrient; skeletal muscle tissue regeneration	ADP binding; ATP binding; magnesium ion binding; potassium ion binding; protein binding; pyruvate kinase activity
Lambda-crystallin homolog	Q811X6	917	1.1	0.013	30.19	3	11.91	5.22E-09	fatty acid metabolic process; oxidation-reduction process	L-gulonate 3-dehydrogenase activity; NAD+ binding
Transaldolase	Q9EQS0	842	1.1	.029	20.18	2	6.6	0.000252	fructose 6-phosphate metabolic process; glyceraldehyde-3-phosphate metabolic process; pentose-phosphate shunt, non-oxidative branch	monosaccharide binding; sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glyceronetransferase activity
Phosphoglycerate kinase 1	P16617	227	1.2	0.00000944	20.18	2	6.5	1.97E-09	gluconeogenesis; glycolysis	ADP binding; ATP binding; phosphoglycerate kinase activity
		834	1.1	0.003	20.17	2	6	5.61E-6		
D-3-phosphoglycerate dehydrogenase	O08651	515	1.2	0.004	20.17	2	4.5	2.36E-6	L-serine biosynthetic process; oxidation-reduction process	NAD binding; phosphoglycerate dehydrogenase activity
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	P49432	913	1.2	0.000437	120.33	12	38.72	5.52E-11	acetyl-CoA biosynthetic process from pyruvate; glycolysis; oxidation-reduction process	pyruvate dehydrogenase (acetyl-transferring) activity
TRANSPORT										
Transitional endoplasmic reticulum ATPase	P46462	317	1.1	0.000618	30.17	3	4.6	0.0000701	ER to Golgi vesicle-mediated transport; positive regulation of proteasomal ubiquitin-dependent protein catabolic process; protein	ATP binding; ATPase activity; identical protein binding; lipid binding; protein complex binding; receptor binding
		859	1.1	0.012	40.16	4	5.8	7.82E-7		
		227	1.2	0.00000944	80.19	8	12.4	4.49E-7		

									homooligomerization	
Acyl-protein thioesterase 1	P70470	824	1.1	0.00095	20.17	2	10.4	6.48E-7	fatty acid metabolic process	lysophospholipase activity
Voltage-dependent anion-selective channel protein 1	Q9Z2L0	917	1.1	0.013	10.13	1	4.59	4.21E-7	apoptosis; mitochondrial calcium ion transport	nucleotide binding; porin activity; protein complex binding; voltage-gated anion channel activity
Voltage-dependent anion-selective channel protein 2	P81155	917	1.1	0.013	10.13	1	4.07	0.000475	transport; ion transport; transmembrane transport	nucleotide binding; porin activity; voltage-gated anion channel activity
Phosphatidylinositol transfer protein alpha isoform	P16446	917	1.1	0.013	10.17	1	3.69	0.00004	transport; phospholipid transport	phosphatidylinositol transporter activity; phospholipid binding
HEAT SHOCK FAMILY										
Stress-induced-phosphoprotein 1	O35814	423	1.2	0.002	20.16	2	3.3	0.0000427		Hsp70 protein binding; chaperone binding; protein C-terminus
Heat shock 70 kDa protein 1-like	P55063	859	1.1	0.012	10.13	1	2	3.96E-7	response to stress	ATP binding
78 kDa glucose-regulated protein	P06761	859	1.1	0.012	10.15	1	1.7	0.000123	anti-apoptosis; ER overload response; activation of signaling protein activity involved in unfolded protein response; cerebellum structural organisation; cerebellar Purkinje cell layer development	ATP binding; unfolded protein binding
Heat shock-related 70 kDa protein 2	P14659	526	1.1	0.031	10.14	1	1.8	0.000148	multicellular organismal development; response to stress	ATP binding
60 kDa heat shock protein, mitochondrial	P63039	470	1.1	0.001	10.14	1	1.6	0.0000671	anti-apoptosis; chaperone mediated protein folding requiring cofactor; detection of misfolded protein; positive regulation of anti-apoptosis; positive regulation of inflammatory response; response to ATP;	ATP binding; insulin binding; misfolded protein binding; protease binding; protein complex binding; protein heterodimerization activity

		1143	1.1	5.16R-4	30.16	3	5.8	5.48E-7	response to cocaine; response to drug; response to estrogen stimulus; response to heat; response to hydrogen peroxide; response to hypoxia; response to lipopolysaccharide	
CHAPERONE										
T-complex protein 1 subunit alpha	P28480	834	1.1	0.003	80.22	8	19.6	5.11E-08	protein folding; spermatid development	ATP binding; unfolded protein binding
T-complex protein 1 subunit epsilon	Q68FQ0	470	1.1	0.001	80.25	8	19.2	2.32E-10	protein folding	ATP binding; unfolded protein binding
		859	1.1	0.012	10.28	1	4.1	8.12E-08		
		824	1.1	0.00095	20.15	2	3.7	2.29E-6		
		1143	1.1	5.16R-4	110.27	11	29.6	3.37E-11		
Protein DJ-1	O88767	824	1.1	0.00095	60.23	6	48.7	1.72E-08	autophagy; cellular response to hydrogen peroxide; inflammatory response; mitochondrion organization; negative regulation of cell death; negative regulation of protein binding; Neuroprotection; positive regulation of oxidative phosphorylation uncoupler activity; protein stabilization; regulation of inflammatory response; response to drug; single fertilization	mRNA binding; peptidase activity; peroxidase activity; protein homodimerization activity
		1143	1.1	5.16R-4	30.2	3	29.6	1.57E-7		
GrpE protein homolog 1, mitochondrial	P97576	824	1.1	0.00095	10.14	1	5.1	0.0000337	protein folding; protein import into mitochondrial matrix	adenyl-nucleotide exchange factor activity; chaperone binding; protein homodimerisation activity
NITRIC OXIDE GENERATION										
N(G),N(G)-	O08557	842	1.1	.029	30.15	3	10.5	0.0000664	arginine metabolic process;	amino acid binding;

dimethylarginine dimethylaminohydrolase 1		227	1.2	0.00000944	50.21	5	21.8	6.32E-7	citrulline metabolic process; positive regulation of angiogenesis; positive regulation of nitric oxide biosynthetic process	dimethylargininase activity; metal ion binding
		834	1.1	0.003	70.21	7	27	2.17E-10		
ANTI COAGULANT										
Annexin A3	P14669	917	1.1	0.013	140.25	14	51.54	4.24E-10	hippocampus development; organ regeneration; positive regulation of DNA metabolic process; response to glucocorticoid stimulus; response to growth factor stimulus	calcium ion binding; calcium-dependent phospholipid binding; phospholipase A2 inhibitor activity
SIGNALING MODULATOR										
Guanine nucleotide- binding protein G(I)/G(S)/G(T) subunit beta-2	P54313	913	1.2	0.000437	10.14	1	3.53	0.0000187	signal transduction	calcium channel regulator activity; signal transducer activity
Guanine nucleotide- binding protein G(I)/G(S)/G(T) subunit beta-1	P54311	917	1.1	0.013	10.13	1	2.94	4.16E-6	elevation of cytosolic calcium ion concentration	signal transducer activity
		913	1.2	0.000437	20.16	2	6.76	3.79E-7		
APOPTOSIS/ TRANSCRIPTION										
Steroid receptor RNA activator 1	Q6QGW5	917	1.1	0.013	10.13	1	4.33	0.000112	apoptosis; cell differentiation; cell proliferation	protein binding; receptor activity
DIFFERENTIATION/NEUROGENESIS										
Protein RUFY3	Q5FVJ0	1143	1.1	5.16R-4	10.16	1	2.3	9.88E-6	negative regulation of axonogenesis	
Clusterin	P05371	1143	1.1	5.16R-4	10.13	1	2	0.0000927	anti-apoptosis; apoptosis; cellular response to growth factor stimulus; endocrine pancreas development; neuron projection morphogenesis; positive regulation of cell differentiation; positive	misfolded protein binding

									regulation of cell proliferation; response to oxidative stress; response to wounding; spermatogenesis	
Serum albumin	P02770	407	1.1	.012	50.19	5	10.2	2.88E-6	cellular response to starvation; hemolysis by symbiont of host erythrocytes; maintenance of mitochondrion location; negative regulation of apoptosis; positive regulation of circadian sleep/wake cycle, non-REM sleep; response to mercury ion; response to nutrient; response to organic substance; response to platinum ion; Transport; vasodilation	DNA binding; cell surface binding; drug binding; enzyme binding; fatty acid binding; pyridoxal phosphate binding; toxin binding; zinc ion binding
		526	1.1	0.031	170.23	17	33.4	4.16E-08		
Serine racemase	Q76EQ0	842	1.1	.029	20.18	2	7.5	2.66E-08	L-serine metabolic process; Aging; brain development; response to drug; response to morphine	ATP binding; D-serine ammonia-lyase activity; L-serine ammonia-lyase activity; magnesium ion binding; protein homodimerization activity; pyridoxal phosphate binding; serine racemase activity
Mitochondrial inner membrane protein (Fragment)	Q3KR86	576	1.1	0.03	10.14	1	1.5	0.000696	response to cold	

5.3.6 Hippocampus: GO analysis following chronic + 4 weeks ECS

The GO terms found to be significant by DAVID following a search for Fat GO terms for proteins in the biological process, molecular function and cellular component ontologies are listed in Tables 5-13 – 5-15. The number of proteins (count) and their identifier (accession number) as well as the p-value for each term is also listed.

Table 5-13 Biological Processes

Term	Count	P-Value	Accession number
pyruvate metabolic process	8	4.68E-09	P42123, P04642, P07323, P49432, Q76EQ0, P08461, P11980, P16617
glucose catabolic process	8	3.04E-08	P42123, P04642, P07323, Q9EQS0, P49432, P08461, P11980, P16617
hexose catabolic process	8	3.89E-08	P42123, P04642, P07323, Q9EQS0, P49432, P08461, P11980, P16617
monosaccharide catabolic process	8	3.89E-08	P42123, P04642, P07323, Q9EQS0, P49432, P08461, P11980, P16617
cellular carbohydrate catabolic process	8	1.58E-07	P42123, P04642, P07323, Q9EQS0, P49432, P08461, P11980, P16617
alcohol catabolic process	8	1.58E-07	P42123, P04642, P07323, Q9EQS0, P49432, P08461, P11980, P16617
glycolysis	7	1.89E-07	P42123, P04642, P07323, P49432, P08461, P11980, P16617
protein polymerization	6	6.66E-07	Q5XIF6, Q4QRB4, Q6P9V9, P19527, Q6P9T8, P85108, P68370
generation of precursor metabolites and energy	11	8.88E-07	P42123, P04642, Q99NA5, P07323, Q66HF1, P49432, Q68FY0, Q561S0, P08461, P11980, P16617
carbohydrate catabolic process	8	1.32E-06	P42123, P04642, P07323, Q9EQS0, P49432, P08461, P11980, P16617
cellular macromolecular complex assembly	10	7.47E-06	Q5XIF6, Q00715, Q4QRB4, Q6P9V9, P19527, P62853, P15865, P62804, Q6P9T8, P85108, P68370
macromolecular complex assembly	14	1.02E-05	Q4QRB4, Q6P9V9, P19527, Q6P9T8, P68370, Q6P0K8, Q5XIF6, Q00715, O08557, P62853, Q76EQ0, P15865, P62804, P85108, P46462
cellular macromolecular complex subunit organization	10	1.83E-05	Q5XIF6, Q00715, Q4QRB4, Q6P9V9, P19527, P62853, P15865, P62804, Q6P9T8, P85108, P68370
macromolecular complex subunit organization	14	1.88E-05	Q4QRB4, Q6P9V9, P19527, Q6P9T8, P68370, Q6P0K8, Q5XIF6, Q00715, O08557, P62853, Q76EQ0, P15865, P62804, P85108, P46462
glucose metabolic process	8	6.20E-05	P42123, P04642, P07323, Q9EQS0, P49432,

			P08461, P11980, P16617
negative regulation of cellular component organization	7	7.96E-05	P02688, Q9Z0W5, Q5FVJ0, P05371, B2GUZ5, P19332, Q3T1K5
hexose metabolic process	8	1.52E-04	P42123, P04642, P07323, Q9EQS0, P49432, P08461, P11980, P16617
microtubule-based movement	6	1.75E-04	Q5XIF6, Q4QRB4, Q6P9V9, P19527, Q6P9T8, P85108, P68370
programmed cell death	10	2.69E-04	P63039, Q9Z2L0, P05371, P19332, Q66HF1, O08557, Q6QGW5, P46462, P62738, P11980
monosaccharide metabolic process	8	3.26E-04	P42123, P04642, P07323, Q9EQS0, P49432, P08461, P11980, P16617
cellular protein complex assembly	6	4.93E-04	Q5XIF6, Q4QRB4, Q6P9V9, P19527, Q6P9T8, P85108, P68370
cell death	10	5.34E-04	P63039, Q9Z2L0, P05371, P19332, Q66HF1, O08557, Q6QGW5, P46462, P62738, P11980
protein complex biogenesis	10	5.53E-04	Q6P0K8, Q5XIF6, Q4QRB4, O08557, Q6P9V9, P19527, Q76EQ0, Q6P9T8, P46462, P85108, P68370
protein complex assembly	10	5.53E-04	Q6P0K8, Q5XIF6, Q4QRB4, O08557, Q6P9V9, P19527, Q76EQ0, Q6P9T8, P46462, P85108, P68370
intermediate filament-based process	4	5.84E-04	P31000, P21807, P19527, P47819
regulation of axonogenesis	5	6.01E-04	P02688, Q5FVJ0, P19332, P19527, P47942
death	10	6.03E-04	P63039, Q9Z2L0, P05371, P19332, Q66HF1, O08557, Q6QGW5, P46462, P62738, P11980
microtubule-based process	7	8.40E-04	Q5XIF6, Q4QRB4, P19332, Q6P9V9, P19527, Q6P9T8, P85108, P68370
apoptosis	9	0.001128	P63039, Q9Z2L0, P05371, P19332, Q66HF1, O08557, Q6QGW5, P46462, P62738

Table 5-14 Molecular Function

Term	Count	P-Value	Accession number
nucleotide binding	31	8.94E-08	P55063, Q9Z2L0, P63039, Q63570, P42123, O08651, P14659, P06761, P62630, P08461, P68370, P16617, P04642, P28480, Q99NA5, Q811X6, Q63355, Q76EQ0, P62738, P85108, Q4QRB4, Q68FQ0, Q6P9V9, Q561S0, Q6P9T8, P81155, Q5XIF6, P60711, P05197, P97576, P46462, P11980
GTPase activity	7	2.05E-05	Q5XIF6, Q4QRB4, Q6P9V9, P05197, P62630, Q6P9T8, P85108, P68370
structural molecule activity	14	2.18E-05	P02688, P21807, Q4QRB4, Q6P9V9, P19527, P48675, Q6P9T8, P68370, Q6P0K8, Q5XIF6, P31000, P62919, P47819, P62853,

			P85108
purine nucleotide binding	24	2.43E-05	P55063, Q63570, P63039, P14659, P06761, P62630, P08461, P16617, P68370, P28480, Q63355, Q76EQ0, P85108, P62738, Q4QRB4, Q68FQ0, Q6P9V9, Q561S0, Q6P9T8, Q5XIF6, P60711, P05197, P97576, P11980, P46462
Purine ribonucleotide binding	23	3.71E-05	P55063, P63039, Q63570, Q4QRB4, Q68FQ0, P14659, Q6P9V9, P06761, P62630, Q561S0, P08461, Q6P9T8, P68370, P16617, Q5XIF6, P60711, P28480, Q63355, P05197, Q76EQ0, P62738, P46462, P11980, P85108
ribonucleotide binding	23	3.74E-05	P55063, P63039, Q63570, Q4QRB4, Q68FQ0, P14659, Q6P9V9, P06761, P62630, Q561S0, P08461, Q6P9T8, P68370, P16617, Q5XIF6, P60711, P28480, Q63355, P05197, Q76EQ0, P62738, P46462, P11980, P85108
structural constituent of cytoskeleton	4	4.86E-04	P31000, Q4QRB4, P19527, P47819

Table 5-15 Cellular Component

Term	Count	P-Value	Accession number
cytosol	27	2.43E-09	Q5RKI0, P63039, P19332, Q9EQS0, P06761, P62630, Q9WTT6, P16617, P68370, O88767, P04642, P28480, Q811X6, P62853, Q35244, Q68FQ0, P07323, Q6P9V9, P19527, Q6P9T8, Q6P0K8, Q5XIF6, P31000, P62919, P60711, O08557, P46462, P11980
cytoskeleton	21	1.27E-06	Q9QZM5, P21807, Q5RKI0, B2GUZ5, P19332, Q4QRB4, Q6P9V9, P19527, Q3T1K5, P48675, Q6P9T8, P68370, Q6P0K8, Q5XIF6, P31000, P60711, P28480, P47819, Q63355, P47942, P62738, P85108
mitochondrion	24	2.01E-06	P55063, P05371, Q3KR86, Q9Z2L0, P63039, Q63570, P42123, Q68FS4, P14659, P49432, Q68FY0, Q561S0, P08461, P81155, O88767, P70470, P04642, Q99NA5, Q66HF1, O08557, P47942, Q35244, P97576, P11980
cell projection	18	2.75E-06	P02688, P21807, Q5FVJ0, Q9QZM5, P19332, Q4QRB4, P54313, P19527, P54311, P31000, O88767, Q9JHU0, P04642, P60711, Q62950, Q63355, P47942, P11980
intracellular non-membrane-bounded organelle	29	3.72E-06	P21807, Q9QZM5, Q9Z2L0, Q5RKI0, P19332, Q3T1K5, P48675, P68370, Q00715, P28480, P47819, Q63355, P62853, P15865, P62804, P62738, P85108, Q4QRB4, B2GUZ5, Q68FQ0, Q6P9V9, P19527, Q6P9T8, Q6P0K8, Q5XIF6, P81155, P31000, P62919, P60711, P47942
non-membrane-bounded organelle	29	3.72E-06	P21807, Q9QZM5, Q9Z2L0, Q5RKI0, P19332, Q3T1K5, P48675, P68370, Q00715, P28480, P47819, Q63355, P62853, P15865, P62804, P62738, P85108, Q4QRB4, B2GUZ5, Q68FQ0, Q6P9V9, P19527, Q6P9T8, Q6P0K8, Q5XIF6, P81155, P31000, P62919, P60711, P47942
mitochondrial matrix	9	1.65E-05	P81155, P55063, P63039, Q9Z2L0, Q99NA5, P49432, Q561S0, P97576, P08461
mitochondrial lumen	9	1.65E-05	P81155, P55063, P63039, Q9Z2L0, Q99NA5, P49432, Q561S0, P97576, P08461
neuron projection	13	2.08E-05	P02688, Q5FVJ0, Q9QZM5, P21807, P19332, Q4QRB4, P19527,

			P31000, O88767, Q9JHU0, P60711, Q62950, P47942
cytoskeletal part	16	3.08E-05	P21807, B2GUZ5, P19332, Q4QRB4, Q6P9V9, P19527, Q3T1K5, P48675, Q6P9T8, P68370, Q5XIF6, P31000, P28480, Q63355, P47819, P62738, P85108
axon	9	3.87E-05	P02688, P31000, O88767, P21807, Q4QRB4, P19332, P60711, P19527, P47942
type III intermediate filament	3	2.47E-04	P31000, P21807, P48675
mitochondrial part	12	4.45E-04	P81155, P55063, P63039, Q9Z2L0, Q3KR86, Q99NA5, Q66HF1, P49432, Q68FY0, Q561S0, P97576, P08461
soluble fraction	9	6.63E-04	P42123, P04642, P60711, P07323, Q9EQS0, Q76EQ0, P62738, P11980, P16617
cytosolic part	6	6.93E-04	Q68FQ0, P28480, P07323, P19527, P06761, P62853

5.3.7 Distribution of identified proteins

Four proteins were common to all three studies (Figure 5-2). One protein was commonly expressed in the Chronic and Acute study and the Chronic and Chronic + 4 week study. Ten proteins were common to the Acute and Chronic + 4 study. Four, ten and sixty-four proteins were unique to the Chronic, Acute and Chronic+ 4 study respectively in the hippocampus. The commonly expressed protein accession numbers are listed in Table 5-16.

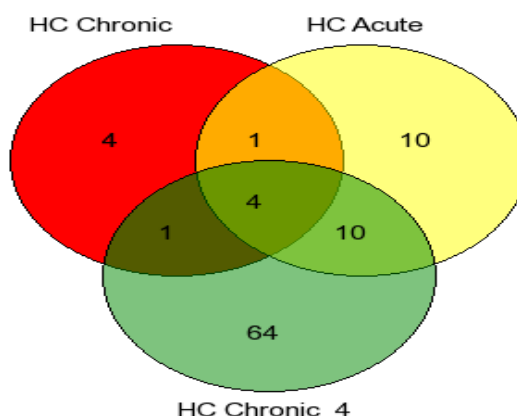


Figure 5-2 Venn diagram of proteins expressed in hippocampus following ECS
HC, hippocampus

Chronic	Chronic & acute	Acute	Acute & chronic+4	Chronic & chronic +4	Chronic & acute & chronic+4	Chronic + 4			
P68035	P16086	O08839	B2GUZ5	P85108	P06761	B0BNF1	P68370	P55063	P08461
P70566		P08413	O88767		P47819	O08557	P70470	P62853	P14659
Q63754		P11232	P05197		P60711	O08651	P81155	P62919	P14669
Q9QXQ0		P28073	P11980		P62738	O35244	P85969	Q9WTT6	P15865
		P35435	P42123			O35814	P97576	Q9Z0W5	P16446
		P62747	P62630			P02688	Q00715	Q9Z2L0	P16617
		Q04940	P62804			P02770	Q3KR86	P38062	P19332
		Q6DGG1	P63039			P04642	Q4QRB4	P46462	P19527
		Q6P7Q4	Q3T1K5			P05371	Q561S0	P47942	P21807
		Q9QUL6	Q68FQ0			P07323	Q5FVJ0	P48675	P28480
						Q5RKI0	Q66HF1	P49432	P31000
						Q5XIF6	Q68FS4	P54311	Q76EQ0
						Q62950	Q68FY0	P54313	Q811X6
						Q63355	Q6P0K8	P54921	Q99NA5
						Q63570	Q6P4Z6	Q6P9V9	Q9EQS0
						Q9QZM5	Q6P9T8	Q6QGW5	Q9JHU0

Table 5-16 Proteins expressed in the hippocampus following acute, chronic and chronic + 4 weeks of ECS

Accession numbers of proteins common to the hippocampus in each study are listed. Corresponding protein identities can be found in the appendix.

5.3.8 Kyoto encyclopaedia of genes and genomes (KEGG) analysis

Analysis revealed that proteins were involved in KEGG pathways following DAVID GO analysis. The statistical criteria used to exclude less relevant entries on the GO categories biological process, molecular function and cellular component were not applied here, as they were overly stringent for this data. The KEGG pathway identifier code, the KEGG pathway and the relevant gene products involved in these pathways are listed in Table 5-17- 5-19. However, due to the low levels of significance obtained for most of these gene products in the KEGG pathways, another form of pathway analysis, Ingenuity Pathway Analysis (IPA), was investigated to better understand the data.

Table 5-17 Hippocampus: KEGG pathway analysis following acute ECS

KEGG Identifier	KEGG pathway	Genes
rno00620	Pyruvate metabolism	Q6P7Q4, P42123, P11980

Table 5-18 Hippocampus: KEGG pathway analysis following chronic ECS

KEGG Identifier	KEGG pathway	Genes
rno04530	Tight junction	P60711, Q9QXQ0, P16086
rno04520	Adherens junction	P60711, Q9QXQ0
rno04670	Leukocyte transendothelial migration	P60711, Q9QXQ0

Table 5-19 Hippocampus: KEGG pathway analysis following chronic + 4 weeks ECS

KEGG Identifier	KEGG pathway	Genes
rno04540	Gap junction	Q5XIF6, Q4QRB4, Q6P9V9, Q6P9T8, P85108, P68370
rno00020	Citrate cycle (TCA cycle)	Q99NA5, P49432, P08461

5.3.9 Pathway analysis

Ingenuity Pathway Analysis (Ingenuity Systems) software was used to investigate the relationships between the potentially significantly changing proteins identified by proteomic analysis following ECS treatment as described in the methods section 2.2.20.2. The networks can consist of direct and indirect relationships and contain different molecules as detailed in the figure legend (Figure 5-3). Only the networks with the highest score in each study are included. As well as taking into account the number of uploaded proteins that were network eligible molecules (focus molecules) and the total number of molecules in the IPA knowledge base, it also considers the number of network eligible molecules in the network and its size (Levin et al. 2010).



Figure 5-3 Legend of shapes that describe nodes in IPA networks

The edges (lines) describe the relationship between nodes. An unbroken line indicates a direct relationship; a broken line indicates an indirect relationship; an edge with an arrow head indicates one node acting on another, a direct edge between two nodes suggests binding of one node to another.

5.3.9.1 Hippocampus: pathway analysis following acute ECS

The hippocampus network contained 18 focus molecules.

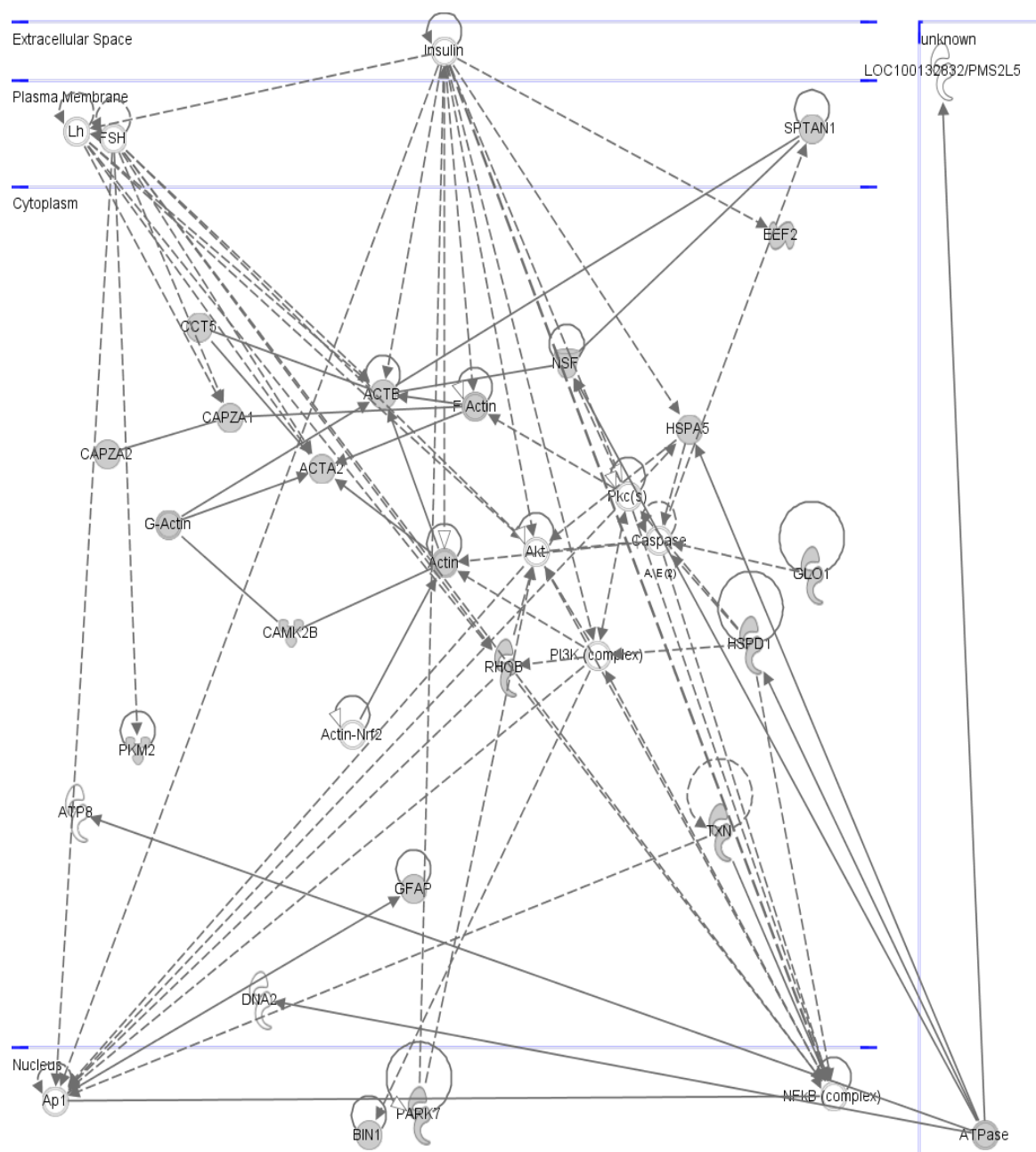


Figure 5-4 Network based on candidate proteins derived from targeted protein spots that were significantly differentially expressed in the hippocampus following acute ECS The network shows possible connections and interactions between molecules based on IPA knowledge base. The nodes shaded in grey were detected in this study.

ACTA2 Actin, aortic smooth muscle; HSPA578kDa glucose regulates protein; ACTB Actin cytoplasmic 1; HSPD1 60kDa heat shock protein, mitochondrial; Akt RAC serine/threonine-protein kinase; Lh Lutenising hormone AP1 Activator protein 1; NFkB Nuclear factor kappa B; ATP8 ATP synthase protein 8; ATPASE NSF Vesicle-fusing ATPase; PARK7 Protein DJ1; BIN1 Myc box-dependent-interacting protein 1; PI3K Phosphatidylinositol 3-kinase; CAMK2B Calcium/calmodulin-dependent protein kinase type II subunit beta; Pkc(s) Protein kinase C; CAPZA1 F-actin-capping protein subunit alpha-1; PKM2 Pyruvate kinase; CAPZA2 F-actin-capping protein subunit alpha-2; RHOA Rho-related GTP-binding protein RhoA; CCT5 T-complex protein 1 subunit epsilon; SPTAN 1 Spectrin alpha chain 1; DNA2 Uncharacterized protein; TXN Thioredoxin

5.3.9.2 Hippocampus: pathway analysis following chronic ECS

The hippocampus chronic ECS network contained 10 focus molecules.

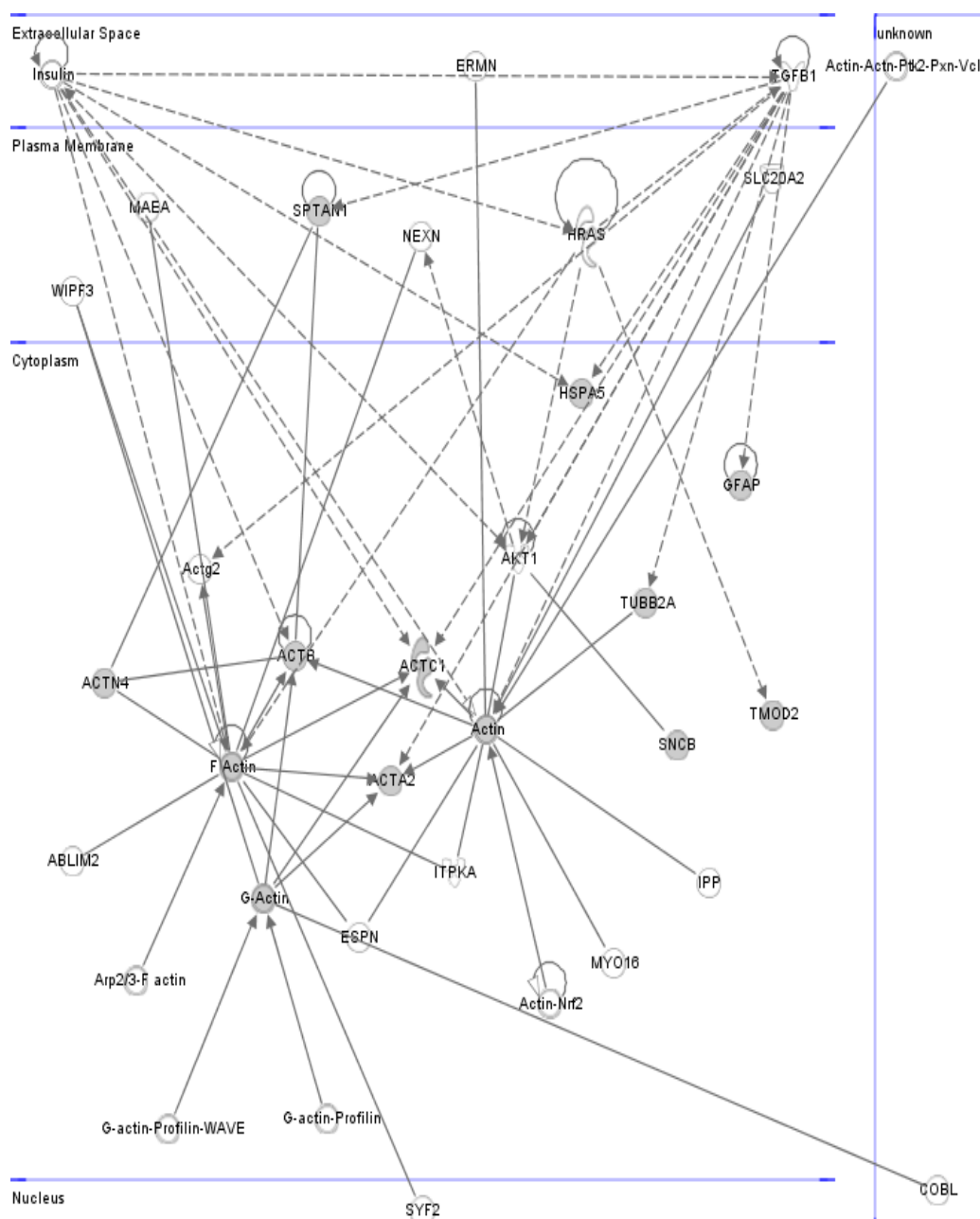


Figure 5-5 Network based on candidate proteins from target protein spots that were significantly differentially expressed in the hippocampus following chronic ECS

The network shows possible connections and interactions between molecules based on IPA knowledge base. The nodes shaded in grey were detected in this study.

ABLIM2 Actin-binding LIM protein 2; HSPA578 kDa glucose-regulated protein; ACTA2 Actin, aortic smooth muscle; IPP Protein phosphatase inhibitor; ACTB Actin, cytoplasmic 1; ITPKA Inositol-trisphosphate 3-kinase A; ACTC1 Actin, alpha cardiac muscle 1; MAEA Macrophage erythroblast attacher; ACTG2 Actin, gamma-enteric smooth muscle; MYO16 Myosin-XVI; ACTN4 Alpha actinin 4; NEXN Nexilin; AKT1 RAC-alpha serine/threonine-protein kinase; SLC2DA2 Sodium-dependent phosphate transporter 2; ARP2/3 Actin-related protein 2/3 complex subunit; SNCB Beta synuclein; COBL Cordon-bleu (Predicted), isoform CRA_a; SPTAN1 Spectrin alpha chain; ERMN Ermin; SYF2 Pre-mRNA-splicing factor SYF2; ESPN Espin; TMOD2 Tropomodulin 2; FGFB1 Transforming growth factor beta-1; TUBB2A Tubulin beta-2A chain; GFAP Glial fibrillary acidic protein; WIPF3 WAS/WASL-interacting protein family member 3; HRAS GTPase HRas.

5.3.9.3 Hippocampus: pathway analysis following chronic + 4 weeks ECS

The hippocampus chronic + 4 week ECS network contained 25 focus molecules.

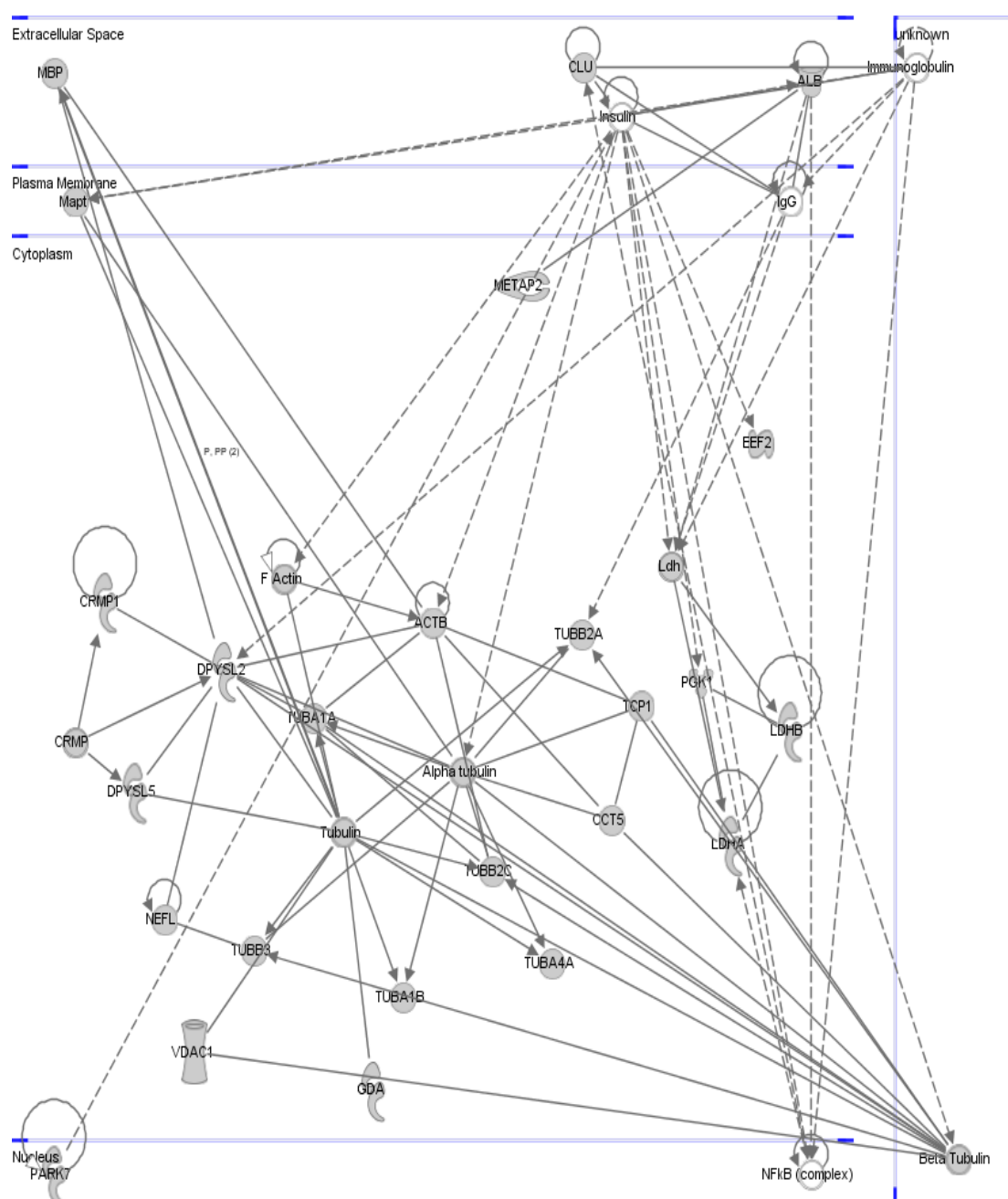


Figure 5-6 Network based on proteins from target protein spots that were significantly differentially expressed in the hippocampus following chronic + 4 week ECS The network shows possible connections and interactions between molecules based on IPA knowledge base. The nodes shaded in grey were detected in this study.

ACTB Actin, cytoplasmic 1; Mapt Microtubule-associated protein tau; ALB Serum albumin; MBP Myelin basic protein S; CCT5 T-complex protein 1 subunit epsilon; Metap2 Methionine aminopeptidase 2; CLU Clusterin; NEFL Neurofilament light polypeptide; CRMP Dihydropyrimidinase-related protein; PARK7 Protein DJ1; CRMP1 Dihydropyrimidinase-related protein 1; PGK Phosphoglycerate kinase; DPYSL2 Dihydropyrimidinase-related protein 2; TCP1 T-complex protein 1 subunit alpha; DPYSL5 Dihydropyrimidinase-related protein 5; TUBA1A Tubulin alpha-1A chain; EEF2 Elongation factor 2; TUBA1B Tubulin alpha-1B chain; GDA Guanine deaminase; TUBA4 Tubulin alpha-4A chain; IGG Immunoglobulin G; TUBB2 Tubulin beta-2 chain; Ldh L-lactate dehydrogenase; TUBB2A Tubulin beta-2A chain; LDHA L-lactate dehydrogenase A chain; TUBB3 Tubulin beta-3 chain; LDHB L-lactate dehydrogenase B chain; VDAC1 Voltage-dependent anion-selective channel protein 1

5.3.10 Confirmation studies

A number of proteins were selected for confirmation by semi-quantitative immunoblotting. These proteins were differentially expressed following ECS. Proteins detected in the Chronic study were of particular interest due to their potential role in the mechanisms of ECS action, however, proteins with cytoskeletal or signalling functions were considered for confirmation.

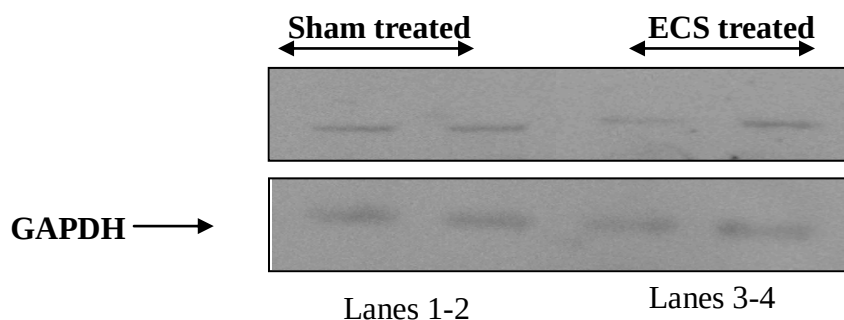
5.3.10.1 Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)

Target protein bands were normalised to the GAPDH bands which were run on the same gels. GAPDH also acted as a loading control for sample variation. Representative GAPDH band images are included next to the target protein blot images below.

5.3.10.2 Tropomodulin 2

Chronic treatment with ECS did not significantly affect relative expression levels of Tropomodulin-2 in the hippocampus (Mann-Whitney $p=0.7396$, $n=7-10$ per group) (Figure 5-7). The average relative % density of Tropomodulin-2 was lower in ECS treated animals ($84\pm18\%$) than in control animals ($100\pm32\%$). Tropomodulin-2 has a predicted molecular weight of 40kDa and was found to run at approximately this value ($\sim 44\text{kDa}$).

(A)



(B)

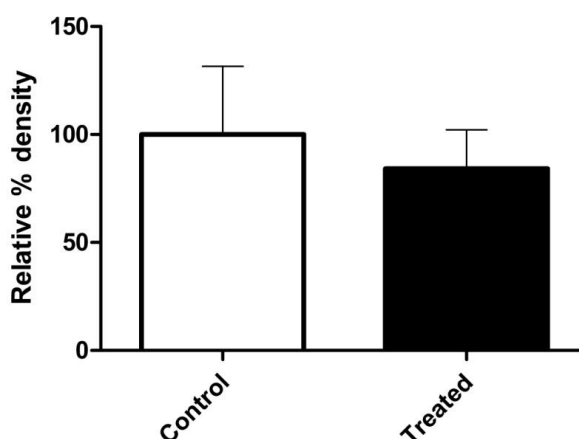


Figure 5-7 Tropomodulin-2 expression in the hippocampus following chronic ECS

(A) Lanes 1 and 2 contain representative hippocampal chronic sham treated sample. Lanes 3 and 4 contain hippocampal chronic ECS treated sample, representative GAPDH bands are below.

(B) There was no significant difference in the relative % density of Tropomodulin-2 following chronic ECS treatment compared to controls. Mann-Whitney test, data expressed as % of controls $\pm$ SEM, $n=7-10$ per group.

5.3.11 Calcium calmodulin kinase II beta (CamKII β)

Acute treatment with ECS resulted in a non-significant decrease in the relative % density of CamKII β compared to control (Student's *t*-test $p=0.596$, $n=10$ per group) (Figure 5-8). The average relative % density of the control group was $100 \pm 12\%$ and of the ECS treated group was $92 \pm 9.9\%$. CamKII β was found to run at its predicted molecular weight of 60kDa.

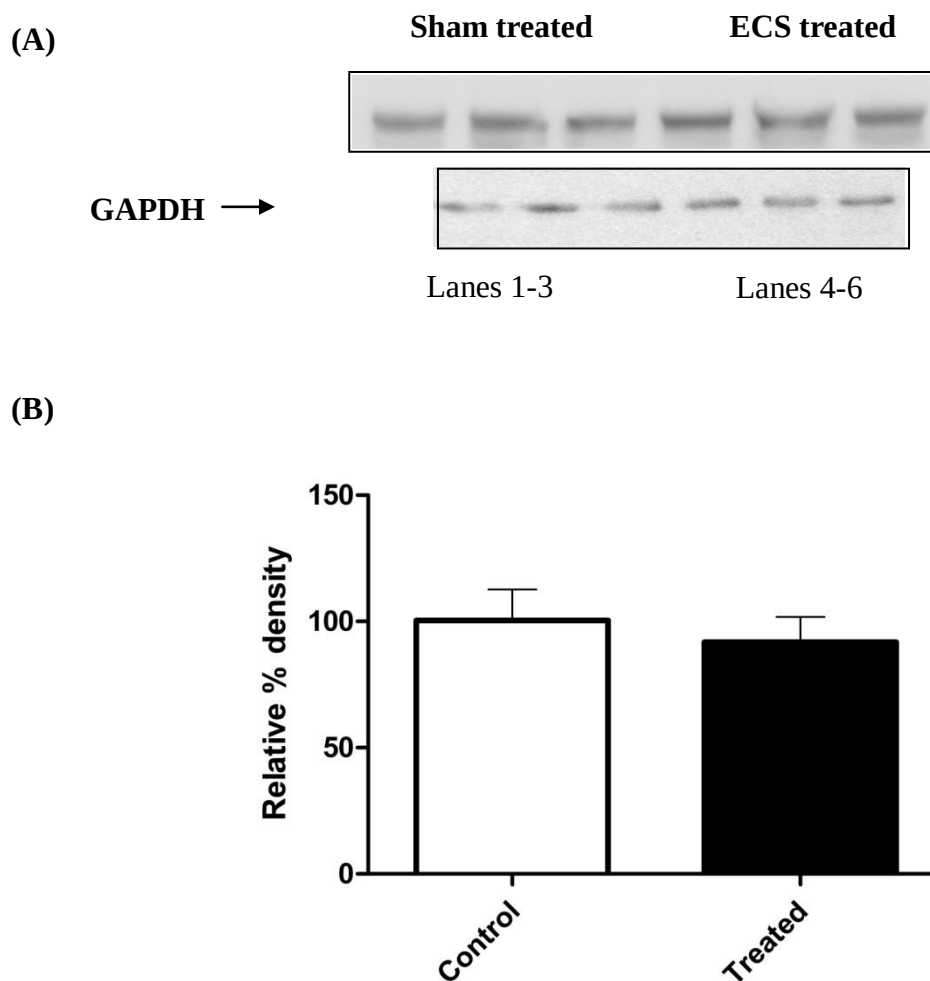


Figure 5-8 CamKII β expression in the hippocampus following acute ECS

(A) Lanes 1-3 contain representative hippocampal acute sham treated samples. Lanes 4-6 contain hippocampal acute ECS treated sample. Representative GAPDH bands are below.

(B) There was no significant difference in the relative % density of CamKII β following acute ECS treatment compared to controls. Student's *t*-test, data expressed as % of controls $\pm$ SEM, $n=10$ per group.

5.3.12 Neurogranin

The Acute study found an increase in the relative % density of ECS treated animals compared to controls (Student's *t*-test, $p=0.0768$, $n=10$). There was an increase in the expression of neurogranin in treated animals compared to controls (Figure 5-9); mean relative percentage density: $100 \pm 4.4\%$ control: $112 \pm 4.9\%$ treated. As a trend towards a significant increase in Neurogranin expression was seen in ECS treated animals in the acute study, the expression changes in the hippocampus following chronic treatment with ECS were investigated.

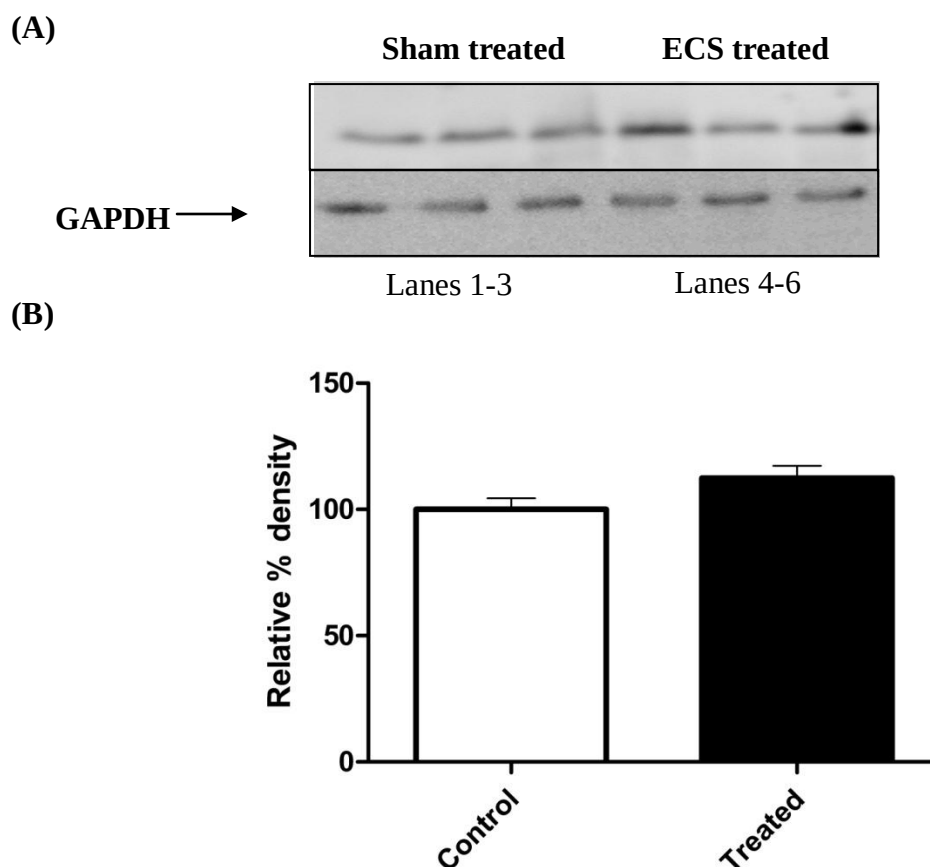


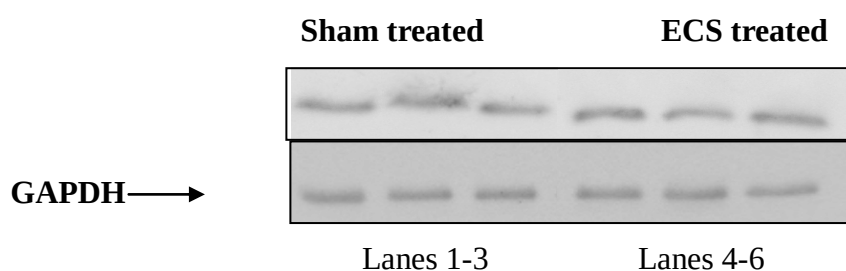
Figure 5-9 Neurogranin expression in the hippocampus following acute ECS

(A) Lanes 1-3 contain representative acute sham treated hippocampal samples. Lanes 4-6 contain acute ECS treated hippocampal samples, representative GAPDH bands below.

(B) There was no significant difference in the relative % density of Neurogranin expression following acute ECS treatment compared to controls. Student's *t*-test, data expressed as % of controls $\pm$ SEM, $n = 10$ per group.

There was no significant difference in the expression levels of Neurogranin in the hippocampus following chronic ECS treatment (Mann-Whitney test, $p=0.3277$; $n=6-9$). The average relative % density of Neurogranin expression decreased in the ECS treated group (control $100 \pm 21\%$; treated $72 \pm 14\%$; Figure 5-10). Neurogranin was found to run at its predicted molecular weight of 15kDa.

(A)



(B)

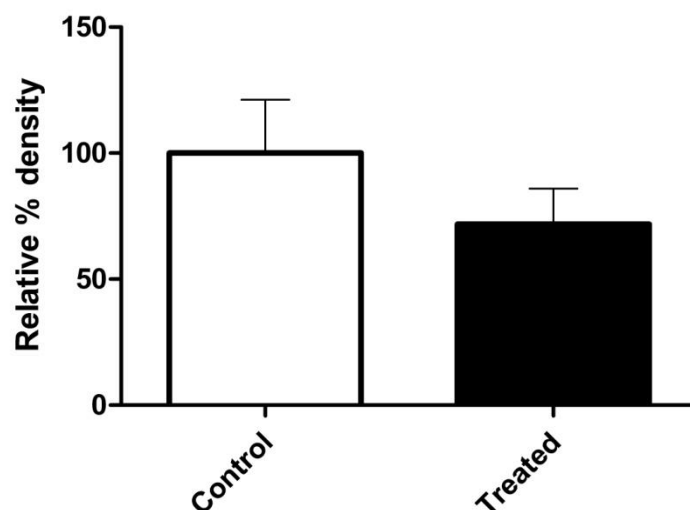


Figure 5-10 Neurogranin expression in the hippocampus following chronic ECS

(A) Lanes 1-3 contain representative chronic sham treated hippocampal samples. Lanes 4-6 contain chronic ECS treated hippocampal samples with representative GAPDH controls below.

(B) There was no significant difference in the relative % density of neurogranin expression following ECS treatment compared to controls. Mann-Whitney test, data expressed as % of controls $\pm$ SEM, $n=6-9$ per group.

5.3.13 Spectrin

Spectrin expression was increased in the hippocampus following acute ECS treatment (Figure 5-11). The study found an increase in the relative % density of ECS treated animals compared to controls (Mann Whitney test, $p = 1$, $n=7-8$ per group); mean relative percentage density: $100 \pm 16\%$ control: $109 \pm 28\%$ treated).

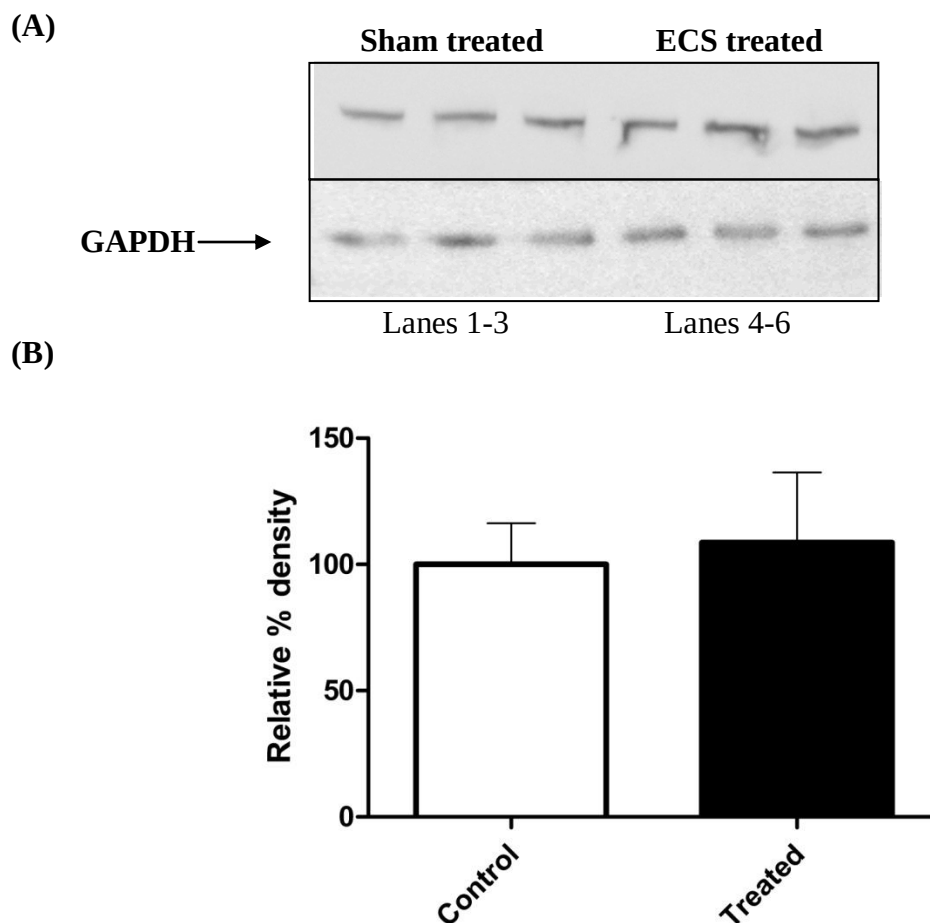


Figure 5-11 Spectrin expression in the hippocampus following acute ECS treatment

(A) Lanes 1-3 contain representative acute sham treated hippocampal samples. Lanes 4-6 contain acute ECS treated hippocampal samples with representative GAPDH bands below.

(B) There was no significant difference in the relative % density of Spectrin expression following ECS treatment compared to controls. Mann-Whitney test, data expressed as % of controls $\pm$ SEM, $n=7-8$ per group.

Spectrin was also identified in the Chronic study (Figure 5-12). There was a non-significant increase in spectrin expression detected following immunoblotting (Student's *t*-test, $p=0.4488$, $n=5-7$). The mean relative percentage density of the control group was $100 \pm 19\%$ and the treated group was $120 \pm 2.7\%$.

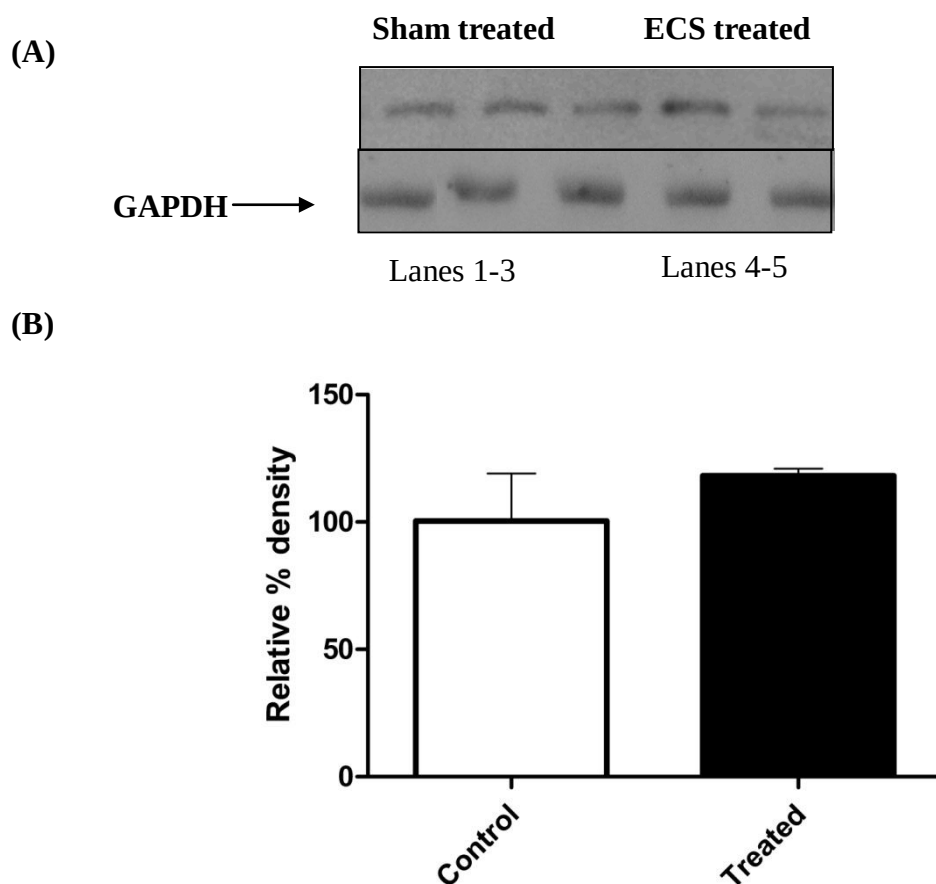


Figure 5-12 Spectrin expression in the hippocampus following chronic ECS

(A) Lanes 1-3 contain representative chronic sham treated hippocampal samples. Lanes 4-5 contain chronic ECS treated hippocampal samples, representative GAPDH are below.

(B) There was no significant difference in the relative % density of Spectrin expression following ECS treatment compared to controls. Student's *t*-test test, data expressed as % of controls $\pm$ SEM, $n=5-7$ per group.

5.3.14 Actin (beta)

Actin expression was studied in the Chronic + 4 week study (Figure 5-13). There was a significant decrease in expression detected following immunoblotting (Student's *t*-test, $p=0.0053$, $n=6-9$). The mean relative percentage density of the control group was $100 \pm 15\%$ and the treated group was $38 \pm 3.8\%$.

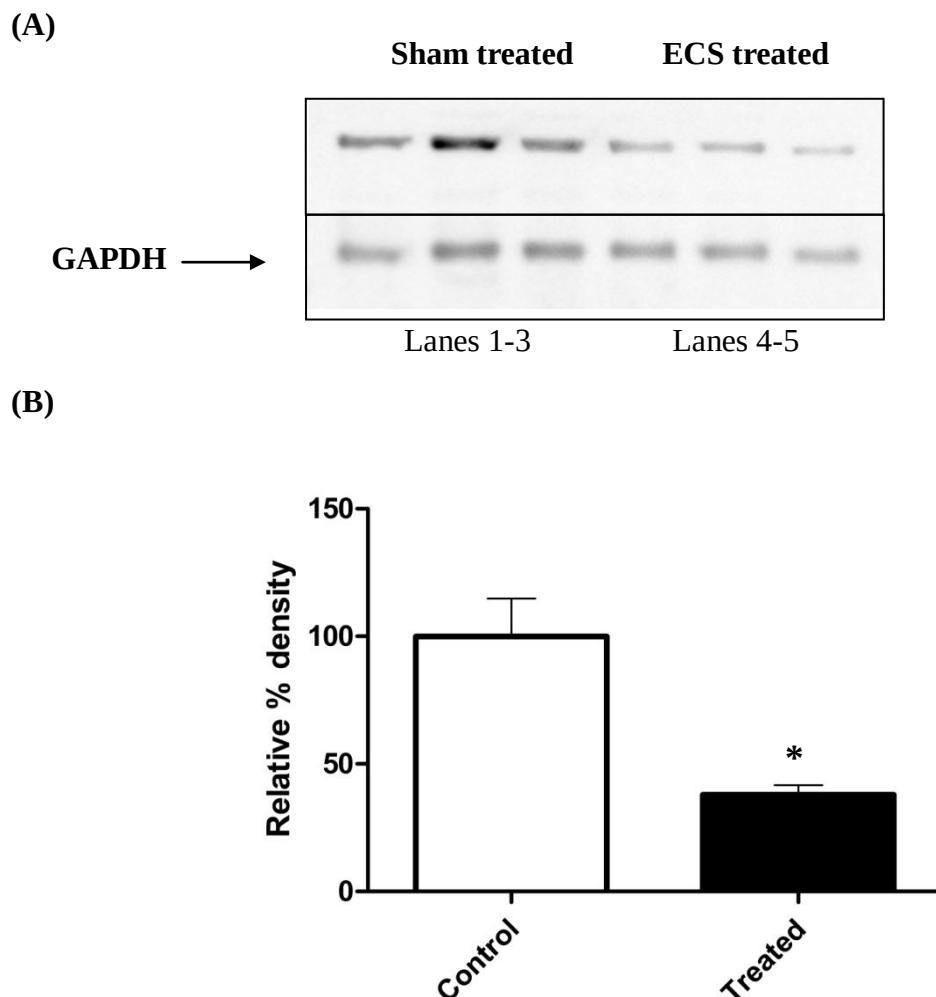


Figure 5-13 Actin expression in the hippocampus following chronic + 4 weeks of ECS treatment

(A) Lanes 1-3 contain representative chronic + 4 week sham treated hippocampal samples. Lanes 4-6 contain chronic + 4 weeks of ECS treated hippocampal samples, representative GAPDH bands are below.

(B) There was a significant difference in the relative % density of β -actin expression following ECS treatment compared to controls. Student's *t*-test test, data expressed as % of controls $\pm$ SEM, $n=6-9$ per group * $p<0.05$.

5.4 Discussion

Many of the proteins identified by mass spectrometry are associated with roles in metabolism or cell structure. Although each protein cannot be discussed individually, a brief summary of the main protein results is considered.

Gene ontology (GO) analysis revealed that many proteins identified in the Acute and Chronic study were involved in biological processes relating to the actin cytoskeleton, its organisation, polymerisation, depolymerisation and actin capping (section 5.4.2; 5.4.4). In the Chronic study in particular, the molecular function and cellular component ontologies highlighted proteins that are related to the cytoskeleton. GO analysis following chronic + 4 weeks of ECS revealed a role for cytoskeletal related proteins in each ontology. However metabolism related proteins appeared to be more significant in this study (section 5.4.6).

5.4.1 Cytoskeletal proteins

The neuronal cytoskeleton consists of three different structural components, microfilaments, neurofilaments and microtubules (Siegel et al. 1999), all of which were altered following ECS treatment.

The microtubule protein tubulin, both alpha and beta isoforms, underwent an upregulation in expression following chronic and chronic + 4 weeks of ECS in the hippocampus.

A number of intermediate filaments were identified following acute, chronic and chronic + 4 weeks of ECS treatment including glial fibrillary acidic protein (GFAP), which was upregulated following acute and chronic + 4 week ECS but downregulated following chronic ECS treatments. The intermediate filaments vimentin, desmin, peripherin and neurofilament light chain protein were upregulated in the Chronic + 4 week study.

Actin was the main microfilament detected in all studies. It was found in multiple spots in each study and its expression was both up and downregulated depending on the spot it was identified in. Spectrin, a microfilament protein chosen for confirmation studies, was identified in the hippocampus in the Acute and Chronic study.

Actin and GFAP were differentially expressed following acute, chronic and chronic + 4 week ECS treatment. These cytoskeletal proteins commonly undergo changes in expression in the brain. Differences in cerebral expression of the actin family of proteins is one of the most commonly reported findings in both human and rodent proteomic studies (Petrak et al. 2008).

GFAP expression changes have previously been reported in rat hippocampus (Samara et al. 2011; Fountoulakis et al. 2005). It has also been identified following antidepressant pharmacotherapy (Carboni et al. 2006a) and antidepressant pharmacotherapy in an animal model of depression (Piubelli et al. 2011a, c). GFAP was also differentially expressed in other psychiatric disorders, including the CSF of patients with schizophrenia (Martins de Souza et al. 2009).

Actin undergoes significant changes in expression even more commonly than GFAP. It has been reported in proteomic studies of the rat hippocampus (Fountoulakis et al. 2005; Samara et al. 2011; McNair et al. 2006) and mouse hippocampus (Robinson et al. 2009), in animal models of depression (Mallei et al. 2011) and following antidepressant treatment (Carboni et al. 2006a) or both (Piubelli et al. 2011 a, b, c). Actin expression in the hippocampus following chronic + 4 weeks of ECS was significantly reduced ($p < 0.05$) following immunoblotting (Figure 5-13). Actin expression at other time points has yet to be studied.

Due to the frequency with which the cytoskeletal proteins are identified as having undergone significant differential expression and the range of studies in which they are identified, it may be difficult to interpret the relevance of finding these proteins following ECS treatment. It seems likely that proteins involved in the regulation and control of cytoskeletal protein expression following ECS treatment may be of particular interest in understanding the effects of this treatment in the brain.

5.4.2 Metabolic proteins

A number of metabolism-related proteins were identified in the hippocampus following ECS treatment. For example, acute ECS induced a reduction in the expression of adenosine triphosphate (ATP) synthase, a protein involved in ATP production. In proteomic studies, ATP synthase was downregulated in the hippocampus following acute ECS and following fluoxetine administration (Carboni et al. 2006a). Equally, many more of the metabolic proteins identified in the hippocampus following acute and chronic + 4 weeks of ECS treatment were also detected by proteomic analysis following escitalopram treatment. Isocitrate dehydrogenase (citric acid cycle), phosphoglycerate kinase (glycolysis), NADH dehydrogenase (electron transport chain) and L-lactate dehydrogenase (catalysed conversion of pyruvate to lactate) were upregulated chronically in the hippocampus following antidepressant treatment (Piubelli et al. 2011a, b). While ECS treatment has affected the levels of expression of a number of metabolism related proteins, it is important to note that 2DE proteomics is biased towards the detection of abundant

proteins such as those with a role in metabolism (Parker et al. 2009). Gene ontology (GO) analysis suggested that many of the proteins identified in the hippocampus were involved in biological processes relating to metabolism, including nucleotide metabolism and glucose catabolic processes. However, this was only seen following chronic + 4 weeks of ECS.

5.4.3 Pathway analysis

The pathway analysis networks created for each study highlight the central roles of actin, tubulin and the numerous interactions surrounding these cytoskeletal proteins (Figure 5-4 – 5-6). Pathway analysis (IPA) contributed to the network of proteins identified following acute, chronic and chronic + 4 weeks of ECS by adding proteins to the networks that were not identified by proteomic analysis. Therefore, although these additional proteins were included in the networks based on findings in the literature, they can be speculative in nature. Of potential interest in the hippocampus was the identification and addition by IPA of actin related protein 2/3 (Arp 2/3) in the Chronic study and insulin in the Chronic and Chronic + 4 week study. Arp 2/3 is an actin nucleating protein complex that is responsible for initiating new actin filament polymerisation, allowing for efficient actin assembly (Goley et al. 2006). The addition of insulin to the networks may be related to evidence suggesting that treatments targeting metabolic networks may be used to treat depression (McIntyre et al. 2007; McIntyre et al. 2009). Insulin has been implicated in other IPA network pathways following antidepressant treatment (Piubelli et al. 2011c).

A large number of proteins were identified by mass spectrometry as being of potential interest in the study of the effects of ECS on the hippocampus. Several of these proteins were chosen for confirmation and are discussed in greater detail below.

5.4.4 Confirmation study proteins

The fold changes in protein expression following 2D proteome analysis were subtle (Pennington et al. 2009; McHugh et al. 2010) and did not highlight any particular protein spots for further study. Proteins were selected for confirmation based on their potential role in the mechanisms of antidepressant action; hence the selection of several cytoskeletal related proteins. The availability of suitable antibodies, as well as the availability of other methods of confirmation (e.g. ELISA) was also a limiting factor when selecting proteins for confirmation.

The significant expression changes of few of the selected proteins were confirmed by semi-quantitative immunoblotting, although trends in changes in expression were

uncovered, as discussed below. As a result of the low fold expression changes detected following proteomic analysis, it is possible that only subtle differences in protein expression could be expected following immunoblotting. Immunoblotting may not be sufficiently sensitive to detect these changes in expression between control and treated groups. The results obtained for the proteins selected for confirmation and their potential role in the mechanisms of ECS are discussed below.

5.4.5 Tropomodulin-2

Tropomodulins are a family of proteins, containing four isoforms (tropomodulin 1-4), encoded by genes Tmod1-4. Tropomodulin 2 is expressed specifically in the brain, although tropomodulin 1 and 3 are expressed in both neuronal and non-neuronal cells (Watakabe et al. 1996; Cox and Zoghbi 2000). Tropomodulins act as capping proteins for the slow-growing, pointed ends of actin filaments (Weber et al. 1994). Tropomodulins prevent the extension and depolymerisation of the pointed end of tropomyosin-containing actin filaments, maintaining the narrow length distribution of actin filaments (Weber et al. 1994).

Tropomodulin 2 knockout mice exhibited impaired learning and memory, although levels of tropomodulin 1 may have been increased in compensation for the loss of tropomodulin 2 (Cox et al. 2003). Tropomodulin 1 has previously been shown to regulate actin filament length and stability in muscle by interacting with tropomyosin at thin filament pointed ends (Gokhin et al. 2010; Mudry et al. 2003). More recent studies have demonstrated that tropomodulin-2, which has a mainly cytoplasmic localisation in neurons, acts to inhibit neurite outgrowth, by inhibiting neurite extension and the ability of cells to initiate neuritogenesis (Fath et al. 2011). It is possible that, as tropomodulin 2 binds actin monomers (Yamashiro et al. 2010), it may sequester actin during neuritogenesis, reducing the availability of monomers and inhibiting neurite outgrowth.

Kainic-acid induced seizure activity has shown an increase in tropomodulin 2 mRNA in the brain that lasts for at least one week (Sussman et al. 1994). Tropomodulin 2 protein expression was also found to be increased in an ischaemic rat model following proteomic analysis (Chen et al. 2007b), and was proposed to be involved in neuronal repair 24 hrs after ischaemia.

In the present study, tropomodulin 2 was located in a protein spot that had a 1.1 fold increase in protein expression in the hippocampus following chronic ECS treatment. Due to its role in neurite outgrowth and the actin cytoskeleton this protein expression change was selected for confirmation using semi-quantitative immunoblotting. No

statistically significant difference was found between the chronic control and ECS treated samples, however a decrease in tropomodulin 2 expression was detected in ECS treated animals (Figure 5-7). As tropomodulin 2 regulates neurite formation, a decrease in tropomodulin 2 may result in a reduction of neurite inhibition and therefore an increase in neuritogenesis.

Tropomodulin 2 knockout animals experience deficits in learning and memory (Cox et al. 2003). Reductions in tropomodulin 2 may therefore also be related to the side effects associated with ECS treatment; these include memory deficits in behavioural paradigms including the water plus maze and passive avoidance protocols (Fochtman, 1994).

An increase in tropomodulin 2 expression was seen following kainic acid seizure induction, a model of epilepsy that is associated with damage to the brain (Sussman et al. 1994; Olney et al. 1983). Unlike the induction of epileptic seizures, ECS is a model of ECT, a method that therapeutically induces seizures (UK ECT Review Group, 2003). Therefore, although the decrease in tropomodulin 2 in the Chronic study was not statistically significantly different, reduced levels of this protein may contribute to the changes in plasticity associated with ECS (Stewart et al. 2000). Further work will be required to determine the effects of tropomodulin 2 following ECS treatment.

However, overall, no statistically significant difference in tropomodulin 2 expression in the hippocampus was found and therefore, its expression at other time points or in other tissues was not investigated.

5.4.6 Ca^{2+} -calmodulin kinase II β (CaMKII β)

CaMKII β was identified in a protein spot which had an increase in expression of 1.2 fold in the hippocampus following acute ECS treatment. However, in the follow-up confirmation study a non-significant decrease in CaMKII β levels was found (Figure 5-8).

CaMKII is abundant in the brain and represents 1-2% of total protein in the brain (Erondy and Kennedy, 1985). CaMKII is found in large quantities in the post synaptic density, at levels almost comparable to those of the cytoskeletal components and in higher levels than other signal transduction molecules (Peng et al. 2004; Erondy and Kennedy, 1985).

CaMKII can be expressed as multiple isoforms but the α and β forms are most commonly expressed in the brain. They can assemble together to form a dodecameric

holoenzyme (Kolodziej et al. 2000; Lisman et al. 2002; Colbran and Brown et al. 2004; Lee, 2006).

CaMKII acts as a signal transducer and is a member of the serine/threonine protein kinase family that is regulated by Ca^{2+} /calmodulin signalling (Wayman et al. 2008).

CaMKII also plays a more direct role in synaptic plasticity than signal transduction. CaMKII β binds to F-actin which may be necessary for maintaining dendritic spine structure (Okamoto et al. 2007). The ability of CaMKII to bind F-actin is unique to the beta isoform (Fink et al. 2003). CaMKII β was also found localised to the actin cytoskeleton (Shen et al. 1998). As synaptic plasticity involves F-actin dynamics (Lamprecht and LeDoux, 2004) CaMKII appears to have a distinct role in the structural plasticity of dendritic spines. A model of the role of CaMKII in structural plasticity has been proposed: in the synapse resting state, F-actin is bundled by CaMKII, maintaining synaptic structure. CaMKII is activated by influx of Ca^{2+} into the cell and detaches F-actin. F-actin is reorganised by other signalling processes. More CaMKII is recruited to the synapse and acts as a signal transducer, influencing in turn the amount of bundled F-actin and the structure of the dendritic spine. When CaMKII returns to the basal state, it bundles the F-actin and acts to maintain the new spine structure (Okamoto et al. 2007). Therefore, CaMKII β has both a signalling role and an actin-bundling role, influencing synaptic plasticity. In mature neurons CaMKII β promoted dendritic remodelling (Fink et al. 2003). However, many of the studies conducted to investigate the role of CaMKII β used *in vitro* models. Further studies into the *in vivo* effects of CaMKII on mature neurons will be required to understand the potential influence that this plasticity related molecule may have following ECS.

5.4.7 Neurogranin

Neurogranin is a calmodulin-binding postsynaptic protein that concentrates in dendritic spines (Baudier et al. 1989; Watson et al. 1992). It also localises to neuronal soma but is rarely seen in axons (Watson et al. 1992). Neurogranin is thought to play a role in long term potentiation (LTP), one of the best established forms of synaptic plasticity (Zhong and Gerges, 2010).

During neuronal depolarisation, Ca^{2+} enters the cell, activates calmodulin which in turn activates downstream targets including CaMKII, leading to LTP induction (Huang et al. 2004; Xia et al. 2005). Therefore, the availability of calmodulin can influence synaptic plasticity induction. There are thought to be two mechanisms that regulate neurogranin-

calmodulin binding and the availability of calmodulin. Firstly, an increase in intracellular Ca^{2+} concentration dissociates calmodulin from neurogranin in a reversible manner, resulting in unbound calmodulin that can act to induce LTP (Huang et al. 1993; Gerendasy et al. 1995). Secondly, protein kinase C (PKC) mediated phosphorylation of neurogranin at its serine 36 residue prevents calmodulin from rebinding to phosphorylated neurogranin, again resulting in free-calmodulin that can induce changes in synaptic plasticity (Ramakers et al. 1995).

Control of neurogranin phosphorylation may act as a method to fine-tune synaptic plasticity (Zhong et al. 2011). The magnitude of LTP could be controlled by the length of time that PKC mediated neurogranin phosphorylation prevents neurogranin-calmodulin binding, allowing free-calmodulin to induce maximal or submaximal LTP expression (Zhong et al. 2011).

Neurogranin acts as a buffer, binding and sequestering calmodulin and controlling its availability in dendritic spines (Sanabria et al. 2008). Neurogranin enhances synaptic strength by increasing calmodulin recruitment to the synapse (Zhabotinsky et al. 2006). Therefore, as calmodulin availability in the spine is regulated by neurogranin, neurogranin also influences plasticity, particularly LTP (Zhong et al. 2009).

It has been suggested that memory deficits related to cognitive disorders, can result from decreased neurogranin levels and disturbances in signalling for neurogranin phosphorylation (Zhong et al. 2011). These deficits can include those associated with age related disorders, including Alzheimer's disease (Chang et al. 1997; Mons et al. 2001). Neurogranin has also been implicated in Jacobsen syndrome, a genetic disorder associated with learning disability (Coldren et al. 2009).

Neurogranin knockout mice show deficits in hippocampal dependent spatial learning and synaptic plasticity which may result from aberrant regulation of Ca^{2+} and calmodulin levels (Pak et al. 2000). Neurogranin is associated with deficits in psychiatric disorders, particularly schizophrenia. A genome wide association study has identified a relationship between schizophrenia and a single nucleotide polymorphism (SNP) in the neurogranin gene (Stefansson et al. 2009). The hippocampus of healthy carriers of the neurogranin SNP showed less activation during contextual fear activation in functional magnetic resonance imaging (fMRI) studies compared to controls (Pohlack et al. 2011). Contextual fear conditioning is highly hippocampus dependent and impairments of processing have previously been associated with schizophrenia (Hettema et al. 2003; Barch et al. 2003).

Acute ECS treatment resulted in a trend towards an increase in neurogranin expression in the hippocampus (Figure 5-9). As a result, the effects of neurogranin following chronic ECS were also confirmed, however, this trend was not repeated in animals treated with chronic ECS (Figure 5-10). A previous study has looked at the acute effects of ECS on phosphorylated neurogranin levels in the hippocampus (Kim et al. 2010), in an effort to understand the underpinnings of long term effects induced by ECS. Acutely, phosphorylated neurogranin was significantly increased ten minutes after ECS only. However, as discussed above, phosphorylation of neurogranin is only one way in which its interaction with calmodulin is regulated (Huang et al. 1993; Gerendasy et al. 1995). Therefore, further study will be required to determine the relevance of an acute increase in neurogranin levels.

Overall, ECS treatment resulted in changes to the normal expression of neurogranin, which in turn may impact on synaptic plasticity in the hippocampus of treated animals.

5.4.8 Spectrin

Spectrin is a cytoskeletal protein that is encoded by seven genes, one of which, SPTAN1 encodes for the spectrin α II isoform detected in the Acute and Chronic study (Cianci et al. 1999).

Spectrin exists as heterotetramers consisting of various α and β subunit isoforms (Shotton et al. 1979; Machnicka et al. 2011). α and β subunits dimerise side by side in an antiparallel rod shape (Shotton et al. 1979). Dimers associate head to head to form tetramers (Ungewickell et al. 1978) which bind actin microfilaments forming a network of cross linked actin filaments (An et al. 2005; Machnika et al. 2011).

Spectrin provides a scaffold by binding membrane proteins and cytosolic proteins either directly or through adaptor proteins (DeMatteis et al. 2000). Spectrin links membrane and cytosol, providing a framework on which signalling molecules, membrane protein complexes and structural elements can interact. Spectrin links to filament systems and is responsible for linking membrane and cytosolic proteins to the cytoskeleton (De Matteis et al. 2000). Membrane elasticity is dependent on integral protein distribution which is controlled by spectrin (Sleep et al. 1999). Spectrin binds the protein 4.1 complex and stabilises the actin-spectrin interaction, maintaining actin filament length (Machnika et al. 2011) and is therefore implicated in plasma membrane stability, cell morphology and in cell architecture (Bennett et al. 2008; Goodman et al. 1981; Wu et al. 2001).

In the nervous system, α II spectrin interacts with 14-3-3 protein, which is involved in synaptic plasticity and neuronal migration, in a phosphorylation-dependent manner, to regulate neural cell adhesion molecule (NCAM) stimulated neurite outgrowth (Ramser et al. 2010). α II spectrin has also been strongly implicated in actin reorganisation (Metral et al. 2009).

Spectrin has been identified in proteomic studies of the hippocampus (Fountoulakis et al. 2005; Samara et al. 2011) following escitalopram in an animal model of depression (Piubelli et al. 2011b). It has also been found in studies of CNS injuries where loss of spectrin breakdown may act as a marker of ischemic stress (Dutta et al. 2002).

There was a non-significant increase in Spectrin levels in the hippocampus following acute and chronic ECS treatment (Figure 5-11 – 5-12). This suggests that ECS upregulates expression of a molecule that is strongly implicated in changes in the actin cytoskeleton and may contribute to the changes in neuroplasticity associated with antidepressant administration (Stewart et al. 2000).

5.4.8.1 Conclusion

Proteomic studies of the effects of antidepressant treatment in the hippocampus have highlighted a role for cytoskeletal related proteins (Piubelli et al. 2011b). This result was verified following the administration of ECS. A number of cytoskeletal related proteins were differentially expressed in the hippocampus. The expression changes of proteins of particular interest were confirmed. However, further study will be required to determine the roles that these proteins may play in the effects of ECS in the brain.

Overall, the greatest number of protein changes was detected following chronic + 4 weeks of ECS treatment. Following the initial proteomic analysis, univariate and multivariate analysis detected a greater number of significant protein spots in this study than following chronic ECS treatment (Table 4-13) where a greater response to ECS treatment may have been expected. Multivariate analysis modelling revealed that more variation was explained by the data following chronic + 4 weeks of ECS (Figure 4-27) and may have resulted in the larger number of proteins spots selected for identification from this study. The extensive number of protein changes following chronic + 4 weeks of ECS may also be informative of the longer-term changes induced in the hippocampus following ECS. As in all studies, the number of protein spots picked from preparative gels, but based on 2D-DiGE gels, is strongly influenced by the technical differences between gels.

5.4.8.1.1 Limitations

The confirmation studies did not result in statistically significant changes in protein expression. However several of the proteins underwent a change in protein expression following ECS that may be of biological relevance and merit further study. Due to technical difficulties, the sample number of several of the immunoblot studies was reduced and may have contributed to the lack of statistical significance obtained.

The proteomic changes induced in the frontal cortex by antidepressant treatment have not been as extensively studied as those in the hippocampus. Therefore, in Chapter 6 and 7, the acute, chronic and longer term effects of ECS on the frontal cortex proteome have been investigated in the same manner as in the hippocampus.

Analysis of frontal cortex proteome following electroconvulsive stimulation

6.1 Introduction

A number of studies have investigated the effects of antidepressant treatments on the proteome of the frontal cortex, a brain region that is implicated in depression (Drevets, 2001). The proteins identified in studies of depressed patients in the frontal cortex have already been discussed in detail (section 4.1). Briefly, metabolic proteins e.g. ubiquinol cytochrome c reductase, underwent changes in expression while dihydropyrimidinase related protein 2, a protein involved in cytoskeleton remodelling, and glial fibrillary acidic protein (GFAP), an intermediate filament that acts as an astrocytic marker also underwent significant expression changes in the frontal cortex of depressed patients (Johnston-Wilson et al. 2000).

In the rat, a similar range of protein changes have been detected in the frontal cortex as in the hippocampus following proteomic analysis of antidepressant treatment. Antidepressants such as nortriptyline (Piubelli et al. 2011c), fluoxetine and novel antidepressants NK1 and CRF1 receptor antagonists (Carboni et al. 2006a) induce changes in protein expression in the frontal cortex. Although each treatment induces a specific range of protein changes, they share the ability to modulate proteins involved in neuroplasticity and metabolism. Dihydropyrimidinase related protein 2 (1.5-fold change in expression), actin (1.7-4.3-fold change in expression), tubulin (2.3-fold change in expression) and pyruvate dehydrogenase (1.5-fold change in expression) are examples of cytoskeletal, plasticity and metabolic proteins that were found to be differentially expressed in the frontal cortex (Piubelli et al. 2011b). These proteins were previously found also to be differentially expressed following antidepressant treatment in the hippocampus (Piubelli et al. 2011b; Carboni et al. 2006a). In a model of learned helplessness, the synaptoproteome of animals treated with nortriptyline indicated that stress and antidepressant treatment had opposing effects on a protein, stathmin (2 fold change in expression), involved in cellular remodelling (Mallei et al. 2011).

Although antidepressants induce differential expression of a number of the same proteins in the frontal cortex and hippocampus, the impact of different forms of antidepressant treatment, including ECS, on the frontal cortex proteome has not been comprehensively studied.

6.1.1 Aim of study

The aim of this study was to identify proteins that undergo changes in expression following administration of acute or chronic ECS in the frontal cortex. This was achieved using 2-dimensional difference in-gel electrophoresis (2D-DiGE) to separate proteins according to their molecular weight and isoelectric point. Univariate and multivariate statistical techniques were employed to identify the protein spots that were differentially expressed and that can be selected for identification by mass spectrometry following ECS treatment.

6.2 Methods

The frontal cortex proteome was analysed using similar methods to those described for the hippocampus in Chapter 4. The same study design and animals were used for the frontal cortex and hippocampal proteome studies. The 2D-DiGE protocol was conducted for frontal cortex tissue as described (section 4.2.2). Univariate and multivariate statistical analysis techniques were used to analyse the data, as discussed in Chapter 4, to determine both absolute changes in expression and correlations in the data (Pedreschi et al. 2009; Pedreschi et al. 2007). The significantly differentially expressed target protein spots were picked for identification by mass spectrometry using the same methods as described in section 4.2.2.

6.3 Results

6.3.1 Cydye labelled gels

Following protein separation, all gels were scanned using a fluorescent scanner (Typhoon 9400, GE Healthcare) and the images were uploaded and manipulated as described in section 2.2.16. High quality protein sample labelling and gel running were required for accurate analysis of the gels by Progenesis Samespot software (NonLinear Dynamics) for DiGE analysis.

Representative raw images of samples labelled with Cy5 (Figure 6-1) and the internal standard labelled with Cy3 (Figure 6-2) are shown. The scanned images of the Cy3 and Cy5 fluorescently labelled samples were overlaid (Figure 6-3) and converted to black/white images (Figure 6-4) for analysis using Progenesis SameSpot software (NonLinear Dynamics).

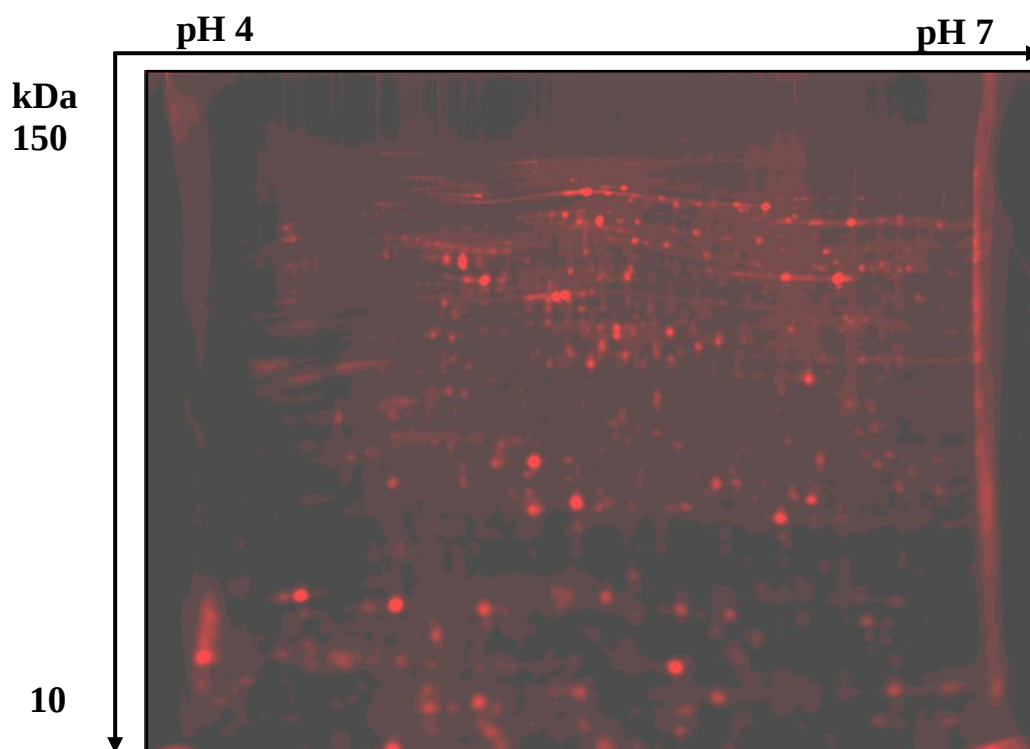


Figure 6-1 Representative fluorescent scan of Cy5 labelled frontal cortex sample

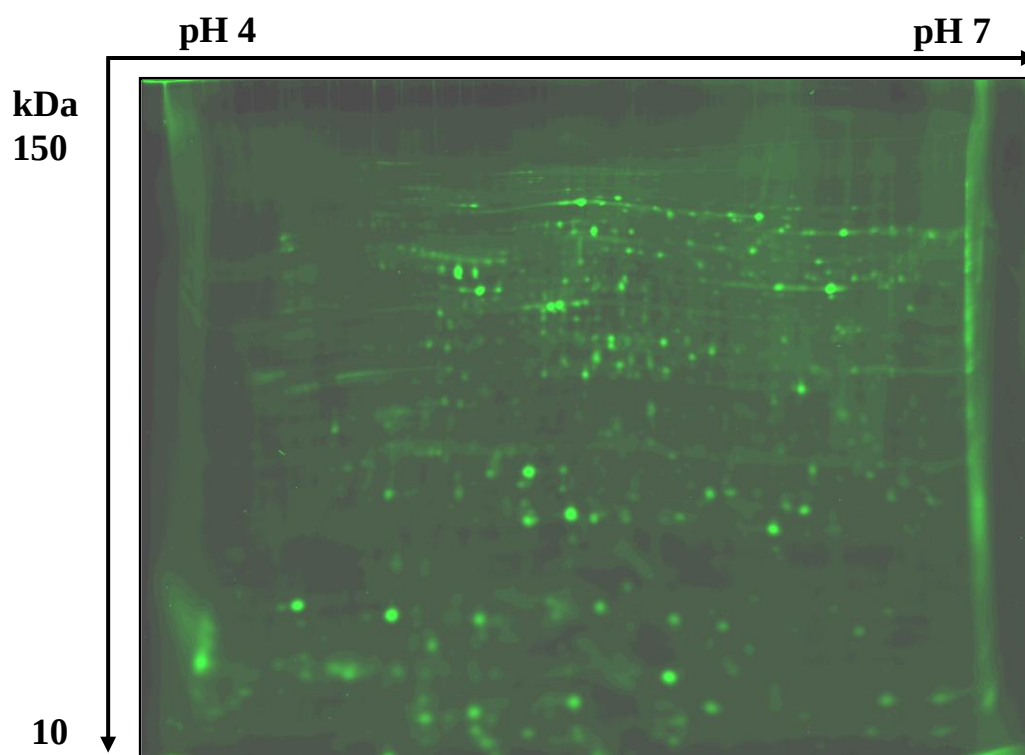


Figure 6-2 Representative fluorescent scan of Cy3 labelled internal standard

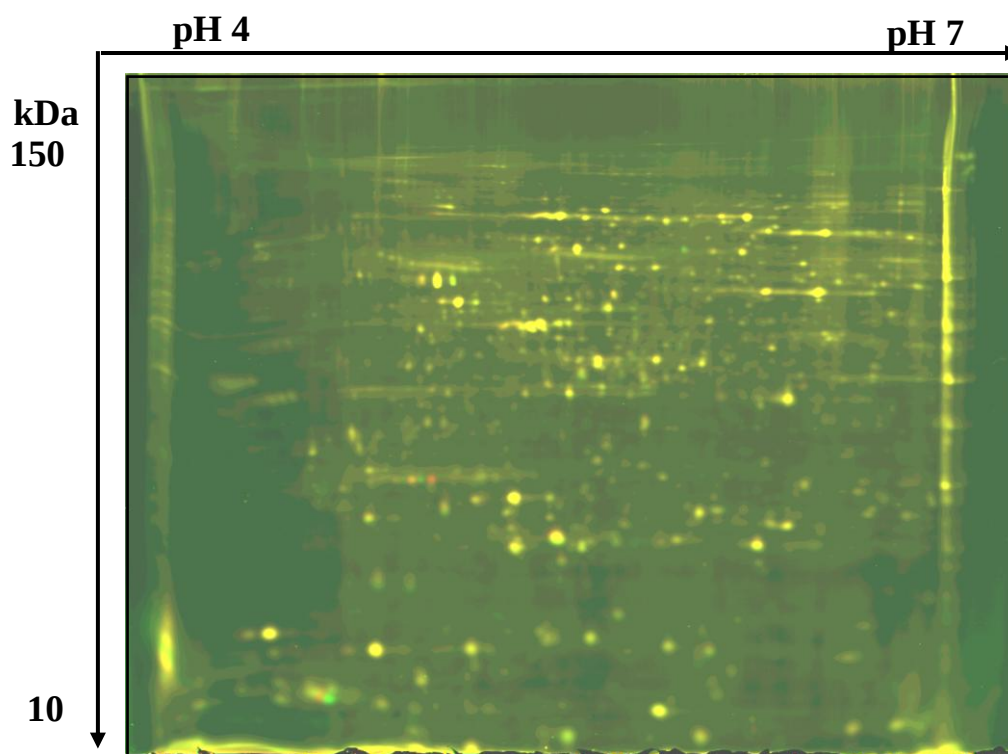


Figure 6-3 Overlay of Cy3 and Cy5 fluorescently scanned gels (representative)
The internal standard containing a pool of all samples overlaid on the frontal cortex sample that is run concurrently on the gel.

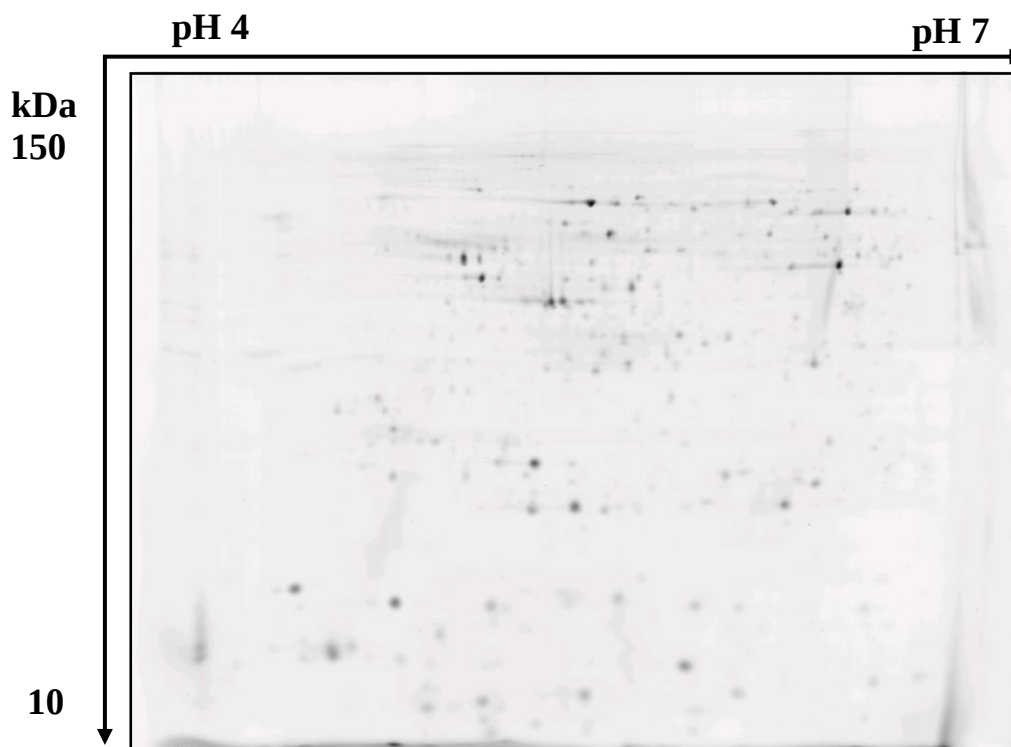


Figure 6-4 Black/white conversion of fluorescently labelled gels (representative)
The fluorescent images are converted to black/white images which were analysed by Progenesis SameSpots software (NonLinear Dynamics).

6.3.2 Frontal cortex: Analysis of the effects of acute ECS

6.3.2.1 Univariate analysis

The univariate results table (Table 6-1) includes the p-value associated with the one-way ANOVA comparison of treated and control ECS samples. The average normalised volume, the fold change between spots, and the q-value, which controls for the false discovery rate (FDR) and the power analysis value, are also included in Table 6-1.

Figure 6-5 shows the location, on a representative frontal cortex acute gel, of the significant spots detected by univariate analysis in Progenesis SameSpot software (NonLinear Dynamics).

The principal components analysis (PCA) conducted in Progenesis SameSpot software (NonLinear Dynamics) was used to detect outlying samples and patterns of clustering in the data. Although it is a multivariate technique, it was applied by Progenesis SameSpot software (NonLinear Dynamics) and was used in to detect outlying gels that could skew the data. The PCA plot, as discussed above, is shown in Figure 6-6.

Table 6-1 List of significantly differentially expressed spots in the frontal cortex following acute ECS, detected by univariate analysis

The table lists the standard spot numbers, ranked according to p-value, based on ANOVA. The fold changes, calculated from the average of the normalised volumes of each spot from treated and control samples, are included in the table. The q-value, a measure of FDR and the power value of the study are also present in the table.

Frontal cortex acute ECS significant spots					Average normalised volumes	
Spot #	Anova (p)	q Value	Power	Fold change	Acute control	Acute treated
394	0.00037	0.348213	0.994024	1.1	1.007	1.111
463	0.00085	0.348213	0.981061	1.2	0.975	1.136
505	0.001172	0.348213	0.971986	1.2	1.182	1.389
768	0.00162	0.348213	0.959585	1.1	1.282	1.434
603	0.005931	0.348213	0.864307	1.2	1.293	1.544
1022	0.006224	0.348213	0.859066	1.1	1.093	1.244
493	0.00722	0.348213	0.842113	1.2	1.174	1.364
659	0.00739	0.348213	0.839348	1.1	0.971	1.106
661	0.008411	0.348213	0.823362	1.1	0.973	1.082
1376	0.00921	0.348213	0.811568	1.4	0.808	1.134
1474	0.010746	0.348213	0.790459	1.2	1.015	1.169
404	0.011285	0.348213	0.783486	1.1	1.018	1.166
1465	0.011348	0.348213	0.782688	1.2	0.973	1.163
711	0.012265	0.348213	0.771299	1.5	0.906	1.332
572	0.012293	0.348213	0.77096	1.2	1.105	1.376
1173	0.012679	0.348213	0.766343	1.3	0.912	0.691

1504	0.013793	0.348213	0.753486	1.2	1.043	0.876
1520	0.014088	0.348213	0.750194	1.9	1.143	2.135
617	0.016309	0.348213	0.726775	1.2	1.019	1.176
1028	0.016681	0.348213	0.723065	1.1	1.045	1.122
909	0.01898	0.348213	0.701367	1.1	1.188	1.256
789	0.025043	0.348213	0.652165	1.1	1.287	1.441
439	0.026121	0.348213	0.644397	1.1	1.013	1.151
935	0.027474	0.348213	0.634994	1.1	1.072	1.158
311	0.028109	0.348213	0.630704	1.2	1.038	0.846
502	0.029295	0.348213	0.622907	1.4	1.108	1.56
839	0.030507	0.348213	0.615193	1.1	1.135	1.254
1479	0.030758	0.348213	0.613632	1.8	1.346	0.768
960	0.03105	0.348213	0.611821	1.2	1	0.869
1233	0.033637	0.348213	0.596384	1.2	1.046	0.861
1179	0.035494	0.348213	0.585907	1.1	1.071	1.216
1241	0.03675	0.348213	0.579082	1.4	1.04	0.763
943	0.040512	0.348213	0.559777	1.2	0.972	1.191
596	0.040746	0.348213	0.558629	1.3	0.867	1.143
1247	0.046832	0.348213	0.53064	1.6	0.723	1.169
808	0.047278	0.348213	0.52872	1.1	1.284	1.447
629	0.049058	0.348213	0.521219	1.4	1.027	0.753
230	0.049989	0.348213	0.517394	1.2	0.97	0.816

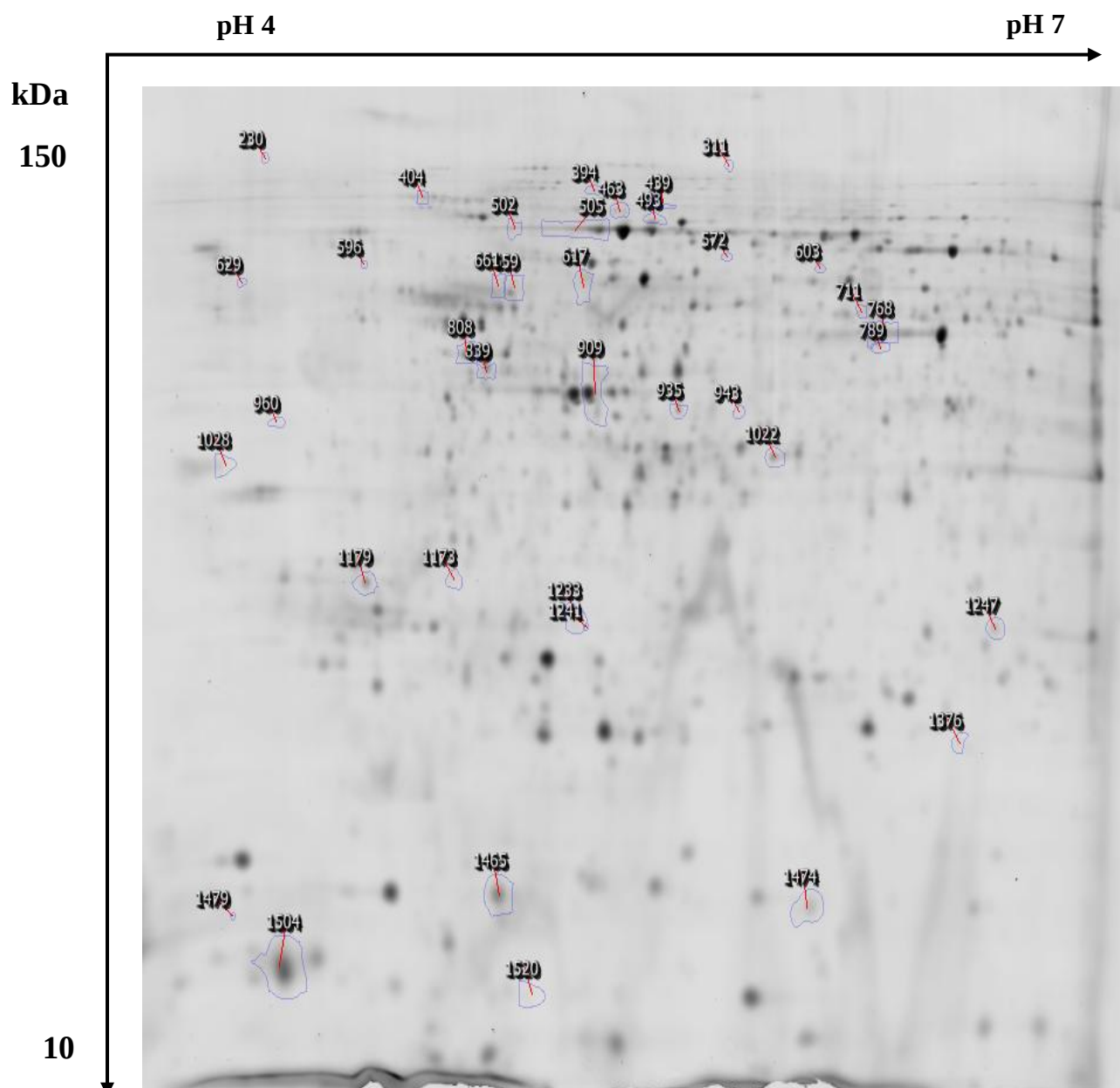


Figure 6-5 Frontal cortex: Location of spots significant spots following univariate analysis and acute ECS

Following analysis by Progenesis SameSpot software (NonLinear Dynamics) the spots highlighted on the gel were identified as being significantly up- or down-regulated ($p \leq 0.05$, one-way ANOVA).

6.3.2.1.1 Principal component analysis

Principal component analysis (PCA), conducted by Progenesis SameSpot software (NonLinear Dynamics) for the detection of outliers, was applied to the data. There is strong clustering of treated gels (blue) together and control gels (pink) together as ECS treatment clearly differentiates the two groups (Figure 6-6). There were no outlying gels in the study.

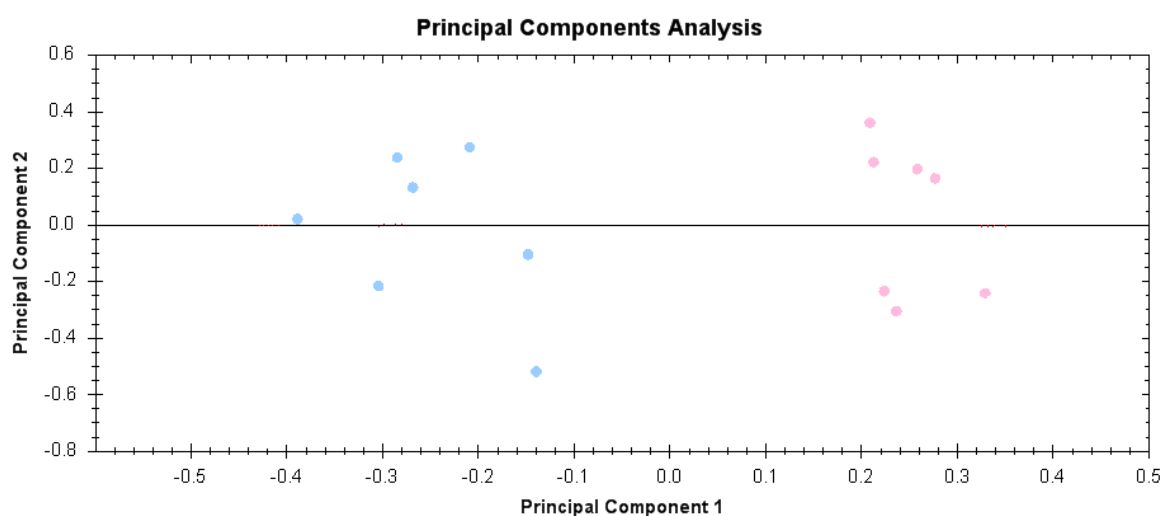


Figure 6-6 PCA plot of treated and control frontal cortex samples following acute ECS

Gel clustering according to ECS treatment (blue spots) and sham treatment (pink spots) can clearly be seen.

6.3.2.2 Multivariate analysis

The protein spots found to contribute to variation in the frontal cortex following acute ECS treatment following multivariate analysis are listed according to p-value below (Table 6-2). Those spots found to be significant at $p \leq 0.05$ are listed in the adjoining columns. Only those spots detected to be significant at this level were considered for further analysis. The different plots used to analyse and detect the sources of variation in each study are shown. The plots containing all variables as well as the final plots where the spots that contribute to noise are removed are shown.

Variables that contribute to explain variation in the frontal cortex acute ECS		Significant variables $p \leq 0.05$	
Spot number	P-values	Spot number	$p \leq 0.05$
195	0.24445	330	0.00056
202	0.43892	385	0.01067
219	0.16495	394	0.00486
227	0.19179	439	0.02687
274	0.06838	446	0.04788
356	0.0988	463	0.0426
380	0.14526	493	0.00931
386	0.27275	661	0.03002
404	0.16189	755	0.04412
457	0.08449	1313	0.03355
502	0.06712	1465	0.05394
505	0.06677		
566	0.23634		
603	0.23595		
617	0.10098		
659	0.14146		
705	0.17561		
711	0.07406		
768	0.10226		
789	0.28979		
889	0.18271		
909	0.2249		
981	0.16461		
996	0.23303		
1002	0.11457		
1004	0.17847		
1022	0.13769		
1173	0.2238		
1194	0.81999		
1376	0.13476		
1386	0.11954		
1468	0.35016		
1470	0.10275		
1474	0.06989		
1487	0.33052		
1504	0.07676		
1523	0.82145		

Table 6-2 Protein spots identified as being statistically significant following multivariate analysis and acute ECS treatment

6.3.2.2.1 PLS-DA diagnostic plots

Representative plots for the models drawn with all data are included for the X-Y correlation loadings and weighted regression coefficients (Figure 6-9, 6-10). These demonstrate the effect of removing non-significant variables in order to detect significant variables in the data. The scores plots (Figure 6-7) and explained variance plots (Figure 6-8) that contain all the variables and the redrawn models containing significant variables only are shown below. The “before” and “after” plots demonstrate the improvement in explained variance (Figure 6-8 (A) and (B)) using the significant data only as well as detecting sample clustering patterns and outliers (Figure 6-7 (A) and (B)).

In Scores plots Figure 6-7 (A) and (B), no samples fall outside the Hotelling T^2 ellipse, therefore no samples were removed as outliers. Based on these plots there is evidence of control samples clustering together and treated samples clustering together.

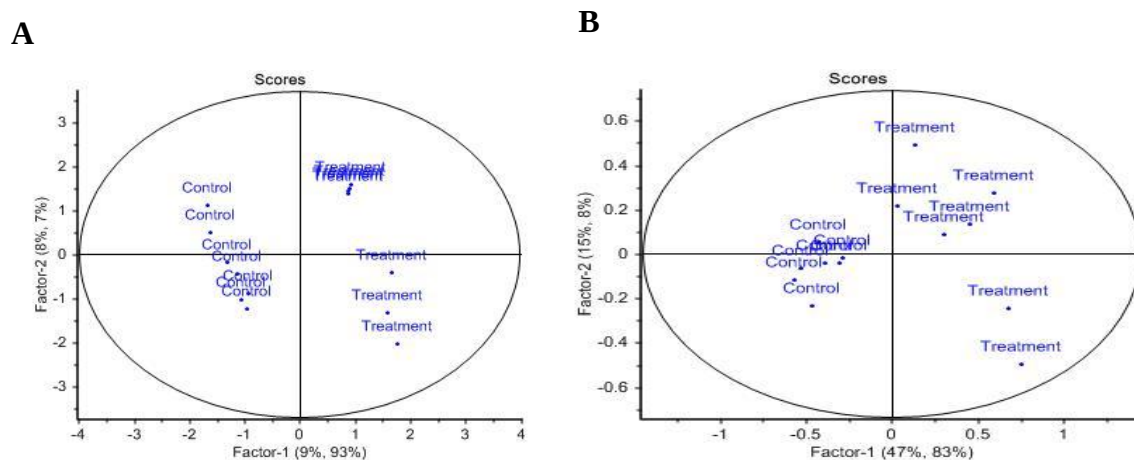
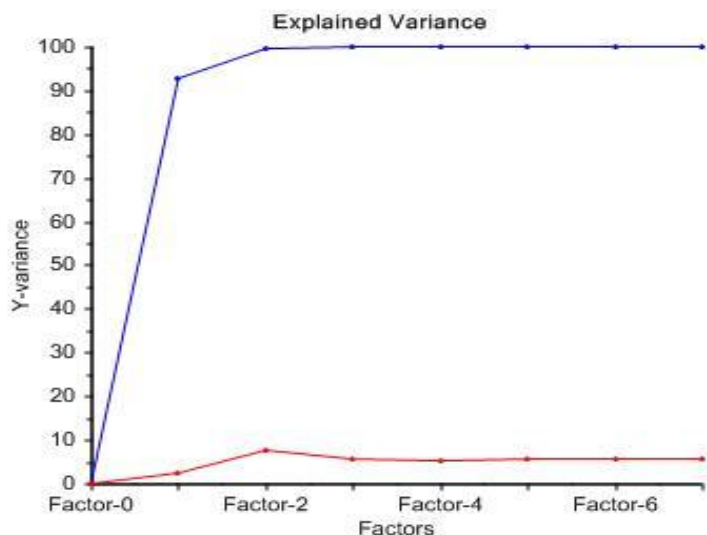


Figure 6-7 Scores plot following acute ECS treatment of the frontal cortex

(A) Scores plot with no variables removed; (B) Scores plot following removal of non-significant variables.

Little variance is explained in plot **(A)** (Figure 6-8) as a result of the masking effect of non-significant variables. In **(B)**, two factors formed the optimal model dimension by full cross validation. Cross validation created 37 submodels, where 1 sample was left out in each. The validation curve showed ~80% explained variance for two factors.

A



B

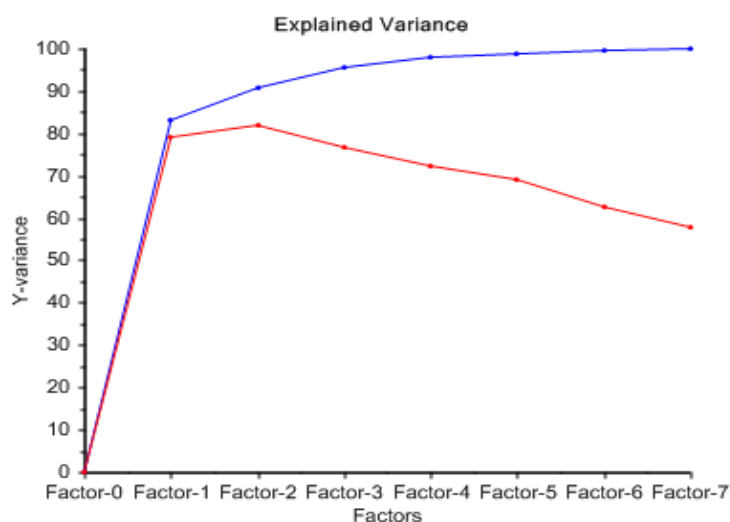


Figure 6-8 Plots of explained variance following acute ECS of the frontal cortex

(A) Explained variance plot, all variables included; **(B)** Explained variance of significant variables only.

B

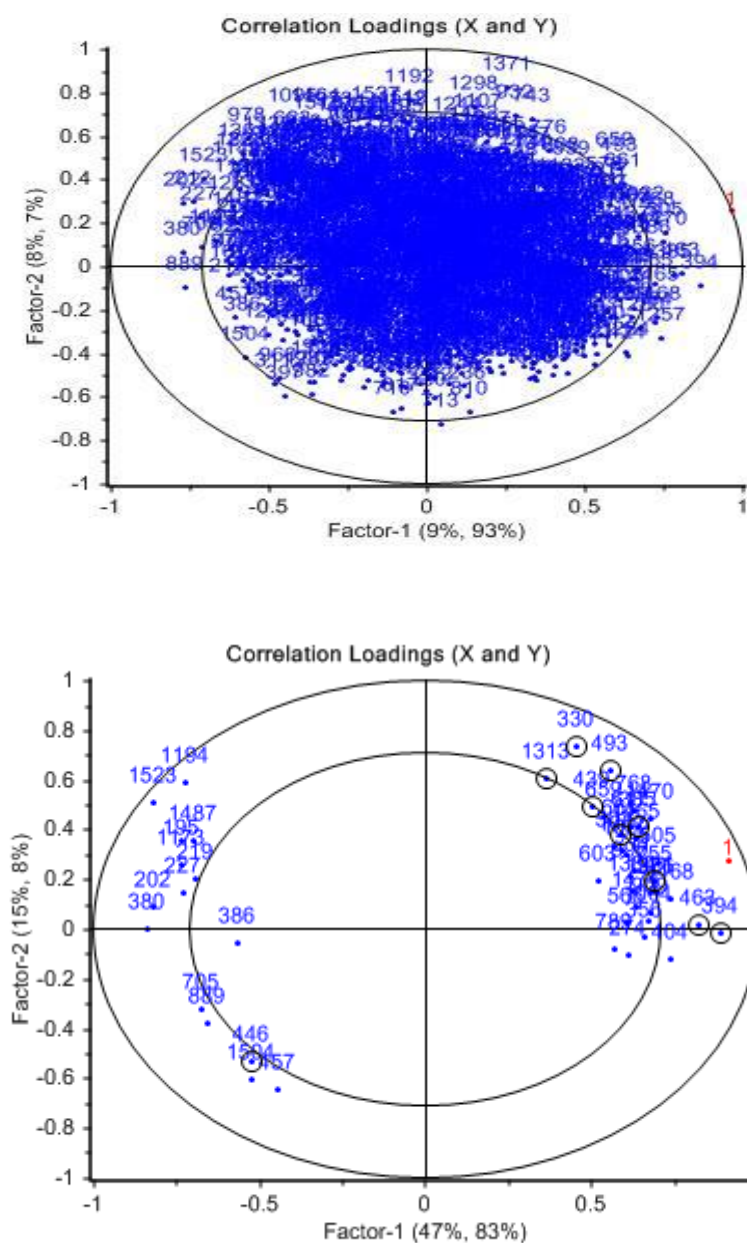
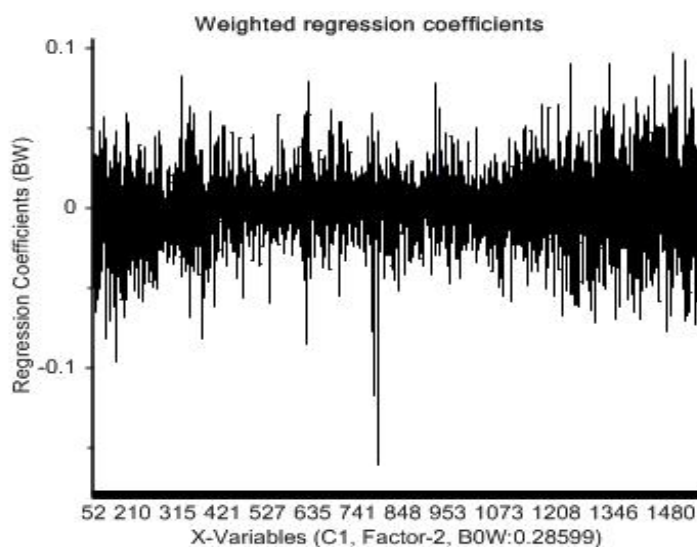


Figure 6-9 X-Y correlation loadings plot following acute ECS treatment of the frontal cortex

(A) Correlation loadings with all X-variables present; **(B)** Correlation loadings with significant variables only. Y variable ("1", red) is located in the top right quadrant.

All variables (**A**) in Figure 6-10 are difficult to interpret but the relevance of different variables can be seen. The significant variables plotted in (**B**) are more relevant to the interpretation of the importance of variables for modelling. The significant variables ($p \leq 0.05$) are circled.

A



B

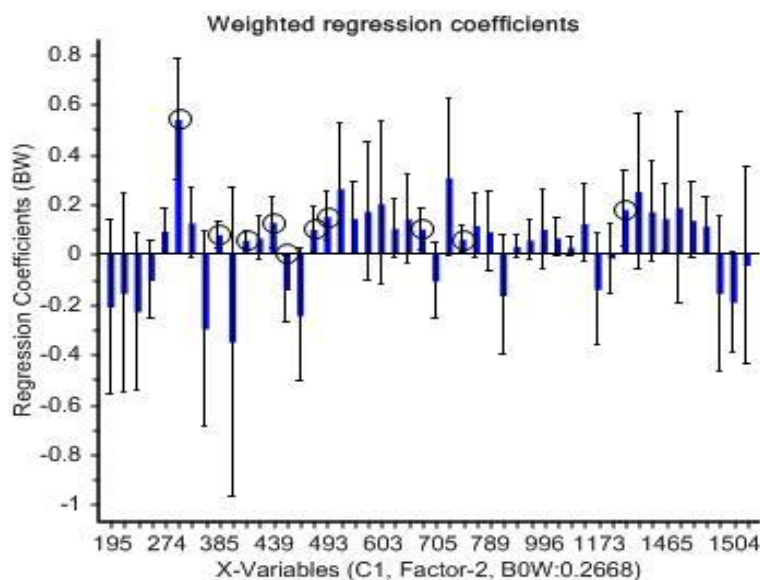


Figure 6-10 Plot of weighted regression coefficients

(A) Weighted regression coefficients all variables; **(B)** weighted regression coefficients of significant variables only. A 95% confidence interval estimates the uncertainty limits.

6.3.3 Comparison of univariate and multivariate significant spot findings

A collection of the spots found to be statistically different following both univariate and multivariate analysis in the frontal cortex following acute ECS was compiled. A Venn diagram describes the number of spots common to both univariate and multivariate analysis as well as unique to each analysis (Figure 6-11). The significant spot numbers from each form of analysis are listed in Table 6-3.

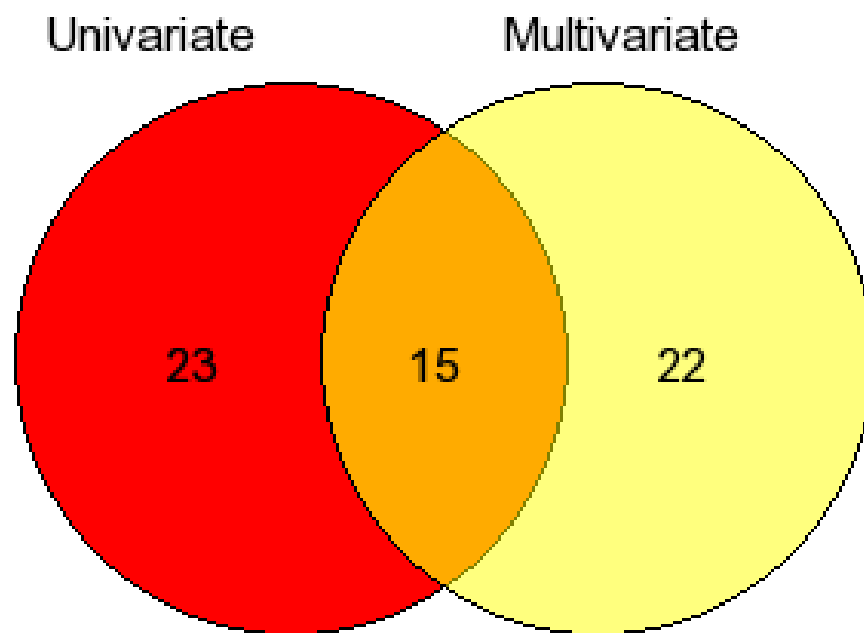


Figure 6-11 Venn diagram of significant spot distribution following univariate and multivariate analysis in the frontal cortex following acute ECS

Drawn in www.genevenn.com

Significant spot number by univariate analysis	Significant spot numbers common to both	Significant spot numbers by multivariate analysis
230	394	195
311	404	202
572	439	219
596	463	227
629	493	274
808	502	330
839	505	356
935	603	380
943	617	385
960	659	386
1028	661	446
1179	711	457
1233	768	566
1241	789	705
1247	909	755
1479	1022	889
1520	1173	981
	1376	996
	1465	1002
	1474	1004
	1504	1194
		1313
		1386
		1468
		1470
		1487
		1523

Table 6-3 List of significant spots following univariate and multivariate analysis of the frontal cortex following acute ECS treatment

6.3.4 Protein spots picked for mass spectrometry

A number of significant spots from the frontal cortex following acute ECS were picked from preparative gels for identification by mass spectrometry. The location of the spots is highlighted in Figure 6-12. These spots, their fold change, p-value and the statistical method by which they were detected are listed in Table 6-4.

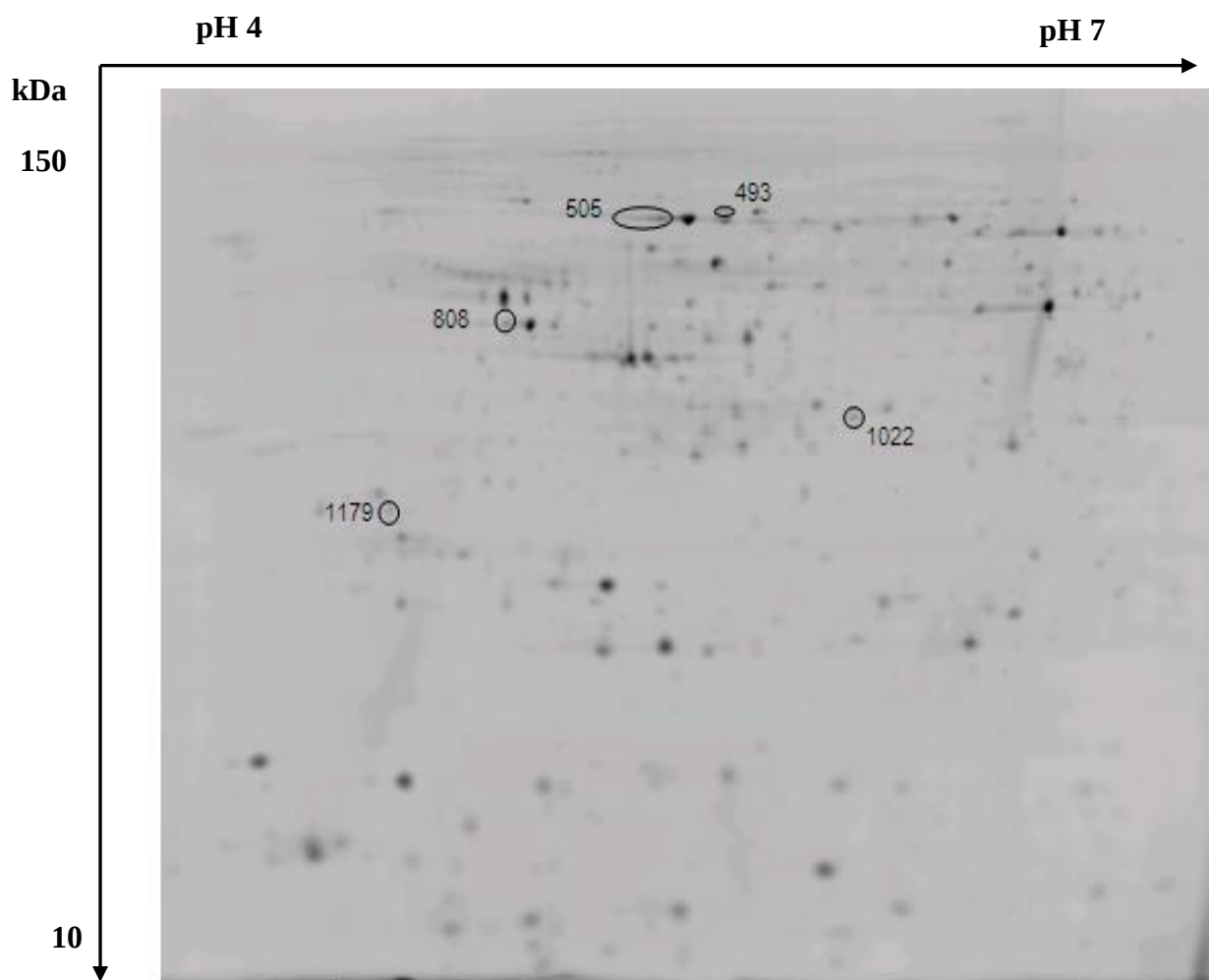


Figure 6-12 Preparative gels indicating significant spots that were picked for identification from the frontal cortex following acute ECS treatment

Frontal cortex	Standard spot no.	Fold change	Up / down regulated	p-value	MVA/UVA
Acute	505	1.2	Up	P=0.011	UVA
	1179	1.1	Up	P=0.035	UVA
	1022	1.1	Up	P=0.006	UVA
	808	1.1	Up	P=0.047	UVA
	493	1.2	Up	P=0.009	MVA/UVA

Table 6-4 Protein spots picked for identification by mass spectrometry

6.3.5 Frontal cortex: Analysis following chronic ECS treatment

6.3.5.1 Univariate analysis

The univariate results table following chronic ECS includes the p-value associated with the one way ANOVA comparison of treated and control ECS samples, the average normalised volume, the fold change between spots, and the q-value, which controls for the FDR and the power analysis value (Table 6-5).

The location of the significant spots detected by univariate analysis in Progenesis SameSpot software (NonLinear Dynamics) is shown on a representative frontal cortex chronic gel, Figure 6-13.

Principal component analysis (PCA) was used to detect outlying samples and patterns of clustering in the data. The PCA plot is shown in Figure 6-14.

Table 6-5 List of significantly differentially expressed spots in the frontal cortex following chronic ECS, detected by univariate analysis

The table lists the standard spot numbers, ranked according to p-value, based on ANOVA. The fold change, calculated from the average of the normalised volumes of each spot from treated and control samples are included in the table. The q-value, a measure of FDR and the power value of the study are also present in the table.

Frontal cortex chronic ECS					Average normalised volumes	
Spot #	Anova (p)	q Value	Power	Fold change	Chronic control	Chronic treated
709	1.60E-05	0.018106	0.999993	1.1	1.072	1.221
770	0.000588	0.172975	0.988345	1.3	1.003	1.27
1356	0.000631	0.172975	0.987169	1.3	1.086	1.362
749	0.000767	0.172975	0.983383	1.3	1.11	1.489
728	0.001763	0.267873	0.955744	1.1	1.164	1.305
1064	0.001901	0.267873	0.952059	1.2	1.109	1.283
1146	0.002902	0.305285	0.92695	1.3	0.857	1.075
1085	0.00299	0.305285	0.924863	1.5	0.836	1.292
1078	0.004694	0.362982	0.887839	1.1	0.928	1.065
1129	0.004925	0.362982	0.883261	1.2	1.123	1.357
938	0.005054	0.362982	0.880743	1.1	1.091	1.246
1006	0.005398	0.362982	0.874148	1.3	0.962	0.723
550	0.005858	0.362982	0.865627	1.1	1.142	1.268
814	0.006733	0.362982	0.850251	1.2	1.186	1.394
759	0.006848	0.362982	0.8483	1.2	1.213	1.01
839	0.007346	0.362982	0.840056	1.1	1.124	1.277
835	0.008174	0.362982	0.826979	1.1	1.076	1.195
1103	0.009701	0.362982	0.804622	1.2	1.056	1.241
1566	0.010912	0.362982	0.788292	1.1	1.23	1.373
1050	0.010979	0.362982	0.787423	1.1	1.2	1.073
773	0.0111	0.362982	0.785858	1.2	1.136	1.418

1474	0.011481	0.362982	0.781004	1.2	1.081	1.263
1016	0.013344	0.362982	0.758581	1.3	0.871	0.696
1001	0.01377	0.362982	0.753744	1.1	1.146	1.259
910	0.013844	0.362982	0.752907	1.1	1.233	1.338
696	0.014764	0.362982	0.742818	1.2	1.124	1.304
808	0.015461	0.362982	0.735445	1.2	1.32	1.595
510	0.01621	0.362982	0.727776	1.1	1.11	1.229
927	0.016466	0.362982	0.725205	1.1	1.118	1.209
702	0.016974	0.362982	0.720192	1.2	1.116	1.29
746	0.017428	0.362982	0.715796	1.1	1.12	1.204
1325	0.017557	0.362982	0.714563	1.2	0.951	1.108
556	0.017914	0.362982	0.711188	1.1	1.136	1.271
621	0.018817	0.362982	0.702843	1.1	1.154	1.271
555	0.019071	0.362982	0.700555	1.1	1.2	1.314
805	0.019753	0.362982	0.694501	1.1	0.968	1.059
748	0.020361	0.362982	0.689229	1.2	1.181	1.396
1359	0.020402	0.362982	0.688874	1.1	1.206	1.368
533	0.021379	0.362982	0.680664	1.1	1.118	1.193
884	0.023642	0.362982	0.662655	1.1	1.019	1.115
802	0.023867	0.362982	0.660942	1.1	1.054	1.167
1036	0.024014	0.362982	0.659824	1.3	1.16	0.899
1496	0.024443	0.362982	0.656601	1.1	1.109	1.203
511	0.024643	0.362982	0.655115	1.1	1.086	1.226
854	0.025123	0.362982	0.651576	1.1	1.085	0.979
1314	0.025605	0.362982	0.64808	1.1	1.135	1.2
1193	0.026721	0.362982	0.640181	1.2	1.2	0.97
929	0.028298	0.362982	0.629448	1.2	0.938	1.136
1094	0.030222	0.362982	0.616985	1.2	1.065	1.287
1476	0.034604	0.362982	0.590871	1.1	1.203	1.341
493	0.036407	0.362982	0.580929	1.1	1.285	1.409
703	0.036416	0.362982	0.580878	1.1	1.282	1.458
1301	0.0371	0.362982	0.577217	1.1	1.07	1.167

1513	0.039606	0.362982	0.564277	1.1	1.029	1.178
1188	0.040884	0.362982	0.557951	1.4	0.717	0.97
291	0.041039	0.362982	0.5572	1.2	1.22	1.019
850	0.04615	0.362982	0.53361	1.1	1.151	1.293
1091	0.047431	0.362982	0.528064	1.1	0.924	0.804
477	0.048346	0.362982	0.52419	1.1	1.14	1.289
604	0.049449	0.362982	0.519601	1.3	1.371	1.087
1226	0.049603	0.362982	0.518971	1.3	0.935	1.184
1155	0.049942	0.362982	0.517585	1.1	1.123	0.978
1463	0.050062	0.362982	0.515796	1.1	1.195	1.355
494	0.050191	0.362982	0.514563	1.1	1.169	1.276
909	0.050264	0.362982	0.511188	1.1	1.223	1.308
281	0.050322	0.362982	0.51604	1.3	1.117	0.851
706	0.050431	0.362982	0.502843	1.1	1.191	1.298

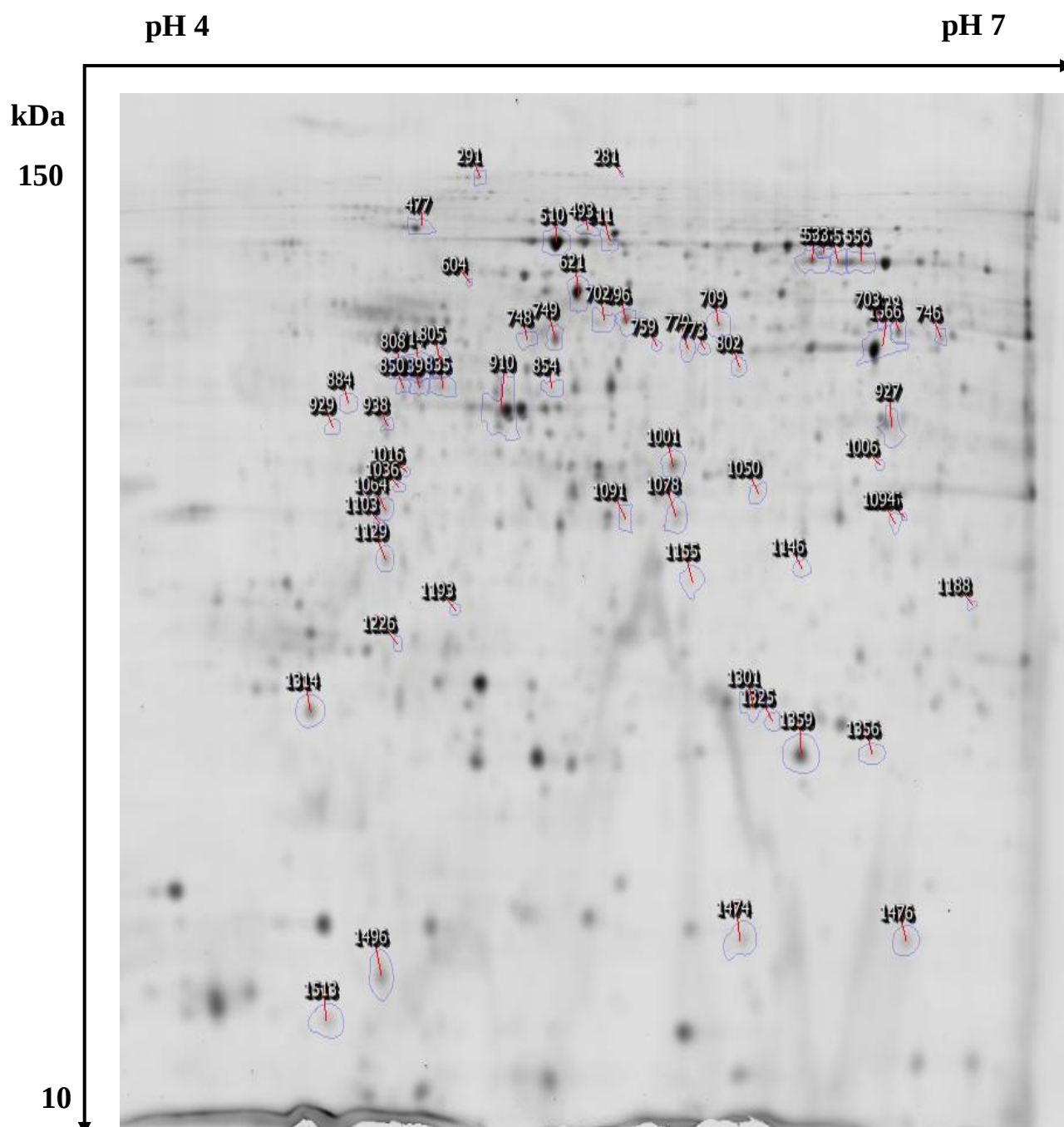


Figure 6-13 Frontal cortex: Location of significant spots following univariate analysis and chronic ECS

Following analysis by Progenesis SameSpot software the spots highlighted on the gel were identified as being significantly up- or down-regulated ($p \leq 0.05$, one-way ANOVA).

6.3.5.2 Principal component analysis

Principal component analysis (PCA) was conducted in Progenesis SameSpot software (NonLinear Dynamics) to detect outliers in the data (Figure 6-14). The ECS treated and control gels cluster according to treatment. There is greater spread among the ECS treated gels, however, no gels were removed as outliers.

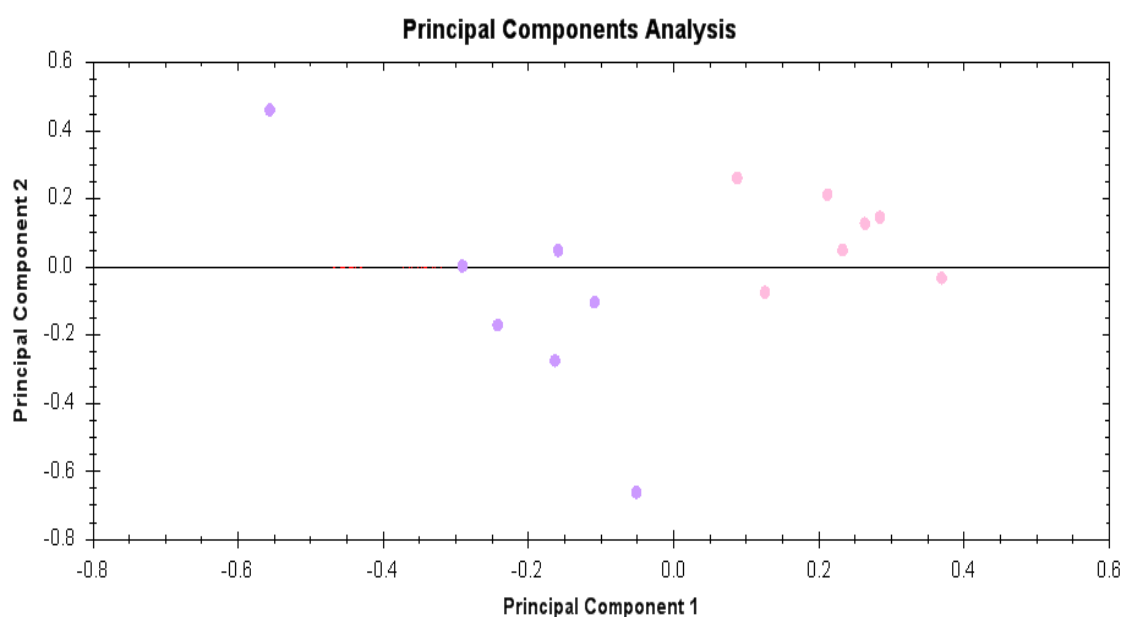


Figure 6-14 PCA plot of treated and control frontal cortex samples following chronic ECS treatment

Control samples are represented by pink spots; ECS treated gels are represented by purple spots.

6.3.5.3 Multivariate analysis

The protein spots found to contribute to variation in the data and that are statistically significant following multivariate analysis are listed according to p-value below (Table 6-6). Those spots found to be significant at $p \leq 0.05$ are listed in the adjoining columns and were considered for further analysis. The different plots used to analyse and detect the sources of variation in each study are shown.

Table 6-6 Protein spots identified as being statistically significant following multivariate analysis and chronic ECS

List is based on p-values of β coefficients determined to be significant; $p \leq 0.05$ values are listed separately.

Variables that contribute to variation in the frontal cortex chronic ECS		Significant variables $p \leq 0.05$	
Spot number	P-value	Spot number	P-value
167	0.62769	283	0.01678
244	0.80409	366	0.0524
284	0.13772	371	0.04298
370	0.41932	769	0.05694
416	0.09091	886	0.01358
417	0.13593	1036	0.0285
495	0.66699	1090	0.02107
539	0.57751	1099	0.05741
541	0.18016	1357	0.04283
543	0.50155	1551	0.01779
544	0.15479		
602	0.86996		
607	0.32799		
685	0.11667		
689	0.67805		
715	0.30598		
729	0.27462		
741	0.50607		
753	0.69967		
766	0.93573		
769	0.05694		
786	0.22328		
791	0.8223		

793	0.77732		
836	0.88328		
849	0.14388		
850	0.3916		
851	0.43337		
914	0.91146		
928	0.09906		
949	0.09454		
993	0.10615		
1011	0.45347		
1018	0.06378		
1044	0.24051		
1065	0.25137		
1099	0.05741		
1102	0.19836		
1127	0.64887		
1142	0.41021		
1298	0.15359		
1317	0.77799		
1319	0.08212		
1343	0.13679		
1374	0.56146		
1381	0.72966		
1403	0.63774		
1468	0.41629		
1471	0.54033		
1482	0.18416		
1522	0.23363		
1552	0.11359		
1553	0.18688		
1566	0.14986		

6.3.5.3.1 PLS-DA diagnostic plots

Plots representing the X-Y correlation loadings and weighted regression coefficients following the removal of non-significant spots during multivariate analysis are shown. The scores plots and explained variance plots that contain all the variables (Figure 6-15(A), 6-16(A)) and the redrawn models containing significant variables only (Figure 6-15(B), 6-16(B)) are shown below. The before and after plots demonstrate the improvement in explained variance using the significant data only as well as detecting sample clustering patterns and outliers.

Based on the score plot (Figure 6-15 **A** and **B**) there is evidence of control sample clustering and treated samples clustering together. No samples fall outside the Hotelling T^2 ellipse and therefore, none are marked as outliers. In plot (**B**), a single treated sample is on the border of the ellipse; however, it was retained in the redrawn model containing significant only spots.

A

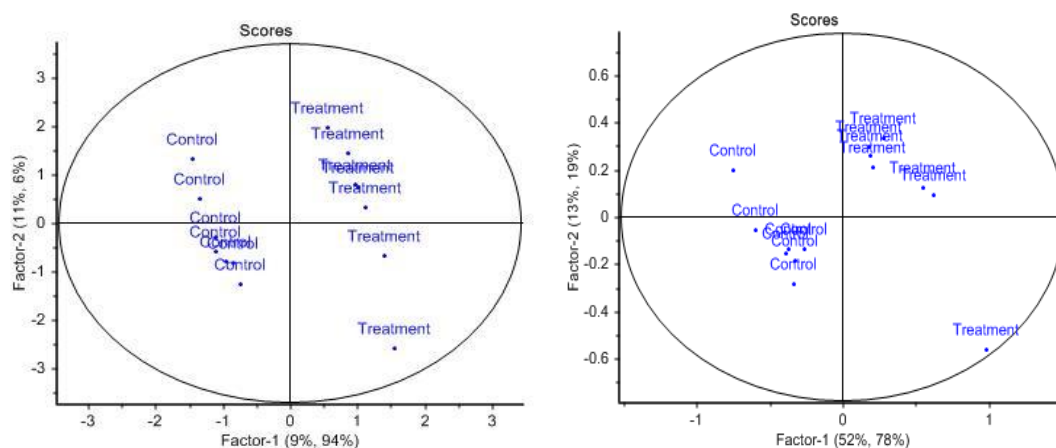
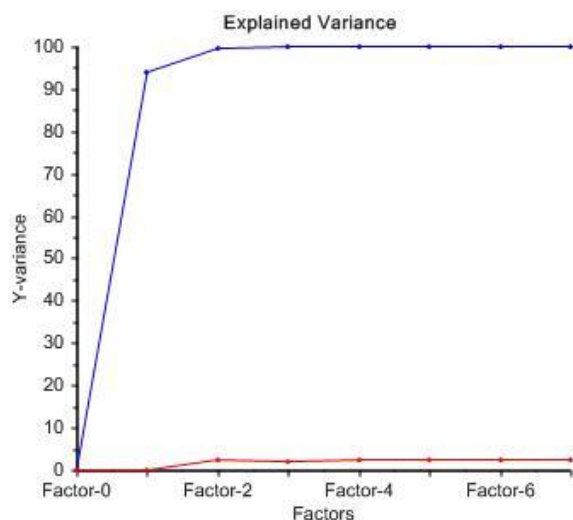


Figure 6-15 Scores plot following chronic ECS treatment of the frontal cortex

(A) Scores plot no data removed; **(B)** Scores plot significant data only.

In Figure 6-16 (B), the model indicated two factors were the optimal model dimension by full cross validation. Cross validation created 64 submodels, where 1 sample was left out in each. The plot showed 90% explained variance for two factors, compared to plot (A).

A



B

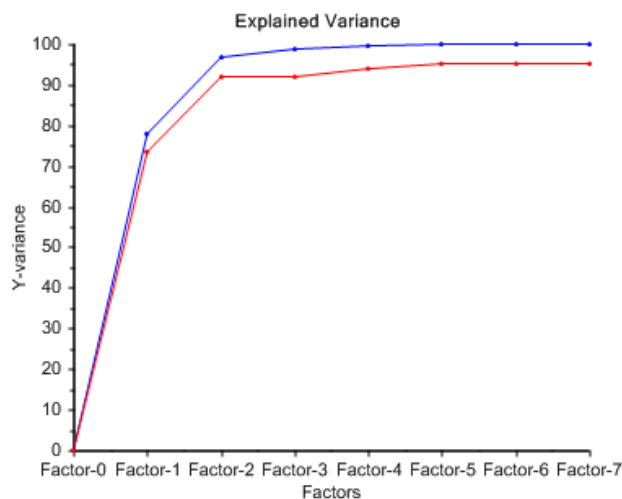


Figure 6-16 Plots of explained variance following chronic ECS treatment of the frontal cortex

(A) Explained variance based on all variables; **(B)** explained variance plot based on significant variables only.



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Figure 6-18 shows the weighted regression coefficients plot of the significant variables only. The importance of individual variables can be seen in this plot. A 95% confidence interval estimates the uncertainty limits. Variables with uncertainty limits that cross zero are not significant at $p \leq 0.05$. The variables significant at this level are circled.

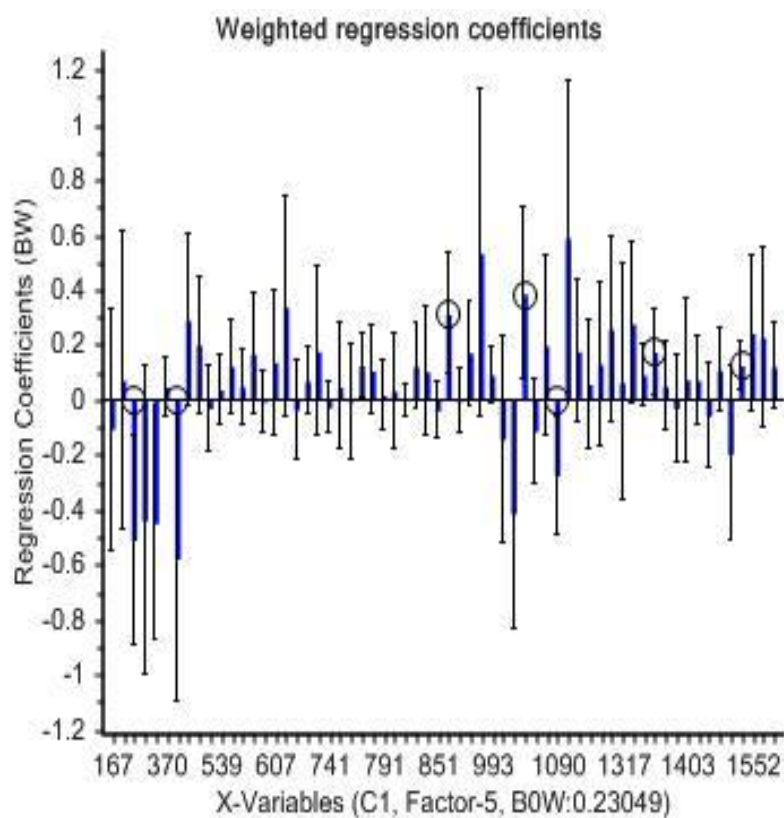


Figure 6-18 Plot of weighted regression coefficients

BW, weighted regression coefficients.

6.3.6 Comparison of univariate and multivariate significant spot findings

A Venn diagram describes the number of spots common to both univariate and multivariate analysis as well as unique to each analysis (Figure 6-19). The significant spot numbers from each form of analysis are listed in Table 6-7.

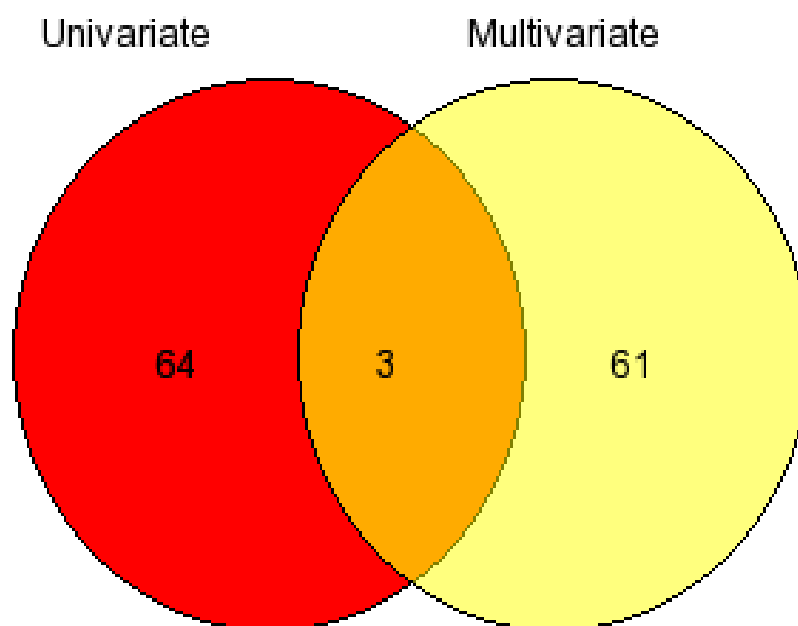


Figure 6-19 Venn diagram of significant spot distribution following univariate and multivariate analysis in the frontal cortex following chronic ECS treatment

Drawn in www.genevenn.com

Table 6-7 List of significant spots following univariate and multivariate analysis of the frontal cortex following chronic ECS treatment

Significant spots univariate analysis	Significant spots common to both	Significant spots multivariate analysis
281	850	167
291	1036	244
477	1566	283
493		284
494		366
510		370
511		371
533		416
550		417
555		495
556		539
604		541
621		543
696		544
702		602
703		607
706		685
709		689
728		715
746		729
748		741
749		753
759		766
770		769
773		769
802		786
805		791

808		793
814		836
835		849
839		851
854		886
884		914
909		928
910		949
927		993
929		1011
938		1018
1001		1044
1006		1065
1016		1090
1050		1099
1064		1099
1078		1102
1085		1127
1091		1142
1094		1298
1103		1317
1129		1319
1146		1343
1155		1357
1188		1374
1193		1381
1226		1403
1301		1468
1314		1471
1325		1482
1356		1522
1359		1551

1463		1552
1474		1553
1476		
1496		
1513		

6.3.7 Protein spots picked for mass spectrometry

A number of significant candidate protein spots from the frontal cortex following chronic ECS were picked from densitometer scanned silver-stained preparatory gels for identification by mass spectrometry. The location of the picked spots is highlighted in Figure 6-20. These spots, their fold change, p-value and the statistical method by which they were detected are listed in Table 6-8.

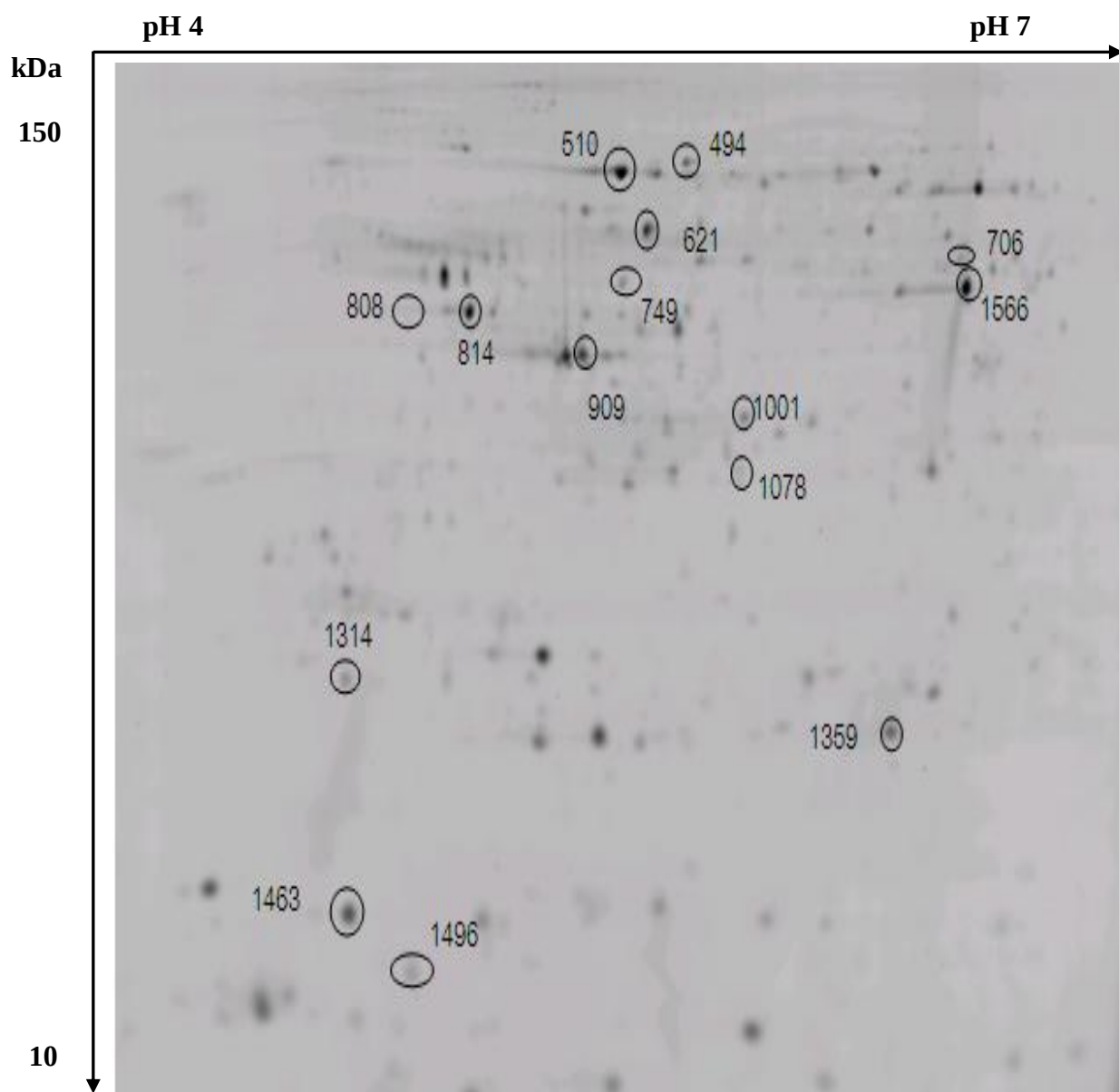


Figure 6-20 Preparative gel indicating significant spots that were picked for identification from the frontal cortex following chronic ECS treatment

Frontal Cortex	Standard spot no.	Fold change	Up / down regulated	p-value	MVA/UVA
Chronic	808	1.2	Up	P=0.015	UVA
	814	1.2	Up	P=0.006	UVA
	706	1.1	Up	P=0.050	UVA
	477	1.1	Up	P=0.048	UVA
	1496	1.1	Up	P=0.024	UVA
	1001	1.1	Up	P=0.013	UVA
	510	1.1	Up	P=0.016	UVA
	1463	1.1	Up	P=0.050	UVA
	909	1.1	Up	P=0.050	UVA
	1314	1.1	Up	P=0.025	UVA
	749	1.1	Up	P=0.0007	UVA
	1359	1.1	Up	P=0.020	UVA
	494	1.1	Up	P=0.050	UVA
	1566	1.1	Up	P=0.010	MVA/UVA
	621	1.1	Up	P=0.018	UVA
	1078	1.1	Up	P=0.004	UVA
	493	1.1	Up	P=0.036	UVA

Table 6-8 Spots picked for identification by mass spectrometry

6.3.8 Frontal cortex: Analysis of the effects of chronic + 4 week ECS treatment

6.3.8.1 Univariate analysis

The univariate results table from the chronic + 4 week ECS treatment study includes the p-value associated with the one way ANOVA comparison of treated and control ECS samples, the average normalised volume, the fold change between spots, the q-value, which controls for the FDR and the power analysis value (Table 6-9).

Figure 6-21 shows a representative frontal cortex Chronic gel and the location of the significant spots detected by univariate analysis in Progenesis SameSpot software (NonLinear Dynamics).

Principal component analysis (PCA) was used to detect outlying samples and patterns of clustering in the data (Figure 6-22).

Table 6-9 List of significantly differentially expressed protein spots in the frontal cortex following chronic + 4 weeks of ECS, detected by univariate analysis

The table lists the standard spot numbers, ranked according to p-value, based on ANOVA. The fold change, calculated from the average of the normalised volumes of each spot from treated and control samples are included in the table. The q-value (FDR) and the power value of the study are also present in the table.

Frontal cortex chronic + 4 study					Average normalised volumes	
Spot #	Anova (p)	q Value	Power	Fold change	Chronic+4 control	Chronic+4 treated
233	0.000282	0.318324	0.996089	1.4	0.736	1.06
1388	0.004446	0.377842	0.89287	1.4	0.968	0.706
311	0.005826	0.377842	0.866212	1.3	0.817	1.037
321	0.007466	0.377842	0.838124	1.3	1.181	0.931
1011	0.008	0.377842	0.829664	1.2	0.942	1.158
1513	0.008496	0.377842	0.822075	1.2	1.034	1.24
1233	0.008569	0.377842	0.820976	1.1	0.815	0.721
1116	0.008593	0.377842	0.820623	1.2	0.902	1.052
1122	0.010679	0.377842	0.791343	1.3	0.75	1.009
303	0.011842	0.377842	0.77648	1.3	1.128	0.89
1053	0.012435	0.377842	0.769258	1.1	1.167	1.035
1044	0.013318	0.377842	0.758884	1.1	1.151	1.012
1315	0.015456	0.377842	0.735497	1.3	0.997	0.782
1308	0.016084	0.377842	0.729042	1.4	0.828	1.146
1266	0.01662	0.377842	0.723669	1.2	1.265	1.083
521	0.017086	0.377842	0.719096	1.2	1.208	1.042
525	0.018101	0.377842	0.709436	1.2	1.251	1.086
1336	0.018475	0.377842	0.705974	1.4	0.684	0.479
596	0.020538	0.377842	0.687717	1.4	0.899	1.216

749	0.021249	0.377842	0.68174	1.1	1.094	1.218
305	0.022214	0.377842	0.673855	1.3	0.794	1.052
1252	0.025959	0.377842	0.645542	1.2	1.313	1.134
955	0.026411	0.377842	0.64235	1.3	0.877	1.121
1337	0.026511	0.377842	0.641645	1.1	1.251	1.386
236	0.029328	0.377842	0.622694	1.3	0.807	1.079
708	0.031128	0.377842	0.611341	1.2	1.147	0.925
462	0.0333	0.377842	0.59834	1.2	0.948	1.17
410	0.036077	0.377842	0.582716	1.5	0.747	1.101
826	0.036798	0.377842	0.578826	1.3	0.98	1.231
892	0.036967	0.377842	0.577924	1.1	1.067	1.19
746	0.038585	0.377842	0.569464	1.1	1.266	1.153
1145	0.040214	0.377842	0.561249	1.2	0.913	1.066
332	0.041166	0.377842	0.55658	1.2	0.914	1.063
573	0.04121	0.377842	0.556367	1.2	1.022	1.24
589	0.042363	0.377842	0.550845	1.1	1.171	1.056
1426	0.046048	0.377842	0.534057	1.5	0.755	1.095
406	0.047491	0.377842	0.527811	1.2	1.087	1.282
1037	0.050294	0.377842	0.516154	1.1	1.171	1.022

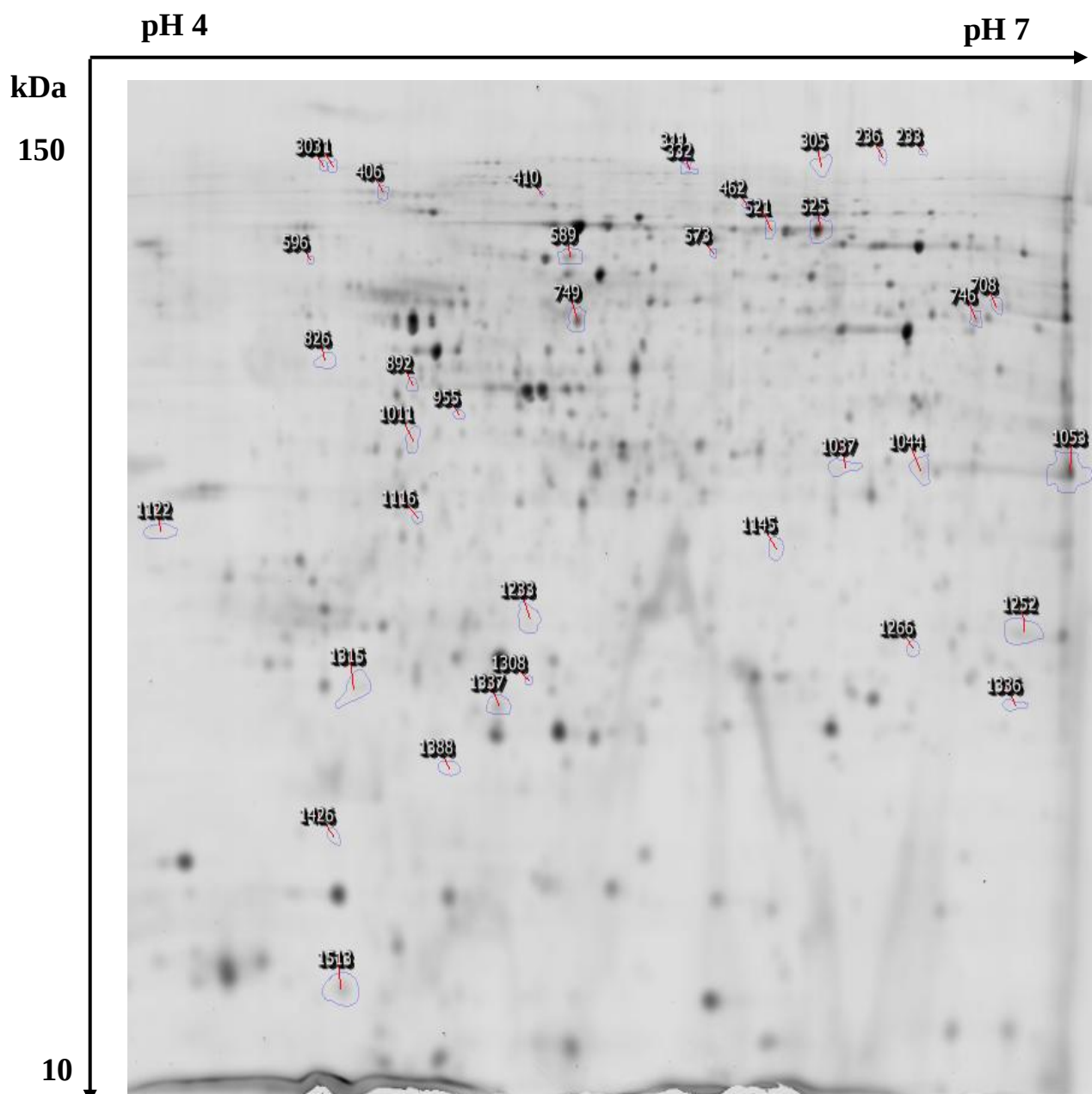


Figure 6-21 Frontal cortex: Location of significant spots following univariate analysis and chronic + 4 weeks ECS treatment

Following analysis by Progenesis SameSpot software the spots highlighted on the gel were identified as being significantly up- or down-regulated ($p \leq 0.05$, one way ANOVA).

6.3.8.2 Principal component analysis

Principal component analysis (PCA), conducted by Progenesis SameSpot software (NonLinear Dynamics) for the detection of outliers, was applied following chronic + 4 weeks of ECS treatment. The ECS treated gels cluster together and the control gels cluster together with no interaction between the two groups, suggesting a strong treatment effect (Figure 6-22). No gels were removed as outliers based on this graph.

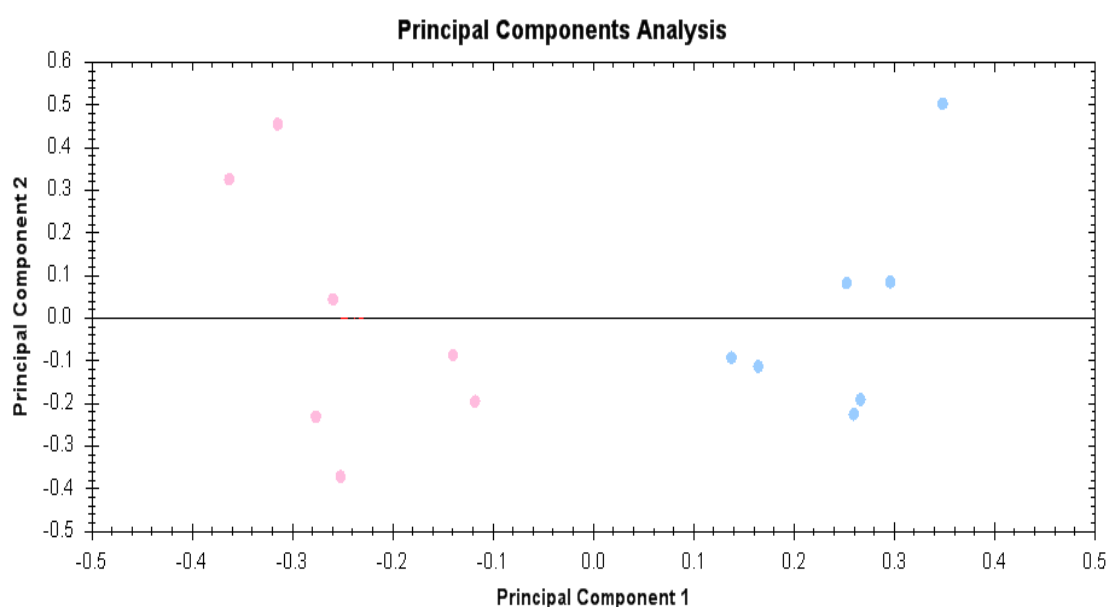


Figure 6-22 PCA plot of treated and control frontal cortex samples following chronic + 4 weeks of ECS

Control gels are represented by blue spots, ECS treated gels are represented by pink spots.

6.3.8.3 Multivariate analysis

The protein spots that contributed to variation in the frontal cortex following chronic + 4 weeks of ECS and those found to be statistically significant following multivariate analysis are listed according to p-value below (Table 6-10). Those spots found to be significant at $p \leq 0.05$ are listed in the adjoining columns. Only those spots that are significant at this level will be considered for further analysis.

Variables that contribute to variation in the frontal cortex after chronic+4 weeks of ECS		Significant variables $p \leq 0.05$	
Spot number	P-value	Spot number	P-value
303	0.07635	233	0.00343
337	0.28417	320	0.00051
410	0.06488	311	0.01983
525	0.35501	321	0.00491
521	0.11492	312	0.00206
566	0.33795	360	0.02106
596	0.08768	509	0.00096
749	0.22492	612	0.0101
748	0.20968	944	0.00463
955	0.21275	1011	0.00378
960	0.19648	1044	0.017
1053	0.25558	1116	0.02446
1122	0.05592	1122	0.05592
1315	0.45945	1212	0.00641
1308	0.08704	1233	0.04021
1336	0.20725	1388	0.00894
1340	0.46785	1524	0.02645
1343	0.95867		
1374	0.07026		
1404	0.14484		
1426	0.28517		
1513	0.08332		

Table 6-10 Protein spots identified as being statistically significant following multivariate analysis and chronic + 4 weeks ECS treatment

List is based on p-values of β coefficients after outliers have been removed, determined significant. $p \leq 0.05$ values listed in column on right.

6.3.8.3.1 PLS-DA diagnostic plots

A number of different plots were used to analyse and detect the sources of variation in each study. The scores plots and explained variance plots that contain all the variables, and the redrawn models containing significant variables only are shown below. The before and after non-significant variable removal plots demonstrate the improvement in explained variance (Figure 6-24) using the significant data only, as well as detecting sample clustering patterns and outliers (Figure 6-23). Figure 6-25 and 6-26 are representative plots for the X-Y correlation loadings and weighted regression coefficients following the removal of non-significant spots.

There is evidence of control sample clustering and treated sample clustering in Figure 6-23 Scores plots **(A)** and **(B)**. There are no apparent outliers in either plot. All samples fall within the Hotelling T^2 ellipse and therefore none were excluded from analysis.

A

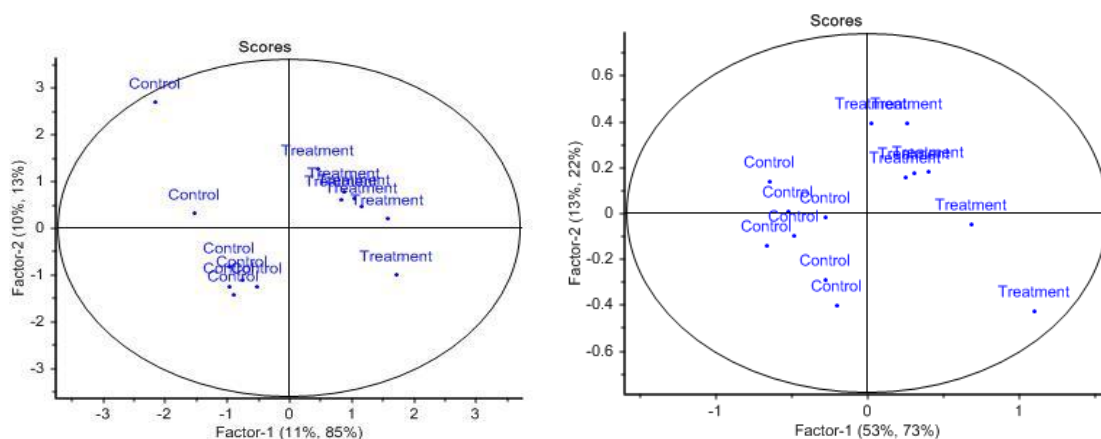
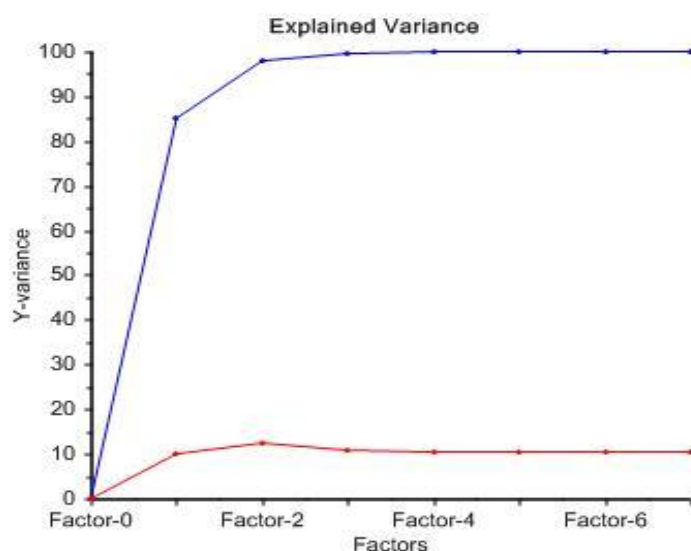


Figure 6-23 Scores plot following chronic + 4 weeks of ECS treatment of the frontal cortex

(A) Scores plot based on all data; **(B)** Scores plot following removal of non-significant data.

In Figure 6-24 (B), removing non-significant variables increases the explained variance. The model indicated two factors were the optimal model dimension by full cross validation. Cross validation created 38 submodels, where 1 sample was left out in each. The plot showed 90% explained variance for two factors, compared to Figure (A).

A



B

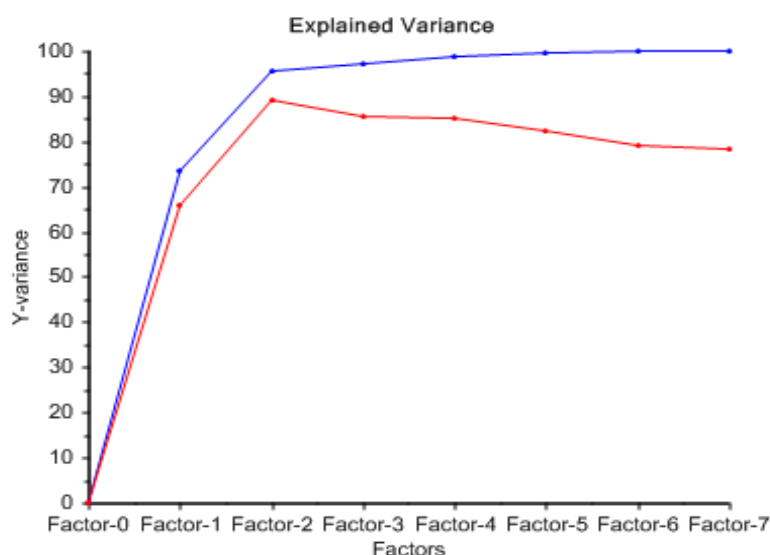


Figure 6-24 Plots of explained variance following chronic+4 weeks of ECS treatment on the frontal cortex

(A) Explained variance plot based on all data; **(B)** explained variance plot based on significant data only.

The relationship between the X variables and Y variable can be seen in Figure 6-25. Those variables located closest to Y are more strongly correlated to it than variables that are further away. Variables that are opposite Y are negatively correlated to it. Circled variables were found to be significant at $p \leq 0.05$.

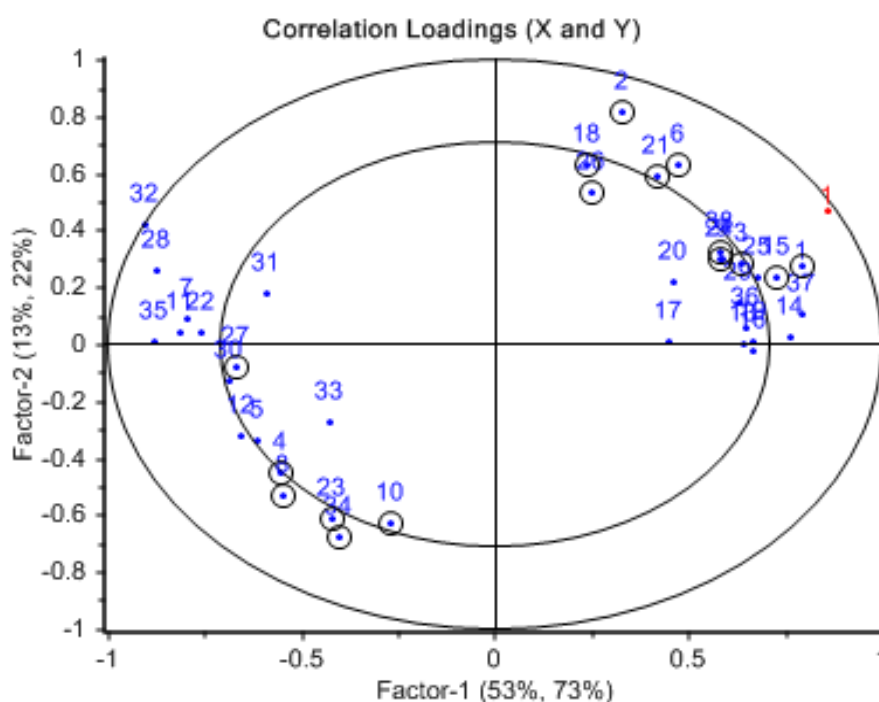


Figure 6-25 X-Y correlation loadings following chronic+4 weeks of ECS treatments on the frontal cortex

Correlations loading plot with significant data only. Y is represented as “1” (red) in the top right quadrant.

The importance of individual variables can be seen in Figure 6-26. A 95% confidence interval estimates the uncertainty limits. If the variable uncertainty limit crosses zero, the variable is not significant at $p \leq 0.05$. The variables that are significant at $p \leq 0.05$ are circled.

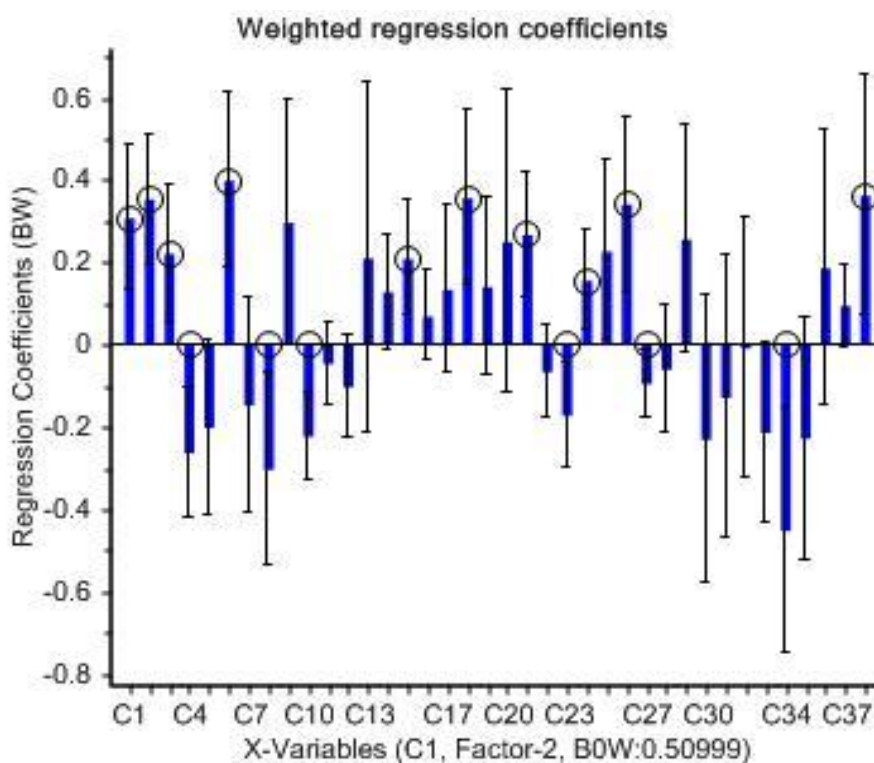


Figure 6-26 Plot of weighted regression coefficients

Regression coefficient plot for significant variables only.

BW, weighted regression coefficients.

6.3.8.4 Comparison of univariate and multivariate significant spot findings

Figure 6-27, a Venn diagram, shows the number of spots common to both univariate and multivariate analysis as well as unique to each analysis. The significant spot numbers from each form of analysis are listed in Table 6-11.

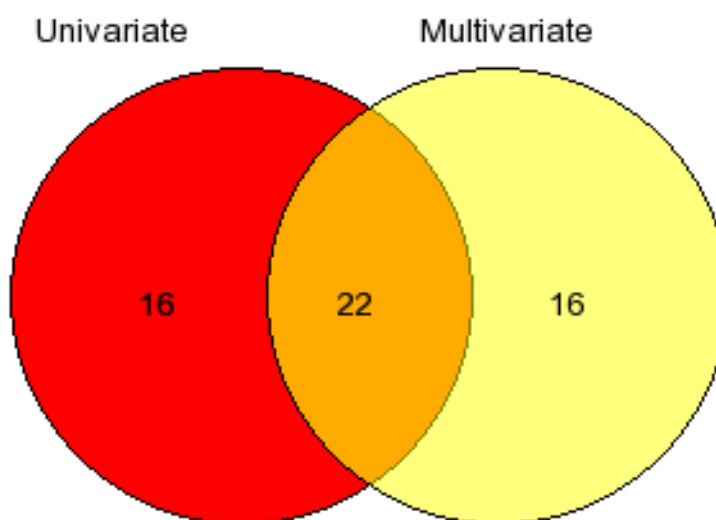


Figure 6-27 Venn diagram of significant spot distribution following univariate and multivariate analysis in the frontal cortex following chronic + 4 weeks of ECS treatment

Drawn in www.genevenn.com

Significant spots unique to univariate analysis	Significant spots common to both	Significant spots unique to multivariate analysis
236	233	312
305	303	320
332	311	337
406	321	360
462	410	509
573	521	566
589	525	612
708	596	748
746	749	944
826	955	960
892	1011	1212
1037	1044	1340
1145	1053	1343
1252	1116	1374
1266	1122	1404
1337	1233	1524
	1308	
	1315	
	1336	
	1388	
	1426	
	1513	

Table 6-11 List of significant spots following univariate and multivariate analysis of the frontal cortex following chronic + 4 weeks of ECS treatment

6.3.9 Protein spots picked for mass spectrometry

A number of significant spots from the frontal cortex following chronic +4 weeks of ECS treatment were picked from silver-stained preparatory gels for identification by mass spectrometry. The location of the picked spots is highlighted in Figure 6-28X. These spots, their fold change, p-value and the statistical method by which they were detected are listed in Table 6-12.

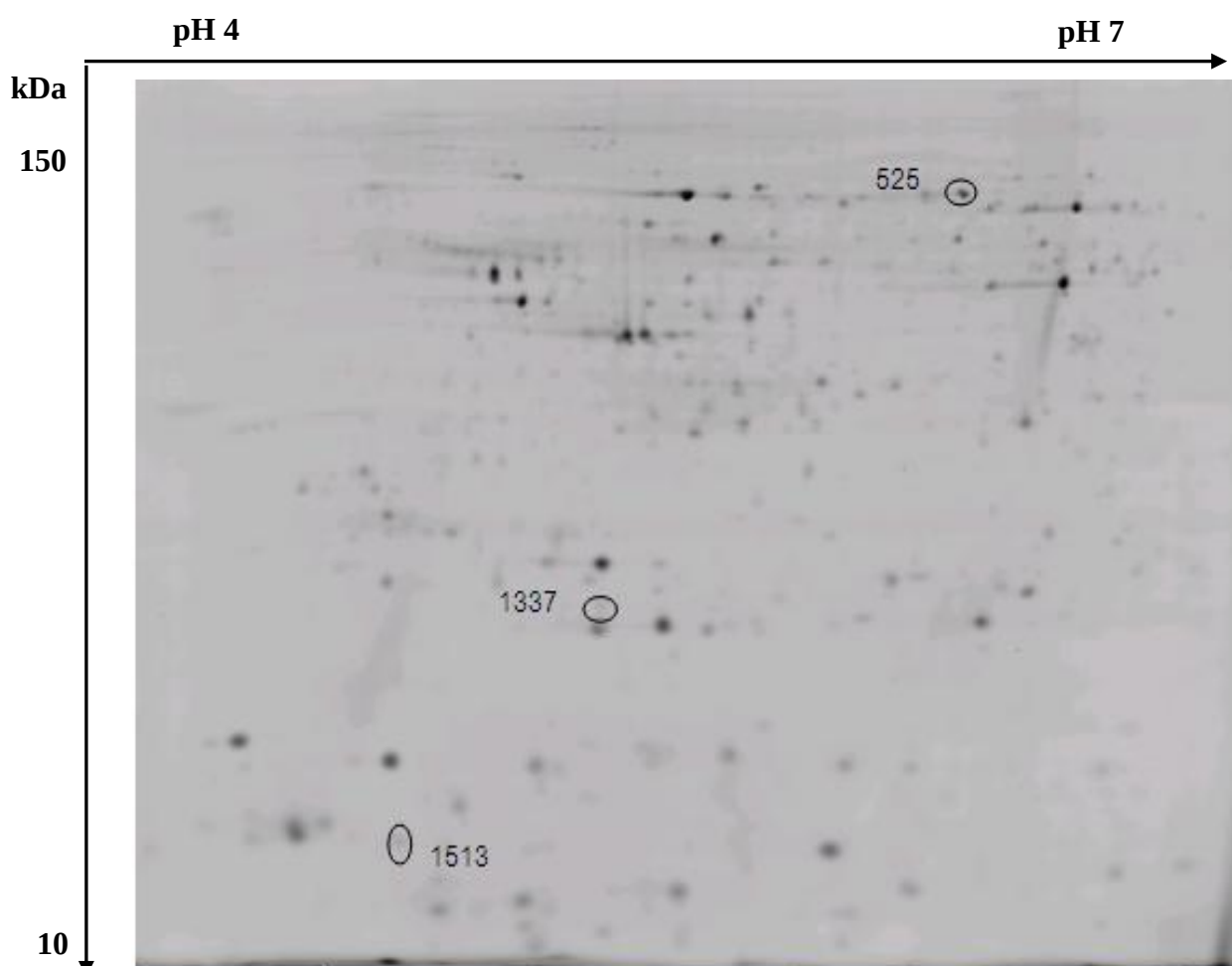


Figure 6-28 Preparative gel indicating significant spots that were picked for identification from the frontal cortex following chronic + 4 weeks of ECS treatment

Frontal Cortex	Standard spot no.	Fold change	Up / down regulated	p-value	MVA/UVA
Chronic + 4	1513	1.1	Up	P=0.008	MVA/UVA
	1337	1.1	Up	P=0.026	UVA
	525	1.2	Down	P=0.018	MVA/UVA

Table 6-12 Spots picked for identification by mass spectrometry

MVA multivariate analysis UVA univariate analysis, MVA/UVA common to both

6.4 Discussion

The techniques used to distinguish significant variables from a large data set will determine the quality of later results. Therefore, 2D-DiGE, a method which minimises variation compared to traditional 2DE techniques (Alban et al. 2003; Karp et al. 2005a) was used to separate proteins based on their molecular weight and isoelectric point as discussed in Chapter 4 (section 4-4). Two types of statistical analysis were applied to the dataset to encapsulate different forms of variation in the data. An awareness of the limitations of the statistical methods is essential to prevent over-interpretation of the results. For example, although univariate analysis detected dozens of significant spots in each study, the FDR must also be considered (Di Carli et al. 2011). A relatively high proportion of significant spots may be false positives (e.g. Table 6-9) which must be taken into consideration at later stages of analysis. While more stringent post hoc measures, for example the Bonferroni test, could be applied to further reduce the FDR it is also important not to apply methods that exclude excessive, potentially real, variables (Dubitzky et al. 2007).

6.4.1 Univariate analysis

Large changes in protein expression among spots could not be seen on the gels following ECS administration. The maximum fold change among significant spots following univariate analysis was 1.9 in the frontal cortex following acute ECS. One way ANOVA directly compares the volume of a protein spot from a treated sample to the same spot from a control sample to determine if ECS treatment affects the level of protein expression in that spot. Significance was set at $p \leq 0.05$. The volume of protein of a number of spots from each study was found to be significantly different (Figure 6-13).

Study	Number of significant spots
acute ECS	38
chronic ECS	67
chronic+4 ECS	38

Table 6-13 The number of significantly different spots determined by univariate analysis

6.4.2 Across study comparisons

A number of spots were found to be significantly expressed in more than one study. Although they will not be directly compared due to differences in animal age and weight at time of sacrifice, they may be of qualitative interest following spot identification. A single spot, 566, was found following acute and chronic + 4 weeks of ECS. The acute and chronic group also shared a number of differentially regulated spots including 493, 909, 1468 and 1474. It is possible that these spots represent proteins that undergo an acute response to ECS but may not have longer-term effects. The chronic and chronic + 4 week group shared spots 746, 748, 749, 1011, 1044, 1343, 1374 and 1513. These spots may have undergone significant expression changes following ECS that persisted four weeks after treatment. Protein identification will be required before the relevance of these common spots is fully understood.

6.4.3 Multivariate analysis

Multivariate statistical methods offer an alternative way to analyse data as they use information from many variables simultaneously to more fully understand the data. This gives a more complete view of the data set when used in conjunction with univariate methods (Pedreschi et al. 2007). Univariate analysis looks at one variable at a time while multivariate analysis addresses the issue that many variables may contribute to variation seen in the data (CAMO V, 2011). Applying multivariate statistics can find patterns and relationships between variables and allows for deeper insights into the data (Marengo et al. 2006; Norden et al. 2005; Kjaersgard et al. 2006; CAMO V, 2011).

6.4.3.1 The effects of acute ECS on the frontal cortex

The scores plot and PCA plot were used to detect outlying gels in the study. The scores plot drawn based on all variables (Figure 6-6 (A)) did not show any gels that required removal as outliers. Therefore, all gels were included when the model was being redrawn with the significant X variables only. The newly drawn scores plot and the PCA plot (Figure 6-6 (B), 6-5) showed strong clustering of control samples with control samples and treated samples with treated samples. This indicates a robust treatment effect. In these plots, as in Figure 6-6 (A), control sample group clustering was stronger than that of treated samples. No gels were considered outliers in the significant-only variable plots.

The model created with the significant variables is robust and representative of the data as described in the explained variance plot. The initial plot drawn, with all the data included (Figure 6-7 A)) explains less than 10% of the variation in the data.

However, the explained variance plot based on the significant variables only (Figure 6-7 (B)), describes approximately 80% of the variance in the original data with a simple model of two factors. This suggests the model is representative of the data.

The presence of all the X variables in the correlations loading plot makes it difficult to interpret, demonstrating the importance of removing variables that mask variation in the data. The relationship between the significant X variables and Y can be seen in Figure 6-8. A number of variables are positively correlated with Y (1) whereas other variables, located in the opposite quadrant, are negatively correlated with Y.

The weighted regression coefficient plots visualise the individual effect of the X variables. The variables that are significant at $p \leq 0.05$ are circled (Figure 6-9). Ten variables were significant at this level, variables (corresponding to spots) 330, 385, 394, 439, 446, 463, 493, 661, 755 and 1313 (Table 6-2). Variable 446 was negatively correlated to Y in Figure 6-8 and the corresponding weighted regression coefficient can be seen in Figure 6-9. The other variables were positively correlated to Y. These spots will be highlighted as being of interest for identification by mass spectrometry.

Fifteen variables were found to be common to both univariate and multivariate analysis, five of these were significant at $p \leq 0.05$ following multivariate analysis (Table 6-3).

Significant spots ($p \leq 0.05$) detected by both univariate and multivariate analysis were highlighted for possible identification by mass spectrometry.

Although spots with the highest level of significance were prioritised for picking from preparative gels, only those spots which could be reliably identified were picked. As a result of the different protein loads and staining techniques employed in preparative gel running, there were many differences in the final gels compared to the DiGE gels. As the sensitivity of the fluorescent Cydyes used in DiGE gels is not matched by silver staining or Coomassie blue staining (section 2.2.15), spots that appear on DiGE gels may not be visible on the preparative gels. Therefore, those spots which appeared clearly on multiple preparative gels and could be consistently picked were the ones chosen from the list of significant spots for reliable identification.

6.4.3.2 The effects of chronic ECS on the frontal cortex

The scores plot of the initial data (Figure 6-15 (A)) showed strong clustering of the control group gels and the treated group gels. No samples were excluded as outliers. The scores plot and PCA plot based on the significant variables only (Figure 6-15 (B), 6-14) also showed robust clustering of control samples and treated samples. A single treatment

sample is close to the Hotelling T^2 ellipse border (circled) in the scores plot, suggesting it is a borderline outlying gel. Although there are treated gels that do not cluster as closely with the rest of the group in the PCA plot (circled), it is more difficult to determine which, if any, of these gels is an outlier. As it is not strictly necessary to remove this gel from the analysis, although it may be advisable if further models were to be drawn, the gel remained in the study.

The validation curve drawn from the significant variables (Figure 6-16) suggests over 90% variance can be explained by a two factor model. This model is very robust, as a simple model design with two factors can explain a large proportion of the variation in the responses. The closely fitted calibration and validation curves mean that the model describes new data well and can be trusted.

The correlation loadings plot shows the relationship of the significant variables to the Y variable (Figure 6-17). Circled variables are significant at a level $p \leq 0.05$. Variables that are positively correlated are located close to Y while negatively correlated variables are located in the quadrant opposite Y.

The importance of individual variables is seen in the weighted regression coefficients plot (Figure 6-18). Seven variables are significant at $p \leq 0.05$. Three of these, variables 283, 371 and 1090 are negatively correlated to Y while variables 886, 1036, 1357 and 1551 are positively correlated to Y. The variable number corresponds to the spot number. These spots will be of particular interest for further identification and analysis.

Three spots only were found to be common to both univariate and multivariate analysis (850, 1036, 1566) (Figure 6-19, Table 6-7). Of these, only variable 1036 was significant at $p \leq 0.05$ following multivariate analysis. Spots found to be significant with a p-value of less than 0.05 following either type of analysis were considered for picking and identification.

A large number of spots were visible and easily picked from multiple frontal cortex preparative gels. Again, a single spot was significant following univariate and multivariate analysis, spot number 1566, although it was not significant at $p \leq 0.05$ by multivariate analysis. The picked spots are found throughout the gel and may represent a range of proteins of different molecular weights and pH values.

6.4.3.3 The effects of chronic + 4 weeks of ECS on the frontal cortex

The scores plot of the initial data (Figure 6-23 (A)) shows that most control samples and treated samples cluster well together in their groups. A single control sample lies close to the Hotelling T^2 ellipse border. Although it is not technically an outlier and was therefore not eliminated from the study, it may be sufficiently removed from the remaining control gels to skew the data. However, the scores plot and PCA plot of the significant variables also show strong group clustering, with no gels acting as outliers.

The explained variance plot shows that a two factor model explains almost 90% of the variance following removal of the non-significant data (Figure 6-24). The close fitted calibration and validation curves suggest this is a robust model that can be trusted.

The correlation loadings plots describe the relationship between the significant X variables and the Y variable (Figure 6-25). The circled variables are significant at $p \leq 0.05$.

The influence of individual variables on variation in the data can be seen with the weighted regression plots (Figure 6-26). A number of variables are significant at $p \leq 0.05$ (circled) and are positively (variables 1, 2, 3, 6, 15, 18, 21, 24, 26 and 38) or negatively (variables 4, 8, 10, 23, 27 and 34) correlated to Y. These variables correspond to spots 233, 320, 311, 612, 944 1011, 1116 1212, 1524 and 312, 360, 509, 1044, 1233 and 1388 respectively.

22 spots are common to both multivariate and univariate analysis, nine of which are significant at $p \leq 0.05$ following multivariate analysis. Spots that are significant following both types of analysis are particularly robust findings and are of interest for further identification by mass spectrometry.

Three spots were picked from this study for identification by mass spectrometry. Two spots were identified following multivariate and univariate analysis (1513 and 525) and a single spot by univariate analysis alone (1337).

In general, PLS-DA models discriminated well between treated and control samples. Removal of variables that contributed to noise and rebuilding models based on variables deemed significant, led to models that robustly explained variation. Several variables which contribute significantly to variation were selected for potential identification by mass spectrometry.

6.4.4 Summary

Univariate and multivariate statistical analysis allowed for the detection of absolute changes in expression of proteins and for correlations and concerted changes in expression. As univariate and multivariate analysis take different approaches to analysing the data, they select different spots as being statistically significant. This is because multivariate analysis takes correlations in the data into account. Therefore, highly correlated proteins will be detected by PLS-DA even if the spot protein expression levels were not significantly changed, as detected by ANOVA (Karp et al. 2005b). Spots that were found to be significant by both forms of analysis are particularly robust findings (Pedreschi et al. 2009).

However, although several spots that were picked for analysis by mass spectrometry were identified as being significant by univariate and multivariate analysis, these spots were generally not significant following multivariate analysis at $p \leq 0.05$. Due to technical difficulties with reliably picking spots identified by multivariate analysis alone, the spots identified throughout the frontal cortex studies were mainly picked following ANOVA.

A range of spots from the frontal cortex of animals treated with ECS were picked for mass spectrometric identification in an effort to find the protein changes that may contribute to the antidepressant effect of ECS. The mass spectrometry results and the analysis of the identified proteins are discussed in Chapter 7.

Identification and confirmation of frontal cortex proteins following electroconvulsive stimulation

7.1 Introduction

Following 2D-DIGE analysis of the frontal cortex, a number of differentially expressed proteins were identified that may contribute to the effects of ECS treatment. In line with other proteomic studies of the effects of antidepressant treatment in the frontal cortex, many of the identified proteins were associated with the cytoskeleton and metabolism (Carboni et al. 2006a). The proteomic changes induced in the frontal cortex following antidepressant administration are not as well studied as those in the hippocampus. However, as discussed in Chapter 6, the identified proteins are similar in nature to those detected in previous proteomic studies of antidepressant therapy. Cytoskeletal and metabolic-related protein expression was particularly prevalent. Changes in cytoskeletal proteins are of particular interest as the effects of antidepressant therapies may result, in part, from changes in plasticity (Pittenger and Duman, 2008; Duman et al. 1999). Gene ontology (GO) analysis also highlighted the cytoskeleton and metabolism as biological processes in which the identified proteins were involved.

As discussed in Chapter 5, the potential changes in expression of a number of hippocampal proteins identified by 2DE analysis were studied by alternative methods to verify the changes in expression of particular proteins of interest. This was important to understand the nature of the protein changes detected following ECS treatment. The identification, analysis and confirmation of proteins identified in the frontal cortex following ECS administration is discussed below.

7.1.1 Aims of the experiment

The purpose of this study was to identify the frontal cortex proteins that are differentially expressed following ECS treatment using mass spectrometry. The identified proteins will be classified using the gene ontology (GO) database, according to their molecular function, the biological processes they are involved in and the cellular components they are found in. The potential interactions and pathways that the proteins may be involved in were also considered using KEGG database and Ingenuity pathway analysis (IPA). Proteins that were identified as being of particular interest were selected for confirmation studies using semi-quantitative immunoblotting.

7.2 Methods

7.2.1 Study design and animal treatment

A study design similar to that used in Chapter 4 (Figure 4.1) was repeated to confirm the presence of selected proteins:

Male Sprague-Dawley rats (150g-200g) were housed four per cage under a 12hr light-dark cycle (lights on 08:00) with food and water available *ad libitum* throughout the experiment. Control animals were handled identically to treated animals but no charge was delivered (section 2.2.2). ECS was administered using the following parameters: 100 pulses per second; 0.5ms pulse width; 0.7s duration; 75mAmp current.

Animals (n=7-10 per group) were administered an acute or chronic course of ECS according to the timeline and protocols discussed in Chapter 5, section 5.2. A subset of chronically treated animals was sacrificed four weeks after the final ECS treatment.

7.2.2 Experimental procedure

All protocols were conducted as described in sections 2.2 and 5.2. Briefly, proteins were identified by LTQ Orbitrap mass spectrometry. MS/MS results were searched against the Uniprot/Swiss-Prot rat database) using SEQUEST (Bioworks, ThermoFinnigan, UK).

Bioinformatic analysis was conducted using the Database for Annotation, Visualisation and Integrated Discovery (DAVID). FatGO terms were listed for gene ontology (GO) categories molecular function, biological process, cellular component and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway. Ingenuity pathway analysis (IPA) software was used to draw maps of protein interactions drawn based on the protein accession numbers of proteins identified by mass spectrometry in each study.

For the confirmation studies, following ECS treatment the animals were sacrificed as previously described (section 2.2.13). Frontal cortex tissue was collected for analysis by semi-quantitative immunoblotting (section 2.2.21). Semi-quantitative immunoblots were visualised and compared by densitometry using ImageJ software. The relative % density of ECS treated samples as a percentage of control samples are presented.

7.3 Results

7.3.1 Frontal cortex: protein identification and classification following acute ECS

The proteins identified by mass spectrometry in the frontal cortex are listed below (Tables 7-1 and 7-2). Table 7-1 lists the protein name, Uniprot (www.uniprot.org) protein accession number, the number of the 2-D gel protein spot in which the protein was found (e.g. Figure 6-5) and the theoretical and experimental molecular weights and isoelectric points of each protein. The proteins are loosely classified based on the function of the protein as described in the Uniprot Knowledgebase (UniprotKB) protein homepage.

A more detailed classification of the proteins, by the gene ontology (GO) categories biological process and molecular function that are listed in Uniprot, is listed in Table 7-2. Mass spectrometry information including the protein cross correlation (XCorr) score, the number of peptides, and percentage coverage, the mass spectrometry p-value, the fold change in protein expression and the one way ANOVA (univariate analysis) p-value for that spot is included in Table 7-2. XCorr is cross correlation score between the observed peptide mass fragment spectrum and the predicted one (ThermoScientific, 2007). The percentage coverage is based on the amino acid sequence of the protein (ThermoScientific, 2007).

Protein Name	Accession Number	Standard Spot No.	pI Experimental	pI Theoretical	MW (kDa) Experimental	MW (kDa) Theoretical
HEAT SHOCK FAMILY						
Heat shock cognate 71 kDa protein	P63018	505	5.4	5.37	71651.28773	70739.87
		1179	4.7		33101.52482	
		493	5.7		73297.30386	
78 kDa glucose-regulated protein	P06761	505	5.4	5.01	71651.28773	70474.59
		493	5.7		73297.30386	
Heat shock 70 kDa protein 1-like	P55063	505	5.4	5.91	71651.28773	70549.18
		493	5.7		73297.30386	
Stress-70 protein, mitochondrial	P48721	493	5.7	5.51	73297.30386	68844.11
Heat shock-related 70 kDa protein 2	P14659	505	5.4	5.51	71651.28773	69641.66
CYTOSKELETON						
Actin, aortic smooth muscle	P62738	1022	6	5.29	43472.63852	41737
		808	5.1		54557.69329	
Dihydropyrimidinase-related protein 2	P47942	505	5.4	5.95	71651.28773	62277.57
Tubulin beta-2C chain	Q6P9T8	1022	6	4.79	43472.63852	49800.98
Tubulin beta-5 chain	P69897	1022	6	4.78	43472.63852	49670.82
Glial fibrillary acidic protein	P47819	493	5.7	5.35	73297.30386	49957.09
Neurofilament light polypeptide	P19527	505	5.4	4.63	71651.28773	61204.09
Tropomyosin alpha-3 chain	Q63610	1179	4.7	4.75	33101.52482	28875.38
Isoform Brain TMB-2 of Tropomyosin alpha-1 chain	P04692-4	1179	4.7	4.81	33101.52482	28704.1
Tubulin beta-2A chain	P85108	1022	6	4.78	43472.63852	49906.97
Tropomyosin alpha-1 chain	P04692	1179	4.7	4.69	33101.52482	32680.56
		1022	6	4.78	43472.63852	
		808	5.1	5.03	54557.69329	
METABOLISM/RESPIRATION						
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Q66HF1	505	5.4	5.28	71651.28773	76860.16
Phosphoglycerate kinase 1	P16617	1022	6	8.02	43472.63852	44407.26
Gamma-enolase	P07323	808	5.1	5.03	54557.69329	47009.34
Beta-enolase	P15429	808	5.1	7.23		46882.71
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	P62138	1022	6	5.94	43472.63852	37380.89
SIGNALING						
14-3-3 protein epsilon	P62260	1179	4.7	4.63	33101.52482	29173.9
REGULATION NO GENERATION						
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	O08557	1022	6	5.76	43472.63852	31294.82
TRANSCRIPTION REGULATION						
Histone H4	P62804	1022	6	11.36	43472.63852	11236.15
Serum albumin	P02770	493	5.7	5.8	73297.30386	65916.36
Zona pellucida sperm-binding protein 4	Q8CH34	1179	4.7	5.90	33101.52482	48566.09

Table 7-1 Frontal cortex protein identification following acute ECS

This table includes information on the protein identity, its theoretical and experimental weights and isoelectric points. MW, molecular weight; kDa, kilo Dalton; pI, isoelectric point.

Table 7-2 Frontal cortex: mass spectrometry results following acute ECS

This table contains information from the mass spectrometry analysis of each protein and the protein classification according to biological process and molecular function as derived from GO in Uniprot database. The MS p-value is the significance value assigned to the protein following mass spectrometry identification. The STAT p-value refers to the one-way ANOVA p-value of the protein spot following univariate analysis (Chapter 4 section 4.2.4). MS, mass spectrometry; STAT, statistical (one way ANOVA p-value); XC, cross correlation (Xcorr).

Protein Name	Accession number	Standard spot no.	Protein score (XC)	No. peptides	Coverage (%)	MS p-value	Fold change	STAT p-value	Gene Ontology (Biological Process)	Gene Ontology (Molecular Function)
HEAT SHOCK FAMILY										
Heat shock cognate 71 kDa protein	P63018	505	110.29	11	18.4	1.63E-12	1.2	0.0006002	chaperone mediated protein folding requiring cofactor; response to stress	ADP binding; ATP binding; ATPase activity, coupled; receptor binding; unfolded protein binding
		1179	10.26	1	3.6	2.71E-09	1.1	0.035		
		493	70.2	7	14.1	5.02E-09	1.1	0.011		
78 kDa glucose-regulated protein	P06761	505	20.21	2	4.1	1.19E-10	1.2	0.0006002	activation of signaling protein activity involved in unfolded protein response; positive regulation of protein ubiquitination	ATP binding; unfolded protein binding
		493	30.27	3	6.9	3.13E-13	1.1	0.011		
Heat shock 70 kDa protein 1-like	P55063	505	20.14	2	3.4	4.07E-7	1.2	0.0006002	response to stress	ATP binding
		493	10.15	1	2	5.52E-08	1.1	0.011		
Stress-70 protein, mitochondrial	P48721	493	120.26	12	21.8	3.56E-10	1.1	0.011	protein folding	ATP binding; unfolded protein binding
Heat shock-related 70 kDa protein 2	P14659	505	10.17	1	1.6	0.000394	1.2	0.0006002	multicellular organismal development; response to stress	ATP binding
CYTOSKELETON										
Actin, aortic smooth	P62738	1022	10.14	1	2.7	0.000232	1.1	0.045	muscle	ATP binding

muscle		808	10.13	1	2.7	0.000358	1.1	0.031	contraction	
Dihydropyrimidinase-related protein 2	P47942	505	50.24	5	11.9	1.29E-7	1.2	0.0006002	olfactory bulb development; positive regulation of glutamate secretion; regulation of axon extension; response to amphetamine; response to cocaine; response to drug; spinal cord development; synaptic vesicle transport	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds; protein binding
Tubulin beta-2C chain	Q6P9T8	1022	30.18	3	8.8	9.83E-08	1.1	0.045	microtubule-based movement; protein polymerization	GTP binding; GTPase activity; structural molecule activity
Tubulin beta-5 chain	P69897	1022	10.15	1	2.7	0.0000028	1.1	0.045	microtubule-based movement; protein polymerization	GTP binding; GTPase activity; protein complex binding
Glial fibrillary acidic protein	P47819	493	10.15	1	2.6	3.52E-7	1.1	0.011	response to wounding	structural constituent of cytoskeleton
Neurofilament light polypeptide	P19527	505	10.15	1	1.8	0.0000317	1.2	0.0006002	protein polymerization; response to corticosterone stimulus; response to organic nitrogen; response to peptide hormone stimulus; response to toxin	phospholipase binding; protein domain specific binding; structural molecule activity

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Tropomyosin alpha-3 chain	Q63610	1179	40.26	4	14.9	9.57E-09	1.1	0.035	brain development	actin binding
Isoform Brain TMBr-2 of Tropomyosin alpha-1 chain	P04692-4	1179	20.18	2	9.6	0.00000124	1.1	0.035	muscle filament sliding; negative regulation of cell migration; positive regulation of ATPase activity; positive regulation of cell adhesion; positive regulation of stress fiber assembly; ruffle organization; wound healing	actin binding
Tubulin beta-2A chain	P85108	1022	90.18	9	24	1.42E-11	1.1	0.045	microtubule-based movement; protein polymerization	GTP binding; GTPase activity; structural molecule activity
Tropomyosin alpha-1 chain	P04692	1179	10.18	1	3.5	0.0000218	1.1	0.035	muscle filament sliding; negative regulation of cell migration; positive regulation of ATPase activity; positive regulation of cell adhesion; positive regulation of stress fiber assembly; ruffle organization; wound healing	actin binding
		1022	10.14	1	3.5	0.000202	1.1	0.045		
		808	10.14	1	3.5	0.00000334	1.1	0.031		
METABOLIC PROCESSES										
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Q66HF1	505	10.14	1	1.4	0.000451	1.2	0.0006002	ATP metabolic process; ATP synthesis coupled electron transport; apoptosis; regulation of mitochondrial membrane potential; transport	2 iron, 2 sulfur cluster binding; 4 iron, 4 sulfur cluster binding; NADH dehydrogenase (ubiquinone) activity; electron carrier activity; metal ion binding

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Phosphoglycerate kinase 1	P16617	1022	20.17	2	6	6.26E-5	1.1	0.045	Gluconeogenesis; glycolysis	ADP binding; ATP binding; phosphoglycerate kinase activity
Gamma-enolase	P07323	808	60.23	6	20.3	1.76E-09	1.1	0.031	Gluconeogenesis; Glycolysis; response to drug; response to estradiol stimulus; response to organic cyclic compound	magnesium ion binding; phosphopyruvate hydratase activity; protein heterodimerization activity; protein homodimerization activity
Beta-enolase	P15429	808	10.16	1	3	2.12E-7	1.1	0.031	Aging; Glycolysis; response to drug; skeletal muscle tissue regeneration	magnesium ion binding; phosphopyruvate hydratase activity; protein heterodimerization activity; protein homodimerization activity
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	P62138	1022	10.13	1	2.7	6.58E-5	1.1	0.045	cell cycle; cell division; glycogen metabolic process; protein dephosphorylation; regulation of glycogen biosynthetic process; regulation of glycogen catabolic process	metal ion binding; protein binding; protein serine/threonine phosphatase activity; ribonucleoprotein binding
SIGNALING										
14-3-3 protein epsilon	P62260	1179	30.16	3	13.7	8.96E-6	1.1	0.035	signal transduction	protein complex binding; protein kinase C inhibitor activity
REGULATION NITRIC OXIDE GENERATION										
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	O08557	1022	60.21	6	27.4	6.51E-11	1.1	0.045	arginine metabolic process; citrulline metabolic process;	amino acid binding; dimethylargininase

									positive regulation of angiogenesis; positive regulation of nitric oxide biosynthetic process	e activity; metal ion binding
TRANSCRIPTION REGULATION										
Histone H4	P62804	1022	20.14	2	19.4	8.59E-6	1.1	0.045	cell differentiation; multicellular organismal development; nucleosome assembly; ossification	DNA binding
Serum albumin	P02770	493	40.23	4	9	6.71E-08	1.1	0.011	cellular response to starvation; hemolysis by symbiont of host erythrocytes; maintenance of mitochondrion location; negative regulation of apoptosis; positive regulation of circadian sleep/wake cycle, non-REM sleep; response to mercury ion; response to nutrient; response to organic substance; response to platinum ion; Transport; vasodilation	DNA binding; cell surface binding; drug binding; enzyme binding; fatty acid binding; pyridoxal phosphate binding; toxin binding; zinc ion binding
Zona pellucida sperm-binding protein 4	Q8CH34	1179	4.08	1	5.5	7.27E-4	1.1	0.035	single fertilization	receptor activity

7.3.2 Frontal cortex: GO analysis following acute ECS

The GO terms found to be significant by DAVID following a search for FatGO terms for proteins in the (i) biological process, (ii) molecular function and (iii) cellular component ontologies are listed below in tables 7-3 –7-5. The number of proteins (count) and their identifier (accession number) as well as the p-value for each term following GO classification is also listed. The (i) biological process (ii) molecular function or (iii) cellular component each protein is involved in is listed under Term.

Table 7-3 Biological Processes

Term	Count	P-Value	Genes
protein polymerization	4	1.78E-05	P69897, P19527, Q6P9T8, P85108
microtubule-based movement	4	5.13E-04	P69897, P19527, Q6P9T8, P85108
cellular macromolecular complex assembly	5	6.62E-04	P69897, P19527, P62804, Q6P9T8, P85108
generation of precursor metabolites and energy	5	6.73E-04	P15429, P07323, Q66HF1, P62138, P16617
cellular protein complex assembly	4	9.84E-04	P69897, P19527, Q6P9T8, P85108
cellular macromolecular complex subunit organization	5	0.001006	P69897, P19527, P62804, Q6P9T8, P85108
intracellular transport	6	0.001294	P48721, P63018, P04692, P19527, P62260, P62738
cytoskeleton-dependent intracellular transport	3	0.001558	P04692, P19527, P62738
macromolecular complex assembly	6	0.00202	P69897, O08557, P19527, P62804, Q6P9T8, P85108
macromolecular complex subunit organization	6	0.002596	P69897, O08557, P19527, P62804, Q6P9T8, P85108
glycolysis	3	0.002719	P15429, P07323, P16617
glucose metabolic process	4	0.003324	P15429, P07323, P62138, P16617

Table 7-4 Molecular Function

Term	Count	P-Value	Genes
purine ribonucleotide binding	10	7.06E-04	P55063, P48721, P63018, P69897, P14659, P06761, Q6P9T8, P85108, P62738, P16617
ribonucleotide binding	10	7.09E-04	P55063, P48721, P63018, P69897, P14659, P06761, Q6P9T8, P85108, P62738, P16617
structural constituent of cytoskeleton	3	0.001003	P69897, P19527, P47819
purine nucleotide binding	10	0.00101	P55063, P48721, P63018, P69897, P14659, P06761, Q6P9T8, P85108, P62738, P16617
nucleotide binding	10	0.003669	P55063, P48721, P63018, P69897, P14659, P06761, Q6P9T8, P85108, P62738, P16617

Table 7-5 Cellular Components

Term	Count	P-Value	Genes
cytosol	11	4.48E-05	P15429, P63018, P07323, P69897, O08557, P19527, P06761, P62138, P62260, Q6P9T8, P16617
cytoskeleton	9	4.86E-04	Q63610, P04692, P69897, P19527, P47819, P47942, Q6P9T8, P85108, P62738
cytosolic part	4	0.001539	P15429, P07323, P19527, P06761
non-membrane-bounded organelle	11	0.002537	Q63610, P48721, P04692, P69897, P19527, P47819, P47942, P62804, Q6P9T8, P85108, P62738
intracellular non-membrane-bounded organelle	11	0.002537	Q63610, P48721, P04692, P69897, P19527, P47819, P47942, P62804, Q6P9T8, P85108, P62738
cytoskeletal part	7	0.002755	Q63610, P69897, P19527, P47819, Q6P9T8, P85108, P62738

7.3.3 Frontal cortex: protein identification and classification following chronic ECS

Table 7-6 and 7-7 below are organised according to the tables described in section 7.4.1. Table 7-6 lists the protein name, accession number, the number of the spot in which the protein was found and the theoretical and experimental molecular weights and isoelectric points of each protein.

Proteins are assigned a more detailed classification, by biological process and molecular function in Table 7-7. Mass spectrometry information including the protein score (XCorr), the number of peptides, and percentage coverage, the mass spectrometry p-value, the fold change in protein expression and the one way ANOVA p-value for that spot are in Table 7-7.

Table 7-6 Frontal cortex protein identification following chronic ECS

The table includes information on the protein identity, its theoretical and experimental weights and isoelectric points. MW, molecular weight; kDa, kilo Dalton; pI, isoelectric point.

Protein Name	Accession Number	Standard Spot no.	pI Experimental	pI Theoretical	MW (kDa) Experimental	MW (kDa) Theoretical
HEAT SHOCK FAMILY						
Heat shock cognate 71 kDa protein	P63018	510	5.6	5.37	70842.19352	70739.87
		1463	4.9		16746.65217	
		621	5.7		64689.82853	
		1078	6		40150.59633	
60 kDa heat shock protein, mitochondrial	P63039	510	5.6	5.35	70842.19352	57925.78
		1463	4.9		16746.65217	
		1566	4.9		57093.14934	
		621	5.7		64689.82853	
		1078	6		40150.59633	
Heat shock 70 kDa protein 1A/1B	Q07439	510	5.6	5.61	70842.19352	70054.12
78 kDa glucose-regulated protein	P06761	477	5.2	5.01	74134.43807	70474.59
		510	5.6		70842.19352	
		1463	4.9		16746.65217	
		494	5.9		72469.62266	
		621	5.7		64689.82853	
Heat shock 70 kDa protein 1-like	P55063	510	5.6	5.91	70842.19352	70549.18
		1463	4.9		16746.65217	
		494	5.9		72469.62266	
		621	5.7		64689.82853	
Heat shock-related 70 kDa protein 2	P14659	510	5.6	5.51	70842.19352	69641.66
		749	5.6		58404.72723	
Stress-70 protein, mitochondrial	P48721	494	5.9	5.51	72469.62266	68844.11

		1566	4.9		57093.14934	
CYTOSKELETON						
Actin, cytoplasmic 1	P60711	909	5.6	5.29	50388.56611	41737
		1314	5		26375.94327	
		749	5.6		58404.72723	
		1359	6.2		24085.29666	
Actin, aortic smooth muscle	P62738	808	5.1	5.29	54557.69329	41737
		814	5.7		58404.72723	
		909	5.6		50388.56611	
		1314	5		26375.94327	
		1566	4.9		57093.14934	
Dihydropyrimidinase-related protein 2	P47942	510	5.6	5.95	70842.19352	62277.57
		621	5.7		64689.82853	
Tubulin alpha-1A chain	P68370	510	5.6	4.94	70842.19352	50135.63
		621	5.7		64689.82853	
Tubulin alpha-4A chain	Q5XIF6	510	5.6	4.95	70842.19352	49924.4
		621	5.7		64689.82853	
Tubulin alpha-1B chain	Q6P9V9	510	5.6	4.94	70842.19352	50151.63
		621	5.7		64689.82853	
Tubulin beta-3 chain	Q4QRB4	1001	6	4.82	44979.22829	50418.65
Tubulin beta-2A chain	P85108	1001	6	4.78	44979.22829	49906.97
		749	5.6		58404.72723	
		621	5.7		64689.82853	
Tubulin beta-2C chain	Q6P9T8	1314	5	4.79	26375.94327	49800.98
Tropomyosin alpha-1 chain	P04692	808	5.1	4.69/5.03	54557.69329	32680.56
Glial fibrillary acidic protein	P47819	1496	5	5.35	15119.5895	49957.09
		1001	6		44979.22829	
		909	5.6		50388.56611	
		1314	5		26375.94327	
		749	5.6		58404.72723	
		1359	6.2		24085.29666	
		494	5.9		72469.62266	
		1566	4.9		57093.14934	
		1078	6		40150.59633	
Fascin	P85845	706	6.5	6.29	61118.96624	54359.8
Myristoylated alanine-rich C-kinase substrate	P30009	477	5.2	4.32	74134.43807	29663.32
Septin-11	B3GNI6	1566	4.9	6.26	57093.14934	49563.45
Translationally-controlled tumor protein	P63029	1463	4.9	4.76	16746.65217	19462.17
Calponin-3	P37397	1001	6	5.47	44979.22829	36434.67
Brain acid soluble protein 1	Q05175	814	5.7	4.50	58404.72723	21659.08
Alpha-internexin	P23565	749	5.6	5.2	58404.72723	5615.38
ANGIOGENESIS/PROTEIN BIOSYNTHESIS						
Tryptophanyl-tRNA synthetase, cytoplasmic	Q6P7B0	706	6.5	6.04	61118.96624	54143.56
DNA REPAIR						
Uncharacterized protein C9orf119 homolog/ DNA repair protein SWI5 homolog	Q63ZV7	1496	5	4.37	15119.5895	10278.55
TRANSCRIPTION/REGULATION						
Transcriptional activator protein Pur-beta	Q68A21	1001	6	5.35	44979.22829	33286.75
CHAPERONE						
T-complex protein 1 subunit beta	Q5XIM9	706	6.5	6.02	61118.96624	57327.14
T-complex protein 1 subunit epsilon	Q68FQ0	510	5.6	5.51	70842.19352	59405.58
		621	5.7		64689.82853	
DA RELEASE AND TRANSPORT						
Alpha-synuclein	P37377	1463	4.9	4.74	16746.65217	14515.25
		909	5.6		50388.56611	
		1314	5		26375.94327	

		621	5.7		64689.82853	
		1314	5		26375.94327	
VESICLE/ENDOCYTOSIS						
Endophilin-A1	O35179	909	5.6	5.26	50388.56611	39899.28
Endophilin-A2	O35964	909	5.6	5.45	50388.56611	41492.01
Endophilin-B2	Q5PPJ9	749	5.6	5.44	58404.72723	44852.78
Synapsin-1	P09951	494	5.9	9.81	72469.62266	73988.38
DIFFERENTIATION/NEUROGENESIS						
Protein NDRG2	Q8VBU2	909	5.6	5.16	50388.56611	40647.99
TRANSPORT						
Transitional endoplasmic reticulum ATPase	P46462	909	5.6	5.14	50388.56611	89217.64
NUCLEOSOME						
Histone H2A type 1-C	P0C169	1314	5	11.05	26375.94327	13974.28
Histone H4	P62804	1566	4.9	11.36	57093.14934	11236.15
TRANSLATION						
Ubiquitin-60S ribosomal protein L40	P62986	1314	5	10.32	26375.94327	6181.49
ANTIOXIDANT						
Superoxide dismutase [Cu-Zn]	P07632	749	5.6	5.89	58404.72723	15780.47
METABOLIC PROCESSES						
L-lactate dehydrogenase B chain	P42123	1078	6	5.7	40150.59633	36481.18
L-lactate dehydrogenase A chain	P04642	1078	6	8.47	40150.59633	36319.31
ATP synthase subunit beta, mitochondrial	P10719	749	5.6	4.95	58404.72723	51710.12
ATP synthase subunit d, mitochondrial	P31399	1359	6.2	6.21	24085.29666	18632.25
Gamma-enolase	P07323	808	5.1	5.03	54557.69329	47009.34
		814	5.7		58404.72723	
		706	6.5		61118.96624	
Beta-enolase	P15429	808	5.1	7.23		46882.71
		1566	4.9		57093.14934	
Alpha-enolase	P04764	814	5.7	6.16	58404.72723	46996.67
		706	6.5		61118.96624	
		1566	4.9		57093.14934	
		621	5.7		64689.82853	
		1078	6		40150.59633	
Serine racemase	Q76EQ0	1078	6	5.68	40150.59633	35693.09
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	P62138	1078	6	5.94	40150.59633	37380.89
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Q99NA5	1496	5	5.72	15119.5895	36682.24
CELL ADHESION						
Cell adhesion molecule 3	Q1WIM3	1078	6	5.5	40150.59633	40814.68
PROTEASE INHIBITOR						
Phosphatidylethanolamine-binding protein 1	P31044	1359	6.2	5.48	24085.29666	20670.2
HYDROLASE						
Pyridoxal phosphate phosphatase	Q8VD52	1463	4.9	5.45	16746.65217	33114.77
Serum albumin	P02770	1078	6	5.8	40150.59633	65916.36
		1001	6		44979.22829	

Table 7-7 Frontal cortex: mass spectrometry results following chronic ECS

This table contains information from the mass spectrometry analysis of each protein and the protein classification according to biological process and molecular function as derived from GO in Uniprot database (section 4.4.2). MS, mass spectrometry; STAT, statistical (one way ANOVA p-value); XC, cross correlation (Xcorr).

Protein Name	Accession Number	Standard Spot No.	Protein Score (XC)	No. Peptides	Coverage (%)	MS p-value	fold change	STAT p-value	Gene Ontology (Biological Process)	Gene Ontology (Molecular Function)
HEAT SHOCK FAMILY										
Heat shock cognate 71 kDa protein	P63018	510	140.3	14	23.4	3.76E-11	1.2	6.002E-3	chaperone mediated protein folding requiring cofactor; response to stress	ADP binding; ATP binding; ATPase activity, coupled; receptor binding; unfolded protein binding
		1463	40.28	4	11.1	1.27E-13	1.1	0.050		
		621	150.24	15	19.3	9.03E-11	1.1	0.019		
		1078	10.2	1	3.6	0.000772	1.1	0.005		
60 kDa heat shock protein, mitochondrial	P63039	510	160.29	12	32.6	5.26E-13	1.2	6.002E-3	anti-apoptosis; chaperone mediated protein folding requiring cofactor; detection of misfolded protein; positive regulation of anti-apoptosis; positive regulation of inflammatory response; response to ATP; response to cocaine; response to drug; response to estrogen stimulus; response to heat; response to hydrogen peroxide; response to hypoxia; response to lipopolysaccharide	ATP binding; insulin binding; misfolded protein binding; protease binding; protein complex binding; protein heterodimerization activity
		1463	30.23	3	6.1	6.03E-09	1.1	0.050		
		1566	10.15	1	2.1	0.00000331	1.1	0.011		
		621	220.3	22	52.7	2.46E-10	1.1	0.019		
		1078	80.28	8	26.2	2.22E-16	1.1	0.005		
Heat shock 70 kDa protein	Q07439	510	10.14	1	2.2	0.0000483	1.2	0.000600	defense response; mRNA	ATP binding; NF-kappaB

1A/1B								2	catabolic process; negative regulation of apoptosis; negative regulation of caspase activity; negative regulation of cell growth; negative regulation of cell proliferation; negative regulation of vasoconstriction; positive regulation of T cell mediated cytotoxicity; response to unfolded protein	binding; protease binding
78 kDa glucose-regulated protein	P06761	477	170.22	17	30.3	1.02E-12	1.1	0.048	activation of signaling protein	ATP binding; unfolded protein binding
		510	30.27	3	4.3	3.12E-10	1.2	6.002E-3	activity involved in unfolded	
		1463	20.2	2	4.1	1.18E-08	1.1	0.050	protein response; positive	
		494	10.15	1	2.4	0.0000133	1.1	0.0501	regulation of protein	
		621	30.25	3	4.3	5.41E-12	1.1	0.019	ubiquitination	
Heat shock 70 kDa protein 1-like	P55063	510	20.13	2	3.4	0.000000542	1.2	6.002E-3	response to stress	ATP binding
		1463	10.14	1	2	4.41E-08	1.1	0.050		
		494	10.14	1	2	0.00000272	1.1	0.0501		
		621	10.14	1	1.4	0.00633	1.1	0.019		
Heat shock-related 70 kDa protein 2	P14659	510	10.17	1	1.6	0.000229	1.2	6.002E-3	multicellular organismal development; response to stress	ATP binding
		749	10.16	1	1.6	0.00056	1.3	7.669E-3		
Stress-70 protein, mitochondrial	P48721	494	200.28	20	37.8	5.52E-10	1.1	0.0501	protein folding	ATP binding; unfolded protein binding
		1566	20.21	2	3.8	0.0000784	1.1	0.011		
CYTOSKELETON										
Actin, cytoplasmic 1	P60711	909	100.28	10	36	0.000000104	1.1	0.0502	axonogenesis	ATP binding; protein kinase

		1314	30.26	3	16.5	8.11E-09	1.1	0.026		binding
		749	20.26	2	9.1	0.0000302	1.3	7.669E-3		
		1359	20.2	2	9.1	0.00000327	1.1	0.02		
Actin, aortic smooth muscle	P62738	808	10.13	1	2.7	0.000358	1.1	0.031	muscle contraction	ATP binding
		814	10.14	1	2.9	0.0000531	1.2	0.007		
		909	60.23	6	17	5.52E-09	1.1	0.0502		
		1314	30.21	3	9.8	6.8E-09	1.1	0.026		
		1566	10.13	1	2.9	0.0000289	1.1	0.011		
Dihydropyrimidinase-related protein 2	P47942	510	20.19	2	4.5	5.59E-09	1.2	6.002E-3	olfactory bulb development; positive regulation of glutamate secretion; regulation of axon extension; response to amphetamine; response to cocaine; response to drug; spinal cord development; synaptic vesicle transport	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds; protein binding
		621	10.13	1	1.9	0.0000971	1.1	0.019		
Tubulin alpha-1A chain	P68370	510	10.21	1	3.3	0.00008	1.2	6.002E-3	microtubule-based movement; protein polymerization	GTP binding; GTPase activity; protein domain specific binding; protein heterodimerization activity; structural molecule activity
		621	60.22	6	17.3	1.87E-09	1.1	0.019		
Tubulin alpha-4A chain	Q5XIF6	510	10.14	1	2.2	0.000267	1.2	0.0006002	microtubule-based movement; protein polymerization	GTP binding; GTPase activity; structural molecule activity
		621	10.18	1	3.3	8.1E-09	1.1	0.019		
Tubulin alpha-1B chain	Q6P9V9	510	10.2	1	2.9	0.0000417	1.2	6.002E-3	microtubule-based movement; protein polymerization	GTP binding; GTPase activity; structural molecule activity
		621	20.17	2	6	0.00000212	1.1	0.019		
Tubulin beta-3 chain	Q4QRB4	1001	10.14	1	2.7	0.0000228	1.1	0.014	axon guidance; microtubule-	GTP binding; GTPase activity;

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									based movement; Mitosis; protein polymerization	peptide binding; structural constituent of cytoskeleton
Tubulin beta-2A chain	P85108	1001	10.15	1	2.2	0.0000228	1.1	0.014	microtubule-based movement; protein polymerization	GTP binding; GTPase activity; structural molecule activity
		749	30.16	3	7.9	0.00000917	1.3	7.669E-3		
		621	20.13	2	4.5	0.0000304	1.1	0.019		
Tubulin beta-2C chain	Q6P9T8	1314	10.17	1	2.7	0.000468	1.1	0.026	microtubule-based movement; protein polymerization	GTP binding; GTPase activity; structural molecule activity
Tropomyosin alpha-1 chain	P04692	808	10.14	1	3.5	0.00000334	1.1	0.031	muscle filament sliding; negative regulation of cell migration; positive regulation of ATPase activity; positive regulation of cell adhesion; positive regulation of stress fiber assembly; ruffle organization; wound healing	actin binding
Glial fibrillary acidic protein	P47819	1496	10.14	1	2.6	0.000473	1.1	0.024	response to wounding	structural constituent of cytoskeleton
		1001	10.16	1	2.6	0.000000487	1.1	0.014		
		909	10.15	1	2.6	0.0000103	1.1	0.0502		
		1314	10.15	1	2.6	0.000004	1.1	0.026		
		749	190.22	19	50	0.000000057	1.3	7.669E-3		
		1359	30.17	3	7.9	0.000415	1.1	0.02		

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		494	20.2	2	5.8	0.000000202	1.1	0.0501		
		1566	10.15	1	2.6	0.00000144	1.1	0.011		
		1078	10.16	1	2.6	0.000000689	1.1	0.005		
		706	10.16	1	1.8	0.0000116	1.1	0.05		
Fascin	P85845	477	10.13	1	3.6	0.000441	1.1	0.048	cell motility; cellular response to cell-matrix adhesion; liver development	actin filament binding; protein binding, bridging
Myristoylated alanine-rich C-kinase substrate	P30009	1566	10.17	1	2.6	0.000692	1.1	0.011		actin binding; calmodulin binding
Septin-11	B3GNI6	1463	10.19	1	7.6	0.000000751	1.1	0.05	cell cycle; cell division	GTP binding
Translationally-controlled tumor protein	P63029	1001	10.15	1	3.9	0.00046	1.1	0.014	cell proliferation; spermatogenesis	
Calponin-3	P37397	814	10.14	1	6.4	0.000019	1.2	0.007	actomyosin structure organization; negative regulation of ATPase activity	actin binding; calmodulin binding; microtubule binding; protein binding, bridging
Brain acid soluble protein 1	Q05175	749	10.18	1	2	0.0000737	1.3	0.0007669	negative regulation of gene-specific transcription	calmodulin binding; promoter binding; specific transcriptional repressor activity; transcription corepressor activity
Alpha-internexin	P23565	706	40.18	4	10.4	0.000000091	1.1	0.05	cell differentiation; nervous system development; tissue regeneration	structural molecule activity
ANGIOGENESIS/PROTEIN BIOSYNTHESIS										
Tryptophanyl-tRNA	Q6P7B0	1496	10.13	1	12.4	0.000138	1.1	0.024	Angiogenesis; tryptophanyl-	ATP binding; tryptophan-

synthetase, cytoplasmic									tRNA aminoacylation	tRNA ligase activity
DNA REPAIR										
Uncharacterized protein C9orf119 homolog/ DNA repair protein SWI5 homolog	Q63ZV7	1001	10.13	1	2.9	0.0000746	1.1	0.014	DNA repair; response to DNA damage stimulus; double- strand break repair via homologous recombination	
TRANSCRIPTION/REGULATION										
Transcriptional activator protein Pur-beta	Q68A21	706	90.28	9	22.1	2.39E-11	1.1	0.05	negative regulation of transcription, DNA- dependent	mRNA binding; purine-rich negative regulatory element binding; single-stranded DNA binding; specific transcriptional repressor activity; transcription factor binding; translation repressor activity, nucleic acid binding
CHAPERONE										
T-complex protein 1 subunit beta	Q5XIM9	510	10.13	1	1.8	0.000019	1.2	6.002E-3	protein folding	ATP binding; unfolded protein binding
T-complex protein 1 subunit epsilon	Q68FQ0	621	10.13	1	1.8	0.00000773	1.1	0.019	protein folding	ATP binding; unfolded protein binding
DA RELEASE AND TRANSPORT										
Alpha-synuclein	P37377	1463	50.31	5	44.3	1.57E-09	1.1	0.05	behavioral response to cocaine; negative regulation of dopamine metabolic process; negative regulation of neuron apoptosis; negative regulation of protein phosphorylation; regulation of dopamine secretion	microtubule binding; phospholipase binding; phospholipid binding; protein N-terminus binding; protein domain specific binding
		909	20.27	2	25.7	8.67E-10	1.1	0.0502		
		1314	50.31	5	42.9	3.17E-10	1.1	0.026		
		621	10.2	1	11.4	5.82E-10	1.1	0.019		
		1314	20.22	2	14	0.00000634	1.1	0.026		

VESICLE/ENDOCYTOSIS										
Endophilin-A1	O35179	909	50.23	5	23	5.32E-12	1.1	0.0502	endocytosis	lipid binding
Endophilin-A2	O35964	909	10.16	1	3.3	0.000161	1.1	0.0502	endocytosis	GTPase binding; lipid binding; phosphatase binding
Endophilin-B2	Q5PPJ9	749	10.18	1	3.2	0.00011	1.3	7.669E-3	filopodium assembly; signal transduction	SH3 domain binding; cytoskeletal adaptor activity
Synapsin-1	P09951	494	10.16	1	1.6	0.000213	1.1	0.0501	neurotransmitter secretion	ATP binding; actin binding; catalytic activity
DIFFERENTIATION/NEUROGENESIS										
Protein NDRG2	Q8VBU2	909	30.25	3	11.9	0.00000387	1.1	0.0502	cell differentiation; nervous system development; signal transduction	
TRANSPORT										
Transitional endoplasmic reticulum ATPase	P46462	909	20.13	2	2.9	0.0000163	1.1	0.0502	ER to Golgi vesicle-mediated transport; positive regulation of proteasomal ubiquitin-dependent protein catabolic process; protein homooligomerization	ATP binding; ATPase activity; identical protein binding; lipid binding; protein complex binding; receptor binding
NUCLEOSOME										
Histone H2A type 1-C	P0C169	1314	10.14	1	6.9	0.0000475	1.1	0.026	nucleosome assembly	DNA binding
Histone H4	P62804	1566	20.16	2	19.4	0.00000197	1.1	0.011	cell differentiation; multicellular organismal development; nucleosome assembly	DNA binding
TRANSLATION										
Ubiquitin-60S ribosomal protein L40	P62986	1314	10.13	1	7	0.0000756	1.1	0.026	translation	structural constituent of ribosome
ANTIOXIDANT										

Superoxide dismutase [Cu-Zn]	P07632	749	10.15	1	7.8	0.000000538	1.3	7.669E-3	DNA fragmentation involved in apoptotic nuclear change; activation of MAPK activity; anti-apoptosis; cell aging; double-strand break repair; myeloid cell homeostasis; negative regulation of neuron apoptosis; neurofilament cytoskeleton organization; oxidation-reduction process; peripheral nervous system myelin maintenance; positive regulation of cytokine production; regulation of mitochondrial membrane potential; regulation of multicellular organism growth; relaxation of vascular smooth muscle; removal of superoxide radicals; response to axon injury;	chaperone binding; copper ion binding; protein phosphatase 2B binding; superoxide dismutase activity; zinc ion binding
METABOLIC PROCESSES										
L-lactate dehydrogenase B chain	P42123	1078	50.23	5	21	1.85E-10	1.1	0.005	NAD metabolic process; Glycolysis; lactate metabolic process; oxidation-reduction process	L-lactate dehydrogenase activity; NAD binding; identical protein binding
L-lactate dehydrogenase A chain	P04642	1078	22.23	3	8.7	0.00000049	1.1	0.005	NAD metabolic process; Glycolysis; lactate metabolic process; oxidation-reduction	

									process; positive regulation of apoptosis; post-embryonic organ development; response to cAMP; response to drug; response to estrogen stimulus; response to glucose stimulus; response to hydrogen peroxide; response to hypoxia	
ATP synthase subunit beta, mitochondrial	P10719	749	10.16	1	3	0.00000378	1.3	7.669E-3	ADP biosynthetic process; ATP synthesis coupled proton transport; receptor-mediated endocytosis	ATP binding; calcium ion binding; hydrogen ion transporting ATP synthase activity, rotational mechanism; hydrogen-exporting ATPase activity, phosphorylative mechanism; lipoprotein particle receptor activity
ATP synthase subunit d, mitochondrial	P31399	1359	110.23	11	76.4	9.62E-08	1.1	0.02	ATP synthesis coupled proton transport	hydrogen ion transmembrane transporter activity
Gamma-enolase	P07323	808	60.23	6	20.3	1.76E-09	1.1	0.031	Gluconeogenesis; Glycolysis; response to drug; response to estradiol stimulus; response to organic cyclic compound	magnesium ion binding; phosphopyruvate hydratase activity; protein heterodimerization activity; protein homodimerization activity
		814	128.28	13	38.2	1.48E-11	1.2	0.007		
		706	70.33	7	30	1.75E-10	1.1	0.05		
Beta-enolase	P15429	808	10.16	1	3	0.000000212	1.1	0.031	Aging; Glycolysis; response to drug; skeletal muscle tissue	magnesium ion binding; phosphopyruvate hydratase
		1566	10.28	1	3.5	3.55E-08	1.1	0.011		

									regeneration	activity; protein heterodimerization activity; protein homodimerization activity
Alpha-enolase	P04764	814	10.2	1	4.1	0.00000775	1.2	0.007	glycolysis	magnesium ion binding; phosphopyruvate hydratase activity; protein heterodimerization activity; protein homodimerization activity; serine-type endopeptidase activity
		706	20.25	2	8.5	0.00000335	1.1	0.05		
		1566	144.31	15	27.6	2.6E-10	1.1	0.011		
		621	10.17	1	3.5	0.000000932	1.1	0.019		
		1078	20.24	2	8.1	0.000000143	1.1	0.005		
Serine racemase	Q76EQ0	1078	10.2	1	4.8	0.00000907	1.1	0.005	L-serine metabolic process; Aging; brain development; response to drug; response to morphine	ATP binding; D-serine ammonia-lyase activity; L-serine ammonia-lyase activity; magnesium ion binding; protein homodimerization activity; pyridoxal phosphate binding; serine racemase activity
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	P62138	1078	10.15	1	3	0.000789	1.1	0.005	cell cycle; cell division; glycogen metabolic process; protein dephosphorylation; regulation of glycogen biosynthetic process; regulation of glycogen catabolic process	metal ion binding; protein binding; protein serine/threonine phosphatase activity; ribonucleoprotein binding
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Q99NA5	1496	70.28	7	21	1.15E-08	1.1	0.024	2-oxoglutarate metabolic process; NADH metabolic process; isocitrate metabolic	NAD binding; isocitrate dehydrogenase (NAD+) activity; magnesium ion

									process; tricarboxylic acid cycle	binding
CELL ADHESION										
Cell adhesion molecule 3	Q1WIM3	1078	30.16	3	8.3	0.00000122	1.1	0.005	cell adhesion; protein localisation; heterophilic cell-cell adhesion; homophilic cell adhesion	protein homodimerisation activity
PROTEASE INHIBITOR										
Phosphatidylethanolamine-binding protein 1	P31044	1359	10.2	1	7	2.95E-09	1.1	0.02	MAPKKK cascade; Aging; brain development; eating behaviour; negative regulation of MAPKKK cascade; negative regulation of protein phosphorylation; positive regulation of acetylcholine metabolic process; positive regulation of cAMP-mediated signalling; positive regulation of mitosis; regulation of neurotransmitter levels; response to cAMP; response to calcium ion; response to corticosterone stimulus; response to drug; response to electrical stimulus; response to oxidative stress; response to toxin; response to wounding	ATP binding; lipid binding; mitogen-activated protein kinase binding; receptor binding; serine-type endopeptidase inhibitor activity

HYDROLASE										
Pyridoxal phosphate phosphatase	Q8VD52	1463	10.16	1	3.6	0.00077	1.1	0.05	actin rod assembly; cellular response to ATP	metal ion binding; pyridoxal phosphatase activity
Serum albumin	P02770	1078	80.21	8	17.4	0.000000103	1.1	0.005	cellular response to starvation; hemolysis by symbiont of host erythrocytes; maintenance of mitochondrion location; negative regulation of apoptosis; positive regulation of circadian sleep/wake cycle, non-REM sleep; response to mercury ion; response to nutrient; response to organic substance; response to platinum ion; Transport; vasodilation	DNA binding; cell surface binding; drug binding; enzyme binding; fatty acid binding; pyridoxal phosphate binding; toxin binding; zinc ion binding
		1001	120.23	12	39.1	5.29E-08	1.1	0.014		

7.3.4 Frontal cortex: GO analysis following chronic ECS

The GO terms found to be significant by DAVID following a search for FatGO terms for proteins in the biological process (Table 7-8), molecular function (Table 7-9) and cellular component (Table 7-10) ontologies are listed below. The number of proteins (gene products), their identifier (accession number) and the p-value for each term is also listed.

Table 7-8 Biological Processes

Term	Count	P-Value	Genes
generation of precursor metabolites and energy	10	1.48E-07	P31399, P15429, P10719, P42123, P04642, Q99NA5, P07323, P04764, P62138, P37377
protein polymerization	5	3.90E-06	Q5XIF6, Q4QRB4, Q6P9V9, Q6P9T8, P85108, P68370
response to drug	9	1.99E-05	P15429, P63039, P07632, P04642, P07323, P31044, Q76EQ0, P62738, P37377
glycolysis	5	2.04E-05	P15429, P42123, P04642, P07323, P04764
glucose catabolic process	5	5.23E-05	P15429, P42123, P04642, P07323, P04764
monosaccharide catabolic process	5	6.00E-05	P15429, P42123, P04642, P07323, P04764
hexose catabolic process	5	6.00E-05	P15429, P42123, P04642, P07323, P04764
macromolecular complex assembly	10	1.22E-04	Q5XIF6, Q4QRB4, B3GNI6, P0C169, Q6P9V9, Q76EQ0, P62804, Q6P9T8, P46462, P85108, P68370
cellular carbohydrate catabolic process	5	1.31E-04	P15429, P42123, P04642, P07323, P04764
alcohol catabolic process	5	1.31E-04	P15429, P42123, P04642, P07323, P04764
macromolecular complex subunit organization	10	1.88E-04	Q5XIF6, Q4QRB4, B3GNI6, P0C169, Q6P9V9, Q76EQ0, P62804, Q6P9T8, P46462, P85108, P68370
cellular macromolecular complex assembly	7	2.01E-04	Q5XIF6, Q4QRB4, P0C169, Q6P9V9, P62804, Q6P9T8, P85108, P68370
microtubule-based movement	5	3.38E-04	Q5XIF6, Q4QRB4, Q6P9V9, Q6P9T8, P85108, P68370
cellular macromolecular complex subunit organization	7	3.67E-04	Q5XIF6, Q4QRB4, P0C169, Q6P9V9, P62804, Q6P9T8, P85108, P68370
glucose metabolic process	6	4.23E-04	P15429, P42123, P04642, P07323, P04764, P62138
carbohydrate catabolic process	5	4.30E-04	P15429, P42123, P04642, P07323, P04764
pyruvate metabolic process	4	5.36E-04	P42123, P04642, P07323, Q76EQ0
protein complex assembly	8	6.81E-04	Q5XIF6, Q4QRB4, B3GNI6, Q6P9V9, Q76EQ0, Q6P9T8, P46462, P85108, P68370
protein complex biogenesis	8	6.81E-04	Q5XIF6, Q4QRB4, B3GNI6, Q6P9V9, Q76EQ0, Q6P9T8, P46462, P85108, P68370

cellular protein complex assembly	5	7.86E-04	Q5XIF6, Q4QRB4, Q6P9V9, Q6P9T8, P85108, P68370
hexose metabolic process	6	8.10E-04	P15429, P42123, P04642, P07323, P04764, P62138
response to organic cyclic substance	6	9.63E-04	P63039, P04642, P07323, P31044, Q76EQ0, P37377
protein folding	5	0.001073	P48721, P63039, Q68FQ0, P63018, Q5XIM9
negative regulation of apoptosis	7	0.001217	P63029, Q07439, P63039, P07632, P06761, P02770, P37377
negative regulation of cell death	7	0.001334	P63029, Q07439, P63039, P07632, P06761, P02770, P37377

Table 7-9 Molecular Function

Term	Count	P-Value	Genes
nucleotide binding	26	1.21E-08	P55063, P63039, P10719, P63018, P42123, P14659, P06761, P68370, P04642, Q99NA5, Q76EQ0, Q6P7B0, P62738, P85108, P48721, Q07439, Q4QRB4, Q68FQ0, B3GNI6, Q5XIM9, Q6P9V9, P31044, Q6P9T8, Q5XIF6, P09951, P60711, P46462
purine ribonucleotide binding	23	2.64E-08	Q07439, P48721, P55063, P63039, P10719, P63018, Q4QRB4, Q68FQ0, P14659, B3GNI6, Q5XIM9, Q6P9V9, P31044, P06761, Q6P9T8, P68370, Q5XIF6, P09951, P60711, Q76EQ0, Q6P7B0, P62738, P85108, P46462
ribonucleotide binding	23	2.67E-08	Q07439, P48721, P55063, P63039, P10719, P63018, Q4QRB4, Q68FQ0, P14659, B3GNI6, Q5XIM9, Q6P9V9, P31044, P06761, Q6P9T8, P68370, Q5XIF6, P09951, P60711, Q76EQ0, Q6P7B0, P62738, P85108, P46462
purine nucleotide binding	23	6.41E-08	Q07439, P48721, P55063, P63039, P10719, P63018, Q4QRB4, Q68FQ0, P14659, B3GNI6, Q5XIM9, Q6P9V9, P31044, P06761, Q6P9T8, P68370, Q5XIF6, P09951, P60711, Q76EQ0, Q6P7B0, P62738, P85108, P46462
ATP binding	17	1.53E-05	P55063, P48721, Q07439, P63039, P10719, P63018, Q68FQ0, Q5XIM9, P14659, P31044, P06761, P09951, P60711, Q76EQ0, Q6P7B0, P62738, P46462
adenyl ribonucleotide binding	17	1.97E-05	P55063, P48721, Q07439, P63039, P10719, P63018, Q68FQ0, Q5XIM9, P14659, P31044, P06761, P09951, P60711, Q76EQ0, Q6P7B0, P62738, P46462
adenyl nucleotide binding	17	4.07E-05	P55063, P48721, Q07439, P63039, P10719, P63018, Q68FQ0, Q5XIM9, P14659, P31044, P06761, P09951, P60711, Q76EQ0, Q6P7B0, P62738, P46462
purine nucleoside binding	17	5.06E-05	P55063, P48721, Q07439, P63039, P10719, P63018, Q68FQ0, Q5XIM9, P14659, P31044, P06761, P09951, P60711, Q76EQ0, Q6P7B0, P62738, P46462
nucleoside binding	17	5.58E-05	P55063, P48721, Q07439, P63039, P10719, P63018, Q68FQ0, Q5XIM9, P14659, P31044, P06761, P09951, P60711, Q76EQ0, Q6P7B0, P62738, P46462

phosphopyruvate hydratase activity	3	9.41E-05	P15429, P07323, P04764
GTPase activity	5	6.25E-04	Q5XIF6, Q4QRB4, Q6P9V9, Q6P9T8, P85108, P68370

Table 7-10 Cellular Components

Term	Count	P-Value	Genes
non-membrane-bounded organelle	25	7.74E-08	P10719, P04692, P0C169, P37397, P23565, P37377, P68370, P30009, P47819, P62804, P85108, P62738, P63029, P48721, Q4QRB4, Q68FQ0, Q8VBU2, B3GNI6, Q6P9V9, Q68A21, Q6P9T8, Q5XIF6, P09951, P60711, P62986, P47942
intracellular non- membrane-bounded organelle	25	7.74E-08	P10719, P04692, P0C169, P37397, P23565, P37377, P68370, P30009, P47819, P62804, P85108, P62738, P63029, P48721, Q4QRB4, Q68FQ0, Q8VBU2, B3GNI6, Q6P9V9, Q68A21, Q6P9T8, Q5XIF6, P09951, P60711, P62986, P47942
cytosol	19	4.12E-07	Q07439, P63039, P63018, Q68FQ0, P07632, P07323, Q5XIM9, Q6P9V9, P06761, P04764, P62138, Q6P9T8, P37377, P68370, Q5XIF6, P15429, P04642, P60711, P62986, P46462
cytoskeleton	17	1.11E-06	P63029, Q4QRB4, Q8VBU2, P04692, B3GNI6, P37397, Q6P9V9, P23565, Q6P9T8, P68370, P37377, Q5XIF6, P60711, P30009, P47819, P47942, P62738, P85108
cytosolic part	7	7.15E-06	P15429, Q68FQ0, P07323, P62986, Q5XIM9, P06761, P04764
cytoskeletal part	13	3.03E-05	P63029, Q4QRB4, Q8VBU2, B3GNI6, P37397, Q6P9V9, P23565, Q6P9T8, P68370, Q5XIF6, P30009, P47819, P62738, P85108
soluble fraction	9	3.83E-05	P42123, P04642, P60711, P07323, P31044, P04764, Q76EQ0, P62738, P37377
neuron projection	10	9.26E-05	Q05175, Q4QRB4, P07632, P60711, Q8VBU2, P37397, P31044, P47942, P62138, P37377
phosphopyruvate hydratase complex	3	1.11E-04	P15429, P07323, P04764
microtubule	6	7.03E-04	Q5XIF6, Q4QRB4, P37397, Q6P9V9, Q6P9T8, P85108, P68370
cell soma	7	7.56E-04	P07632, P07323, P37397, P31044, P47942, P62138, Q76EQ0
cell projection	11	9.68E-04	Q05175, Q4QRB4, P07632, P04642, P60711, Q8VBU2, P37397, P31044, P47942, P62138, P37377
microtubule cytoskeleton	8	0.001071	Q5XIF6, Q4QRB4, P30009, Q8VBU2, P37397, Q6P9V9, Q6P9T8, P85108, P68370

7.3.5 Frontal cortex: protein identification and classification following chronic + 4 weeks of ECS

The proteins identified by mass spectrometry in the frontal cortex are detailed below (Table 7-11, Table 7-12) as described in section 7.4.1. The protein name, accession number, the number of the spot in which the protein was found and the theoretical and experimental molecular weights and isoelectric points of each protein.

Table 7-12 includes a more detailed classification of the proteins, by biological process and molecular function. Mass spectrometry information including the protein score (XCorr) the number of peptides, and percentage coverage, the mass spectrometry p-value, the fold change in protein expression and the one way ANOVA p-value for that spot are included.

Protein Name	Accession number	Standard spot no.	pI Experimental	pI Theoretical	MW (kDa) Experimental	MW (kDa) Theoretical
HEAT SHOCK FAMILY						
Heat shock cognate 71 kDa protein	P63018	525	6.2	5.37	70842.19352	70739.87
78 kDa glucose-regulated protein	P06761	525	6.2	5.01	70842.19352	70474.59
Heat shock 70 kDa protein 1-like	P55063	525	6.2	5.91	70842.19352	70549.18
Heat shock-related 70 kDa protein 2	P14659	525	6.2	5.51	70842.19352	69641.66
Stress-70 protein, mitochondrial	P48721	1337	5.4	5.51	25204.61105	68844.11
60 kDa heat shock protein, mitochondrial	P63039	525	6.2	5.35	70842.19352	57925.78
CYTOSKELETON						
Actin, cytoplasmic 1	P60711	1337	5.4	5.29	25204.61105	41737
Septin-11	B3GNI6	1337	5.4	6.26	25204.61105	49563.45
Dihydropyrimidinase-related protein 2	P47942	525	6.2	5.95	70842.19352	62277.57
Tubulin alpha-1A chain	P68370	525	6.2	4.94	70842.19352	50135.63
Glial fibrillary acidic protein	P47819	1513	4.8	5.35	13650.60815	49957.09
METABOLISM						
Alpha-enolase	P04764	1513	4.8	6.16	13650.60815	46996.67
		1337	5.4		25204.61105	
		525	6.2		70842.19352	
Beta-enolase	P15429	1337	5.4	7.23	25204.61105	46882.71
Gamma-enolase	P07323	1513	4.8	5.03	13650.60815	47009.34
CHAPERONE						
T-complex protein 1 subunit beta	Q5XIM9	1513	4.8	6.02	13650.60815	57327.14
		1337	5.4		25204.61105	
DA RELEASE AND TRANSPORT						
Alpha-synuclein	P37377	1337	5.4	4.74	25204.61105	14515.25
METABOLIC PROCESSES						
ATP synthase subunit d, mitochondrial	P31399	1337	5.4	6.21	25204.61105	18632.25
Serum albumin	P02770	525	6.2	5.8	70842.19352	65916.36

Table 7-11 Frontal cortex protein identification following chronic + 4 weeks of ECS

This table includes information on the protein identity; its theoretical and experimental weights and isoelectric points. MW, molecular weight; kDa, kilo Dalton; pI, isoelectric point.

Table 7-12 Frontal cortex: mass spectrometry results following chronic + 4 weeks ECS

This table contains information from the mass spectrometry analysis of each protein and the protein classification according to biological process and molecular function as derived from GO in Uniprot database. MS, mass spectrometry; STAT, one way ANOVA p-value; XC, cross correlation (Xcorr).

Protein Name	Accession Number	Standard Spot No.	Protein Score (XC)	No. Peptides	Coverage (%)	MS p-value	Fold change	STAT p-value	Gene Ontology (Biological Process)	Gene Ontology (Molecular Function)
HEAT SHOCK FAMILY										
Heat shock cognate 71 kDa protein	P63018	525	70.29	7	17.2	1.02E-10	1.2	0.018	chaperone mediated protein folding requiring cofactor; response to stress	ADP binding; ATP binding; ATPase activity, coupled; receptor binding; unfolded protein binding
78 kDa glucose-regulated protein	P06761	525	20.26	2	4.1	3.56E-09	1.2	0.018	activation of signaling protein activity involved in unfolded protein response; positive regulation of protein ubiquitination	ATP binding; unfolded protein binding
Heat shock 70 kDa protein 1-like	P55063	525	10.13	1	2	0.000000082	1.2	0.018	response to stress	ATP binding
Heat shock-related 70 kDa protein 2	P14659	525	10.13	1	1.4	0.00015	1.2	0.018	multicellular organismal development; response to stress	ATP binding
Stress-70 protein, mitochondrial	P48721	1337	50.23	5	11.3	5.93E-10	1.1	0.027	protein folding	ATP binding; unfolded protein binding
60 kDa heat shock protein, mitochondrial	P63039	525	160.33	16	36.3	1.37E-12	1.2	0.018	anti-apoptosis; chaperone mediated protein folding requiring cofactor; detection of misfolded protein; positive regulation of anti-apoptosis; positive regulation of inflammatory response; response to ATP; response to cocaine; response to drug; response to estrogen stimulus; response to	ATP binding; insulin binding; misfolded protein binding; protease binding; protein complex binding; protein heterodimerization activity

									heat; response to hydrogen peroxide; response to hypoxia; response to lipopolysaccharide	
CYTOSKELETON										
Actin, cytoplasmic 1	P60711	1337	10.21	1	6.1	0.0000056	1.1	0.027	axonogenesis	ATP binding; protein kinase binding
Septin-11	B3GNI6	1337	20.16	2	6	0.000000371	1.1	0.027	cell cycle; cell division	GTP binding
Dihydropyrimidinase-related protein 2	P47942	525	20.19	2	3.7	0.000047	1.2	0.018	olfactory bulb development; positive regulation of glutamate secretion; regulation of axon extension; response to amphetamine; response to cocaine; response to drug; spinal cord development; synaptic vesicle transport	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds; protein binding
Tubulin alpha-1A chain	P68370	525	20.24	2	7.3	0.00000685	1.2	0.018	microtubule-based movement; protein polymerization	GTP binding; GTPase activity; protein domain specific binding; protein heterodimerization activity; structural molecule activity
Glial fibrillary acidic protein	P47819	1513	10.15	1	2.6	0.000000221	1.1	0.04	response to wounding	structural constituent of cytoskeleton
METABOLIC PROCESSES										
Alpha-enolase	P04764	1513	30.28	3	12.7	0.000000085	1.1	0.04	glycolysis	magnesium ion binding; phosphopyruvate hydratase activity; protein heterodimerization activity; protein homodimerization activity; serine-type endopeptidase activity
		1337	156.33	16	38.5	1.24E-10	1.1	0.027		
		525	10.17	1	3.5	0.000000894	1.2	0.018		
Beta-enolase	P15429	1337	10.26	1	3.5	3.36E-08	1.1	0.027	Aging; Glycolysis; response to drug; skeletal muscle tissue regeneration	magnesium ion binding; phosphopyruvate hydratase activity; protein heterodimerization

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										activity; protein homodimerization activity
ATP synthase subunit d, mitochondrial	P31399	1337	10.18	1	7.5	0.0000235	1.1	0.027	ATP synthesis coupled proton transport	hydrogen ion transmembrane transporter activity
Gamma-enolase	P07323	1513	78.24	8	13.6	7.65E-10	1.1	0.04	Gluconeogenesis; Glycolysis; response to drug; response to estradiol stimulus; response to organic cyclic compound	magnesium ion binding; phosphopyruvate hydratase activity; protein heterodimerization activity; protein homodimerization activity
CHAPERONE										
T-complex protein 1 subunit beta	Q5XIM9	1513	20.2	2	6.2	0.00000404	1.1	0.04	protein folding	ATP binding; unfolded protein binding
		1337	10.14	1	2.6	0.00018	1.1	0.027		
DA RELEASE AND TRANSPORT										
Alpha-synuclein	P37377	1337	10.22	1	14.3	0.0000626	1.1	0.027	behavioral response to cocaine; negative regulation of dopamine metabolic process; negative regulation of neuron apoptosis; negative regulation of protein phosphorylation; regulation of dopamine secretion	microtubule binding; phospholipase binding; phospholipid binding; protein N-terminus binding; protein domain specific binding
OTHER										
Serum albumin	P02770	525	200.26	20	37.8	1.42E-09	1.2	0.018	cellular response to starvation; hemolysis by symbiont of host erythrocytes; maintenance of mitochondrion location; negative regulation of apoptosis; positive regulation of circadian sleep/wake cycle, non-REM sleep; response to nutrient; Transport; vasodilation	DNA binding; cell surface binding; drug binding; enzyme binding; fatty acid binding; pyridoxal phosphate binding; toxin binding; zinc ion binding

7.3.6 Frontal cortex: GO analysis following chronic + 4 weeks ECS

The GO terms found to be significant by DAVID following a search for FatGO terms for proteins in the biological process, molecular function and cellular component ontologies are listed in Table 7-13 – 7-15. The number of proteins (count) and their identifier (accession number) as well as the p-value for each term is also listed.

Table 7-13 Biological Processes

Term	Count	P-Value	Genes
generation of precursor metabolites and energy	5	2.21E-04	P31399, P15429, P07323, P04764, P37377
protein folding	4	5.47E-04	P48721, P63039, P63018, Q5XIM9
glycolysis	3	0.001572	P15429, P07323, P04764
glucose catabolic process	3	0.002511	P15429, P07323, P04764
hexose catabolic process	3	0.002688	P15429, P07323, P04764
monosaccharide catabolic process	3	0.002688	P15429, P07323, P04764

Table 7-14 Molecular Function

Term	Count	P-Value	Genes
phosphopyruvate hydratase activity	3	1.00E-05	P15429, P07323, P04764
purine ribonucleotide binding	10	5.06E-05	P55063, P48721, P63039, P63018, P60711, Q5XIM9, P14659, B3GNI6, P06761, P68370
ribonucleotide binding	10	5.09E-05	P55063, P48721, P63039, P63018, P60711, Q5XIM9, P14659, B3GNI6, P06761, P68370
purine nucleotide binding	10	7.48E-05	P55063, P48721, P63039, P63018, P60711, Q5XIM9, P14659, B3GNI6, P06761, P68370
protein heterodimerization activity	5	1.92E-04	P15429, P63039, P07323, P04764, P68370
nucleotide binding	10	3.11E-04	P55063, P48721, P63039, P63018, P60711, Q5XIM9, P14659, B3GNI6, P06761, P68370
ATP binding	8	6.37E-04	P55063, P48721, P63039, P63018, P60711, Q5XIM9, P14659, P06761
adenyl ribonucleotide binding	8	7.20E-04	P55063, P48721, P63039, P63018, P60711, Q5XIM9, P14659, P06761
adenyl nucleotide binding	8	0.001025	P55063, P48721, P63039, P63018, P60711, Q5XIM9, P14659, P06761
purine nucleoside binding	8	0.00114	P55063, P48721, P63039, P63018, P60711, Q5XIM9, P14659, P06761
hydro-lyase activity	3	0.001146	P15429, P07323, P04764

nucleoside binding	8	0.001197	P55063, P48721, P63039, P63018, P60711, Q5XIM9, P14659, P06761
protein dimerization activity	5	0.004468	P15429, P63039, P07323, P04764, P68370
unfolded protein binding	3	0.004987	P48721, P63018, Q5XIM9

Table 7-15 Cellular component

Term	Count	P-Value	Genes
phosphopyruvate hydratase complex	3	1.40E-05	P15429, P07323, P04764
cytosol	10	1.51E-05	P15429, P63039, P63018, P60711, P07323, Q5XIM9, P06761, P04764, P37377, P68370
cytosolic part	5	2.13E-05	P15429, P07323, Q5XIM9, P06761, P04764
cell surface	5	0.001655	P48721, P63039, P63018, P14659, P06761

7.3.7 Distribution of identified proteins

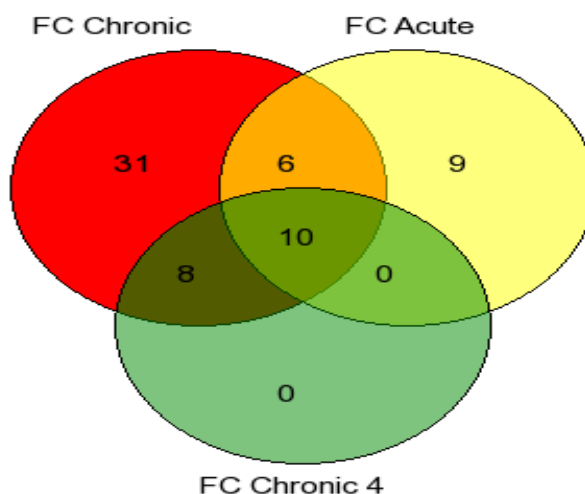


Figure 7-1 Venn diagram of proteins expressed in the frontal cortex following ECS
FC, frontal cortex

Chronic		Chronic & acute	Acute	Chronic +4	Acute & Chronic+4	Acute & Chronic & Chronic+4	Chronic & Chronic+4
O35179	P85845	P04692	O08557			P02770	B3GNI6
O35964	Q05175	P62138	P04692-4			P06761	P04764
P04642	Q07439	P62738	P16617			P07323	P31399
P07632	Q1WIM3	P62804	P19527			P14659	P37377
P09951	Q4QRB4	P85108	P62260			P15429	P60711
P0C169	Q5PPJ9	Q6P9T8	P69897			P47819	P63039
P10719	Q5XIF6		Q63610			P47942	P68370
P23565	Q63ZV7		Q66HF1			P48721	Q5XIM9
P30009	Q68A21		Q8CH34			P55063	
P31044	Q68FQ0					P63018	
P37397	Q6P7B0						
P42123	Q6P9V9						
P46462	Q76EQ0						
P62986	Q8VBU2						
P63029	Q8VD52						
	Q99NA5						

Table 7-16 Proteins expressed in the frontal cortex following acute, chronic and chronic + 4 weeks of ECS

Accession numbers of proteins common to the frontal cortex in each study are listed; corresponding proteins in the Appendix.

7.3.8 Kyoto encyclopaedia of genes and genomes (KEGG) analysis

Analysis revealed that proteins were involved in KEGG pathways following DAVID GO analysis. As in Chapter 5, section 5.4.8, the KEGG pathway identifier code, the KEGG pathway and the relevant gene products involved in these pathways are listed for each study. However, due to the low levels of significance obtained for most of these gene products in the KEGG pathways, other forms of pathway analysis were investigated to better understand the data.

Table 7-17 Frontal cortex KEGG pathway analysis following acute ECS

KEGG Identifier	KEGG pathway	Genes
rno04612	Antigen processing and presentation	P55063, P63018, P14659, P06761
rno03018	RNA degradation	P48721, P15429, P07323
rno04540	Gap junction	P69897, Q6P9T8, P85108
rno00010	Glycolysis / Gluconeogenesis	P15429, P07323, P16617
rno03040	Spliceosome	P55063, P63018, P14659

Table 7-18 Frontal cortex KEGG pathway analysis following chronic ECS

KEGG Identifier	KEGG pathway	Genes
rno03018	RNA degradation	P48721, P15429, P63039, P07323, P04764
rno04540	Gap junction	Q5XIF6, Q4QRB4, Q6P9V9, Q6P9T8, P85108, P68370
rno00010	Glycolysis/ Gluconeogenesis	P15429, P42123, P04642, P07323, P04764
rno04612	Antigen processing and presentation	P55063, Q07439, P63018, P14659, P06761
rno04144	Endocytosis	Q5PPJ9, P55063, Q07439, P63018, O35964, P14659
rno03040	Spliceosome	P55063, P63018, P14659

Table 7-19 Frontal cortex KEGG pathway analysis following chronic+4 weeks of ECS

KEGG Identifier	KEGG pathway	Genes
rno03018	RNA degradation	P48721, P15429, P63039, P07323, P04764 14
rno04612	Antigen processing and presentation	P55063, P63018, P14659, P06761
rno00010	Glycolysis/ Gluconeogenesis	P15429, P07323, P04764
rno03040	Spliceosome	P55063, P63018, P14659
rno04144	Endocytosis	P55063, P63018, P14659

7.3.9 Pathway analysis

Ingenuity Pathway Analysis (Ingenuity Systems) software was used to investigate the relationships between the potentially significantly changing proteins identified by proteomic analysis following ECS treatment as described in section 2.2.20.2. The networks can consist of direct and indirect relationships and contain of different molecules as detailed in the figure legend (Figure 7-2). As in Chapter 5, section 5.4.9, the number of focus molecules, the uploaded proteins that were network eligible, in each study are also listed.



Figure 7-2 Legend of shapes that describe nodes in IPA networks

The edges (lines) describe the relationship between nodes. An unbroken line indicates a direct relationship; a broken line indicates an indirect relationship; an edge with an arrow head indicates one node acting on another, a direct edge between two nodes suggests binding of one node to another.

7.3.9.1 Frontal cortex pathway analysis following acute ECS

The frontal cortex network following acute ECS contained 18 focus molecules.

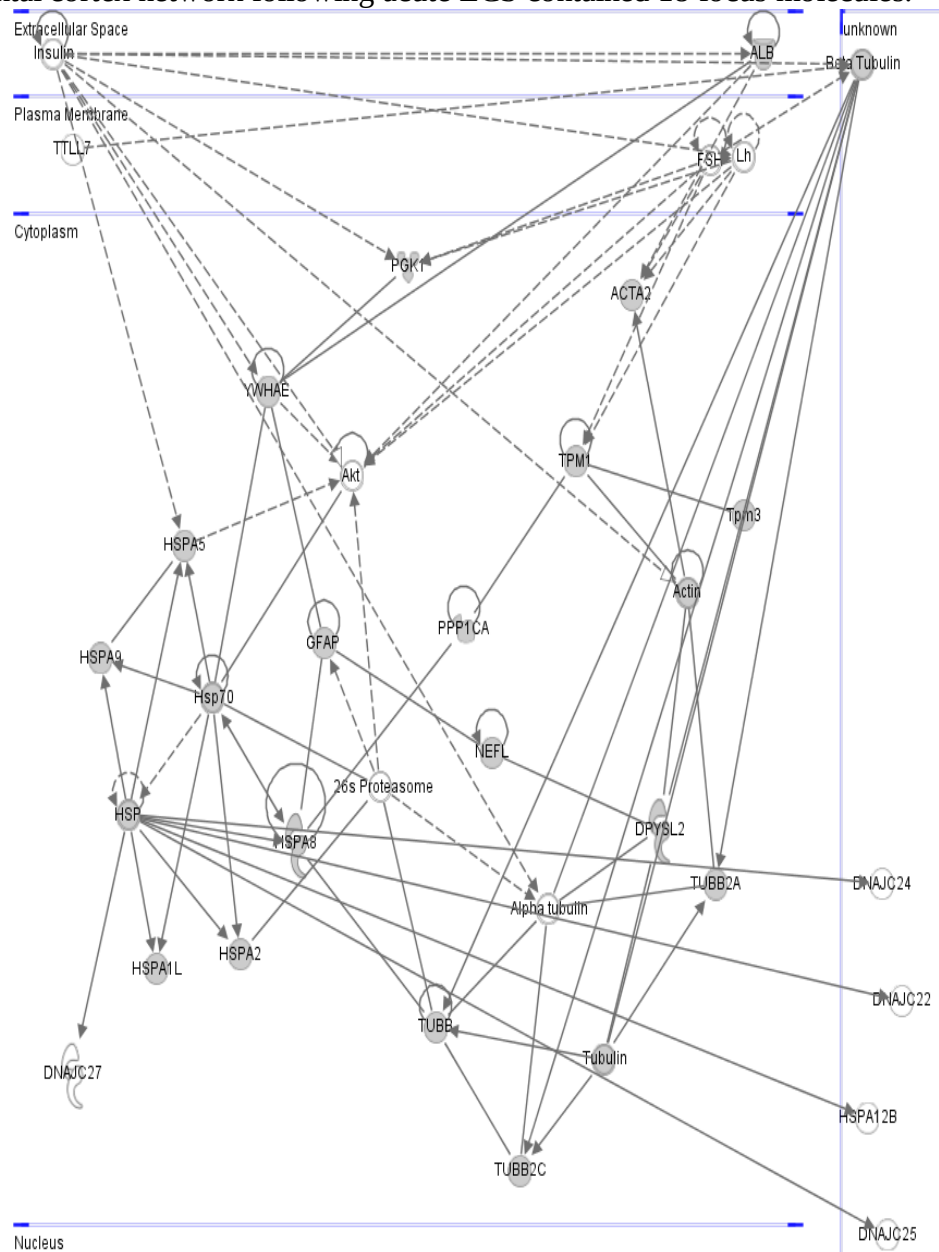


Figure 7-3 Network based on candidate proteins derived from targeted protein spots that were significantly differentially expressed in the frontal cortex following acute ECS

The network shows possible connections and interactions between molecules based on IPA knowledge base. The nodes shaded in grey were detected in this study.

ACTA2 Actin aortic smooth muscle; AK1 Adenylate kinase isoenzyme 1; ALB Serum albumin; DNAJC22 DnaJ homolog subfamily C member 22; DNAJC24 DnaJ homolog subfamily C member 24 F1M2S2; DNAJC25 DnaJ homolog subfamily C member 25; DNAJC27 DnaJ homolog subfamily C member 27; DPYSL2 Dihydropyrimidinase-related protein 2; FSH Follicle stimulating hormone; GFAP Glial fibrillary acidic protein; HSP Heat shock protein; Hsp70 Heat shock protein 70; HSPA9 Heat shock protein hsp9 (not in rat/animals); HSPA12B25D3ZVM5 uncharacterised protein; HSPA1L Heat shock 70kDa protein-1 like; HSPA2 Heat shock related 70kDa protein 2; HSPA578kDa glucose regulates protein; HSPA8 Heat shock cognate 71kDa protein; Lh Lutenising hormone; NEFL Neurofilament light polypeptide; PGK1 Phosphoglycerate kinase 1; PPP1CA Serine/threonine-protein phosphatase PP1-alpha catalytic subunit; TPM1 Tropomyosin alpha 1 chain; Tpm3 Tropomyosin alpha-3 chain; TLL7 D4ACG4 uncharacterised protein (tubulin polyglutamylase); TUBB Tubulin beta-2B chain; TUBB2A Tubulin beta 2A chain; TUBB2C Tubulin beta-2C chain; YWHAE14-3-3 protein epsilon

7.3.9.2 Frontal cortex analysis following chronic ECS

The hippocampus network following chronic ECS contained 24 focus molecules.

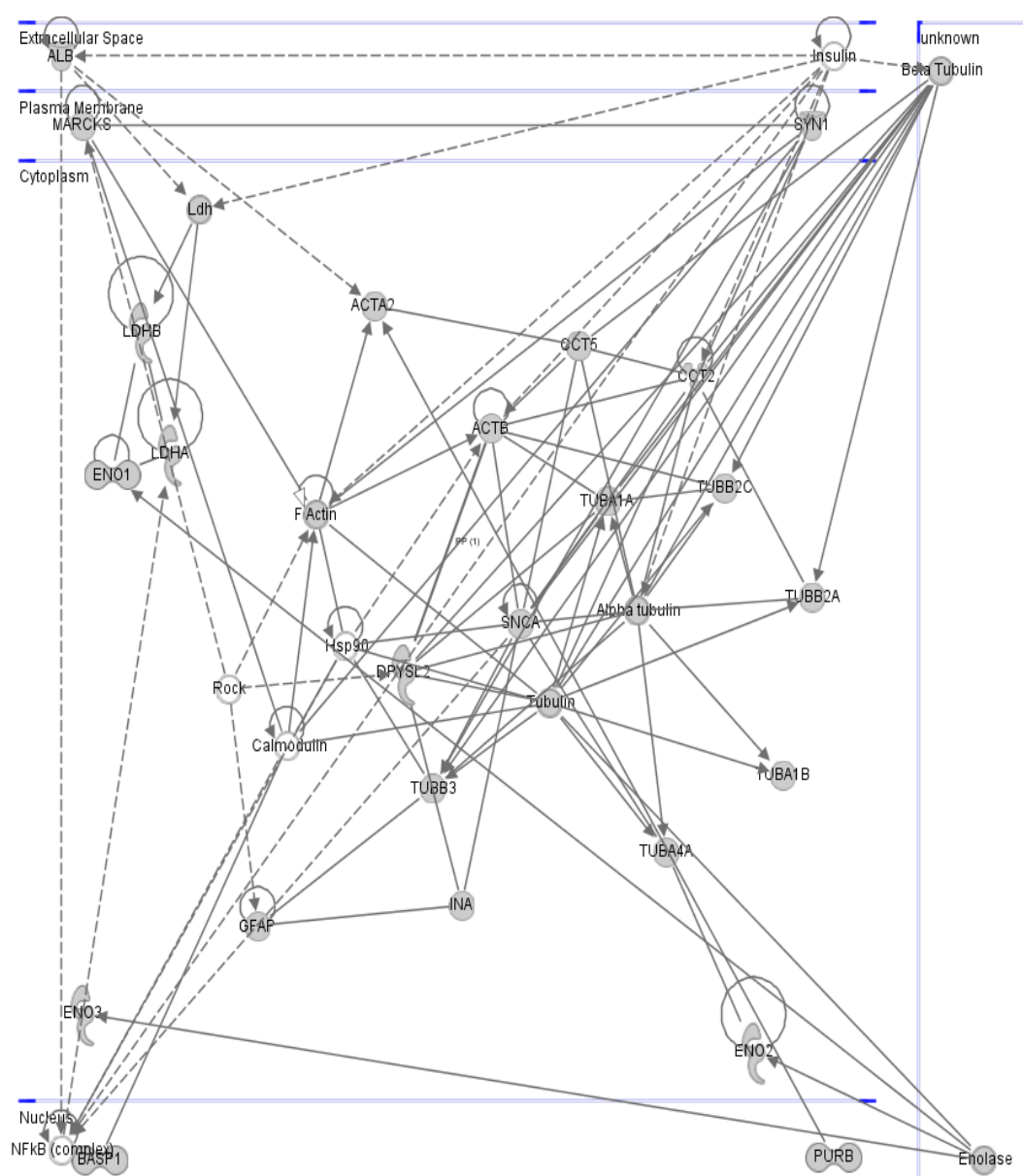


Figure 7-4 Network based on proteins that were significantly differentially expressed in the frontal cortex following chronic ECS

The network shows possible connections and interactions between molecules based on IPA knowledge base. The nodes shaded in grey were detected in this study.

ACTA2 Actin, aortic smooth muscle; ACTB Actin cytoplasmic 1; BASP1 Brain acid soluble protein 1; CCT2 T complex protein subunit 1 beta; CCT5 T complex protein 1 subunit epsilon; DPYSL2 Dihydropyrimidinase-related protein 2; ENO1 Alpha enolase; ENO3 Beta enolase; Hsp90 Heat shock protein 90; INA Alpha internexin; Ldh L-lactate dehydrogenase; LDHA L-lactate dehydrogenase A chain; LDHB L-lactate dehydrogenase B chain; MARCKS Myristoylated alanine-rich C-kinase substrate; NFkB Nuclear factor kappa B; PURB Transcriptional activator protein Pur-beta; Rock Rho-associated protein kinase; SNCA Alpha synuclein; SYN1 Synapsin 1; TUB1A Tubulin alpha-1A chain; TUBA1B Tubulin alpha-1B chain; TUBA4A Tubulin alpha 4A chain; TUBB2A Tubulin beta-2A chain; TUBB2C Tubulin beta-2C chain; TUBB3 Tubulin beta-3 chain

7.3.9.3 Frontal cortex pathway analysis following chronic + 4 weeks ECS

The frontal cortex following chronic + 4 weeks of ECS contained 16 focus molecules.

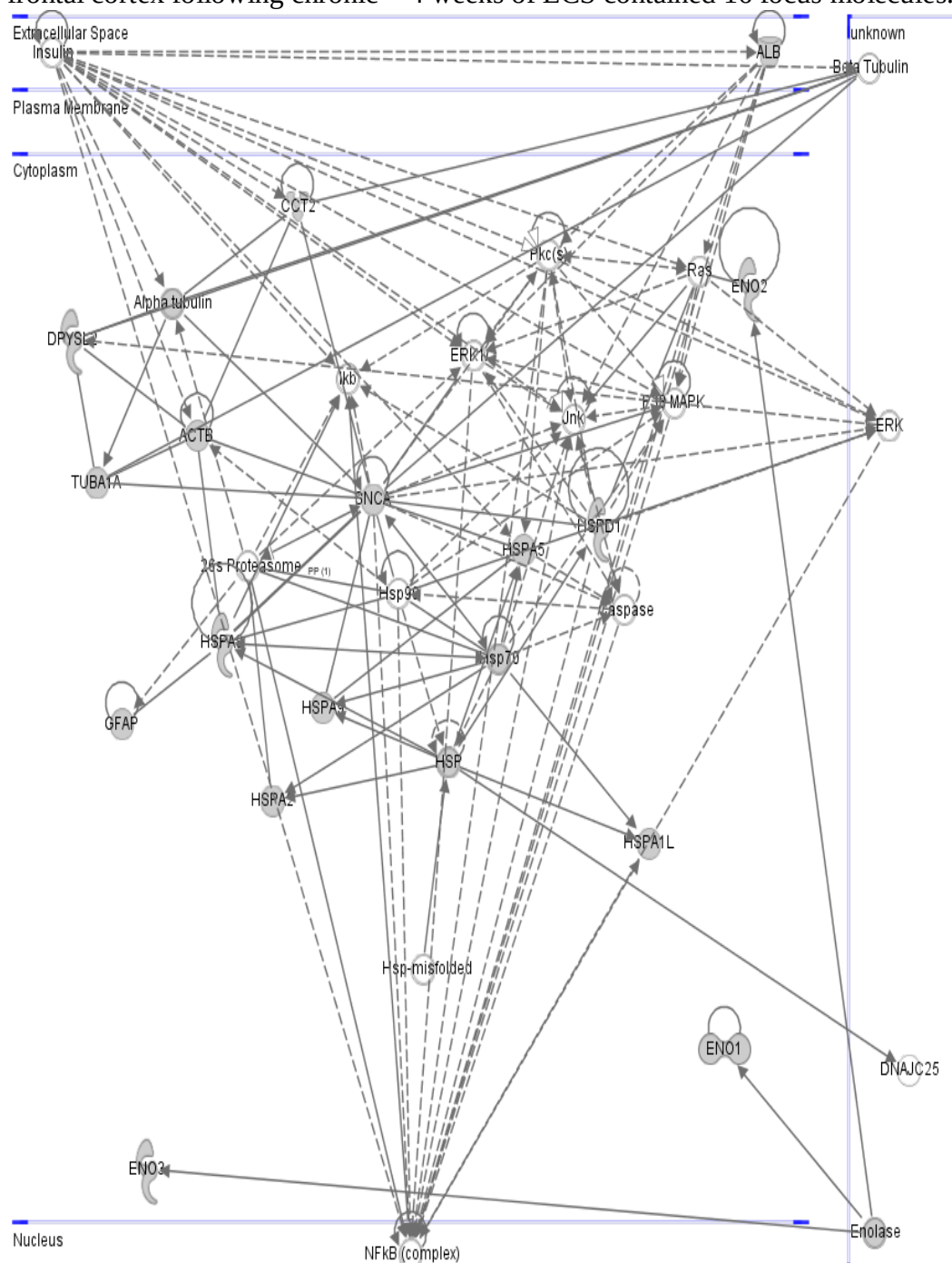


Figure 7-5 Network based on candidate proteins that were significantly differentially expressed in the frontal cortex following chronic + 4 weeks of ECS

The network shows possible connections and interactions between molecules based on IPA knowledge base. The nodes shaded in grey were detected in this study.

ACTB Actin cytoplasmic 1; ALB Serum albumin; CCT2 T complex protein subunit 1 beta; DNAJC25 DnaJ homolog subfamily C member 25; DPYSL2 Dihydropyrimidinase-related protein 2; ENO1 Alpha enolase; ENO2 Gamma enolase; ENO3 Beta enolase; ERK Mitogen activated protein kinase; ERK1 Mitogen-activated protein kinase 3; GFAP Glial fibrillary acidic protein; HSP Heat shock protein; Hsp78 Heat shock protein 78 (not animals-fungus, yeast); Hsp90 Heat shock protein 90; HSPA1L Heat shock 70kDa protein-1 like; HSPA2 Heat shock 70kDa protein 2; HSPA4 Heat shock 70kDa protein 4; HSPA578kDa glucose regulates protein; HSPD160kDa heat shock protein, mitochondrial; Ikb NF-kappa-B inhibitor; Jnk Mitogen-activated protein kinase; MAPK Mitogen activated protein kinase; Nfkb Nuclear factor kappa B; Pkc(s) Protein kinase c; Ras Rat sarcoma; SNCA Alpha synuclein; TUBA1 Tubulin alpha 1A chain

7.3.10 Confirmation studies

The following two proteins were selected for initial confirmation by immunoblotting. They were detected in the frontal cortex following chronic ECS treatment and as a result, may be of particular relevance in the mechanisms of action of ECS. Both proteins have plasticity-related roles in the brain and have been detected relatively infrequently in antidepressant related proteomic studies. As discussed in section 5.5.4, no proteins were emphasised for further analysis based on fold change in expression following proteomic analysis. Therefore, protein selection for confirmation was again based on the theoretical interest of the protein based on the current literature and technical considerations including antibody availability.

7.3.10.1 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH was used as the “housekeeping” protein in all studies. All targets were normalised to GAPDH as GAPDH expression did not vary between treatment and control tissues. It also acted as a loading control. GAPDH was run on all target blots and representative GAPDH bands are included with the blots described below.

7.3.11 Brain acid soluble protein 1 (BASP-1)

Chronic treatment with ECS did not significantly affect the relative % density of BASP-1 in the frontal cortex (Student's t-test, $p=0.9001$, $n=7-10$) (Figure 7-6). The average relative % density of BASP-1 did not differ between ECS treated and control animals: control $100 \pm 12\%$, ECS treated $97 \pm 22\%$).

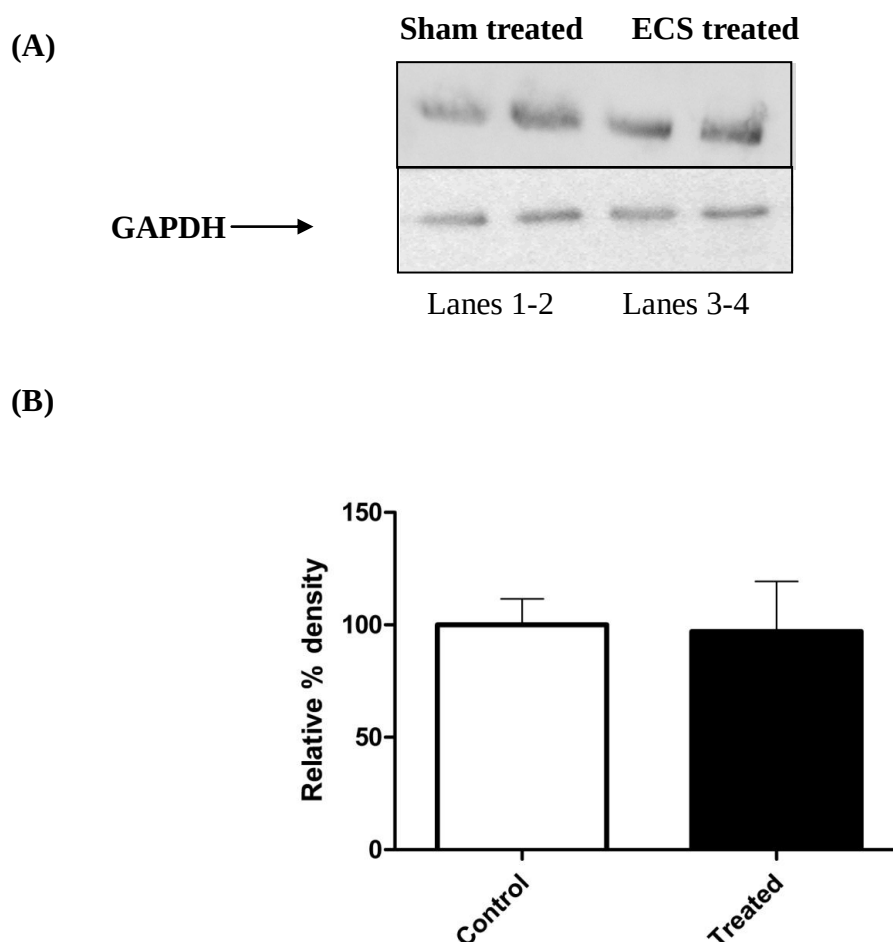


Figure 7-6 BASP-1 expression in the frontal cortex following chronic ECS

(A) The first two lanes contain representative sham treated frontal cortex tissue. The remaining two lanes contain ECS treated frontal cortex tissue. Representative GAPDH bands are below.

(B) There was no significant difference in the relative % density of BASP-1 following chronic ECS treatment compared to controls. Mann-Whitney test, data expressed as % of controls $\pm$ SEM, $n=7-9$ per group.

7.3.12 Calponin 3

Chronic treatment with ECS did not significantly affect relative expression levels of Calponin 3 in the frontal cortex (Mann-Whitney $p=0.1416$, $n=7-9$) (Figure 7-7). The average relative % density of Calponin 3 was lower in ECS treated animals than in control animals (control $100 \pm 16\%$, ECS treated $65 \pm 9\%$).

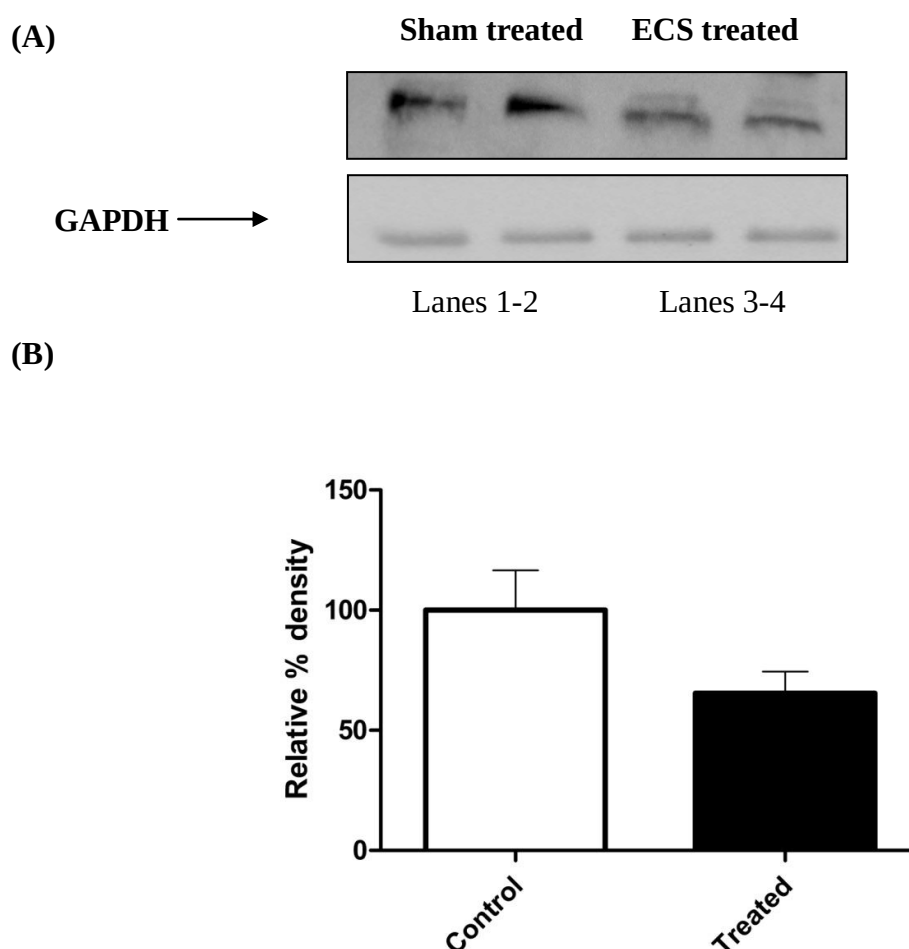


Figure 7-7 Calponin 3 expression in the frontal cortex following chronic ECS

(A) The first two lanes contain representative sham treated frontal cortex tissue. The remaining two lanes contain ECS treated frontal tissue, representative GAPDH bands below

(B) There was no significant difference in the relative % density of Calponin 3 following chronic ECS treatment compared to controls. Mann-Whitney test, data expressed as % of controls $\pm$ SEM, $n=7-9$ per group.

7.4 Discussion

The differentially expressed proteins identified in the frontal cortex following ECS are largely cytoskeletal or metabolic related, as previously seen in proteomic studies of antidepressant action in the same brain region (Piubelli et al. 2011a, b).

Gene ontology (GO) analysis was applied to each study to assist in categorising the identified proteins. Following acute ECS, proteins were involved in biological processes mainly relating to the cytoskeleton, including protein polymerisation and microtubule movement (section 7.4.2). A number of proteins had a role in glycolysis. Following chronic ECS, the biological processes again included both metabolic (e.g. glycolysis, glucose and carbohydrate catabolic processes) and cytoskeletal (protein polymerisation, protein complex assembly and microtubule based movement). The cellular components proteins involved following chronic ECS treatment included neuron projection, cytoskeletal parts and the microtubule skeleton (section 7.4.2). GO analysis following chronic + 4 weeks of ECS revealed a role for metabolism related proteins in each ontology. For example, several proteins identified in the frontal cortex were involved in biological processes including glycolysis, glucose catabolic processes and metabolite and energy generation. A brief overview of the cytoskeletal and metabolic proteins identified in the frontal cortex is discussed below.

7.4.1 Cytoskeletal proteins

As discussed in section 5.5.1, the neuronal cytoskeleton consists of three different structural components, microfilaments, neurofilaments and microtubules (Siegel et al. 1999), all of which were possibly altered following ECS treatment.

Tubulin alpha and beta, microtubule proteins, were upregulated in the frontal cortex following acute and chronic ECS but tubulin alpha was downregulated following chronic + 4 weeks of ECS. Tubulin upregulation has been detected following proteomic analysis of antidepressant pharmacotherapy (Carboni et al. 2006a; Piubelli et al. 2011b).

GFAP, an intermediate filament and astrocyte marker (Middledorp and Hol, 2011), was upregulated following acute, chronic and chronic + 4 weeks of ECS treatment. GFAP was expressed in a number of spots following chronic and chronic + 4 weeks of ECS. In other proteomic studies of antidepressant effect on the frontal cortex, GFAP expression was also upregulated (Carboni et al. 2006a; Piubelli et al. 2011b). GFAP was significantly downregulated in the frontal cortex of depressed patients following proteomic analysis (Johnston-Wilson et al. 2000). Neurofilament light chain intermediate filament protein

was also significantly upregulated in the frontal cortex following acute ECS while alpha-internexin was upregulated in the frontal cortex following chronic ECS, as has been previously seen following antidepressant treatment (Carboni et al. 2006a; Piubelli et al. 2011c).

The microfilament protein actin was upregulated following acute, chronic and chronic + 4 weeks of ECS treatment in the frontal cortex. Actin has previously been identified in proteomic studies of the frontal cortex following antidepressant treatment (Carboni et al. 2006a; Piubelli et al. 2011c; Piubelli et al. 2011b). Tropomyosin alpha, a microfilament that, in combination with tropomodulin, acts to regulate actin filament length and stability (Gokhin et al. 2010), was upregulated in the frontal cortex Acute and Chronic studies. Tropomyosin was also upregulated following antidepressant treatment in an animal model of depression (Piubelli et al. 2011a; Mu et al. 2007).

As discussed in Chapter 5 (section 5.5.1), cytoskeletal proteins are frequently identified as having undergone significant differential expression in a range of proteomic studies. Therefore, proteins that are involved in the regulation and control of the cytoskeleton may be of particular interest in understanding the effects of ECS treatment in the frontal cortex.

7.4.2 Metabolic proteins

A number of metabolism-related proteins were identified in the frontal cortex following ECS treatment. Adenosine triphosphate (ATP) synthase expression was increased following chronic and chronic + 4 weeks of ECS treatment. ATP synthase β is one of the most commonly identified differentially expressed proteins in proteomic studies (Petrak et al. 2008). It was detected following ECS treatment in the hippocampus (section 5.5.2) and in proteomic studies of the frontal cortex following antidepressant treatment (Piubelli et al. 2011c; Carboni et al. 2006).

As previously seen in the hippocampus, ECS treatment induced changes in the expression of a number of metabolic proteins in the frontal cortex that are frequently and abundantly detected using a 2DE experimental approach (Parker et al. 2009).

7.4.3 Pathway analysis

The cytoskeletal proteins play an essential role in the networks created by pathway analysis. Tubulin, in particular, is involved in multiple interactions within the networks (Figure 7-3 - 7-5) as are the heat shock proteins, a family of chaperone proteins involved in protein aggregation (Romi et al. 2011; Figure 7-5). A number of proteins were added to the

network of proteins identified following acute, chronic and chronic + 4 weeks of ECS following pathway analysis (IPA). These proteins were not identified by proteomic analysis and are speculative in nature. Of potential interest in the frontal cortex was the identification and addition by IPA of mitogen activated protein kinase (MAPK) following chronic + 4 weeks of ECS treatment. MAPK is a signalling molecule that has been implicated in antidepressant action and the mechanisms of depression (Duman et al. 2007). Other MAPK pathway proteins connected to MAPK in the IPA network included mitogen-activated protein kinase 3 (ERK1). This may suggest a role for this signalling cascade in the mechanisms of ECS action.

Two proteins were selected from the Chronic study for further confirmation studies due to their cytoskeletal-related roles and the relative infrequency with which they have been detected in proteomic studies.

7.4.4 BASP-1

BASP1 is a 22kDa protein that is highly expressed in the brain (Maekawa et al. 1993). It is also known as 22kDa neuronal tissue enriched acidic protein (NAP-22) and 23kDa cortical cytoskeleton associated protein (CAP-23). BASP1 is functionally related to other growth associated proteins, growth associated protein 43 (GAP-43) and myristoylated alanine-rich C kinase substrate (MARCKS) (Maekawa et al. 1993). GAP43 can functionally substitute for BASP1 in vivo (Frey et al. 2000).

BASP1 depletion in neuronal culture leads to thinner neurites with bulbous growth cones which has been specifically linked to a deficit in the actin cytoskeleton, resulting from the absence of BASP1 (Frey et al. 2000). Neuronal growth cones changed the direction they were extending leading to an abnormal winding pattern in neurite outgrowth.

BASP1 expression, with that of GAP-43 and MARCKS enhanced neurite outgrowth in neuronal culture. These growth associated proteins were shown to correlate with cell cortex dynamics. This has led to the proposal of a model whereby BASP1, GAP-43 and MARCKS affect local levels of phosphatidyl inositol (4,5) biphosphate (PIP₂), a signalling substrate, at the plasma membrane which in turn controls actin regulating proteins (Laux et al. 2000).

Proteomic analysis has shown that, in normally reared animals, BASP1 expression was increased following chronic escitalopram treatment although this result was not found in animals exposed to a maternal separation paradigm and was not detected in the frontal cortex but in the hippocampus of treated animals (Marais et al. 2009). BASP1 expression

increases have also been found in the brains of patients with schizophrenia (Behan et al. 2009; Prabakaran et al. 2004) and bipolar disorder (Behan et al. 2009).

Chronic ECS treatment did not have any significant effect on the expression of BASP-1 in the frontal cortex as determined by immunoblotting (Figure 7-6) although it was significantly upregulated by 1.2 fold in a protein spot following proteomic analysis.

7.4.5 Calponin 3

The calponin family consists of three isoforms, calponin 1-3. Calponins are actin filament associated proteins of 34-37kDa and are found in smooth muscle and non-muscle cells (Jin et al. 2008). Calponin molecules range from 297 to 329 amino acids in length. The first 273 amino acids display a high level of sequence homology but the C terminal regions differ significantly (Rozenblum et al. 2008). This leads to variation in isoelectric point and molecular mass. Calponin 1 has an isoelectric point (pI) of 8.99 and is also known as basic calponin. Calponin 2 has a pI of 7.58 and is also named neutral calponin while calponin 3 is the acidic isoform with a pI of 5.51 (Strasser et al. 1993; Applegate et al. 1994; Jin et al. 2003). Calponins were initially identified as being involved in regulating smooth muscle cell contraction and relaxation (Gimona and Small, 1996) and in organising the actin cytoskeleton of non-muscle cells (Kake et al. 1995; Danninger and Gimona, 2000; Ferhat et al. 2001; Fukui et al. 1997).

Calponin 3 is known to regulate actin filaments during neuronal remodelling (Rami et al. 2006). Calponin 3 has been localised to dendritic spines (Agassandian et al. 2000). Spine density is increased in Calponin 3 expressing neurons which may result from the polymerisation of actin or the stabilisation of actin filaments (Rami et al. 2006). Calponin 3 expressing neurons also saw changes in spine morphology with an elongation of dendritic spines, suggesting that Calponin 3 binds actin filaments in spines to induce these morphological changes (Rami et al. 2006).

The increased expression of Calponin 3 in an animal model of epilepsy, patient tissue and cerebrospinal fluid samples, as well as its function as an actin binding protein have led to suggestions for a role in epilepsy (Han et al. 2011). Changes in spine morphology and density similar to those discussed above were observed in pilocarpine and kainate models of temporal lobe epilepsy (Represa et al 1993; Isokawa et al. 2000). Calponin 3 levels were also increased in a pilocarpine model of temporal lobe epilepsy (Ferhat et al. 2003).

The increased spinal density and elongated dendritic spines associated with Calponin 3 expression are also seen in fragile X syndrome, a genetic disorder that results in mental retardation. Patients and FRM1 knock-out mouse models of fragile X syndrome display a similar change in spine morphology as is seen in Calponin 3 expressing neurons (Rami et al. 2006; Irwin et al. 2000; Irwin et al. 2001; Comery et al. 1997; Nimchinsky et al. 2001). It has been speculated that misregulation of actin through Calponin 3 may occur in neurons and contribute to the pathophysiology of, for example, epilepsy (Rami et al. 2006; Han et al. 2011).

Chronic ECS treatment led to a non-significant decrease in calponin-3 expression in the frontal cortex (Figure 7-7). Differential expression of Calponin-3 appears to be associated with dendritic plasticity and disease pathophysiology, however further study will be required to determine whether this protein plays a role in the mechanisms of ECS action.

7.4.6 Conclusion

BASP-1 and Calponin-3 were selected from the frontal cortex study for confirmation, in part because they were unique to the frontal cortex proteome. Although semi-quantitative immunoblotting did not reveal any significant differences following ECS treatment, further investigation of cytoskeletal-related proteins detected by proteomic analysis may reveal a role for the proteins involved in the effects of ECS. As discussed in section 5.5.8.1, the lack of significance detected following immunoblotting may result from the study sample number or difficulty detecting the subtle changes in protein expression.

7.4.6.1 Compare and contrast frontal cortex and hippocampus results

Following proteomic analysis, there is strong evidence for the differential expression of cytoskeletal related proteins in the mechanisms of action of ECS. Many of these proteins were expressed, to the same extent (1.1-1.3 fold change) in both the hippocampus and frontal cortex. However, while proteins such as actin and tubulin may play a role in the mechanisms of ECS treatment, they may not prove useful for determining the specific effects induced by ECS. Changes in the expression of these proteins are common to proteomic studies of antidepressant treatment (Carboni et al. 2006a; Piubelli et al. 2011c).

While many of the same proteins were differentially expressed in the frontal cortex and hippocampus, each brain region also had a specific protein expression profile. The

frontal cortex proteins selected for confirmation were unique to the frontal cortex proteome as were those confirmed in the hippocampus, following analysis. Further investigation of proteins such as these may be relevant for the development of markers of the effects of ECS.

ECS was associated with global protein changes in the brain when administered acutely, chronically or over a longer period of time. Analysis of the hippocampus and frontal cortex determined a number of proteins that may be relevant in understanding the mechanisms of action of ECS treatment.

Conclusions

Electroconvulsive therapy (ECT) is the most effective treatment available for severe depression (UK ECT Review Group, 2003). However its mechanisms of action are not yet fully understood. Using an animal model of ECT, electroconvulsive stimulation (ECS), the cellular, molecular and behavioural effects of altering stimulus parameters were investigated. Additionally, the protein changes induced in the brain by ECS administration were characterised in an effort to understand the effects of ECS and potentially the therapeutic actions of ECT.

8.1 Brief pulse and ultra brief pulse electroconvulsive stimulation

ECT treatment is associated with cognitive side-effects that may be overcome while therapeutic efficacy is maintained by altering ECT stimulus parameters (Sackeim et al. 2008). While changing stimulus waveform successfully reduced treatment side effects without impacting on the antidepressant effects of ECT, the benefits of adjusting other treatment parameters, including pulse width, are now being investigated (Sackeim et al. 2000; Loo et al. 2008). To address this, a study was conducted that compared the effects of different ECS pulse widths. The standard brief pulse of 0.5ms and an ultra brief pulse width of 0.3ms were administered to determine whether the different ECS stimulus parameters were as effective at inducing cellular, behavioural and molecular effects.

Overall, ultrabrief pulse ECS was not as effective a treatment as brief pulse ECS. It did not induce a significant increase in cell proliferation in the dentate gyrus, a well established effect of brief pulse ECS treatment (Scott et al. 2000). It also failed to induce significant changes in the expression of brain derived neurotrophic factor (BDNF), a neurotrophin whose expression increases in the hippocampus following chronic treatment with ECS and antidepressant pharmacotherapy (Jacobsen and Mork, 2004).

The superior antidepressant behavioural effect of brief pulse ECS was established using the forced swim test, where brief pulse treated animals spent significantly less time immobile than the ultra brief pulse treated animals.

However, the cognitive effects of ultra brief pulse ECS compared to brief pulse ECS were more difficult to discern. Results from the water plus maze showed that while neither brief pulse nor ultra brief pulse ECS treated animals navigated the maze as effectively as sham treated animals, there was no significant difference between the three groups or between the different pulse widths. This may be due to the time delay between

ECS treatment and conducting the water plus maze trials. Further investigation will be required to determine whether or not ultra brief pulse ECS offers an improvement in the cognitive side effects associated with treatment. However, ultra brief pulse ECS is not as effective as brief pulse ECS at inducing the molecular and cellular changes in the brain which may be associated with the superior antidepressant efficacy of brief pulse ECS. The results of this preclinical study, the first to investigate the effects of reducing ECS pulse width, are in line with those obtained in more recent ultra brief pulse ECT trials (Quante et al. 2011; Sienaert et al. 2009) and may be informative for future clinical trials into the efficacy of ultra brief pulse ECT for patients with depression.

8.2 The effects of electroconvulsive stimulation on the rat proteome

While brief pulse ECS is an effective inducer of antidepressant-associated behaviour as well as molecular and cellular changes in the brain, the mechanisms by which it brings about these changes are not fully understood. To understand better the effects of ECS, proteomic analysis of the acute, chronic and longer term effects of treatment on the brain were investigated.

ECS induced changes in global protein expression in both the hippocampus and frontal cortex at all three time points investigated. Univariate (one-way ANOVA) and multivariate (PLS-DA) analyses of 2D-DiGE gels were used in combination to detect alterations in the expression of protein spots between ECS and sham treated samples. There was a clear and strong differentiation between both groups, as demonstrated by the principal component analysis plots and scores plots in each study. This confirmed that ECS resulted in significant changes in protein expression at all time points and brain regions studied. The low magnitude changes in protein spot expression ranged from 1.1 to 1.9 fold. Expression changes of this order are in line with a number of antidepressant proteomic studies (Pennington et al. 2009; McHugh et al. 2011). This suggests that the effects of ECS may result from a multitude of subtle changes in protein expression.

Many of the protein spots picked from 2D gels of ECS-treated samples contained proteins that were identified by mass spectrometry as having cytoskeletal-related roles. Cytoskeletal and plasticity related proteins are often the main proteins of interest detected in the hippocampus and frontal cortex following antidepressant treatment (McNair et al. 2006; Kedracka-Krok et al. 2010; Piubelli et al. 2011a). Although commonly expressed proteins (e.g. actin and tubulin) may play a role in antidepressant action, they are not useful

in differentiating the effects of ECS and other antidepressant treatments due to the lack of specificity of their expression. Therefore, as a first phase strategy to confirm putative changes in candidate proteins, I investigated other cytoskeletal-related proteins expressed following ECS that could lead to further insights into the specific effects of ECS treatment.

8.2.1 Confirmation of protein expression changes

To verify the results of 2D-DiGE and mass spectrometry analyses, proteins were selected for confirmation in a new sample set and analysed using semi-quantitative immunoblotting. Cytoskeletal-related proteins that may contribute to the effects of ECS were of particular interest. In the hippocampus Acute study, a trend towards an increase in neurogranin expression was detected, in contrast to the downregulation in expression of the protein spot in which it was found (Chapter 4, Table 4-4). Neurogranin expression was then investigated in the Chronic study but no significant difference was found between treated and control groups. Actin was expressed in the proteome in all studies and a down regulation in its expression in the hippocampus Chronic + 4 week study was confirmed. Changes in the expression of other cytoskeletal proteins, selected based on their theoretical function and antibody availability, were not confirmed by immunoblotting.

The lack of statistically significant protein expression changes found following immunoblotting may be explained by several factors. Significant variations in expression of proteins identified by 2D-DiGE proteomic analysis may not be detected if the change occurs in a post-translationally modified isoform of the protein that is not identified by the antibody (Seo and Link, 2004; Piubelli et al. 2011b). The fold change in protein expression following ECS was subtle (e.g. 1.1 fold change) and detection of such a change is dependent on antibody sensitivity. The sample size, although in line with many other proteomic studies (McHugh et al. 2010; Mu et al. 2007; Marais et al. 2009), may also contribute to the lack of significance detected by immunoblotting. A statistically significant result may be observed with a larger sample number. Also, due to the large number of comparisons conducted during proteomic analyses, Type I errors may result in the detection and identification of proteins where no real change in expression existed. The multiple testing problem was controlled for during analysis (false discovery rate (q-value) Chapter 4, section 4.2.3). However, the possibility of false positive results must be considered.

8.2.2 Study design

The effects of ECS were extensively investigated in parallel but separate studies in the hippocampus and frontal cortex at three different time points. This was the first comprehensive study of the protein changes induced by ECS; a previous study has looked only at whole-brain changes following ECS (Lee et al. 2009). The hippocampal and frontal cortex proteomes are regions that have previously been investigated with regards to the effect of antidepressant action (Carboni et al. 2006). The protein changes induced by ECS were also studied acutely, chronically and four weeks after chronic treatment. The changes induced following chronic ECS treatment may be particularly relevant in understanding the mechanisms of action of ECS, as chronic ECS treatment has significant antidepressant efficacy in models such as the forced swim test (O'Donovan et al. 2011). Overall, the protein changes induced by ECS administration were methodically studied in order to understand the potential mechanisms of this antidepressant treatment.

8.3 Future work

It has been established that ECS treatment induces an array of proteome changes that may be informative of its mechanism of action. A number of the cytoskeletal proteins identified by proteomic and mass spectrometry analysis merits further analysis. For example, neurofilament light protein, detected in the frontal cortex, has previously been differentially expressed in the proteome following antidepressant pharmacotherapy administration and confirmed by western blotting (Guest et al. 2004). Neurofilament proteins are associated with synaptic remodelling. A reduction in expression was previously reported in conjunction with an increase in mossy fibre sprouting following chronic ECS (Vaidya et al. 2000).

Confirmation of protein expression changes using an alternate technique is essential to verify the results of 2D-DiGE analysis. Future confirmation work may require larger sample sizes to ensure statistically significant changes in protein expression are detected. If the altered levels of expression of a protein were confirmed in the study where the protein was initially detected, an investigation of the protein's expression at other time points (acute, chronic and chronic + 4 weeks) or in other brain regions (hippocampus and frontal cortex) may be informative of its pattern of expression. In addition, BDNF expression, which was not detected by proteome analysis probably because its isoelectric point fell outside the examined range, was significantly affected by ECS. Changes in BDNF

expression could be verified in the different studies to further understand its action in the brain.

Previous proteomic studies of antidepressant action examined the effects of pharmacotherapy in normal animals (Carboni et al. 2006a) before conducting experiments into the effects in animal models of depression (Piubelli et al. 2011a, c). It may also be of interest to investigate the effects of ECS, or the proteins confirmed to be induced by ECS, in an animal model of depression. Animal models of depression investigated using proteome analysis techniques include learned helplessness (Mallei et al. 2011), maternal separation (Marais et al, 2009) and chronic mild stress (Kedracka-Krok et al. 2010).

The hippocampus and frontal cortex were selected, as in previous proteomic studies (Carboni et al. 2006a), as they are brain regions where antidepressant administration induces a robust effect (D'Sa and Duman, 2002). Analysis of subproteomes of these regions, for example the dentate gyrus and prefrontal cortex, may give a more focused proteomic result. Equally, examining the presence of confirmed proteins of interest in these regions may assist in localising the expression of those proteins.

Following confirmation, proteins of interest could also be investigated in ECT patient plasma samples, collected in a concurrently running clinical trial. Translating significant proteins from preclinical studies and validating them in clinical samples could, potentially, lead to the development of prognostic or diagnostic biomarkers for ECT treatment.

Therefore, the protein confirmation work described in this project is the initial approach in mining the proteomic and mass spectrometry data obtained from the investigation of the effects of ECS. Future work is essential to continue and expand on the knowledge derived from the proteome analysis, in order to better understand the effects of ECS and potentially inform us of the mechanisms of ECT and the neurobiology of depression.

While 2D-DiGE is a sensitive and well established technique for separating complex proteomes like those of the brain, the field of proteomics is a rapidly developing one. New technical developments and advances in mass spectrometry mean that traditional separation methods are constantly being improved and modified. 2D-DiGE followed by mass spectrometry analysis is considered a “bottom-up” approach. Proteins are sorted, broken down and then analysed (Han et al. 2008). Shotgun proteomics separates and identifies peptides by coupling liquid chromatography directly to mass spectrometry

(Delahunty and Yates, 2006), removing the need for gel electrophoresis separation. Future proteomic studies discussed above, could, with considerations, be conducted more rapidly using shotgun proteomic techniques. However, despite these technical developments, peptide resolution can be limited when analysing complex biological mixtures such as brain samples (Delahunty and Yates, 2006). Overall, the established, time consuming methods of gel based proteomic analysis are still effective in elucidating intervention-induced protein changes.

8.4 Summary

In conclusion, this project was the first to study the cellular, molecular and behavioural affects of ultra brief pulse and brief pulse ECS and following from this, was the first proteomic study to comprehensively investigate the protein changes induced by ECS treatment in the rat hippocampus and frontal cortex. It was determined that ECS influences the proteomes of these regions by inducing wide-ranging but subtle changes in protein expression. As in previous proteomic studies of antidepressant therapies (Piubelli et al. 2011a,b), cytoskeletal proteins were strongly implicated in the effects of ECS. Combining the non-hypothesis driven proteomic-approach with the hypothesis driven methods described in Chapter 3, could, with future work, be instructive regarding the effects of ECS and in turn, ECT and the neurobiology of depression.

References

- Abrams R (1997b) The mortality rate with ECT. *Convulsive Therapy* 13:125-127.
- Abrams R (1997a) *Electroconvulsive Therapy*, 3rd Edition. New York: Oxford University Press.
- Abrams R (2002) Stimulus Titration and ECT Dosing. *J ECT* 18:3-9 discussion 14-15.
- Accornero F (1988) An eyewitness Account of the Discovery of Electroshock. *Convulsive Therapy* 41:40-49.
- Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. *Nature* 422:198-207.
- Agassandian C, Plantier M, Fattoum A, Represa A, der Terrossian E (2000) Subcellular distribution of calponin and caldesmon in rat hippocampus. *Brain Res* 887:444-449.
- Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. *J Neurosci Res* 85:525-535.
- Alamed J, Wilcock DM, Diamond DM, Gordon MN, Morgan D (2006) Two-day radial-arm water maze learning and memory task; robust resolution of amyloid-related memory deficits in transgenic mice. *Nat Protoc* 1:1671-1679.
- Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* 3:36-44.
- Alonso R, Griebel G, Pavone G, Stemmelin J, Le Fur G, Soubrie P (2004) Blockade of CRF(1) or V(1b) receptors reverses stress-induced suppression of neurogenesis in a mouse model of depression. *Mol Psychiatr* 9:278-286, 224.
- Altar CA, Whitehead RE, Chen R, Wortwein G, Madsen TM (2003) Effects of electroconvulsive seizures and antidepressant drugs on brain-derived neurotrophic factor protein in rat brain. *Biol Psychiatry* 54:703-709.
- Altar CA, Laeng P, Jurata LW, Brockman JA, Lemire A, Bullard J, Bukhman YV, Young TA, Charles V, Palfreyman MG (2004) Electroconvulsive seizures regulate gene expression of distinct neurotrophic signaling pathways. *J Neurosci* 24:2667-2677.
- Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol* 124:319-335.
- American Psychiatric Association, ed (1994) *Diagnostic and Statistical Manual of Mental Disorders IV*, 4th Edition. Washington D.C.
- Amersham Biosciences (2002) *Ettan DiGE System User Manual* 18-1173-17.
- Amersham Biosciences (2002) *IEF User Manual*. In: *Ettan IPGphor Isoelectric focusing system 2D electrophoresis and first dimension* In: Amersham Biosciences.
- An X, Debnath G, Guo X, Liu S, Lux SE, Baines A, Gratzner W, Mohandas N (2005) Identification and functional characterization of protein 4.1R and actin-binding sites in erythrocyte beta spectrin: regulation of the interactions by phosphatidylinositol-4,5-bisphosphate. *Biochemistry* 44:10681-10688.

- Angelucci F, Aloe L, Jimenez-Vasquez P, Mathe AA (2002) Electroconvulsive stimuli alter the regional concentrations of nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor in adult rat brain. *Journal of ECT* 18:138-143.
- Angelucci F, Aloe L, Jimenez-Vasquez P, Mathe AA (2003) Electroconvulsive stimuli alter nerve growth factor but not brain-derived neurotrophic factor concentrations in brains of a rat model of depression. *Neuropeptides* 37:51-56.
- Appel K, Schwahn C, Mahler J, Schulz A, Spitzer C, Fenske K, Stender J, Barnow S, John U, Teumer A, Biffar R, Nauck M, Volzke H, Freyberger HJ, Grabe HJ (2011) Moderation of Adult Depression by a Polymorphism in the FKBP5 Gene and Childhood Physical Abuse in the General Population. *Neuropsychopharmacology* 36:1982-1991.
- Appella E, Padlan EA, Hunt DF (1995) Analysis of the structure of naturally processed peptides bound by class I and class II major histocompatibility complex molecules. *Exs* 73:105-119.
- Applegate D, Feng W, Green RS, Taubman MB (1994) Cloning and expression of a novel acidic calponin isoform from rat aortic vascular smooth muscle. *J Biol Chem* 269:10683-10690.
- Arch RH, Gedrich RW, Thompson CB (1998) Tumor necrosis factor receptor-associated factors (TRAFs)--a family of adapter proteins that regulates life and death. *Genes Dev* 12:2821-2830.
- Arnone D, McIntosh AM, Ebmeier KP, Munafo MR, Anderson IM (2011) Magnetic resonance imaging studies in unipolar depression: Systematic review and meta-regression analyses. *Eur Neuropsychopharmacol*.
- Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 25:25-29.
- Aubry JM, Gervasoni N, Osiek C, Perret G, Rossier MF, Bertschy G, Bondolfi G (2007) The DEX/CRH neuroendocrine test and the prediction of depressive relapse in remitted depressed outpatients. *J Psychiatr Res* 41:290-294.
- Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng PF, Kavalali ET, Monteggia LM (2011) NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature*.
- Aydemir C, Yalcin ES, Aksaray S, Kisa C, Yildirim SG, Uzbay T, Goka E (2006) Brain-derived neurotrophic factor (BDNF) changes in the serum of depressed women. *Prog Neuropsychopharmacol Biol Psychiatry* 30:1256-1260.
- Azima H, Vispo RH (1958) Imipramine; a potent new anti-depressant compound. *Am J Psychiat* 115:245-246.
- Balu DT, Hoshaw BA, Malberg JE, Rosenzweig-Lipson S, Schechter LE, Lucki I (2008) Differential regulation of central BDNF protein levels by antidepressant and non-antidepressant drug treatments. *Brain Res* 1211:37-43.
- Banerjee SB, Rajendran R, Dias BG, Ladiwala U, Tole S, Vaidya VA (2005) Recruitment of the Sonic hedgehog signalling cascade in electroconvulsive seizure-mediated regulation of adult rat hippocampal neurogenesis. *Eur J Neurosci* 22:1570-1580.
- Barch DM, Carter CS, MacDonald AW, 3rd, Braver TS, Cohen JD (2003) Context-processing deficits in schizophrenia: diagnostic specificity, 4-week course, and relationships to clinical symptoms. *Journal of abnormal psychology* 112:132-143.

- Barrientos RM, Sprunger DB, Campeau S, Higgins EA, Watkins LR, Rudy JW, Maier SF (2003) Brain-derived neurotrophic factor mRNA downregulation produced by social isolation is blocked by intrahippocampal interleukin-1 receptor antagonist. *Neuroscience* 121:847-853.
- Baudier J, Bronner C, Kligman D, Cole RD (1989) Protein kinase C substrates from bovine brain. Purification and characterization of neuromodulin, a neuron-specific calmodulin-binding protein. *J Biol Chem* 264:1824-1828.
- Bayes A, Grant SG (2009) Neuroproteomics: understanding the molecular organization and complexity of the brain. *Nat Rev Neurosci* 10:635-646.
- Beasley CL, Pennington K, Behan A, Wait R, Dunn MJ, Cotter D (2006) Proteomic analysis of the anterior cingulate cortex in the major psychiatric disorders: Evidence for disease-associated changes. *Proteomics* 6:3414-3425.
- Beatty WW, Bierley RA, Rush JR (1985) Spatial memory in rats- electroconvulsive shock selectively disrupts working memory but spares reference memory. *Behav Neural Biol* 44:403-414.
- Behan AT, Byrne C, Dunn MJ, Cagney G, Cotter DR (2009) Proteomic analysis of membrane microdomain-associated proteins in the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder reveals alterations in LAMP, STXBP1 and BASP1 protein expression. *Mol Psychiatr* 14:601-613.
- Bennett V, Healy J (2008) Organizing the fluid membrane bilayer: diseases linked to spectrin and ankyrin. *Trends Mol Med* 14:28-36.
- Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science* 311:864-868.
- Berton O, Nestler EJ. (2006) New approaches to antidepressant drug discovery: beyond monoamines. *Nat Rev Neurosci*. 7(2):137-51.
- Besedovsky H, del Rey A, Sorkin E, Dinarello CA (1986) Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science* 233:652-654.
- Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA, Almeida OFX, Sousa N (2009) The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol Psychiatr* 14:764-773.
- Bhagwagar Z, Hafizi S, Cowen PJ (2005) Increased salivary cortisol after waking in depression. *Psychopharmacology (Berl)* 182:54-57.
- Binder EB et al. (2004) Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. *Nature Genet* 36:1319-1325.
- Bland ST, Tamlyn JP, Barrientos RM, Greenwood BN, Watkins LR, Campeau S, Day HE, Maier SF (2007) Expression of fibroblast growth factor-2 and brain-derived neurotrophic factor mRNA in the medial prefrontal cortex and hippocampus after uncontrollable or controllable stress. *Neuroscience* 144:1219-1228.
- Blendy JA (2006) The role of CREB in depression and antidepressant treatment. *Biological Psychiatry* 59:1144-1150.

- Bloch RG, Dooneief AS, Buchberg AS, Spellman S (1954) The clinical effect of isoniazid and iproniazid in the treatment of pulmonary tuberculosis. *Annals of Internal Medicine* 40:881-900.
- Bocchio-Chiavetto L, Zanardini R, Bortolomasi M, Abate M, Segala M, Giacomuzzi M, Riva MA, Marchina E, Pasqualetti P, Perez J, Gennarelli M (2006) Electroconvulsive Therapy (ECT) increases serum Brain Derived Neurotrophic Factor (BDNF) in drug resistant depressed patients. *Eur Neuropsychopharmacol* 16:620-624.
- Bocchio-Chiavetto L, Bagnardi V, Zanardini R, Molteni R, Nielsen MG, Placentino A, Giovannini C, Rilloso L, Ventriglia M, Riva MA, Gennarelli M (2010) Serum and plasma BDNF levels in major depression: a replication study and meta-analyses. *World J Biol Psychiatry* 11:763-773.
- Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* 286:1358-1362.
- Bowley MP, Drevets WC, Ongur D, Price JL (2002) Low glial numbers in the amygdala in major depressive disorder. *Biological Psychiatry* 52:404-412.
- Bradford MM, (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-54.
- Bremner JD, Narayan M, Anderson ER, Staib LH, Miller HL, Charney DS (2000) Hippocampal volume reduction in major depression. *Am J Psychiat* 157:115-118.
- Calverley RK, Jones DG (1990) Contributions of dendritic spines and perforated synapses to synaptic plasticity. *Brain Research Brain Research Reviews* 15:215-249.
- Cameron HA, McKay RD (2001) Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *J Comp Neurol* 435:406-417.
- CAMO I (2009) Introduction to Partial Least Squares regression (PLS) Help Section, PLS Procedure, PLS Theory Chapter. In: PLS Procedure The Unscrambler X. Norway: CAMO A/S www.camo.com.
- CAMO II (2009) Predefined PLS Plots: Regression Overview Help Section, PLS Procedure, PLS Theory Chapter. In: Unscrambler X: PLS Procedure Manual. Norway: CAMO A/S www.camo.com.
- CAMO III (2009) Variable selection and model stability (Tutorial M) Help Section, PLS Procedure, PLS Theory Chapter. In: UnscramblerX: PLS Procedure Manual. Norway: CAMO A/S www.camo.com.
- CAMO IV (2009) PLS Discriminant Analysis (PLS-DA): Tutorial H Help Section, PLS Procedure, PLS Theory Chapter. In: Unscrambler X: PLS Procedure Manual. Norway: CAMO A/S www.camo.com.
- CAMO V (2011) What is Multivariate Analysis? An introduction to the principles and common models used in multivariate analysis. In: Norway: CAMO A/S www.camo.com.
- Campbell S, Macqueen G (2004a) The role of the hippocampus in the pathophysiology of major depression. *J Psychiatry Neurosci* 29:417-426.
- Campbell S, Marriott M, Nahmias C, MacQueen GM (2004b) Lower hippocampal volume in patients suffering from depression: a meta-analysis. *Am J Psychiat* 161:598-607.
- Cao L, Jiao X, Zuzga DS, Liu Y, Fong DM, Young D, During MJ (2004) VEGF links hippocampal activity with neurogenesis, learning and memory. *Nature Genet* 36:827-835.

- Carboni L, Vighini M, Piubelli C, Castelletti L, Milli A, Domenici E (2006b) Proteomic analysis of rat hippocampus and frontal cortex after chronic treatment with fluoxetine or putative novel antidepressants: CRF1 and NK1 receptor antagonists. *Eur Neuropsychopharmacol* 16:521-537.
- Carboni L, Piubelli C, Pozzato C, Astner H, Arban R, Righetti PG, Hamdan M, Domenici E (2006a) Proteomic analysis of rat hippocampus after repeated psychosocial stress. *Neuroscience* 137:1237-1246.
- Carboni L, Becchi S, Piubelli C, Mallei A, Giambelli R, Razzoli M, Mathe AA, Popoli M, Domenici E (2010) Early-life stress and antidepressants modulate peripheral biomarkers in a gene-environment rat model of depression. *Progress in Neuropsychopharmacology and Biological Psychiatry* 34:1037-1048.
- Carboni L, Piubelli C, Vighini M, El Khoury A, Gruber S, Anderssen W, Mathe AA, Aitchison KJ, Mallei A, Giambelli R, Popoli M, Domenici E (2006c) Proteomic analysis of hippocampus and frontal cortex in a rat model of depression with gene-environment interaction and antidepressant treatment. *Eur Neuropsychopharmacol* 16:S336-S337.
- Carek PJ, Laibstein SE, Carek SM (2011) Exercise for the treatment of depression and anxiety. *International journal of psychiatry in medicine* 41:15-28.
- Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, McClay J, Mill J, Martin J, Braithwaite A, Poulton R (2003) Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 301:386-389.
- Castren E, Rantamäki T (2009) The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. *Dev Neurobiol.* 70(5):289-97.
- Castrén E, Rantamäki T (2010). Role of brain-derived neurotrophic factor in the aetiology of depression: implications for pharmacological treatment. *CNS Drugs.* 24(1):1-7
- Cecconi D, Mion S, Astner H, Domenici E, Righetti PG, Carboni L (2007) Proteomic analysis of rat cortical neurons after fluoxetine treatment. *Brain Res* 1135:41-51.
- Celada P, Siuciak JA, Tran TM, Altar CA, Tepper JM (1996) Local infusion of brain-derived neurotrophic factor modifies the firing pattern of dorsal raphe serotonergic neurons. *Brain Res* 712:293-298.
- Cerqueira JJ, Mailliet F, Almeida OF, Jay TM, Sousa N (2007) The prefrontal cortex as a key target of the maladaptive response to stress. *J Neurosci* 27:2781-2787.
- Chang JW, Schumacher E, Coulter PM, 2nd, Vinters HV, Watson JB (1997) Dendritic translocation of RC3/neurogranin mRNA in normal aging, Alzheimer disease and fronto-temporal dementia. *J Neuropathol Exp Neurol* 56:1105-1118.
- Chao HM, Sakai RR, Ma LY, McEwen BS (1998) Adrenal steroid regulation of neurotrophic factor expression in the rat hippocampus. *Endocrinology* 139:3112-3118.
- Chen F, Madsen TM, Wegener G, Nyengaard JR (2008a) Changes in rat hippocampal CA1 synapses following imipramine treatment. *Hippocampus* 18:631-639.
- Chen F, Madsen TM, Wegener G, Nyengaard JR (2009) Repeated electroconvulsive seizures increase the total number of synapses in adult male rat hippocampus. *Eur Neuropsychopharmacol* 19:329-338.

- Chen B, Dowlathshahi D, MacQueen GM, Wang JF, Young LT (2001b) Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. *Biological Psychiatry* 50:260-265.
- Chen WQ, Viidik A, Skalicky M, Hoger H, Lubec G (2007) Hippocampal signaling cascades are modulated in voluntary and treadmill exercise rats. *Electrophoresis* 28:4392-4400.
- Chen A, Liao WP, Lu Q, Wong WS, Wong PT (2007b) Upregulation of dihydropyrimidinase-related protein 2, spectrin alpha II chain, heat shock cognate protein 70 pseudogene 1 and tropomodulin 2 after focal cerebral ischemia in rats—a proteomics approach. *Neurochem Int* 50:1078-1086.
- Chen ACH, Eisch AJ, Sakai N, Takahashi M, Nestler EJ, Duman RS (2001) Regulation of GFR alpha-1 and GFR alpha-2 mRNAs in rat brain by electroconvulsive seizure. *Synapse* 39:42-50.
- Chen L, Lawlor DA, Lewis SJ, Yuan W, Abdollahi MR, Timpon NJ, Day IN, Ebrahim S, Smith GD, Shugart YY (2008b) Genetic association study of BDNF in depression: finding from two cohort studies and a meta-analysis. *American Journal of Medical Genetics Part B Neuropsychiatric Genetics* 147B:814-821.
- Chin PC, Majdzadeh N, D'Mello SR (2005) Inhibition of GSK3beta is a common event in neuroprotection by different survival factors. *Brain Research Molecular Brain Research* 137:193-201.
- Chitteti BR, Liu Y, Srour EF (2011) Genomic and proteomic analysis of the impact of mitotic quiescence on the engraftment of human CD34+ cells. *PLoS One* 6:e17498.
- Churchill R, Hunot V, Corney R, Knapp M, McGuire H, Tylee A, Wessely S (2001) A systematic review of controlled trials of the effectiveness and cost-effectiveness of brief psychological treatments for depression. *Health Technology Assessment* 5:1-173.
- Cianci CD, Zhang Z, Pradhan D, Morrow JS (1999) Brain and muscle express a unique alternative transcript of alphaII spectrin. *Biochemistry* 38:15721-15730.
- Cipriani A, Furukawa TA, Salanti G, Geddes JR, Higgins JP, Churchill R, Watanabe N, Nakagawa A, Omori IM, McGuire H, Tansella M, Barbui C (2009) Comparative efficacy and acceptability of 12 new-generation antidepressants: a multiple-treatments meta-analysis. *Lancet* 373:746-758.
- Coe CL, Kramer M, Czeh B, Gould E, Reeves AJ, Kirschbaum C, Fuchs E (2003) Prenatal stress diminishes neurogenesis in the dentate gyrus of juvenile rhesus monkeys. *Biological Psychiatry* 54:1025-1034.
- Colbran RJ, Brown AM (2004) Calcium/calmodulin-dependent protein kinase II and synaptic plasticity. *Curr Opin Neurobiol* 14:318-327.
- Coldren CD, Lai Z, Shragg P, Rossi E, Glidewell SC, Zuffardi O, Mattina T, Ivy DD, Curfs LM, Mattson SN, Riley EP, Treier M, Grossfeld PD (2009) Chromosomal microarray mapping suggests a role for BSX and Neurogranin in neurocognitive and behavioral defects in the 11q terminal deletion disorder (Jacobsen syndrome). *Neurogenetics* 10:89-95.
- Cole J, Costafreda SG, McGuffin P, Fu CH (2011) Hippocampal atrophy in first episode depression: A meta-analysis of magnetic resonance imaging studies. *J Affect Disord* 134:483-487.
- Cole J, Toga AW, Hojatkashani C, Thompson P, Costafreda SG, Cleare AJ, Williams SC, Bullmore ET, Scott JL, Mitterschiffthaler MT, Walsh ND, Donaldson C, Mirza M, Marquand A, Nosarti C, McGuffin P, Fu CH (2010) Subregional hippocampal deformations in major depressive disorder. *J Affect Disord* 126:272-277.

- Comery TA, Harris JB, Willems PJ, Oostra BA, Irwin SA, Weiler IJ, Greenough WT (1997) Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. *Proc Natl Acad Sci U S A* 94:5401-5404.
- Conrad CD (2010) A critical review of chronic stress effects on spatial learning and memory. *Prog Neuropsychopharmacol Biol Psychiatry* 34:742-755.
- Conti AC, Cryan JF, Dalvi A, Lucki I, Blendy JA (2002) cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or endocrine responses to antidepressant drugs. *J Neurosci* 22:3262-3268.
- Cook SC, Wellman CL (2004) Chronic stress alters dendritic morphology in rat medial prefrontal cortex. *Journal of Neurobiology* 60:236-248.
- Coppen A, Wood K (1978) Tryptophan and depressive illness. *Psychol Med* 8:49-57.
- Costafreda SG, Chu C, Ashburner J, Fu CH (2009) Prognostic and diagnostic potential of the structural neuroanatomy of depression. *PLoS ONE* 4:e6353.
- Cotter D, Hudson L, Landau S (2005) Evidence for orbitofrontal pathology in bipolar disorder and major depression, but not in schizophrenia. *Bipolar Disord* 7:358-369.
- Cotter D, Mackay D, Landau S, Kerwin R, Everall I (2001) Reduced glial cell density and neuronal size in the anterior cingulate cortex in major depressive disorder. *Arch Gen Psychiatry* 58:545-553.
- Cox PR, Zoghbi HY (2000) Sequencing, expression analysis, and mapping of three unique human tropomodulin genes and their mouse orthologs. *Genomics* 63:97-107.
- Cox PR, Fowler V, Xu B, Sweatt JD, Paylor R, Zoghbi HY (2003) Mice lacking Tropomodulin-2 show enhanced long-term potentiation, hyperactivity, and deficits in learning and memory. *Mol Cell Neurosci* 23:1-12.
- Crews L, Ruf R, Patrick C, Dumaop W, Trejo-Morales M, Achim CL, Rockenstein E, Masliah E (2011) Phosphorylation of collapsin response mediator protein-2 disrupts neuronal maturation in a model of adult neurogenesis: Implications for neurodegenerative disorders. *Molecular neurodegeneration* 6:67.
- Crossley NA, Bauer M (2007) Acceleration and augmentation of antidepressants with lithium for depressive disorders: two meta-analyses of randomized, placebo-controlled trials. *Journal of Clinical Psychiatry* 68:935-940.
- Crow TJ (2011) 'The missing genes: what happened to the heritability of psychiatric disorders?'. *Mol Psychiatry* 16:362-364.
- Cryan JF, Markou A, Lucki I (2002) Assessing antidepressant activity in rodents: recent developments and future needs. *Trends Pharmacol Sci* 23:238-245.
- Cuijpers P, van Straten A, Andersson G, van Oppen P (2008) Psychotherapy for depression in adults: a meta-analysis of comparative outcome studies. *Journal of Consulting and Clinical Psychology* 76:909-922.
- Cuijpers P, Smit F, Bohlmeijer E, Hollon SD, Andersson G (2010) Efficacy of cognitive-behavioural therapy and other psychological treatments for adult depression: meta-analytic study of publication bias. *Br J Psychiatry* 196:173-178.

- Czeh B, Michaelis T, Watanabe T, Frahm J, de Biurrun G, van Kampen M, Bartolomucci A, Fuchs E (2001) Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. *Proc Natl Acad Sci U S A* 98:12796-12801.
- Dahl A, Eriksson PS, Persson AI, Karlsson G, Davidsson P, Ekman R, Westman-Brinkmalm A (2003) Proteome analysis of conditioned medium from cultured adult hippocampal progenitors. *Rapid Commun Mass Spectrom* 17:2195-2202.
- Danninger C, Gimona M (2000) Live dynamics of GFP-calponin: isoform-specific modulation of the actin cytoskeleton and autoregulation by C-terminal sequences. *Journal of cell science* 113 Pt 21:3725-3736.
- Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* 9:46-56.
- Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91:231-241.
- David DJ, Samuels BA, Rainer Q, Wang JW, Marsteller D, Mendez I, Drew M, Craig DA, Guiard BP, Guilloux JP, Artymyshyn RP, Gardier AM, Gerald C, Antonijevic IA, Leonardo ED, Hen R (2009) Neurogenesis-Dependent and -Independent Effects of Fluoxetine in an Animal Model of Anxiety/Depression. *Neuron* 62:479-493.
- De Matteis MA, Morrow JS (2000) Spectrin tethers and mesh in the biosynthetic pathway. *Journal of cell science* 113 (Pt 13):2331-2343.
- de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 396:474-477.
- Dechant G, Barde YA (2002) The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat Neurosci* 5:1131-1136.
- Delahunty C, Yates JR, 3rd (2005) Protein identification using 2D-LC-MS/MS. *Methods* 35:248-255.
- d'Elia G (1970) Unilateral Electroconvulsive Therapy. *Acta Psychiatr Scand Suppl*:1-98.
- Denicoff KD, Rubinow DR, Papa MZ, Simpson C, Seipp CA, Lotze MT, Chang AE, Rosenstein D, Rosenberg SA (1987) The neuropsychiatric effects of treatment with interleukin-2 and lymphokine-activated killer cells. *Annals of Internal Medicine* 107:293-300.
- Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome biology* 4:P3.
- Desmyter S, van Heeringen C, Audenaert K (2011) Structural and functional neuroimaging studies of the suicidal brain. *Progress in Neuropsychopharmacology and Biological Psychiatry* 35:796-808.
- Detke MJ, Rickels M, Lucki I (1995) Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology (Berl)* 121:66-72.
- Di Carli M, Zamboni A, Pe ME, Pezzotti M, Lilley KS, Benvenuto E, Desiderio A (2011) Two-dimensional differential in gel electrophoresis (2D-DIGE) analysis of grape berry proteome during postharvest withering. *J Proteome Res* 10:429-446.

- Diamond DM, Park CR, Heman KL, Rose GM (1999) Exposing rats to a predator impairs spatial working memory in the radial arm water maze. *Hippocampus* 9:542-552.
- Dias BG, Banerjee SB, Duman RS, Vaidya VA (2003) Differential regulation of brain derived neurotrophic factor transcripts by antidepressant treatments in the adult rat brain. *Neuropharmacology* 45:553-563.
- Diez-Guerra FJ (2010) Neurogranin, a link between calcium/calmodulin and protein kinase C signaling in synaptic plasticity. *IUBMB life* 62:597-606.
- Dimmer EC, Huntley RP, Barrell DG, Binns D, Draghici S, Camon EB, Hubank M, Talmud PJ, Apweiler R, Lovering RC (2008) The Gene Ontology - Providing a Functional Role in Proteomic Studies. *Proteomics*.
- Ditzen C, Tang N, Jastorff AM, Teplytska L, Yassouridis A, Maccarrone G, Uhr M, Bronisch T, Miller CA, Holsboer F, Turck CW (2011) Cerebrospinal Fluid Biomarkers for Major Depression Confirm Relevance of Associated Pathophysiology. *Neuropsychopharmacology*.
- Dong J, Min S, Wei K, Li P, Cao J, Li Y (2010) Effects of electroconvulsive therapy and propofol on spatial memory and glutamatergic system in hippocampus of depressed rats. *J ECT* 26:126-130.
- Dranovsky A, Hen R (2006) Hippocampal neurogenesis: regulation by stress and antidepressants. *Biological Psychiatry* 59:1136-1143.
- Drevets WC (2001) Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. *Current Opinions in Neurobiology* 11:240-249.
- Drevets WC, Price JL (2005) Neuroimaging and Neuropathological Studies of Mood Disorders. . In: *Biology of Depression: From Novel Insights to Therapeutic Strategies*. (Lininio JWM, ed). Weinheim: Wiley-VCH Verlag GmbH & Co.
- Drevets WC, Bogers W, Raichle ME (2002) Functional anatomical correlates of antidepressant drug treatment assessed using PET measures of regional glucose metabolism. *Eur Neuropsychopharmacol* 12:527-544.
- Drevets WC, Gadde K, Krishnan KRR (2004) Neuroimaging Studies of Depression. In: *The neurobiological foundation of mental illness*, 2nd Edition (Charney DS, Nestler EJ, Bunney BS, eds). New York: Oxford University Press.
- D'Sa C, Duman RS (2002) Antidepressants and neuroplasticity. *Bipolar Disord* 4:183-194.
- Dubitzky W, Granzow M, Berrar D, P. (2007) Introduction to Genomic and Proteomic Data Analysis. In: *Fundamentals of data mining in genomics and proteomics* (Dubitzky W, Granzow M, Berrar D, P., eds). New York: Springer.
- Duman RS (2004) Depression: A case of neuronal life and death? *Biological Psychiatry* 56:140-145.
- Duman RS, Monteggia LM (2006) A neurotrophic model for stress-related mood disorders. *Biological Psychiatry* 59:1116-1127.
- Duman RS, Malberg J, Thome J (1999) Neural plasticity to stress and antidepressant treatment. *Biological Psychiatry* 46:1181-1191.
- Duman RS, Malberg J, Nakagawa S (2001) Regulation of adult neurogenesis by psychotropic drugs and stress. *J Pharmacol Exp Ther* 299:401-407.

- Duman CH, Schlesinger L, Kodama M, Russell DS, Duman RS (2007) A role for MAP kinase signaling in behavioral models of depression and antidepressant treatment. *Biological Psychiatry* 61:661-670.
- Dunn AJ, Swiergiel AH, de Beaurepaire R (2005) Cytokines as mediators of depression: what can we learn from animal studies? *Neurosci Biobehav Rev* 29:891-909.
- Dutta S, Chiu YC, Probert AW, Wang KK (2002) Selective release of calpain produced alphaII-spectrin (alpha-fodrin) breakdown products by acute neuronal cell death. *Biological chemistry* 383:785-791.
- Dwivedi Y, Roberts R, Conley R, Pandey GN (2006a) Extracellular signal-regulated kinase (ERK)-5 in postmortem brain of suicide subjects. *J Neurochem* 98:29-29.
- Dwivedi Y, Rizavi HS, Conley RR, Pandey GN (2006b) ERK MAP kinase signaling in post-mortem brain of suicide subjects: differential regulation of upstream Raf kinases Raf-1 and B-Raf. *Mol Psychiatry* 11:86-98.
- Dwivedi Y, Conley RR, Roberts RC, Tamminga CA, Pandey GN (2003a) Tyrosine receptor kinase B expression in postmortem brain of suicide victims. *Biological Psychiatry* 53:409.
- Dwivedi Y, Mondal AC, Shukla PK, Rizavi HS, Lyons J (2004) Altered protein kinase A in brain of learned helpless rats: Effects of acute and repeated stress. *Biological Psychiatry* 56:30-40.
- Dwivedi Y, Rizavi HS, Roberts RC, Conley RC, Tamminga CA, Pandey GN (2001) Reduced activation and expression of ERK1/2 MAP kinase in the post-mortem brain of depressed suicide subjects. *J Neurochem* 77:916-928.
- Dwivedi Y, Rizavi HS, Conley RR, Roberts RC, Tamminga CA, Pandey GN (2003c) Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase B in postmortem brain of suicide subjects. *Arch Gen Psychiatry* 60:804-815.
- Dwivedi Y, Rizavi HS, Zhang H, Roberts RC, Conley RR, Pandey GN (2009) Aberrant extracellular signal-regulated kinase (ERK)1/2 signalling in suicide brain: role of ERK kinase 1 (MEK1). *Int J Neuropsychopharmacol* 12:1337-1354.
- Dwivedi Y, Rao JS, Rizavi HS, Kotowski J, Conley RR, Roberts RC, Tamminga CA, Pandey GN (2003b) Abnormal expression and functional characteristics of cyclic adenosine monophosphate response element binding protein in postmortem brain of suicide subjects. *Arch Gen Psychiatry* 60:273-282.
- Dwivedi Y, Rizavi HS, Teppen T, Sasaki N, Chen H, Zhang H, Roberts RC, Conley RR, Pandey GN (2007) Aberrant extracellular signal-regulated kinase (ERK) 5 signaling in hippocampus of suicide subjects. *Neuropsychopharmacology* 32:2338-2350.
- Edgar PF, Schonberger SJ, Dean B, Faull RL, Kydd R, Cooper GJ (1999) A comparative proteome analysis of hippocampal tissue from schizophrenic and Alzheimer's disease individuals. *Mol Psychiatry* 4:173-178.
- Eisch AJ, Bolanos CA, de Wit J, Simonak RD, Pudiak CM, Barrot M, Verhaagen J, Nestler EJ (2003) Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: A role in depression. *Biological Psychiatry* 54:994-1005.
- Eldridge LL, Engel SA, Zeineh MM, Bookheimer SY, Knowlton BJ (2005) A dissociation of encoding and retrieval processes in the human hippocampus. *J Neurosci* 25:3280-3286.
- English JA, Dicker P, Focking M, Dunn MJ, Cotter DR (2009) 2-D DIGE analysis implicates cytoskeletal abnormalities in psychiatric disease. *Proteomics* 9:3368-3382.

- Eranti S, Mogg A, Pluck G, Landau S, Purvis R, Brown RG, Howard R, Knapp M, Philpot M, Rabe-Hesketh S, Romeo R, Rothwell J, Edwards D, McLoughlin DM (2007) A randomized, controlled trial with 6-month follow-up of repetitive transcranial magnetic stimulation and electroconvulsive therapy for severe depression. *Am J Psychiat* 164:73-81.
- Ernfors P, Lee KF, Jaenisch R (1994) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* 368:147-150.
- Erondou NE, Kennedy MB (1985) Regional distribution of type II Ca²⁺/calmodulin-dependent protein kinase in rat brain. *J Neurosci* 5:3270-3277.
- Fath T, Fischer RS, Dehmelt L, Halpain S, Fowler VM (2011) Tropomodulins are negative regulators of neurite outgrowth. *Eur J Cell Biol* 90:291-300.
- Fedorova I, Salem N (2006) Omega-3 fatty acids and rodent behavior. *Prostaglandins Leukot Essent Fatty Acids* 75:271-289.
- Ferhat L, Rami G, Medina I, Ben-Ari Y, Represa A (2001) Process formation results from the imbalance between motor-mediated forces. *Journal of cell science* 114:3899-3904.
- Ferhat L, Esclapez M, Represa A, Fattoum A, Shirao T, Ben-Ari Y (2003) Increased levels of acidic calponin during dendritic spine plasticity after pilocarpine-induced seizures. *Hippocampus* 13:845-858.
- Ficarro SB, McClelland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat Biotechnol* 20:301-305.
- Fink M (2009) *Electroconvulsive Therapy A Guide for Professionals and their Patients* New York: Oxford University Press.
- Fink CC, Bayer KU, Myers JW, Ferrell JE, Jr., Schulman H, Meyer T (2003) Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. *Neuron* 39:283-297.
- Finkbeiner S. (2000) CREB couples neurotrophin signals to survival messages. *Neuron* 25(1):11-4.
- Fochtmann LJ (1994) Animal studies of electroconvulsive therapy: foundations for future research. *Psychopharmacology bulletin* 30:321-444.
- Folkerts HW, Michael N, Tolle R, Schonauer K, Mucke S, Schulze-Monking H (1997) Electroconvulsive therapy vs. paroxetine in treatment-resistant depression -- a randomized study. *Acta Psychiatr Scand* 96:334-342.
- Fountoulakis M, Tsangaris GT, Maris A, Lubec G (2005) The rat brain hippocampus proteome. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences* 819:115-129.
- Fox HA, Rosen A, Campbell RJ (1989) Are brief pulse and sine wave ECT equally efficient? *J Clin Psychiatry* 50:432-435.
- Frenois F, Moreau M, O'Connor J, Lawson M, Micon C, Lestage J, Kelley KW, Dantzer R, Castanon N (2007) Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior. *Psychoneuroendocrinology* 32:516-531.

- Frey D, Laux T, Xu L, Schneider C, Caroni P (2000) Shared and unique roles of CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomical plasticity. *J Cell Biol* 149:1443-1454.
- Frodl T, Jager M, Smajstrlova I, Born C, Bottlender R, Palladino T, Reiser M, Moller HJ, Meisenzahl EM (2008a) Effect of hippocampal and amygdala volumes on clinical outcomes in major depression: a 3-year prospective magnetic resonance imaging study. *Journal of Psychiatry and Neuroscience* 33:423-430.
- Frodl T, Meisenzahl EM, Zetsche T, Born C, Groll C, Jager M, Leinsinger G, Bottlender R, Hahn K, Moller HJ (2002) Hippocampal changes in patients with a first episode of major depression. *Am J Psychiat* 159:1112-1118.
- Frodl T, Meisenzahl EM, Zetsche T, Hohne T, Banac S, Schorr C, Jager M, Leinsinger G, Bottlender R, Reiser M, Moller HJ (2004) Hippocampal and amygdala changes in patients with major depressive disorder and healthy controls during a 1-year follow-up. *Journal of Clinical Psychiatry* 65:492-499.
- Frodl T, Koutsouleris N, Bottlender R, Born C, Jager M, Morgenthaler M, Scheuerecker J, Zill P, Baghai T, Schule C, Rupprecht R, Bondy B, Reiser M, Moller HJ, Meisenzahl EM (2008b) Reduced gray matter brain volumes are associated with variants of the serotonin transporter gene in major depression. *Mol Psychiatry* 13:1093-1101.
- Fujita A, Nakaaki S, Segawa K, Azuma H, Sato K, Arahata K, Otsuki K, Hori M, Mochida Y, Uchida M, Yamada T, Nakamura C, Akechi T, Furukawa TA (2006) Memory, attention, and executive functions before and after sine and pulse wave electroconvulsive therapies for treatment-resistant major depression. *J ECT* 22:107-112.
- Fukui Y, Masuda H, Takagi M, Takahashi K, Kiyokane K (1997) The presence of h2-calponin in human keratinocyte. *Journal of dermatological science* 14:29-36.
- Gabbard GO, ed (2007a) Gabbards treatment of psychiatric disorders, 4th Edition. Arlington: American Psychiatric Association Publishing Inc.
- Gabbard GO, Beck JS, Homlmes J, eds (2007b) Oxford Textbook of Psychotherapy. Oxford: Oxford University Press.
- Gage FH (2000) Mammalian neural stem cells. *Science* 287:1433-1438.
- Garfin DE (2003) Gel Electrophoresis of Proteins. In: *Essential Cell Biology: Cell Structure, A Practical Approach* (Davey J, Lord M, eds). Oxford, UK: Oxford University Press.
- Gene Ontology Consortium (2001) Creating the gene ontology resource: design and implementation. *Genome research* 11:1425-1433.
- Gerendasy DD, Herron SR, Jennings PA, Sutcliffe JG (1995) Calmodulin stabilizes an amphiphilic alpha-helix within RC3/neurogranin and GAP-43/neuromodulin only when Ca²⁺ is absent. *J Biol Chem* 270:6741-6750.
- Gerritsen L, Tendolkar I, Franke B, Vasquez AA, Kooijman S, Buitelaar J, Fernandez G, Rijpkema M (2011) BDNF Val66Met genotype modulates the effect of childhood adversity on subgenual anterior cingulate cortex volume in healthy subjects. *Mol Psychiatr*.
- Gersner R, Dar DE, Shabat-Simon M, Zangen A (2005) Behavioral analysis during the forced swimming test using a joystick device. *J Neurosci Methods* 143:117-121.
- Gersner R, Toth E, Isserles M, Zangen A (2009) Site-specific antidepressant effects of repeated subconvulsive electrical stimulation: potential role of brain-derived neurotrophic factor. *Biological Psychiatry* 67:125-132.

- Gharbi S, Gaffney P, Yang A, Zvelebil MJ, Cramer R, Waterfield MD, Timms JF (2002) Evaluation of two-dimensional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. *Molecular & cellular proteomics* : MCP 1:91-98.
- Giepmans BN, Adams SR, Ellisman MH, Tsien RY (2006) The fluorescent toolbox for assessing protein location and function. *Science* 312:217-224.
- Gimona, Small (1996) *Biochemistry of smooth muscle contraction*. San Diego: Academic Press.
- Giovannini MG (2006) The role of the extracellular signal-regulated kinase pathway in memory encoding. *Reviews in the Neurosciences* 17:619-634.
- Gokhin DS, Lewis RA, McKeown CR, Nowak RB, Kim NE, Littlefield RS, Lieber RL, Fowler VM (2010) Tropomodulin isoforms regulate thin filament pointed-end capping and skeletal muscle physiology. *J Cell Biol* 189:95-109.
- Gold PW, Chrousos GP (1999) The endocrinology of melancholic and atypical depression: relation to neurocircuitry and somatic consequences. *Proceedings of the Association of American Physicians* 111:22-34.
- Gold PW, Chrousos GP (2002) Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states. *Mol Psychiatr* 7:254-275.
- Golden SH, Malhotra S, Wand GS, Brancati FL, Ford D, Horton K (2007) Adrenal gland volume and dexamethasone-suppressed cortisol correlate with total daily salivary cortisol in African-American women. *Journal of Clinical Endocrinology and Metabolism* 92:1358-1363.
- Goldman D, Merrill CR, Ebert MH (1980) Two-dimensional gel electrophoresis of cerebrospinal fluid proteins. *Clin Chem* 26:1317-1322.
- Goley ED, Welch MD (2006) The ARP2/3 complex: an actin nucleator comes of age. *Nature reviews Molecular cell biology* 7:713-726.
- Goodman SR, Zagon IS, Kulikowski RR (1981) Identification of a spectrin-like protein in nonerythroid cells. *Proc Natl Acad Sci U S A* 78:7570-7574.
- Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 21:1037-1053.
- Gould E, Tanapat P, Rydel T, Hastings N (2000) Regulation of hippocampal neurogenesis in adulthood. *Biological Psychiatry* 48:715-720.
- Gould E, Reeves AJ, Fallah M, Tanapat P, Gross CG, Fuchs E (1999) Hippocampal neurogenesis in adult Old World primates. *Proceedings of the National Academy of Sciences (PNAS) USA* 96:5263-5267.
- Griffin EW, Bechara RG, Birch AM, Kelly AM (2009) Exercise enhances hippocampal-dependent learning in the rat: evidence for a BDNF-related mechanism. *Hippocampus* 19:973-980.
- Groves JO (2007) Is it time to reassess the BDNF hypothesis of depression? *Mol Psychiatr* 12:1079-1088.
- Guest PC, Knowles MR, Molon-Noblot S, Salim K, Smith D, Murray F, Laroque P, Hunt SP, de Felipe C, Rupniak NM, McAllister G (2004) Mechanisms of action of the antidepressants fluoxetine and the substance P antagonist L-000760735 are associated with altered neurofilaments and synaptic remodeling. *Brain Res* 1002:1-10.

- Hajszan T, MacLusky NJ, Leranath C (2005) Short-term treatment with the antidepressant fluoxetine triggers pyramidal dendritic spine synapse formation in rat hippocampus. *Eur J Neurosci* 21:1299-1303.
- Halim ND, Weickert CS, McClintock BW, Weinberger DR, Lipska BK (2004) Effects of chronic haloperidol and clozapine treatment on neurogenesis in the adult rat hippocampus. *Neuropsychopharmacology* 29:1063-1069.
- Hamidi M, Drevets WC, Price JL (2004) Glial reduction in amygdala in major depressive disorder is due to oligodendrocytes. *Biological Psychiatry* 55:563-569.
- Han X, Aslanian A, Yates JR, 3rd (2008) Mass spectrometry for proteomics. *Curr Opin Chem Biol* 12:483-490.
- Han Y, Yin H, Xu Y, Zhu Q, Luo J, Wang X, Chen G (2011) Increased expression of calponin-3 in epileptic patients and experimental rats. *Exp Neurol*.
- Hartwig JH, Kwiakowski DJ (1991) Actin-binding proteins. *Current opinion in cell biology* 3:87-97.
- Hasler G, Fromm S, Carlson PJ, Luckenbaugh DA, Waldeck T, Geraci M, Roiser JP, Neumeister A, Meyers N, Charney DS, Drevets WC (2008) Neural response to catecholamine depletion in unmedicated subjects with major depressive disorder in remission and healthy subjects. *Arch Gen Psychiatry* 65:521-531.
- Hastings RS, Parsey RV, Oquendo MA, Arango V, Mann JJ (2004) Volumetric analysis of the prefrontal cortex, amygdala, and hippocampus in major depression. *Neuropsychopharmacology* 29:952-959.
- Hauptman JS, DeSalles AA, Espinoza R, Sedrak M, Ishida W (2008) Potential surgical targets for deep brain stimulation in treatment-resistant depression. *Neurosurgical Focus* 25:E3.
- Heckers S, Konradi C (2010) Hippocampal pathology in schizophrenia. *Current Topics in Behavioral Neurosciences* 4:529-553.
- Heine VM, Zareno J, Maslam S, Joels M, Lucassen PJ (2005) Chronic stress in the adult dentate gyrus reduces cell proliferation near the vasculature and VEGF and Flk-1 protein expression. *Eur J Neurosci* 21:1304-1314.
- Hellsten J, Wennstrom M, Mohapel P, Ekdahl CT, Bengzon J, Tingstrom A (2002) Electroconvulsive seizures increase hippocampal neurogenesis after chronic corticosterone treatment. *Eur J Neurosci* 16:283-290.
- Henn FA, Vollmayr B (2004) Neurogenesis and depression: Etiology or epiphenomenon? *Biological Psychiatry* 56:146-150.
- Hensley K, Venkova K, Christov A, Gunning W, Park J (2011) Collapsin response mediator protein-2: an emerging pathologic feature and therapeutic target for neurodegeneration. *Mol Neurobiol* 43:180-191.
- Herholz K, Carter SF, Jones M (2007) Positron emission tomography imaging in dementia. *British Journal of Radiology* 80 Spec No 2:S160-167.
- Hettema JM, Annas P, Neale MC, Kendler KS, Fredrikson M (2003) A twin study of the genetics of fear conditioning. *Arch Gen Psychiatry* 60:702-708.
- Heuser I, Yassouridis A, Holsboer F (1994) The combined dexamethasone/CRH test: a refined laboratory test for psychiatric disorders. *J Psychiatr Res* 28:341-356.

- Heuser IJ, Schweiger U, Gotthardt U, Schmider J, Lammers CH, Dettling M, Yassouridis A, Holsboer F (1996) Pituitary-adrenal-system regulation and psychopathology during amitriptyline treatment in elderly depressed patients and normal comparison subjects. *Am J Psychiat* 153:93-99.
- Hickie I, Naismith S, Ward PB, Turner K, Scott E, Mitchell P, Wilhelm K, Parker G (2005) Reduced hippocampal volumes and memory loss in patients with early- and late-onset depression. *Br J Psychiatry* 186:197-202.
- Holick KA, Lee DC, Hen R, Dulawa SC (2008) Behavioral effects of chronic fluoxetine in BALB/cJ mice do not require adult hippocampal neurogenesis or the serotonin 1A receptor. *Neuropsychopharmacology* 33:406-417.
- Howland RH, Shutt LS, Berman SR, Spotts CR, Denko T (2011) The emerging use of technology for the treatment of depression and other neuropsychiatric disorders. *Annals of Clinical Psychiatry* 23:48-62.
- Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* 24:677-736.
- Huang KP, Huang FL, Chen HC (1993) Characterization of a 7.5-kDa protein kinase C substrate (RC3 protein, neurogranin) from rat brain. *Archives of biochemistry and biophysics* 305:570-580.
- Huang KP, Huang FL, Jager T, Li J, Reymann KG, Balschun D (2004) Neurogranin/RC3 enhances long-term potentiation and learning by promoting calcium-mediated signaling. *J Neurosci* 24:10660-10669.
- Hudmon A, Schulman H (2002) Neuronal CA2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annual review of biochemistry* 71:473-510.
- Husain MM, Rush AJ, Fink M, Knapp R, Petrides G, Rummans T, Biggs MM, O'Connor K, Rasmussen K, Litle M, Zhao WL, Bernstein HJ, Smith G, Mueller M, McClintock SM, Bailine SH, Kellner CH (2004) Speed of response and remission in major depressive disorder with acute electroconvulsive therapy (ECT): A Consortium for Research in ECT (CORE) report. *Journal of Clinical Psychiatry* 65:485-491.
- Ingram A, Saling MM, Schweitzer I (2008) Cognitive Side Effects of Brief Pulse Electroconvulsive Therapy: A Review. *J ECT* 24:3-9.
- Irwin SA, Galvez R, Greenough WT (2000) Dendritic spine structural anomalies in fragile-X mental retardation syndrome. *Cereb Cortex* 10:1038-1044.
- Irwin SA, Patel B, Idupulapati M, Harris JB, Crisostomo RA, Larsen BP, Kooy F, Willems PJ, Cras P, Kozlowski PB, Swain RA, Weiler IJ, Greenough WT (2001) Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination. *Am J Med Genet* 98:161-167.
- Isokawa M (2000) Remodeling dendritic spines of dentate granule cells in temporal lobe epilepsy patients and the rat pilocarpine model. *Epilepsia* 41 Suppl 6:S14-17.
- Ito M, Seki T, Liu J, Nakamura K, Namba T, Matsubara Y, Suzuki T, Arai H (2010) Effects of repeated electroconvulsive seizure on cell proliferation in the rat hippocampus. *Synapse* 64:814-821.
- Iversen L (2005) The Monoamine Hypothesis of Depression. In: *Biology of Depression: From Novel Insights to Therapeutic Strategies* (Licinio J, Wong M, eds). Weinheim: Wiley-VCH.
- Jacobsen JPR, Mork A (2004) The effect of escitalopram, desipramine, electroconvulsive seizures and lithium on brain-derived neurotrophic factor mRNA and protein expression in the rat brain and the correlation to 5-HT and 5-HIAA levels. *Brain Res* 1024:183-192.

- Jansson L, Hellsten J, Tingstrom A (2006) Region specific hypothalamic neuronal activation and endothelial cell proliferation in response to electroconvulsive seizures. *Biol Psychiatry* 60:874-881.
- Jansson L, Wennstrom M, Johanson A, Tingstrom A (2009) Glial cell activation in response to electroconvulsive seizures. *Prog Neuropsychopharmacol Biol Psychiatry* 33:1119-1128.
- Ji B, Zhang Z, Zhang M, Zhu H, Zhou K, Yang J, Li Y, Sun L, Feng G, Wang Y, He L, Wan C (2009) Differential expression profiling of the synaptosome proteome in a rat model of antipsychotic resistance. *Brain Res* 1295:170-178.
- Jin JP, Zhang Z, Bautista JA (2008) Isoform diversity, regulation, and functional adaptation of troponin and calponin. *Critical reviews in eukaryotic gene expression* 18:93-124.
- Jin JP, Wu D, Gao J, Nigam R, Kwong S (2003) Expression and purification of the h1 and h2 isoforms of calponin. *Protein expression and purification* 31:231-239.
- Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA (2002) Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proceedings of the National Academy of Sciences USA* 99:11946-11950.
- Johnston-Wilson NL, Sims CD, Hofmann JP, Anderson L, Shore AD, Torrey EF, Yolken RH (2000) Disease-specific alterations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder. The Stanley Neuropathology Consortium. *Mol Psychiatry* 5:142-149.
- Joliffe IT (2002) *Principal Component Analysis*, 2nd Edition. New York: Springer-Verlag.
- Kake T, Kimura S, Takahashi K, Maruyama K (1995) Calponin induces actin polymerization at low ionic strength and inhibits depolymerization of actin filaments. *Biochem J* 312 (Pt 2):587-592.
- Kamagata C, Tsuboko Y, Okabe T, Sato C, Sakamoto A (2011) Proteomic analysis of rat brains in a model of neuropathic pain following exposure to electroconvulsive stimulation. *Biomed Res* 32:91-102.
- Kandel ER, Schwartz JH, Jessell TM, eds (2000) *Principles of Neural Science*, 4th Edition: McGraw Hill.
- Kanellopoulos D, Gunning FM, Morimoto SS, Hoptman MJ, Murphy CF, Kelly RE, Glatt C, Lim KO, Alexopoulos GS (2011) Hippocampal volumes and the brain-derived neurotrophic factor val66met polymorphism in geriatric major depression. *Am J Geriatr Psychiatry* 19:13-22.
- Kao CF, Fang YS, Zhao Z, Kuo PH (2011) Prioritization and evaluation of depression candidate genes by combining multidimensional data resources. *PLoS ONE* 6:e18696.
- Karege F, Vaudan G, Schwald M, Perroud N, La Harpe R (2005b) Neurotrophin levels in postmortem brains of suicide victims and the effects of antemortem diagnosis and psychotropic drugs. *Brain Research Molecular Brain Research* 136:29-37.
- Karege F, Bondolfi G, Gervasoni N, Schwald M, Aubry JM, Bertschy G (2005a) Low brain-derived neurotrophic factor (BDNF) levels in serum of depressed patients probably results from lowered platelet BDNF release unrelated to platelet reactivity. *Biological Psychiatry* 57:1068-1072.
- Karg K, Burmeister M, Shedden K, Sen S (2011) The serotonin transporter promoter variant (5-HTTLPR), stress, and depression meta-analysis revisited: evidence of genetic moderation. *Arch Gen Psychiatry* 68:444-454.

- Karishma KK, Herbert J (2002) Dehydroepiandrosterone (DHEA) stimulates neurogenesis in the hippocampus of the rat, promotes survival of newly formed neurons and prevents corticosterone-induced suppression. *Eur J Neurosci* 16:445-453.
- Karp NA, Lilley KS (2005a) Maximising sensitivity for detecting changes in protein expression: experimental design using minimal CyDyes. *Proteomics* 5:3105-3115.
- Karp NA, Griffin JL, Lilley KS (2005b) Application of partial least squares discriminant analysis to two-dimensional difference gel studies in expression proteomics. *Proteomics* 5:81-90.
- Kasper S, Anghelescu IG, Szegedi A, Dienel A, Kieser M (2006) Superior efficacy of St John's wort extract WS 5570 compared to placebo in patients with major depression: a randomized, double-blind, placebo-controlled, multi-center trial [ISRCTN77277298]. *BMC medicine* 4:14.
- Kato M, Serretti A (2010) Review and meta-analysis of antidepressant pharmacogenetic findings in major depressive disorder. *Mol Psychiatr* 15:473-500.
- Kedracka-Krok S, Fic E, Jankowska U, Jaciuk M, Gruca P, Papp M, Kusmider M, Solich J, Debski J, Dadlez M, Dziedzicka-Wasylewska M (2010) Effect of chronic mild stress and imipramine on the proteome of the rat dentate gyrus. *J Neurochem* 113:848-859.
- Kellner CH, Knapp R, Husain MM, Rasmussen K, Sampson S, Cullum M, McClintock SM, Tobias KG, Martino C, Mueller M, Bailine SH, Fink M, Petrides G (2010) Bifrontal, bitemporal and right unilateral electrode placement in ECT: randomised trial. *Br J Psychiatry* 196:226-234.
- Kempton MJ, Salvador Z, Munafo MR, Geddes JR, Simmons A, Frangou S, Williams SC (2011) Structural Neuroimaging Studies in Major Depressive Disorder: Meta-analysis and Comparison With Bipolar Disorder. *Arch Gen Psychiatry* 68:675-690.
- Kendler KS, Karkowski LM, Prescott CA (1999) Causal relationship between stressful life events and the onset of major depression. *Am J Psychiat* 156:837-841.
- Kessing LV, Willer IS, Knorr U (2011) Volume of the adrenal and pituitary glands in depression. *Psychoneuroendocrinology* 36:19-27.
- Kessler RC (1997) The effects of stressful life events on depression. *Annual Review of Psychology* 48:191-214.
- Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR, Rush AJ, Walters EE, Wang PS (2003) The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). *JAMA* 289:3095-3105.
- Khaibullina AA, Rosenstein JM, Krum JM (2004) Vascular endothelial growth factor promotes neurite maturation in primary CNS neuronal cultures. *Brain Research Developmental Brain Research* 148:59-68.
- Khan A, Lai H, Ukai Y, Mirolo MH (1994) NS-3, a TRH analog, reverses repeated ECS-induced deficits in water maze performance in the rat. *Pharmacol Biochem Behav* 47:477-481.
- Khawaja X, Xu J, Liang JJ, Barrett JE (2004) Proteomic analysis of protein changes developing in rat hippocampus after chronic antidepressant treatment: Implications for depressive disorders and future therapies. *J Neurosci Res* 75:451-460.
- Kho KH, van Vreeswijk MF, Simpson S, Zwinderman AH (2003) A meta-analysis of electroconvulsive therapy efficacy in depression. *Journal of ECT* 19:139-147.

- Kim HG, Kim KL (2007) Decreased hippocampal cholinergic neurostimulating peptide precursor protein associated with stress exposure in rat brain by proteomic analysis. *J Neurosci Res* 85:2898-2908.
- Kim SH, Kim MK, Yu HS, Kim HS, Park IS, Park HG, Kang UG, Kim YS (2010) Electroconvulsive seizure increases phosphorylation of PKC substrates, including GAP-43, MARCKS, and neurogranin, in rat brain. *Prog Neuropsychopharmacol Biol Psychiatry* 34:115-121.
- Kirchner L, Chen WQ, Afjehi-Sadat L, Viidik A, Skalicky M, Hoyer H, Lubec G (2008) Hippocampal metabolic proteins are modulated in voluntary and treadmill exercise rats. *Exp Neurol* 212:145-151.
- Kjaersgard IV, Norrelykke MR, Jessen F (2006) Changes in cod muscle proteins during frozen storage revealed by proteome analysis and multivariate data analysis. *Proteomics* 6:1606-1618.
- Klose J, Feller M (1981) Genetic variability of proteins from plasma membranes and cytosols of mouse organs. *Biochemical genetics* 19:859-870.
- Klose J, von Wallenberg-Pachaly H (1976) Changes of soluble protein populations during organogenesis of mouse embryos as revealed by protein mapping. *Dev Biol* 51:324-331.
- Kodama M, Fujioka T, Duman RS (2004) Chronic olanzapine or fluoxetine administration increases cell proliferation in hippocampus and prefrontal cortex of adult rat. *Biological Psychiatry* 56:570-580.
- Kodama M, Russell DS, Duman RS (2005) Electroconvulsive seizures increase the expression of MAP kinase phosphatases in limbic regions of rat brain. *Neuropsychopharmacology* 30:360-371.
- Koenigs M, Grafman J (2009) The functional neuroanatomy of depression: distinct roles for ventromedial and dorsolateral prefrontal cortex. *Behav Brain Res* 201:239-243.
- Koenigs M, Huey ED, Calamia M, Raymont V, Tranel D, Grafman J (2008) Distinct regions of prefrontal cortex mediate resistance and vulnerability to depression. *J Neurosci* 28:12341-12348.
- Kolodziej SJ, Hudmon A, Waxham MN, Stoops JK (2000) Three-dimensional reconstructions of calcium/calmodulin-dependent (CaM) kinase II α and truncated CaM kinase II α reveal a unique organization for its structural core and functional domains. *J Biol Chem* 275:14354-14359.
- Kondratyev A, Ved R, Gale K (2002) The effects of repeated minimal electroconvulsive shock exposure on levels of mRNA encoding fibroblast growth factor-2 and nerve growth factor in limbic regions. *Neuroscience* 114:411-416.
- Kornhauser JM, Cowan CW, Shaywitz AJ, Dolmetsch RE, Griffith EC, Hu LS, Haddad C, Xia Z, Greenberg ME (2002) CREB transcriptional activity in neurons is regulated by multiple, calcium-specific phosphorylation events. *Neuron* 34:221-233.
- Krapfenbauer K, Berger M, Lubec G, Fountoulakis M (2001) Changes in the brain protein levels following administration of kainic acid. *Electrophoresis* 22:2086-2091.
- Krystal JH, Tolin DF, Sanacora G, Castner SA, Williams GV, Aikins DE, Hoffman RE, D'Souza DC (2009) Neuroplasticity as a target for the pharmacotherapy of anxiety disorders, mood disorders, and schizophrenia. *Drug Discov Today* 14:690-697.
- Kuhn R (1957) [Treatment of depressive states with an iminodibenzyl derivative (G 22355)]. *Schweiz Med Wochenschr* 87:1135-1140.

- Kumar C, Mann M (2009) Bioinformatics analysis of mass spectrometry-based proteomics data sets. *FEBS Lett* 583:1703-1712.
- Kunugi H et al. (2006) Assessment of the dexamethasone/CRH test as a state-dependent marker for hypothalamic-pituitary-adrenal (HPA) axis abnormalities in major depressive episode: a Multicenter Study. *Neuropsychopharmacology* 31:212-220.
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lamont SR, Paulls A, Stewart CA (2001) Repeated electroconvulsive stimulation, but not antidepressant drugs, induces mossy fibre sprouting in the rat hippocampus. *Brain Res* 893:53-58.
- Lamprecht R, LeDoux J (2004) Structural plasticity and memory. *Nat Rev Neurosci* 5:45-54.
- Larsen MH, Rosenbrock H, Sams-Dodd F, Mikkelsen JD (2007) Expression of brain derived neurotrophic factor, activity-regulated cytoskeleton protein mRNA, and enhancement of adult hippocampal neurogenesis in rats after sub-chronic and chronic treatment with the triple monoamine re-uptake inhibitor tesofensine. *Eur J Pharmacol* 555:115-121.
- Laux T, Fukami K, Thelen M, Golub T, Frey D, Caroni P (2000) GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J Cell Biol* 149:1455-1472.
- Lee HK (2006) Synaptic plasticity and phosphorylation. *Pharmacol Ther* 112:810-832.
- Lee HY, Kim YK (2008) Plasma brain-derived neurotrophic factor as a peripheral marker for the action mechanism of antidepressants. *Neuropsychobiology* 57:194-199.
- Lee R, Kermani P, Teng KK, Hempstead BL (2001b) Regulation of cell survival by secreted proneurotrophins. *Science* 294:1945-1948.
- Lee CS, Kang KR, Lee JY, Park CS, Hahn KH, Sohn JW, Kim BJ (2009) Proteomic analysis of rat brains following exposure to electroconvulsive therapy. *J Korean Med Sci* 24:132-137.
- Lee HJ, Kim JW, Yim SV, Kim MJ, Kim SA, Kim YJ, Kim CJ, Chung JH (2001a) Fluoxetine enhances cell proliferation and prevents apoptosis in dentate gyrus of maternally separated rats. *Mol Psychiatr* 6:610, 725-618.
- Lemaire V, Koehl M, Le Moal M, Abrous DN (2000) Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus. *Proceedings of the National Academy of Science USA* 97:11032-11037.
- Leonard BE (2003) Drug Treatment of Depression. In: *Fundamentals of Psychopharmacology* 3rd Edition, pp 153-192. New York: Wiley.
- Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, Benjamin J, Muller CR, Hamer DH, Murphy DL (1996) Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 274:1527-1531.
- Levin Y, Wang L, Schwarz E, Koethe D, Leweke FM, Bahn S (2010) Global proteomic profiling reveals altered proteomic signature in schizophrenia serum. *Mol Psychiatry* 15:1088-1100.
- Li BJ, Suemaru K, Cui R, Araki H (2007) Repeated electroconvulsive stimuli have long-lasting effects on hippocampal BDNF and decrease immobility time in the rat forced swim test. *Life Sci* 80:1539-1543.

- Li Y, Luikart BW, Birnbaum S, Chen J, Kwon CH, Kernie SG, Bassel-Duby R, Parada LF (2008) TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. *Neuron* 59:399-412.
- Li NX, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, Li XY, Aghajanian G, Duman RS (2010) mTOR-Dependent Synapse Formation Underlies the Rapid Antidepressant Effects of NMDA Antagonists. *Science* 329:959-964.
- Lisanby SH (2007) Electroconvulsive therapy for depression. *New England Journal of Medicine* 357:1939-1945.
- Lisanby SH, Maddox JH, Prudic J, Devanand DP, Sackeim HA (2000) The effects of electroconvulsive therapy on memory of autobiographical and public events. *Arch Gen Psychiatry* 57:581-590.
- Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci* 3:175-190.
- Lisy ME, Jarvis KB, Delbello MP, Mills NP, Weber WA, Fleck D, Strakowski SM, Adler CM (2011) Progressive neurostructural changes in adolescent and adult patients with bipolar disorder. *Bipolar Disord* 13:396-405.
- Liu QR, Lu L, Zhu XG, Gong JP, Shaham Y, Uhl GR (2006) Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. *Brain Res* 1067:1-12.
- Loo CK, Sainsbury K, Sheehan P, Lyndon B (2008) A comparison of RUL ultrabrief pulse (0.3 ms) ECT and standard RUL ECT. *Int J Neuropsychopharmacol* 11:883-890.
- Lopez-Leon S, Janssens AC, Gonzalez-Zuloeta Ladd AM, Del-Favero J, Claes SJ, Oostra BA, van Duijn CM (2008) Meta-analyses of genetic studies on major depressive disorder. *Mol Psychiatry* 13:772-785.
- Lozano AM, Mayberg HS, Giacobbe P, Hamani C, Craddock RC, Kennedy SH (2008) Subcallosal cingulate gyrus deep brain stimulation for treatment-resistant depression. *Biological Psychiatry* 64:461-467.
- Lu B, Pang PT, Woo NH (2005) The yin and yang of neurotrophin action. *Nat Rev Neurosci* 6:603-614.
- Lukoyanov NV, Sa MJ, Madeira MD, Paula-Barbosa MM (2004) Selective loss of hilar neurons and impairment of initial learning in rats after repeated administration of electroconvulsive shock seizures. *Experimental Brain Research* 154:192-200.
- Lynch D, Laws KR, McKenna PJ (2010) Cognitive behavioural therapy for major psychiatric disorder: does it really work? A meta-analytical review of well-controlled trials. *Psychol Med* 40:9-24.
- Lyons DM, Buckmaster PS, Lee AG, Wu C, Mitra R, Duffey LM, Buckmaster CL, Her S, Patel PD, Schatzberg AF (2010) Stress coping stimulates hippocampal neurogenesis in adult monkeys. *Proceedings of the National Academy of Science USA* 107:14823-14827.
- Maayan R, Morad O, Dorfman P, Overstreet DH, Weizman A, Yadid G (2005) The involvement of dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) in blocking the therapeutic effect of electroconvulsive shocks in an animal model of depression. *Eur Neuropsychopharmacol* 15:253-262.
- Machnicka B, Grochowalska R, Boguslawska DM, Sikorski AF, Lecomte MC (2012) Spectrin-based skeleton as an actor in cell signaling. *Cellular and molecular life sciences : CMLS* 69:191-201.

- Madsen TM, Newton SS, Eaton ME, Russell DS, Duman RS (2003) Chronic electroconvulsive seizure up-regulates beta-catenin expression in rat hippocampus: Role in adult neurogenesis. *Biological Psychiatry* 54:1006-1014.
- Madsen TM, Treschow A, Bengzon J, Bolwig TG, Lindvall O, Tingstrom A (2000) Increased neurogenesis in a model of electroconvulsive therapy. *Biological Psychiatry* 47:1043-1049.
- Maekawa S, Maekawa M, Hattori S, Nakamura S (1993) Purification and molecular cloning of a novel acidic calmodulin binding protein from rat brain. *J Biol Chem* 268:13703-13709.
- Majek P, Reicheltova Z, al. e (2011) Plasma proteome changes in cardiovascular disease patients: novel isoforms of apolipoprotein A1. *Journal of Translational Medicine* 9.
- Maki WS (1985) Differential effects of electroconvulsive shock on concurrent spatial memories-old memories are impaired while new memories are spared. *Behav Neural Biol* 43:162-177.
- Malberg JE, Duman RS (2003) Cell proliferation in adult hippocampus is decreased by inescapable stress: Reversal by fluoxetine treatment. *Neuropsychopharmacology* 28:1562-1571.
- Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* 20:9104-9110.
- Malik R, Dulla K, Nigg EA, Korner R (2010) From proteome lists to biological impact--tools and strategies for the analysis of large MS data sets. *Proteomics* 10:1270-1283.
- Mallei A, Shi B, Mocchetti I (2002) Antidepressant treatments induce the expression of basic fibroblast growth factor in cortical and hippocampal neurons. *Mol Pharmacol* 61:1017-1024.
- Mallei A, Giambelli R, Gass P, Racagni G, Mathe AA, Vollmayr B, Popoli M (2011) Synaptoproteomics of learned helpless rats involve energy metabolism and cellular remodeling pathways in depressive-like behavior and antidepressant response. *Neuropharmacology* 60:1243-1253.
- Malone DA, Jr., Dougherty DD, Rezai AR, Carpenter LL, Friehs GM, Eskandar EN, Rauch SL, Rasmussen SA, Machado AG, Kubu CS, Tyrka AR, Price LH, Stypulkowski PH, Giftakis JE, Rise MT, Malloy PF, Salloway SP, Greenberg BD (2009) Deep brain stimulation of the ventral capsule/ventral striatum for treatment-resistant depression. *Biological Psychiatry* 65:267-275.
- Manji HK, Drevets WC, Charney DS (2001) The cellular neurobiology of depression. *Nat Med* 7:541-547.
- Manji HK, Quiroz JA, Sporn J, Payne JL, Denicoff K, N AG, Zarate CA, Jr., Charney DS (2003) Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression. *Biological Psychiatry* 53:707-742.
- Marais L, Hattingh SM, Stein DJ, Daniels WM (2009) A proteomic analysis of the ventral hippocampus of rats subjected to maternal separation and escitalopram treatment. *Metab Brain Dis* 24:569-586.
- Marano CM, Phatak P, Vemulapalli UR, Sasan A, Nalbandyan MR, Ramanujam S, Soekadar S, Demosthenous M, Regenold WT (2007) Increased plasma concentration of brain-derived neurotrophic factor with electroconvulsive therapy: a pilot study in patients with major depression. *Journal of Clinical Psychiatry* 68:512-517.

- Marengo E, Robotti E, Bobba M, Liparota MC, Rustichelli C, Zamo A, Chilosi M, Righetti PG (2006) Multivariate statistical tools applied to the characterization of the proteomic profiles of two human lymphoma cell lines by two-dimensional gel electrophoresis. *Electrophoresis* 27:484-494.
- Marengo E, Robotti E, Bobba M, Milli A, Campostrini N, Righetti SC, Cecconi D, Righetti PG (2008) Application of partial least squares discriminant analysis and variable selection procedures: a 2D-PAGE proteomic study. *Anal Bioanal Chem* 390:1327-1342.
- Markakis EA, Gage FH (1999) Adult-generated neurons in the dentate gyrus send axonal projections to field CA(3) and are surrounded by synaptic vesicles. *J Comp Neurol* 406:449-460.
- Martens H, Martens M (2000) Modified Jack-knife estimation of parameter uncertainty in bilinear modelling by partial least squares regression (PLSR). *Food Qual Prefer* 11:5-16.
- Martinowich K, Manji H, Lu B (2007) New insights into BDNF function in depression and anxiety. *Nat Neurosci* 10:1089-1093.
- Martins-de-Souza D, Harris LW, Guest PC, Turck CW, Bahn S (2010) The role of proteomics in depression research. *Eur Arch Psychiatry Clin Neurosci* 260:499-506.
- Martins-de-Souza D, Maccarrone G, Wobrock T, Zerr I, Gormanns P, Reckow S, Falkai P, Schmitt A, Turck CW (2009) Proteome analysis of the thalamus and cerebrospinal fluid reveals glycolysis dysfunction and potential biomarkers candidates for schizophrenia. *J Psychiatr Res* 44:1176-1189.
- Martinsen EW, Medhus A, Sandvik L (1985) Effects of aerobic exercise on depression: a controlled study. *Br Med J (Clin Res Ed)* 291:109.
- Massa F, Koehl M, Wiesner T, Grosjean N, Revest JM, Piazza PV, Abrous DN, Oliet SH (2011) Conditional reduction of adult neurogenesis impairs bidirectional hippocampal synaptic plasticity. *Proceedings of the National Academy of Science USA* 108:6644-6649.
- Matrisciano F, Bonaccorso S, Ricciardi A, Scaccianoce S, Panaccione I, Wang L, Ruberto A, Tatarelli R, Nicoletti F, Girardi P, Shelton RC (2009) Changes in BDNF serum levels in patients with major depression disorder (MDD) after 6 months treatment with sertraline, escitalopram, or venlafaxine. *J Psychiatr Res* 43:247-254.
- Mayberg HS, Lozano AM, Voon V, McNeely HE, Seminowicz D, Hamani C, Schwalb JM, Kennedy SH (2005) Deep brain stimulation for treatment-resistant depression. *Neuron* 45:651-660.
- McCall WV, Reboussin DM, Weiner RD, Sackeim HA (2000) Titrated moderately suprathreshold vs fixed high-dose right unilateral electroconvulsive therapy: acute antidepressant and cognitive effects. *Arch Gen Psychiatry* 57:438-444.
- McCormick LM, Brumm MC, Benede AK, Lewis JL (2009) Relative ineffectiveness of ultrabrief right unilateral versus bilateral electroconvulsive therapy in depression. *Journal of ECT* 25:238-242.
- McEwen BS, Eiland L, Hunter RG, Miller MM (2011) Stress and anxiety: Structural plasticity and epigenetic regulation as a consequence of stress. *Neuropharmacology*.
- McEwen BS, Chattarji S, Diamond DM, Jay TM, Reagan LP, Svenningsson P, Fuchs E (2010) The neurobiological properties of tianeptine (Stablon): from monoamine hypothesis to glutamatergic modulation. *Mol Psychiatr* 15:237-249.

- McGarvey KA, Zis AP, Brown EE, Nomikos GG, Fibiger HC (1993) ECS-induced dopamine release: effects of electrode placement, anticonvulsant treatment, and stimulus intensity. *Biological Psychiatry* 34:152-157.
- McHugh PC, Rogers GR, Glubb DM, Joyce PR, Kennedy MA (2010) Proteomic analysis of rat hippocampus exposed to the antidepressant paroxetine. *J Psychopharmacol* 24:1243-1251.
- McIntyre RS, Soczynska JK, Konarski JZ, Woldeyohannes HO, Law CW, Miranda A, Fulgosi D, Kennedy SH (2007) Should Depressive Syndromes Be Reclassified as "Metabolic Syndrome Type II"? *Ann Clin Psychiatry* 19:257-264.
- McIntyre RS, Rasgon NL, Kemp DE, Nguyen HT, Law CW, Taylor VH, Woldeyohannes HO, Alsuwaidan MT, Soczynska JK, Kim B, Lourenco MT, Kahn LS, Goldstein BI (2009) Metabolic syndrome and major depressive disorder: co-occurrence and pathophysiologic overlap. *Current diabetes reports* 9:51-59.
- McKay MS, Zakzanis KK, (2010) The impact of treatment on HPA axis activity in unipolar major depression. *J Psychiatr Res* 44(3):183-92.
- McKinnon MC, Yucel K, Nazarov A, MacQueen GM (2009) A meta-analysis examining clinical predictors of hippocampal volume in patients with major depressive disorder. *Journal of Psychiatry and Neuroscience* 34:41-54.
- McNair K, Davies CH, Cobb SR (2006) Plasticity-related regulation of the hippocampal proteome. *Eur J Neurosci* 23:575-580.
- Meeter M, Murre JM, Janssen SM, Birkenhager T, van den Broek WW (2011) Retrograde amnesia after electroconvulsive therapy: A temporary effect? *J Affect Disord* 132:216-222.
- Mello AF, Juruena MF, Pariente CM, Tyrka AR, Price LH, Carpenter LL, Del Porto JA (2007) [Depression and stress: is there an endophenotype?]. *Revista Brasileira de Psiquiatria* 29 Suppl 1:S13-18.
- Mercier G, Lennon AM, Renouf B, Dessouroux A, Ramauge M, Courtin F, Pierre M (2004) MAP kinase activation by fluoxetine and its relation to gene expression in cultured rat astrocytes. *J Mol Neurosci* 24:207-216.
- Metral S, Machnicka B, Bigot S, Colin Y, Dhermy D, Lecomte MC (2009) AlphaII-spectrin is critical for cell adhesion and cell cycle. *J Biol Chem* 284:2409-2418.
- Metz G, Whishaw I, Kolb B (2005) Analysis of behaviour in laboratory rodents. In: *The behavior of the laboratory rat: a handbook with tests* (Whishaw I, Kolb B, eds). England: Oxford University Press.
- Middeldorp J, Hol EM (2011) GFAP in health and disease. *Prog Neurobiol* 93:421-443.
- Middeldorp CM, Sullivan PF, Wray NR, Hottenga JJ, de Geus EJ, van den Berg M, Montgomery GW, Coventry WL, Statham DJ, Andrews G, Slagboom PE, Boomsma DI, Martin NG (2009) Suggestive linkage on chromosome 2, 8, and 17 for lifetime major depression. *American Journal of Medical Genetics Part B Neuropsychiatric Genetics* 150B:352-358.
- Mons N, Enderlin V, Jaffard R, Higeret P (2001) Selective age-related changes in the PKC-sensitive, calmodulin-binding protein, neurogranin, in the mouse brain. *J Neurochem* 79:859-867.
- Morgan D, Diamond DM, Gottschall PE, Ugen KE, Dickey C, Hardy J, Duff K, Jantzen P, DiCarlo G, Wilcock D, Connor K, Hatcher J, Hope C, Gordon M, Arendash GW (2000) A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease *Nature* 408 (6815):982-5.

- Mu J, Xie P, Yang ZS, Yang DL, Lv FJ, Luo TY, Li Y (2007) Neurogenesis and major depression: implications from proteomic analyses of hippocampal proteins in a rat depression model. *Neurosci Lett* 416:252-256.
- Mudry RE, Perry CN, Richards M, Fowler VM, Gregorio CC (2003) The interaction of tropomodulin with tropomyosin stabilizes thin filaments in cardiac myocytes. *J Cell Biol* 162:1057-1068.
- Muglia P, Tozzi F, Galwey NW, Francks C, Upmanyu R, Kong XQ, Antoniadou A, Domenici E, Perry J, Rothen S, Vandeley CL, Mooser V, Waeber G, Vollenweider P, Preisig M, Lucae S, Muller-Myhsok B, Holsboer F, Middleton LT, Roses AD (2010) Genome-wide association study of recurrent major depressive disorder in two European case-control cohorts. *Mol Psychiatr* 15:589-601.
- Munk-Olsen T, Laursen TM, Videbech P, Mortensen PB, Rosenberg R (2007) All-cause mortality among recipients of electroconvulsive therapy: register-based cohort study. *British Journal Psychiatry* 190:435-439.
- Murer MG, Yan Q, Raisman-Vozari R (2001) Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Prog Neurobiol* 63:71-124.
- Murray EA, Wise SP, Drevets WC (2011) Localization of dysfunction in major depressive disorder: prefrontal cortex and amygdala. *Biological Psychiatry* 69:e43-54.
- Nair A, Vadodaria KC, Banerjee SB, Benekareddy M, Dias BG, Duman RS, Vaidya VA (2007) Stressor-specific regulation of distinct brain-derived neurotrophic factor transcripts and cyclic AMP response element-binding protein expression in the postnatal and adult rat hippocampus. *Neuropsychopharmacology* 32:1504-1519.
- Nakagawa S, Kim JE, Lee R, Malberg JE, Chen JS, Steffen C, Zhang YJ, Nestler EJ, Duman RS (2002) Regulation of neurogenesis in adult mouse hippocampus by cAMP and the cAMP response element-binding protein. *J Neurosci* 22:3673-3682.
- National Institute of Mental Health (2008) The numbers count: Mental Disorders in America In. Washington D.C.: NIMH.
- Nemeroff CB, Widerlov E, Bissette G, Walleus H, Karlsson I, Eklund K, Kilts CD, Loosen PT, Vale W (1984) Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. *Science* 226:1342-1344.
- Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002) Neurobiology of depression. *Neuron* 34:13-25.
- Newton SS, Collier EF, Hunsberger J, Adams D, Terwilliger R, Selvanayagam E, Duman RS (2003) Gene profile of electroconvulsive seizures: Induction of neurotrophic and angiogenic factors. *J Neurosci* 23:10841-10851.
- Nibuya M, Morinobu S, Duman RS (1995) Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* 15:7539-7547.
- Nibuya M, Nestler EJ, Duman RS (1996) Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. *J Neurosci* 16:2365-2372.
- NICE NifHaCE (2009) Depression: Treatment and Management of Depression in Adults, including Adults with a Chronic Physical Health Problem.

- Niemantsverdriet L, Birkenhager TK, van den Broek WW (2011) The efficacy of ultrabrief-pulse (0.25 millisecond) versus brief-pulse (0.50 millisecond) bilateral electroconvulsive therapy in major depression. *Journal of ECT* 27:55-58.
- Nikonenko I, Jourdain P, Alberi S, Toni N, Muller D (2002) Activity-induced changes of spine morphology. *Hippocampus* 12:585-591.
- Nimchinsky EA, Oberlander AM, Svoboda K (2001) Abnormal development of dendritic spines in FMR1 knock-out mice. *J Neurosci* 21:5139-5146.
- NonLinear Dynamics I (2011) Progenesis SameSpots: Notes on False Discovery Rate In: www.nonlinear.com.
- NonLinear Dynamics II (2011) Progenesis SameSpots: Notes on Power Analysis. In: www.nonlinear.com.
- NonLinear Dynamics III (2011) Progenesis SameSpots: Notes on Principal Component Analysis. In: www.nonlinear.com.
- NonLinear Dynamics IV (2011) Progenesis SameSpots: Notes on p-values and q-values. In: www.nonlinear.com.
- Norden B, Broberg P, Lindberg C, Plymoth A (2005) Analysis and understanding of high-dimensionality data by means of multivariate data analysis. *Chem Biodivers* 2:1487-1494.
- Novikova SI, He F, Cutrufello NJ, Lidow MS (2006) Identification of protein biomarkers for schizophrenia and bipolar disorder in the postmortem prefrontal cortex using SELDI-TOF-MS ProteinChip profiling combined with MALDI-TOF-PSD-MS analysis. *Neurobiology of disease* 23:61-76.
- O'Donovan S, Kennedy M, Guinan B, O'Mara S, McLoughlin DM (2011) A comparison of brief pulse and ultrabrief pulse electroconvulsive stimulation on rodent brain and behaviour. *Prog Neuropsychopharmacol Biol Psychiatry*.
- O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250:4007-4021.
- Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M (1999) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* 27:29-34.
- Okamoto K, Narayanan R, Lee SH, Murata K, Hayashi Y (2007) The role of CaMKII as an F-actin-bundling protein crucial for maintenance of dendritic spine structure. *Proc Natl Acad Sci U S A* 104:6418-6423.
- Okamoto N, Nakai T, Sakamoto K, Nagafusa Y, Higuchi T, Nishikawa T (2010) Rapid antidepressant effect of ketamine anesthesia during electroconvulsive therapy of treatment-resistant depression: comparing ketamine and propofol anesthesia. *Journal of ECT* 26:223-227.
- Olney JW, de Gubareff T, Labruyere J (1983) Seizure-related brain damage induced by cholinergic agents. *Nature* 301:520-522.
- O'Neill LA, Kaltschmidt C (1997) NF-kappa B: a crucial transcription factor for glial and neuronal cell function. *Trends Neurosci* 20:252-258.
- Organisation WH, ed (2008) World Health Report 2008:Primary Health Care Now More Than Ever. Geneva, Switzerland.

- Pagnin D, de Queiroz V, Pini S, Cassano GB (2004) Efficacy of ECT in depression: A meta-analytic review. *Journal of ECT* 20:13-20.
- Pak JH, Huang FL, Li J, Balschun D, Reymann KG, Chiang C, Westphal H, Huang KP (2000) Involvement of neurogranin in the modulation of calcium/calmodulin-dependent protein kinase II, synaptic plasticity, and spatial learning: a study with knockout mice. *Proc Natl Acad Sci U S A* 97:11232-11237.
- Papiol S, Arias B, Gasto C, Gutierrez B, Catalan R, Fananas L (2007) Genetic variability at HPA axis in major depression and clinical response to antidepressant treatment. *J Affect Disord* 104:83-90.
- Pariante CM (2003) Depression, stress and the adrenal axis. *Journal of Neuroendocrinology* 15:811-812.
- Pariante CM (2004) Glucocorticoid receptor function in vitro in patients with major depression. *Stress* 7:209-219.
- Pariante CM (2006) The glucocorticoid receptor: part of the solution or part of the problem? *J Psychopharmacol* 20:79-84.
- Pariante CM, Miller AH (2001) Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. *Biological Psychiatry* 49:391-404.
- Pariante CM, Lightman SL (2008) The HPA axis in major depression: classical theories and new developments. *Trends Neurosci* 31:464-468.
- Pariante CM, Papadopoulos AS, Poon L, Cleare AJ, Checkley SA, English J, Kerwin RW, Lightman S (2004) Four days of citalopram increase suppression of cortisol secretion by prednisolone in healthy volunteers. *Psychopharmacology (Berl)* 177:200-206.
- Pariante CM, Dazzan P, Danese A, Morgan KD, Brudaglio F, Morgan C, Fearon P, Orr K, Hutchinson G, Pantelis C, Velakoulis D, Jones PB, Leff J, Murray RM (2005) Increased pituitary volume in antipsychotic-free and antipsychotic-treated patients of the AESop first-onset psychosis study. *Neuropsychopharmacology* 30:1923-1931.
- Parker KC, Walsh RJ, Salajegheh M, Amato AA, Krastins B, Sarracino DA, Greenberg SA (2009) Characterization of human skeletal muscle biopsy samples using shotgun proteomics. *J Proteome Res* 8:3265-3277.
- Pattabiraman PP, Tropea D, Chiaruttini C, Tongiorgi E, Cattaneo A, Domenici L (2005) Neuronal activity regulates the developmental expression and subcellular localization of cortical BDNF mRNA isoforms in vivo. *Mol Cell Neurosci* 28:556-570.
- Patton WF (2002) Detection technologies in proteome analysis. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences* 771:3-31.
- Paxinos G, Watson CS (2007) *The rat brain in stereotaxic coordinates*, 6th Edition. Amsterdam: Elsevier.
- Payne NA, Prudic J (2009) Electroconvulsive Therapy: Part I. A Perspective on the Evolution and Current Practice of ECT. *J Psychiatr Pract* 15:346-368.
- Pedreschi R, Hertog M, Robben J, Lilley KS, Karp NA, Baggerman G, Vanderleyden J, Nicolai B (2009) Gel-based proteomics approach to the study of metabolic changes in pear tissue during storage. *J Agric Food Chem* 57:6997-7004.

- Pedreschi R, Vanstreels E, Carpentier S, Hertog M, Lammertyn J, Robben J, Noben JP, Swennen R, Vanderleyden J, Nicolai BM (2007) Proteomic analysis of core breakdown disorder in Conference pears (*Pyrus communis* L.). *Proteomics* 7:2083-2099.
- Peng J, Kim MJ, Cheng D, Duong DM, Gygi SP, Sheng M (2004) Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. *J Biol Chem* 279:21003-21011.
- Pennington K, Focking M, McManus CA, Pariante CM, Dunn MJ, Cotter DR (2009) A proteomic investigation of similarities between conventional and herbal antidepressant treatments. *J Psychopharmacol* 23:520-530.
- Penzes P, Cahill ME, Jones KA, VanLeeuwen JE, Woolfrey KM (2011) Dendritic spine pathology in neuropsychiatric disorders. *Nat Neurosci* 14:285-293.
- Perera TD, Dwork AJ, Keegan KA, Thirumangalakudi L, Lipira CM, Joyce N, Lange C, Higley JD, Rosoklija G, Hen R, Sackeim HA, Coplan JD (2011) Necessity of hippocampal neurogenesis for the therapeutic action of antidepressants in adult nonhuman primates. *PLoS One* 6:e17600.
- Perera TD, Coplan JD, Lisanby SH, Lipira CM, Arif M, Carpio C, Spitzer G, Santarelli L, Scharf B, Hen R, Rosoklija G, Sackeim HA, Dwork AJ (2007) Antidepressant-induced neurogenesis in the hippocampus of adult nonhuman primates. *J Neurosci* 27:4894-4901.
- Petrak J, Ivanek R, Toman O, Cmejla R, Cmejlova J, Vyoral D, Zivny J, Vulpe CD (2008) Deja vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. *Proteomics* 8:1744-1749.
- Petrides G, Fink M, Husain MM, Knapp RG, Rush AJ, Mueller M, Rummans TA, O'Connor KM, Rasmussen KG, Jr., Bernstein HJ, Biggs M, Bailine SH, Kellner CH (2001) ECT remission rates in psychotic versus nonpsychotic depressed patients: a report from CORE. *J ECT* 17:244-253.
- Piccinni A, Del Debbio A, Medda P, Bianchi C, Roncaglia I, Veltri A, Zanello S, Massimetti E, Origlia N, Domenici L, Marazziti D, Dell'Osso L (2009) Plasma Brain-Derived Neurotrophic Factor in treatment-resistant depressed patients receiving electroconvulsive therapy. *Eur Neuropsychopharmacol* 19:349-355.
- Piccoli G, Verpelli C, Tonna N, Romorini S, Alessio M, Nairn AC, Bachi A, Sala C (2007) Proteomic analysis of activity-dependent synaptic plasticity in hippocampal neurons. *J Proteome Res* 6:3203-3215.
- Pine DS, Cohen P, Johnson JG, Brook JS (2002) Adolescent life events as predictors of adult depression. *J Affect Disord* 68:49-57.
- Piosik PA, van Groenigen M, Ponne NJ, Bolhuis PA, Baas F (1995) RC3/neurogranin structure and expression in the caprine brain in relation to congenital hypothyroidism. *Brain Res Mol Brain Res* 29:119-130.
- Pittenger C, Duman RS (2008) Stress, depression, and neuroplasticity: A convergence of mechanisms. *Neuropsychopharmacology* 33:88-109.
- Piubelli C, Vighini M, Mathe AA, Domenici E, Carboni L (2011a) Escitalopram modulates neuron-remodelling proteins in a rat gene-environment interaction model of depression as revealed by proteomics. Part I: genetic background. *Int J Neuropsychopharmacol* 14:796-833.
- Piubelli C, Vighini M, Mathe AA, Domenici E, Carboni L (2011b) Escitalopram affects cytoskeleton and synaptic plasticity pathways in a rat gene-environment interaction model of depression as revealed by proteomics. Part II: environmental challenge. *Int J Neuropsychopharmacol* 14:834-855.

- Piubelli C, Gruber S, El Khoury A, Mathe AA, Domenici E, Carboni L (2011c) Nortriptyline influences protein pathways involved in carbohydrate metabolism and actin-related processes in a rat gene-environment model of depression. *Eur Neuropsychopharmacol* 21:545-562.
- Ploski JE, Newton SS, Duman RS (2007) Electroconvulsive seizure-induced gene expression profile of the hippocampus dentate gyrus granule cell layer (vol 99, pg 1122, 2006). *J Neurochem* 101:863-864.
- Pohlack ST, Nees F, Ruttorf M, Witt SH, Nieratschker V, Rietschel M, Flor H (2011) Risk variant for schizophrenia in the neurogranin gene impacts on hippocampus activation during contextual fear conditioning. *Mol Psychiatry* 16:1072-1073.
- Poland J, Rabilloud T, Sinha P (2005) Silver Staining of 2D Gels. In: *The Proteomics Protocols Handbook* (Walker JM, ed). Totowa New Jersey: Humana Press.
- Porcelli S, Drago A, Fabbri C, Gibiino S, Calati R, Serretti A (2011) Pharmacogenetics of antidepressant response. *Journal of Psychiatry and Neuroscience* 36:87-113.
- Porsolt RD, Brossard G, Hautbois C, Roux S (2001) Rodent models of depression: forced swimming and tail suspension behavioral despair tests in rats and mice. *Curr Protoc Neurosci* Chapter 8:Unit 8 10A.
- Porsolt R.D, Jalfre M., (1977) Depression: A new animal model sensitive to antidepressant treatment. *Nature* 266:730-732.
- Posener JA, Wang L, Price JL, Gado MH, Province MA, Miller MI, Babb CM, Csernansky JG (2003) High-dimensional mapping of the hippocampus in depression. *Am J Psychiat* 160:83-89.
- Prabakaran S, Swatton JE, Ryan MM, Huffaker SJ, Huang JT, Griffin JL, Wayland M, Freeman T, Dudbridge F, Lilley KS, Karp NA, Hester S, Tkachev D, Mimmack ML, Yolken RH, Webster MJ, Torrey EF, Bahn S (2004) Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. *Mol Psychiatry* 9:684-697, 643.
- Prudic J (2005) Electroconvulsive Therapy. In: *Comprehensive Textbook of Psychiatry*, 8 Edition (Saddock BJ, V.J. S, eds). Philadelphia: Lipincott, Williams and Wilkins.
- Prudic J, Peyser S, Sackeim HA (2000) Subjective memory complaints: a review of patient self-assessment of memory after electroconvulsive therapy. *Journal of ECT* 16:121-132.
- Quante A, Luborzewski A, Brakemeier EL, Merkl A, Danker-Hopfe H, Bajbouj M (2011) Effects of 3 different stimulus intensities of ultrabrief stimuli in right unilateral electroconvulsive therapy in major depression: a randomized, double-blind pilot study. *J Psychiatr Res* 45:174-178.
- Quinn CC, Gray GE, Hockfield S (1999) A family of proteins implicated in axon guidance and outgrowth. *J Neurobiol* 41:158-164.
- Ramagli LS, Rodriguez LV (1985) Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 6:559-563.
- Ramakers GM, De Graan PN, Urban IJ, Kraay D, Tang T, Pasinelli P, Oestreicher AB, Gispen WH (1995) Temporal differences in the phosphorylation state of pre- and postsynaptic protein kinase C substrates B-50/GAP-43 and neurogranin during long-term potentiation. *J Biol Chem* 270:13892-13898.

- Rami G, Caillard O, Medina I, Pellegrino C, Fattoum A, Ben-Ari Y, Ferhat L (2006) Change in the shape and density of dendritic spines caused by overexpression of acidic calponin in cultured hippocampal neurons. *Hippocampus* 16:183-197.
- Ramser EM, Buck F, Schachner M, Tilling T (2010) Binding of alphaII spectrin to 14-3-3beta is involved in NCAM-dependent neurite outgrowth. *Mol Cell Neurosci* 45:66-74.
- Ranck JBJ (1975) Which Elements are Excited in Electrical Stimulation of Mammalian Central Nervous System: A Review. *Brain Res* 98:417-440.
- Rantamaki T, Hendolin P, Kankaanpaa A, Mijatovic J, Piepponen P, Domenici E, Chao MV, Mannisto PT, Castren E (2007) Pharmacologically diverse antidepressants rapidly activate brain-derived neurotrophic factor receptor TrkB and induce phospholipase-Cgamma signaling pathways in mouse brain. *Neuropsychopharmacology* 32:2152-2162.
- Rao MS, Shetty AK (2004) Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. *Eur J Neurosci* 19:234-246.
- Rasband WS (1997-2005) ImageJ. In. Bethesda, Maryland, USA: US National Institutes of Health.
- Rasmusson AM, Shi L, Duman R (2002) Downregulation of BDNF mRNA in the hippocampal dentate gyrus after re-exposure to cues previously associated with footshock. *Neuropsychopharmacology* 27:133-142.
- Reed R, Holmes D, Weyers J, Jones A (2003) Practical skills in biomolecular sciences, 2nd Edition. England: Pearson Education Limited.
- Renart J, Reiser J, Stark GR (1979) Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc Natl Acad Sci U S A* 76:3116-3120.
- Renault PF, Hoofnagle JH, Park Y, Mullen KD, Peters M, Jones DB, Rustgi V, Jones EA (1987) Psychiatric complications of long-term interferon alfa therapy. *Archives of Internal Medicine* 147:1577-1580.
- Rencher AC (2002) Methods of Multivariate Analysis: Introduction. In: Methods of Multivariate Analysis 2nd Edition. USA: Wiley-Interscience.
- Represa A, Jorquera I, Le Gal La Salle G, Ben-Ari Y (1993) Epilepsy induced collateral sprouting of hippocampal mossy fibers: does it induce the development of ectopic synapses with granule cell dendrites? *Hippocampus* 3:257-268.
- Rethorst CD, Wipfli BM, Landers DM (2009) The antidepressive effects of exercise: a meta-analysis of randomized trials. *Sports Med* 39:491-511.
- Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science* 286:2358-2361.
- Robinson AA, Westbrook JA, English JA, Boren M, Dunn MJ (2009) Assessing the use of thermal treatment to preserve the intact proteomes of post-mortem heart and brain tissue. *Proteomics* 9:4433-4444.
- Roepke S, Luborzewski A, Schindler F, Quante A, Anghelescu I, Heuser I, Bajbouj M (2011) Stimulus pulse-frequency-dependent efficacy and cognitive adverse effects of ultrabrief-pulse electroconvulsive therapy in patients with major depression. *J ECT* 27:109-113.

- Romi F, Helgeland G, Gilhus NE (2011) Heat-shock proteins in clinical neurology. *European neurology* 66:65-69.
- Rosa MA, Rosa MO, Daltio CS, Abreu LN, Marcolin MA (2006) Open Trial on the Efficacy of Right Unilateral Electroconvulsive Therapy with Titration and High Charge. *Journal of ECT* 22:237-239.
- Rosenthal A, Goeddel DV, Nguyen T, Martin E, Burton LE, Shih A, Laramée GR, Wurm F, Mason A, Nikolics K, et al. (1991) Primary structure and biological activity of human brain-derived neurotrophic factor. *Endocrinology* 129:1289-1294.
- Rozenblum GT, Gimona M (2008) Calponins: adaptable modular regulators of the actin cytoskeleton. *Int J Biochem Cell Biol* 40:1990-1995.
- Rubin RT, Phillips JJ, McCracken JT, Sadow TF (1996) Adrenal gland volume in major depression: relationship to basal and stimulated pituitary-adrenal cortical axis function. *Biological Psychiatry* 40:89-97.
- Rudberg I, Mohebi B, Hermann M, Refsum H, Molden E (2008) Impact of the ultrarapid CYP2C19*17 allele on serum concentration of escitalopram in psychiatric patients. *Clinical Pharmacology and Therapeutics* 83:322-327.
- Rush AJ, Trivedi M, Fava M (2003) Depression, IV: STAR*D treatment trial for depression. *Am J Psychiat* 160:237.
- Rush AJ, Trivedi MH, Wisniewski SR, Nierenberg AA, Stewart JW, Warden D, Niederehe G, Thase ME, Lavori PW, Lebowitz BD, McGrath PJ, Rosenbaum JF, Sackeim HA, Kupfer DJ, Luther J, Fava M (2006) Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. *Am J Psychiat* 163:1905-1917.
- Rybakowski JK (2008) BDNF gene: functional Val66Met polymorphism in mood disorders and schizophrenia. *Pharmacogenomics* 9:1589-1593.
- Sackeim HA (2004) Convulsant and anticonvulsant properties of electroconvulsive therapy: towards a focal form of brain stimulation. *Clin Neurosci Res* 4:39-57.
- Sackeim HA, Prudic J, Devanand DP, Kiersky JE, Fitzsimons L, Moody BJ, McElhiney MC, Coleman EA, Settembrino JM (1993) Effects of Stimulus Intensity and Electrode Placement on the Efficacy and Cognitive Effects of Electroconvulsive Therapy. *New England Journal of Medicine* 328:839-846.
- Sackeim HA, Prudic J, Devanand DP, Nobler MS, Lisanby SH, Peyser S, Fitzsimons L, Moody BJ, Clark J (2000) A prospective, randomized, double-blind comparison of bilateral and right unilateral electroconvulsive therapy at different stimulus intensities. *Arch Gen Psychiatry* 57:425-434.
- Sackeim HA, Prudic J, Nobler MS, Fitzsimons L, Lisanby SH, Payne N, Berman RM, Brakemeier EL, Perera T, Devanand DP (2008) Effects of pulse width and electrode placement on the efficacy and cognitive effects of electroconvulsive therapy. *Brain Stimulation* 1:71-83.
- Sairanen M, O'Leary OF, Knuuttila JE, Castren E (2007) Chronic antidepressant treatment selectively increases expression of plasticity-related proteins in the hippocampus and medial prefrontal cortex of the rat. *Neuroscience* 144:368-374.
- Sairanen M, Lucas G, Ernfors P, Castren M, Castren E (2005) Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus. *J Neurosci* 25:1089-1094.

- Samara A, Vougas K, Papadopoulou A, Anastasiadou E, Baloyanni N, Paronis E, Chrousos GP, Tsangaris GT (2011) Proteomics reveal rat hippocampal lateral asymmetry. *Hippocampus* 21:108-119.
- Sanabria H, Digman MA, Gratton E, Waxham MN (2008) Spatial diffusivity and availability of intracellular calmodulin. *Biophysical journal* 95:6002-6015.
- Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 301:805-809.
- Sartorius A, Hellweg R, Litzke J, Vogt M, Dormann C, Vollmayr B, Danker-Hopfe H, Gass P (2009) Correlations and Discrepancies between Serum and Brain Tissue Levels of Neurotrophins after Electroconvulsive Treatment in Rats. *Pharmacopsychiatry* 42:270-276.
- Sauers DG (1997) Hotelling's T2 statistic for multivariate statistical process control: a nonrigorous approach. *Quality Engineering* 9:627-634.
- Schaaf MJ, Hoetelmans RW, de Kloet ER, Vreugdenhil E (1997) Corticosterone regulates expression of BDNF and trkB but not NT-3 and trkC mRNA in the rat hippocampus. *J Neurosci Res* 48:334-341.
- Schaaf MJ, de Jong J, de Kloet ER, Vreugdenhil E (1998) Downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. *Brain Res* 813:112-120.
- Schildkraut JJ, Kety SS (1967) Biogenic amines and emotion. *Science* 156:21-37.
- Schlicht K, Buttner A, Siedler F, Scheffer B, Zill P, Eisenmenger W, Ackenheil M, Bondy B (2007) Comparative proteomic analysis with postmortem prefrontal cortex tissues of suicide victims versus controls. *J Psychiatr Res* 41:493-501.
- Schmidt HD, Duman RS (2007) The role of neurotrophic factors in adult hippocampal neurogenesis, antidepressant treatments and animal models of depressive-like behavior. *Behav Pharmacol* 18:391-418.
- Schmidt HD, Duman RS (2010) Peripheral BDNF Produces Antidepressant-Like Effects in Cellular and Behavioral Models. *Neuropsychopharmacology* 35:2378-2391.
- Schosser A et al. (2011) Genomewide association scan of suicidal thoughts and behaviour in major depression. *PLoS ONE* 6:e20690.
- Scott BW, Wojtowicz JM, Burnham VM (2000) Neurogenesis in the dentate gyrus of the rat following electroconvulsive shock seizures. *Exp Neurol* 165:231-236.
- Segi-Nishida E, Warner-Schmidt JL, Duman RS (2008) Electroconvulsive seizure and VEGF increase the proliferation of neural stem-like cells in rat hippocampus. *Proc Natl Acad Sci U S A* 105:11352-11357.
- Semkovska M, McLoughlin DM (2010) Objective Cognitive Performance Associated with Electroconvulsive Therapy for Depression: A Systematic Review and Meta-Analysis. *Biological Psychiatry* 68:568-577.
- Semkovska M, Keane D, Babalola O, McLoughlin DM (2011) Unilateral brief-pulse electroconvulsive therapy and cognition: Effects of electrode placement, stimulus dosage and time. *J Psychiatr Res* 45:770-780.
- Seo J, Lee KJ (2004) Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. *Journal of biochemistry and molecular biology* 37:35-44.

- Sheline YI (2000) 3D MRI studies of neuroanatomic changes in unipolar major depression: the role of stress and medical comorbidity. *Biological Psychiatry* 48:791-800.
- Sheline YI, Gado MH, Kraemer HC (2003) Untreated depression and hippocampal volume loss. *Am J Psychiat* 160:1516-1518.
- Sheline YI, Sanghavi M, Mintun MA, Gado MH (1999) Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. *J Neurosci* 19:5034-5043.
- Shen K, Teruel MN, Subramanian K, Meyer T (1998) CaMKII β functions as an F-actin targeting module that localizes CaMKII α / β heterooligomers to dendritic spines. *Neuron* 21:593-606.
- Sheng M, McFadden G, Greenberg ME (1990) Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. *Neuron* 4:571-582.
- Shi J et al. (2011) Genome-wide association study of recurrent early-onset major depressive disorder. *Mol Psychiatry* 16:193-201.
- Shirayama Y, Chen ACH, Nakagawa S, Russell DS, Duman RS (2002) Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *J Neurosci* 22:3251-3261.
- Shiwach RS, Reid WH, Carmody TJ (2001) An analysis of reported deaths following electroconvulsive therapy in Texas, 1993-1998. *Psychiatric Services* 52:1095-1097.
- Shotton DM, Burke BE, Branton D (1979) The molecular structure of human erythrocyte spectrin. Biophysical and electron microscopic studies. *J Mol Biol* 131:303-329.
- Shyn SI, Hamilton SP (2010) The genetics of major depression: moving beyond the monoamine hypothesis. *Psychiatric Clinics of North America* 33:125-140.
- Shyn SI, Shi J, Kraft JB, Potash JB, Knowles JA, Weissman MM, Garriock HA, Yokoyama JS, McGrath PJ, Peters EJ, Scheftner WA, Coryell W, Lawson WB, Jancic D, Gejman PV, Sanders AR, Holmans P, Slager SL, Levinson DF, Hamilton SP (2011) Novel loci for major depression identified by genome-wide association study of Sequenced Treatment Alternatives to Relieve Depression and meta-analysis of three studies. *Mol Psychiatr* 16:202-215.
- Siegel GJ, Agranoff BW, Albers RW, et al (1999) *Basic Neurochemistry: Molecular, Cellular and Medical Aspects*, 6th Edition. Philadelphia: Lippincott-Raven.
- Sienaert P, Vansteelandt K, Demyttenaere K, Peuskens J (2009) Randomized comparison of ultra-brief bifrontal and unilateral electroconvulsive therapy for major depression: Clinical efficacy. *J Affect Disord* 116:106-112.
- Sienaert P, Vansteelandt K, Demyttenaere K, Peuskens J (2010) Randomized comparison of ultra-brief bifrontal and unilateral electroconvulsive therapy for major depression: cognitive side-effects. *J Affect Disord* 122:60-67.
- Sitek B, Scheibe B, Jung K, Schramm A, Stuhler K (2002) Difference Gel Electrophoresis (DIGE): the NEXt Generation of Two-Dimensional Gel Electrophoresis for Clinical Research. In: *Proteomics in drug research* (Hamacher M, ed). Weinheim: Wiley-VCH.
- Siuciak JA, Lewis DR, Wiegand SJ, Lindsay RM (1997) Antidepressant-like effect of brain-derived neurotrophic factor (BDNF). *Pharmacol Biochem Behav* 56:131-137.

- Skynner HA, Amos DP, Murray F, Salim K, Knowles MR, Munoz-Sanjuan I, Camargo LM, Bonnert TP, Guest PC (2006) Proteomic analysis identifies alterations in cellular morphology and cell death pathways in mouse brain after chronic corticosterone treatment. *Brain Res* 1102:12-26.
- Sleep J, Wilson D, Simmons R, Gratzer W (1999) Elasticity of the red cell membrane and its relation to hemolytic disorders: an optical tweezers study. *Biophysical journal* 77:3085-3095.
- Smith RS (1991) The macrophage theory of depression. *Medical Hypotheses* 35:298-306.
- Smith MA, Makino S, Kvetnansky R, Post RM (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci* 15:1768-1777.
- Smith R, Fadok RA, Purcell M, Liu S, Stonnington C, Spetzler RF, Baxter LC (2011) Localizing sadness activation within the subgenual cingulate in individuals: a novel functional MRI paradigm for detecting individual differences in the neural circuitry underlying depression. *Brain Imaging Behav.*
- Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature* 476:458-461.
- Sousa N, Lukoyanov NV, Madeira MD, Almeida OF, Paula-Barbosa MM (2000) Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. *Neuroscience* 97:253-266.
- Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98:503-517.
- Stefansson H et al. (2009) Common variants conferring risk of schizophrenia. *Nature* 460:744-747.
- Stelzhammer V, Rothermundt M, Guest PC, Michael N, Sondermann C, Kluge W, Martins-de-Souza D, Rahmoune H, Bahn S (2011) Proteomic changes induced by anaesthesia and muscle relaxant treatment prior to electroconvulsive therapy. *Proteomics Clin Appl.*
- Stewart CA, Reid IC (2000) Repeated ECS and fluoxetine administration have equivalent effects on hippocampal synaptic plasticity. *Psychopharmacology (Berl)* 148:217-223.
- Strasser P, Gimona M, Moessler H, Herzog M, Small JV (1993) Mammalian calponin. Identification and expression of genetic variants. *FEBS Lett* 330:13-18.
- Strong M, Eisenberg D (2007) The protein network as a tool for finding novel drug targets. *Progress in drug research* 64:191, 193-215.
- Sullivan PF, Neale MC, Kendler KS (2000) Genetic epidemiology of major depression: review and meta-analysis. *Am J Psychiatr* 157:1552-1562.
- Sullivan PF et al. (2009) Genome-wide association for major depressive disorder: a possible role for the presynaptic protein piccolo. *Mol Psychiatr* 14:359-375.
- Surget A, Saxe M, Leman S, Ibarguen-Vargas Y, Chalon S, Griebel G, Hen R, Helzung C (2008) Drug-dependent requirement of hippocampal neurogenesis in a model of depression and of antidepressant reversal. *Biological Psychiatry* 64:293-301.

- Surget A, Tanti A, Leonardo ED, Laugeray A, Rainer Q, Touma C, Palme R, Griebel G, Ibarguen-Vargas Y, Hen R, Belzung C (2011) Antidepressants recruit new neurons to improve stress response regulation. *Mol Psychiatr*.
- Sussman MA, Sakhi S, Tocco G, Najm I, Baudry M, Kedes L, Schreiber SS (1994) Neural tropomodulin: developmental expression and effect of seizure activity. *Brain Res Dev Brain Res* 80:45-53.
- Swatton JE, Prabakaran S, Karp NA, Lilley KS, Bahn S (2004) Protein profiling of human postmortem brain using 2-dimensional fluorescence difference gel electrophoresis (2-D DIGE). *Mol Psychiatr* 9:128-143.
- Tanapat P, Hastings NB, Reeves AJ, Gould E (1999) Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J Neurosci* 19:5792-5801.
- Task Force Report of the American Psychiatric Association (2001) The Practice of Electroconvulsive Therapy: Recommendations for Treatment, Training, and Privileging. In, 2nd Edition. Washington D.C.: American Psychiatric Association.
- Taurines R, Dudley E, Grassl J, Warnke A, Gerlach M, Coogan AN, Thome J (2011) Proteomic research in psychiatry. *J Psychopharmacol* 25:151-196.
- Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen ZY, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. *J Neurosci* 25:5455-5463.
- The UK ECT Review Group (2003) Efficacy and safety of electroconvulsive therapy in depressive disorders: a systematic review and meta-analysis. *Lancet* 361:799-808.
- ThermoFisher Scientific (2008) LTQ Orbitrap XL Hardware Manual.
- ThermoScientific (2007) Xcalibur Bioworks User guide. In: XCALI-97194 Revision B (Scientific T, ed).
- Thomas K, Davies A (2005) Neurotrophins: a ticket to ride for BDNF. *Current Biology* 15:R262-264.
- Thompson JW, Weiner RD, Myers CP (1994) Use of ECT in the United States in 1975, 1980, and 1986. *Am J Psychiat* 151:1657-1661.
- Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H (1993) Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron* 10:475-489.
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76:4350-4354.
- Tsuang MT, Stone WS, Faraone SV (2001) Genes, environment and schizophrenia. *Br J Psychiatry Suppl* 40:s18-24.
- Tsutsumi T, Fujiki M, Akiyoshi J, Horinouchi Y, Isogawa K, Hori S, Nagayama H (2002) Effect of repetitive transcranial magnetic stimulation on forced swimming test. *Prog Neuropsychopharmacol Biol Psychiatry* 26:107-111.
- Tyler WJ, Pozzo-Miller L (2003) Miniature synaptic transmission and BDNF modulate dendritic spine growth and form in rat CA1 neurones. *Journal of Physiology* 553:497-509.
- Ulett GA, Smith K, Gleser GC (1956) Evaluation of convulsive and subconvulsive shock therapies utilizing a control group. *Am J Psychiat* 112:795-802.

- Ungewickell E, Gratzner W (1978) Self-association of human spectrin. A thermodynamic and kinetic study. *Eur J Biochem* 88:379-385.
- Vaidya VA, Terwilliger RZ, Duman RS (2000) Alterations in heavy and light neurofilament proteins in hippocampus following chronic ECS administration. *Synapse* 35:137-143.
- Vakili K, Pillay SS, Lafer B, Fava M, Renshaw PF, Bonello-Cintron CM, Yurgelun-Todd DA (2000) Hippocampal volume in primary unipolar major depression: a magnetic resonance imaging study. *Biological Psychiatry* 47:1087-1090.
- van der Hart MGC, Czeh B, de Biurrun G, Michaelis T, Watanabe T, Natt O, Frahm J, Fuchs E (2002) Substance P receptor antagonist and clomipramine prevent stress-induced alterations in cerebral metabolites, cytochrome in the dentate gyrus and hippocampal volume. *Mol Psychiatry* 7:933-941.
- Van Eyck JE, Dunn M, eds (2008) *Clinical proteomics: from diagnosis to therapy*. Germany: Wiley VCH.
- van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. *Nature* 415:1030-1034.
- van Tol MJ, van der Wee NJ, van den Heuvel OA, Nielen MM, Demenescu LR, Aleman A, Renken R, van Buchem MA, Zitman FG, Veltman DJ (2010) Regional brain volume in depression and anxiety disorders. *Arch Gen Psychiatry* 67:1002-1011.
- Veith RC, Lewis N, Linares OA, Barnes RF, Raskind MA, Villacres EC, Murburg MM, Ashleigh EA, Castillo S, Peskind ER, et al. (1994) Sympathetic nervous system activity in major depression. Basal and desipramine-induced alterations in plasma norepinephrine kinetics. *Arch Gen Psychiatry* 51:411-422.
- Verma R, Holmans P, Knowles JA, Grover D, Evgrafov OV, Crowe RR, Scheftner WA, Weissman MM, DePaulo JR, Jr., Potash JB, Levinson DF (2008) Linkage disequilibrium mapping of a chromosome 15q25-26 major depression linkage region and sequencing of NTRK3. *Biological Psychiatry* 63:1185-1189.
- Vermetten E, Vythilingam M, Southwick SM, Charney DS, Bremner JD (2003) Long-term treatment with paroxetine increases verbal declarative memory and hippocampal volume in posttraumatic stress disorder. *Biological Psychiatry* 54:693-702.
- Vieta E, Colom F (2011) Therapeutic options in treatment-resistant depression. *Ann Med*.
- Vollmayr B, Simonis C, Weber S, Gass P, Henn F (2003) Reduced cell proliferation in the dentate gyrus is not correlated with the development of learned helplessness. *Biological Psychiatry* 54:1035-1040.
- von Bohlen Und Halbach O (2009) Structure and function of dendritic spines within the hippocampus. *Annals of Anatomy* 191:518-531.
- von Gunten A, Fox NC, Cipolotti L, Ron MA (2000) A volumetric study of hippocampus and amygdala in depressed patients with subjective memory problems. *J Neuropsychiatr Clin Neurosci* 12:493-498.
- Voss T, Haberl P (2000) Observations on the reproducibility and matching efficiency of two-dimensional electrophoresis gels: consequences for comprehensive data analysis. *Electrophoresis* 21:3345-3350.
- Wada T, Haigh JJ, Ema M, Hitoshi S, Chaddah R, Rossant J, Nagy A, van der Kooy D (2006) Vascular endothelial growth factor directly inhibits primitive neural stem cell survival but promotes definitive neural stem cell survival. *J Neurosci* 26:6803-6812.

- Wang HD, Dunnavant FD, Jarman T, Deutch AY (2004) Effects of antipsychotic drugs on neurogenesis in the forebrain of the adult rat. *Neuropsychopharmacology* 29:1230-1238.
- Warner-Schmidt JL, Duman RS (2008b) VEGF as a potential target for therapeutic intervention in depression. *Curr Opin Pharmacol* 8:14-19.
- Warner-Schmidt JL, Madsen TM, Duman RS (2008a) Electroconvulsive seizure restores neurogenesis and hippocampus-dependent fear memory after disruption by irradiation. *Eur J Neurosci* 27:1485-1493.
- Wasserman D, Sokolowski M, Rozanov V, Wasserman J (2008) The CRHR1 gene: a marker for suicidality in depressed males exposed to low stress. *Genes Brain Behav* 7:14-19.
- Watakabe A, Kobayashi R, Helfman DM (1996) N-tropomodulin: a novel isoform of tropomodulin identified as the major binding protein to brain tropomyosin. *Journal of cell science* 109 (Pt 9):2299-2310.
- Watson JB, Sutcliffe JG, Fisher RS (1992) Localization of the protein kinase C phosphorylation/calmodulin-binding substrate RC3 in dendritic spines of neostriatal neurons. *Proc Natl Acad Sci U S A* 89:8581-8585.
- Wayman GA, Lee YS, Tokumitsu H, Silva AJ, Soderling TR (2008) Calmodulin-kinases: modulators of neuronal development and plasticity. *Neuron* 59:914-931.
- Weber A, Pennise CR, Babcock GG, Fowler VM (1994) Tropomodulin caps the pointed ends of actin filaments. *J Cell Biol* 127:1627-1635.
- Webster JJ, Carlstedt-Duke J (2002) Involvement of multidrug resistance proteins (MDR) in the modulation of glucocorticoid response. *Journal of Steroid Biochemistry and Molecular Biology* 82:277-288.
- Weiner RD, Rogers HJ, Davidson JR, Squire LR (1986) Effects of stimulus parameters on cognitive side effects. *Ann NY Acad Sci* 462:315-325.
- Weissman MM, ed (2001) *Treatment of depression: bridging the 21st century*. New York: American Psychopathological Publications.
- Wichers MC, Maes M (2004) The role of indoleamine 2,3-dioxygenase (IDO) in the pathophysiology of interferon-alpha-induced depression. *Journal of Psychiatry and Neuroscience* 29:11-17.
- Wilkins MR, Sanchez JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, Williams KL (1996) Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev* 13:19-50.
- Wojtowicz JM, Kee N (2006) BrdU assay for neurogenesis in rodents. *Nat Protoc* 1:1399-1405.
- Wong ML, Licinio J (2001) Research and treatment approaches to depression. *Nat Rev Neurosci* 2:343-351.
- Wong ML, Licinio J (2004) From monoamines to genomic targets: A paradigm shift for drug discovery in depression. *Nature Reviews Drug Discovery* 3:136-151.
- Wong ML, Kling MA, Munson PJ, Listwak S, Licinio J, Prolo P, Karp B, McCutcheon IE, Geraciotti TD, Jr., DeBellis MD, Rice KC, Goldstein DS, Veldhuis JD, Chrousos GP, Oldfield EH, McCann SM, Gold PW (2000) Pronounced and sustained central hypernoradrenergic function in major depression with melancholic features: relation to hypercortisolism and corticotropin-releasing hormone. *Proceedings of the National Academy of Science USA* 97:325-330.

- Wu S, Sangerman J, Li M, Brough GH, Goodman SR, Stevens T (2001) Essential control of an endothelial cell ISOC by the spectrin membrane skeleton. *J Cell Biol* 154:1225-1233.
- Xia Z, Storm DR (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nat Rev Neurosci* 6:267-276.
- Yamashiro S, Speicher KD, Speicher DW, Fowler VM (2010) Mammalian tropomodulins nucleate actin polymerization via their actin monomer binding and filament pointed end-capping activities. *J Biol Chem* 285:33265-33280.
- Yates JR, Ruse CI, Nakorchevsky A (2009) Proteomics by mass spectrometry: approaches, advances, and applications. *Annual review of biomedical engineering* 11:49-79.
- Yirmiya R, Weidenfeld J, Pollak Y, Morag M, Morag A, Avitsur R, Barak O, Reichenberg A, Cohen E, Shavit Y, Ovadia H (1999) Cytokines, "depression due to a general medical condition," and antidepressant drugs. *Advances in Experimental Medicine and Biology* 461:283-316.
- Yuuki N, Ida I, Oshima A, Kumano H, Takahashi K, Fukuda M, Oriuchi N, Endo K, Matsuda H, Mikuni M. (2005) HPA axis normalization, estimated by DEX/CRH test, but less alteration on cerebral glucose metabolism in depressed patients receiving ECT after medication treatment failures. *Acta Psychiatr Scand.* 112 (4):257-65.
- Zagrebelsky M, Holz A, Dechant G, Barde YA, Bonhoeffer T, Korte M (2005) The p75 neurotrophin receptor negatively modulates dendrite complexity and spine density in hippocampal neurons. *J Neurosci* 25:9989-9999.
- Zetterstrom TS, Pei Q, Grahame-Smith DG (1998) Repeated electroconvulsive shock extends the duration of enhanced gene expression for BDNF in rat brain compared with a single administration. *Brain Research Molecular Brain Research* 57:106-110.
- Zhabotinsky AM, Camp RN, Epstein IR, Lisman JE (2006) Role of the neurogranin concentrated in spines in the induction of long-term potentiation. *J Neurosci* 26:7337-7347.
- Zhang ZJ, Kang WH, Li Q, Tan QR (2007) The beneficial effects of the herbal medicine Free and Easy Wanderer Plus (FEWP) for mood disorders: double-blind, placebo-controlled studies. *J Psychiatr Res* 41:828-836.
- Zhong L, Gerges NZ (2010) Neurogranin and synaptic plasticity balance. *Communicative & integrative biology* 3:340-342.
- Zhong L, Kaleka KS, Gerges NZ (2011) Neurogranin phosphorylation fine-tunes long-term potentiation. *Eur J Neurosci* 33:244-250.
- Zhong L, Cherry T, Bies CE, Florence MA, Gerges NZ (2009) Neurogranin enhances synaptic strength through its interaction with calmodulin. *EMBO J* 28:3027-3039.

APPENDIX

1.1 Hippocampal proteins

Accession Number	Protein Name
B0BNF1	Septin-8
B2GUZ5	F-actin-capping protein subunit alpha-1
B2GUZ5	F-actin-capping protein subunit alpha-1
O08557	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1
O08651	D-3-phosphoglycerate dehydrogenase
O08839	Myc box-dependent-interacting protein 1
O35244	Peroxiredoxin-6
O35814	Stress-induced-phosphoprotein 1
O88767	Protein DJ-1
P02688	Myelin basic protein S
P02770	Serum albumin
P04642	L-lactate dehydrogenase A chain
P05197	Elongation factor 2
P05371	Clusterin
P06761	78 kDa glucose-regulated protein
P07323	Gamma-enolase
P08413	Calcium/ calmodulin-dependent protein kinase type II subunit beta
P08461	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial
P11232	Thioredoxin
P11980	Pyruvate kinase isozymes M1/M2
P14659	Heat shock-related 70 kDa protein 2
P14669	Annexin A3
P15865	Histone H1.2
P16086	Spectrin alpha chain, brain
P16446	Phosphatidylinositol transfer protein alpha isoform
P16617	Phosphoglycerate kinase 1
P19332	Microtubule-associated protein tau
P19527	Neurofilament light polypeptide
P21807	Peripherin
P28073	Proteasome subunit beta type-6
P28480	T-complex protein 1 subunit alpha
P31000	Vimentin

P35435	ATP synthase subunit gamma, mitochondrial
P38062	Methionine aminopeptidase 2
P42123	L-lactate dehydrogenase B chain
P46462	Transitional endoplasmic reticulum ATPase
P47819	Glial fibrillary acidic protein
P47942	Dihydropyrimidinase-related protein 2
P48675	Desmin
P49432	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial
P54311	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1
P54313	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2
P54921	Alpha-soluble NSF attachment protein
P55063	Heat shock 70 kDa protein 1-like
P60711	Actin, cytoplasmic 1
P62630	Elongation factor 1-alpha 1
P62738	Actin, aortic smooth muscle
P62747	Rho-related GTP-binding protein RhoB
P62804	Histone H4
P62853	40S ribosomal protein S25
P62919	60S ribosomal protein L8
P63039	60 kDa heat shock protein, mitochondrial
P68035	Actin, alpha cardiac muscle 1
P68370	Tubulin alpha-1A chain
P70470	Acyl-protein thioesterase 1
P70566	Tropomodulin-2
P81155	Voltage-dependent anion-selective channel protein 2
P85108	Tubulin beta-2A chain
P85969	Beta-soluble NSF attachment protein
P97576	GrpE protein homolog 1, mitochondrial
Q00715	Histone H2B type 1
Q04940	Neurogranin
Q3KR86	Mitochondrial inner membrane protein (Fragment)
Q3T1K5	F-actin-capping protein subunit alpha-2
Q3T1K5	F-actin-capping protein subunit alpha-2
Q4QRB4	Tubulin beta-3 chain
Q561S0	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10,

	mitochondrial
Q5FVJ0	Protein RUFY3
Q5RKI0	WD repeat-containing protein 1
Q5XIF6	Tubulin alpha-4A chain
Q62950	Dihydropyrimidinase-related protein 1
Q63355	Myosin-Ic
Q63570	26S Protease Regulatory Subunit 6B
Q63754	Beta-synuclein
Q66HF1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial
Q68FQ0	T-complex protein 1 subunit epsilon
Q68FS4	Cytosol aminopeptidase
Q68FY0	Cytochrome b-c1 complex subunit 1
Q6DGG1	Abhydrolase domain-containing protein 14B
Q6P0K8	Junction plakoglobin
Q6P4Z6	Leucine carboxyl methyltransferase 1
Q6P7Q4	Lactoylglutathione lyase
Q6P9T8	Tubulin beta-2C chain
Q6P9V9	Tubulin alpha-1B chain
Q6QGW5	Steroid receptor RNA activator 1
Q76EQ0	Serine racemase
Q811X6	Lambda-crystallin homolog
Q99NA5	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial
Q9EQS0	Transaldolase
Q9JHU0	Dihydropyrimidinase-related protein 5
Q9QUL6	Vesicle-fusing ATPase
Q9QXQ0	Alpha-actinin-4
Q9QZM5	Abl interactor 1
Q9WTT6	Guanine deaminase
Q9Z0W5	Protein kinase C and casein kinase substrate in neurons protein 1
Q9Z2L0	Voltage-dependent anion-selective channel protein 1

1.2 Frontal cortex

Accession Number	Protein Name
B3GNI6	Septin-11
	N(G),N(G)-dimethylarginine
O08557	dimethylaminohydrolase 1
O35179	Endophilin-A1
O35964	Endophilin-A2
P02770	Serum albumin
P04642	L-lactate dehydrogenase A chain
P04692	Tropomyosin alpha-1 chain
P04692-4	Isoform Brain TMBR-2 of Tropomyosin alpha-1 chain
P04764	Alpha-enolase
P06761	78 kDa glucose-regulated protein
P07323	Gamma-enolase
P07632	Superoxide dismutase [Cu-Zn]
P09951	Synapsin-1
P0C169	Histone H2A type 1-C
P10719	ATP synthase subunit beta, mitochondrial
P14659	Heat shock-related 70 kDa protein 2
P15429	Beta-enolase
P16617	Phosphoglycerate kinase 1
P19527	Neurofilament light polypeptide
P23565	Alpha-internexin
	Myristoylated alanine-rich C-kinase substrate
P30009	Phosphatidylethanolamine-binding protein 1
P31044	ATP synthase subunit d, mitochondrial
P31399	Alpha-synuclein
P37377	Calponin-3
P37397	L-lactate dehydrogenase B chain
P42123	Transitional endoplasmic reticulum ATPase
P46462	Glial fibrillary acidic protein
P47819	Dihydropyrimidinase-related protein 2
P47942	Stress-70 protein, mitochondrial
P48721	Heat shock 70 kDa protein 1-like
P55063	Actin, cytoplasmic 1
P60711	Serine/threonine-protein phosphatase
	PP1-alpha catalytic subunit
P62138	14-3-3 protein epsilon
P62260	Actin, aortic smooth muscle
P62738	

P62804	Histone H4
P62986	Ubiquitin-60S ribosomal protein L40
P63018	Heat shock cognate 71 kDa protein
P63029	Translationally-controlled tumor protein
P63039	60 kDa heat shock protein, mitochondrial
P68370	Tubulin alpha-1A chain
P69897	Tubulin beta-5 chain
P85108	Tubulin beta-2A chain
P85845	Fascin
Q05175	Brain acid soluble protein 1
Q07439	Heat shock 70 kDa protein 1A/1B
Q1WIM3	Cell adhesion molecule 3
Q4QRB4	Tubulin beta-3 chain
Q5PPJ9	Endophilin-B2
Q5XIF6	Tubulin alpha-4A chain
Q5XIM9	T-complex protein 1 subunit beta
Q63610	Tropomyosin alpha-3 chain
	Uncharacterized protein C9orf119
	homolog/ DNA repair protein SWI5
Q63ZV7	homolog
	NADH-ubiquinone oxidoreductase 75
Q66HF1	kDa subunit, mitochondrial
Q68A21	Transcriptional activator protein Pur-beta
Q68FQ0	T-complex protein 1 subunit epsilon
	Tryptophanyl-tRNA synthetase,
Q6P7B0	cytoplasmic
Q6P9T8	Tubulin beta-2C chain
Q6P9V9	Tubulin alpha-1B chain
Q76EQ0	Serine racemase
Q8CH34	Zona pellucida sperm-binding protein 4
Q8VBU2	Protein NDRG2
Q8VD52	Pyridoxal phosphate phosphatase
	Isocitrate dehydrogenase [NAD] subunit
Q99NA5	alpha, mitochondrial

Publications, posters and presentations

Poster presentations

4TH European Proteomic Association (EUPA) and 6th PROCURA Meeting: 2010 Proteomics Odyssey Towards Next Decades

Estoril, Portugal

PROTEOMIC ANALYSIS OF RAT HIPPOCAMPUS AND FRONTAL CORTEX FOLLOWING ACUTE AND CHRONIC ELECTROCONVULSIVE STIMULATION

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THE ANTIDEPRESSANT EFFICACY OF ULTRA BRIEF PULSE STIMULATION COMPARED TO BRIEF PULSE STIMULATION IN THE RODENT

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PROTEOME CHANGES IN THE RAT HIPPOCAMPUS FOLLOWING ACUTE AND CHRONIC ELECTROCONVULSIVE STIMULATION

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RAT HIPPOCAMPAL PROTEOME CHANGES FOLLOWING TREATMENT WITH ELECTROCONVULSIVE STIMULATION: ELUCIDATION OF THE MECHANISMS OF ACTION OF ELECTROCONVULSIVE STIMULATION

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COMPARISON OF ULTRA BRIEF AND BRIEF PULSE ELECTROCONVULSIVE
STIMULATION ON RAT BRAIN AND BEHAVIOUR

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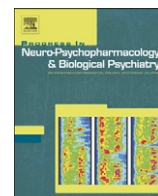
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A comparison of brief pulse and ultrabrief pulse electroconvulsive stimulation on rodent brain and behaviour

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ABSTRACT

Brief pulse electroconvulsive therapy (BP ECT; pulse width 0.5–1.5 ms) is a very effective treatment for severe depression but is associated with cognitive side-effects. It has been proposed that ultrabrief pulse (UBP; pulse width 0.25–0.30 ms) ECT may be as effective as BP ECT but have less cognitive effects because it is a more physiological form of neuronal stimulation. To investigate this further, we treated normal rats with a 10 session course of either BP (0.5 ms), UBP (0.3 ms), or sham electroconvulsive stimulation (ECS) and measured antidepressant-related changes in dentate gyrus cell proliferation and hippocampal BDNF protein levels as well as hippocampal-dependent spatial reference memory using the water plus maze and immobility time on the forced swim test. Both BP and UBP ECS induced very similar types of motor seizures. However, BP ECS but not UBP ECS treatment led to a significant, near 3-fold, increase in cell proliferation ($p = 0.026$) and BDNF levels ($p = 0.01$). In the forced swim test, only BP ECS treated animals had a significantly lower immobility time ($p = 0.046$). There was a trend for similarly reduced hippocampal-dependent memory function in both BP and UBP groups but overall there was not a significant difference between treatment and control animals when tested 10 days after completing allocated treatment. These findings show that, even though both forms of ECS elicited similar motor seizures, UBP ECS was less efficient than BP ECS in inducing antidepressant-related molecular, cellular and behavioural changes.

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1. Introduction

Electroconvulsive therapy (ECT) is the most acutely effective treatment available for severe, often treatment resistant, depression with remission rates of about 60% (Eranti et al., 2007; U.K. ECT Review Group, 2003; Kellner et al., 2010). However, its use is limited by concerns about adverse effects on memory and executive function arising during treatment though these mostly resolve within weeks of finishing a course of ECT (Semkovska and McLoughlin, 2010; Semkovska et al., 2011). Since its introduction over 70 years ago, there have been several modifications to ECT technique to reduce such cognitive side-effects while maintaining its effectiveness. These have focussed mainly on electrode placement and electrical stimulus parameters. While there seems to be little difference between bitemporal and bifrontal electrode placement (Dunne and McLoughlin, 2011), right unilateral electrode placement is associated with less adverse cognitive effects than

bitemporal ECT but has less of an anti-depressant effect (Sackeim et al., 1993). To improve effectiveness of unilateral ECT higher stimulus doses above seizure threshold are required but this is associated with increasing dose-related cognitive deficits (McCall et al., 2000; Semkovska et al., 2011). Recent studies have confirmed that high-dose unilateral ECT is as effective as modestly suprathreshold bitemporal ECT but it is not yet clear that it has substantially less cognitive adverse effects (Kellner et al., 2010).

Changes in electrical stimulus parameters have improved the efficiency of neuronal stimulation. The move from a sine-wave stimulus to brief pulse (i.e. 0.5–1.5 ms) square wave stimulus, requiring less energy, has reduced cognitive deficits but not compromised clinical effectiveness (U.K. ECT Review Group, 2003; Semkovska and McLoughlin, 2010). More recently there has been interest in using ultrabrief (i.e. 0.25–0.3 ms) pulse stimulation as this is more physiological, reducing stimulation of neurones that are already depolarising or in the refractory period (Sackeim, 2004). The first randomised trial reported a high remission rate (77%) and few cognitive deficits following high-dose right unilateral ultrabrief pulse ECT compared to brief pulse ECT (Sackeim et al., 2008). However, this high remission rate has so far not been replicated in other randomised (6–44%; (Quante et al., 2011; Sienaert et al., 2009)) and non-randomised studies (13–42%; (Loo et al., 2007; Loo et al., 2008; Niemantsverdriet et al.,

Abbreviations: ECT, electroconvulsive therapy; ECS, electroconvulsive stimulation; BP, brief pulse; UBP, ultrabrief pulse; BDNF, brain derived neurotrophic factor; FST, forced swim test.

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2011)). Like brief-pulse unilateral ECT, one possibility is that a higher stimulus charge is required and the optimal stimulus parameters remain to be established.

Electroconvulsive stimulation (ECS) is the animal model equivalent of ECT. Much has been reported on molecular and cellular changes induced by courses of repeated brief-pulse ECS in both normal rats and rat models for depression and that are believed to be relevant to antidepressant mechanisms, e.g. up-regulation of neurotrophic factors and hippocampal cell proliferation and neurogenesis (Gersner et al., 2010; Madsen et al., 2000; Newton et al., 2003). In contrast, little has been reported on such effects following ultrabrief-pulse ECS. Here we aimed to compare the effects of brief pulse and ultra-brief pulse ECS on normal rodent brain and behaviour. We sought to determine if both forms of brain stimulation would induce similar antidepressant-related changes in hippocampal cellular proliferation and brain derived neurotrophic factor (BDNF) expression. Additionally, as secondary measures, we sought to identify if there were any differences in antidepressant-related behavioural changes and cognitive function that could be attributed to the difference in stimulus pulse width.

2. Experimental procedures

2.1. Animals and treatment

Male Sprague–Dawley rats (Harlan, UK), weighing 150–200 g at intake, were housed in groups of four with food and water available ad libitum under 12 hour light–dark conditions. Animals were allowed to habituate for 10 days prior to ECS. Experiments were conducted in accordance with EU directive 2010/63/EU and guidelines of the Bioresearch Ethics Committee, Trinity College Dublin.

The experimental design is summarised in Fig. 1A. Animals were randomly allocated to a course of bilateral brief pulse (BP), ultrabrief pulse (UBP), or sham ECS. ECS was delivered thrice weekly (Monday, Wednesday, Friday) for ten sessions via ear-clip electrodes using the ECT Unit 57800 device (Ugo Basile, Italy). Parameters for BP ECS were: 0.5 ms pulse-width; 100 pulses/s; 0.7 s duration; 75 mA current; while for UBP ECS the pulse-width was reduced to 0.3 ms. Sham animals were identically handled but received no charge. Tonic and clonic seizure durations were recorded on the first, fifth and tenth ECS sessions. The tonic phase of the seizure was defined as the period starting at the end of stimulus administration during which an animal's hind legs and forearms were held rigidly against the body with some muscle twitching until the beginning of the clonic phase during which the forearms and legs extended stiffly away from the body and began flexing – extending rapidly until such motor activity was no longer apparent.

2.2. Behavioural studies

2.2.1. Water plus maze

Animals were habituated to the behavioural testing area for 1 week after completing the allocated course of ECS. Hippocampal-dependent spatial reference memory was assessed using a water plus maze (Diamond et al., 1999). The maze had four closed arms in a plus formation with a central rectangular region and was filled with water ($21 \pm 1^\circ\text{C}$) to a depth of 45 cm. A hidden platform, the same colour as the maze, was placed 2.5 cm below the level of the water and located at the distal end of one arm. Visual cues were positioned on surrounding walls. Each animal was placed, facing away from the centre, into the distal end of a pre-selected entry arm on either the left or right of the hidden platform arm for all trials on days 1–3 of testing and completed 10 such trials daily (60 s/trial; 30 s inter-trial intervals). On day 4, animals underwent 30 trials in which platform position did not change but entry into arms was pseudo-randomised so that all animals started 10 trials in each of the other three different arms. Frequency of platform acquisition, as a measure of reference memory, was calculated. Velocity (cm/s) for each treatment group and platform acquisition on each

experiment day was also measured. EthovisionXT (Noldus Information Technology, The Netherlands) was used for data collection.

2.2.2. Forced swim test

The forced swim test, a measure of responsiveness to antidepressant treatments (Porsolt et al., 1977), was conducted in two sessions on days 12–13 after completing the course of ECS, consisting of a 15 minute pre-test followed by a 5 minute test 24 h later. A 50 cm high, 25 cm diameter, clear cylinder was filled 35 cm from the base with water ($23 \pm 1^\circ\text{C}$). The length of time animals spent immobile was measured over the 5 minute test session. Immobility behaviour was recorded when animals were making only movements required to stay afloat. Animals were sacrificed 24 h after the swim test and the hippocampus was immediately dissected, snap-frozen in liquid nitrogen and stored at -80°C .

2.3. BDNF expression

Hippocampal BDNF protein levels were measured using a sandwich ELISA kit according to the manufacturer's instructions (Chemikine, Chemicon (Millipore), USA). Briefly, hippocampal samples were homogenised in the recommended lysis buffer (10% w/v). Homogenates were centrifuged at 14,000 g for 30 min and the supernatant was collected for use in the BDNF assay. The amount of BDNF in each sample was analysed in duplicate and determined from the regression line generated by BDNF standards (range 7.8 pg/ml to 500 pg/ml). Total protein concentrations were measured by Bradford protein assay. BDNF results are presented as pg BDNF per mg of total protein content in each sample.

2.4. Immunohistochemistry

To label proliferating cells in the dentate gyrus, a second set of animals was similarly treated with ECS and administered BrdU (40 mg/kg, i.p.; Sigma-Aldrich) 2–4 h post ECS on treatment days three to ten (see Fig. 1B). Twenty-four hours after the final ECS, animals were anaesthetised with urethane and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde, essentially as previously described (Kesavapany et al., 2002).

Hippocampal coronal sections ($10\ \mu\text{m}$) were mounted on slides for staining. Unless specified otherwise, all procedures took place at room temperature. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide solution. Slides were washed for 10 min under running water, microwaved on full power for 13 min in 10 mM citric acid buffer (pH6.0), washed in TBS, incubated in avidin (Vector Laboratories) for 10 min, washed twice in TBS and then incubated in biotin (Vector Laboratories) for 10 min. Following two washes in TBS and incubation in 10% normal rabbit serum (Dako) for 5 min, sections were incubated with rat anti-BrdU (1:10,000; ab6326, Abcam) in TBS for 1 h. After this, slides were washed thrice in TBS and incubated in biotinylated rabbit anti-rat secondary antibody (1:50; Vector Laboratories) in TBS for 30 min. Following two TBS washes, StrepAB/HRP complex (Vector Laboratories) was incubated on each slide for 30 min. Slides were washed once in TBS and twice in distilled water before a 15 minute DAB (SigmaFAST DAB tablets) incubation to visualise the reaction product. Following a wash with distilled water, sections were counterstained with Haematoxylin. Sections were imaged using an Olympus DP72 at $20\times$ magnification. Numbers of BrdU-positive cells in dentate gyrus subgranular zone and granule cell layer were counted blind on coded slides from every sixth section over a range of 24 sections per animal.

2.5. Statistical analyses

Data are presented as means $\pm$ SEM and were analysed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla California, USA). A

repeated measures ANOVA was used to compare seizure durations between groups. One-way ANOVAs were used to compare ELISA data and probe day platform acquisition for the water plus maze with Newman Keuls post-hoc tests as required. Repeated measures ANOVA, with Day (water plus maze Training days 1, 2, 3) or Treatment (BP ECS, UBP ECS, sham ECS) as factors, was used to analyse platform acquisition during training on the water plus maze with Bonferroni post tests as required. Kruskal–Wallis test with Dunn's multiple comparison post hoc tests was used to analyse BrdU-positive cell counts. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Tonic–clonic seizure durations

Tonic and clonic seizure durations were compared on ECS treatment days 1, 5 and 10 (Fig. 2). Repeated measures ANOVA showed that both tonic ($F_{(2,20)} = 3.88$, $p = 0.038$) and clonic ($F_{(2,20)} = 18.63$, $p < 0.0001$) seizure durations lengthened with increasing number of treatments. Tonic seizure durations have previously been similarly reported to increase over time in a study comparing the effects of ECS using pulse widths of either 0.5 ms or 10 ms (Jansson et al., 2009). However, durations of the tonic ($F_{(1,20)} = 0.27$, $p = 0.616$) or clonic ($F_{(1,20)} = 0.82$, $p = 0.386$) seizure phases did not significantly differ between BP and UBP groups.

3.2. Behavioural changes after ECS

Water plus maze platform acquisition on training and probe days was investigated to determine if animals learned the platform position and could recall this information. Platform acquisition during the training period increased significantly over time ($F_{(2,28)} = 7.23$, $p = 0.0029$) but was not affected by treatment type ($F_{(2,28)} = 0.41$, $p = 0.67$;

Fig. 3A), indicating that all animals learned the task, and there was no group $\times$ time interaction effect ($F_{(4,28)} = 0.48$, $p = 0.7513$). On the probe trial day, both BP and UBP ECS-treated animals obtained the platform less frequently than controls but this difference was not statistically significant ($F_{(2,14)} = 1.8$, $p = 0.21$; Fig. 3B). Of note, there was no difference between the levels of platform acquisition by BP and UBP groups. The swimming velocity (cm/s) at which the animals obtained the platform increased significantly ($F_{(2,42)} = 6.47$, $p = 0.004$) over the three training days but did not differ between the three treatment groups ($F_{(2,42)} = 0.16$, $p = 0.849$), indicating no motor deficit resulted as a consequence of real ECS treatments. Similarly, no significant difference in velocity to finding the platform was found between the three groups ($F_{(2,9)} = 0.033$, $p = 0.967$) on the probe day.

The groups differed with regards to immobility times in the Forced Swim Test ($F_{(2,15)} = 3.8$, $p = 0.046$; Fig. 4). Post hoc analysis revealed that the BP group showed a significant decrease ($p < 0.05$) in immobility time when compared to controls, whereas a non-significant decrease in immobility time was seen in the UBP group.

3.3. BDNF protein levels after ECS

Different levels of hippocampal BDNF were found between the three groups ($F_{(2,15)} = 6.3$, $p = 0.01$) when measured 2 weeks after completing the allocated course of ECS with BP ECS producing significantly higher levels than found in both the control ($p < 0.01$) and UBP ($p < 0.05$) groups (Fig. 5). A smaller, but non-significant, increase was found in the UBP group compared to sham-treated controls.

3.4. Immunohistochemistry

ECS treatment was associated with an increase in cell proliferation compared to sham treatment ($p = 0.026$, Fig. 6) when measured 24 h after completion of the allocated course of ECS. BP ECS induced a

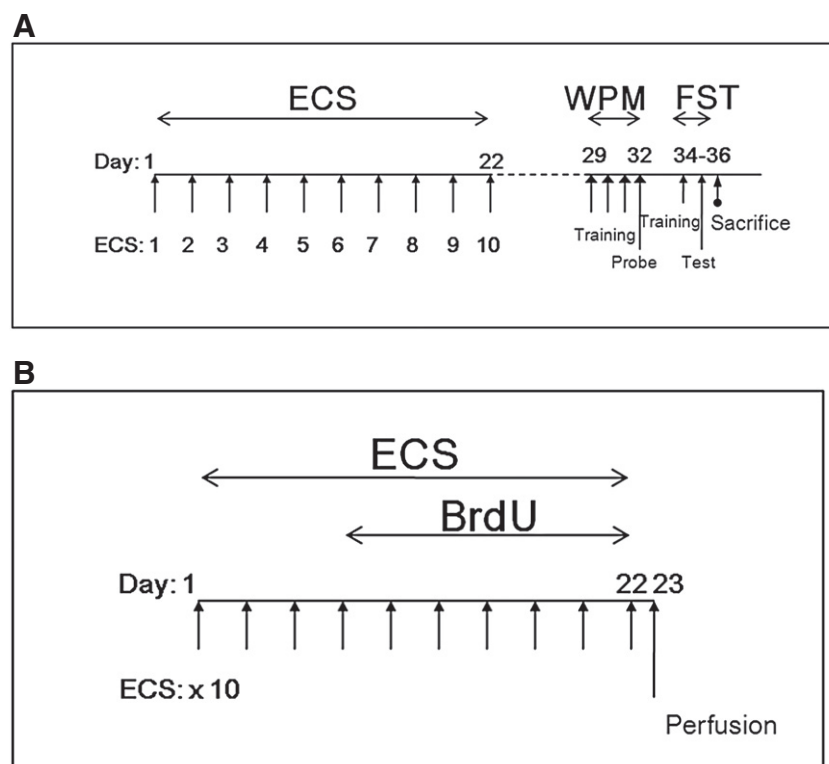


Fig. 1. Experimental design. (A) Animals were administered real or sham ECS thrice weekly for ten sessions over 22 days. Following one week habituation to the behavioural suite, animals were trained and tested in the water plus maze (WPM) followed by the forced swim test (FST) 2 days later. The animals were sacrificed 24 h after the swim test. (B) Another set of animals was similarly treated with ECS and was also injected with BrdU 2–4 h after ECS treatments 4–10. The animals were perfused 24 h after the final ECS session and the brains extracted to examine changes in cell proliferation.

significant ($p < 0.05$), almost 3-fold, increase in the number of proliferating cells compared to sham treated control animals, whereas a much smaller and non-significant increase in cell proliferation was seen following UBP-ECS.

4. Discussion

To our knowledge, this is the first study to systematically compare the effects of UBP (0.3 ms) and BP (0.5 ms) width stimuli on chronic ECS and related antidepressant-related changes in rats. We found that both forms of ECS elicited seizures that were indistinguishable with regards to observable motor seizure activity and the durations of the tonic and clonic phases. However, while BP ECS induced significant increases in recognised markers of antidepressant action, UBP ECS did not have the same effect.

Compared to sham-treated animals, a large, three-fold, and significant increase in cell proliferation was seen within the dentate gyrus 1 day after completing a course of BP ECS while only a much smaller, non-significant, increase was seen following UBP ECS. This degree of increased cell proliferation following BP ECS is similar to that previously found by other groups (Madsen et al., 2000; Segi-Nishida et al., 2008). The majority (75–88%) of these new cells have been reported to be neuronal (Malberg et al., 2000; Scott et al., 2000) and such hippocampal neurogenesis is required for antidepressant behavioural effects in both rats and nonhuman primates (Perera et al., 2011; Santarelli et al., 2003). Additionally, 2 weeks after completing a course, we found that BP ECS resulted in a significantly greater increase in hippocampal levels of BDNF than UBP ECS. Increased BDNF expression has been reported to mediate anti-depressant effects for a range of antidepressant drugs as well as ECS in animals (Castren and Rantamaki, 2010).

Chronic UBP ECS was therefore not as efficient as BP ECS for inducing antidepressant-related cellular and molecular changes in rat hippocampus. In parallel with these differences, we found that BP ECS had a greater antidepressant behavioural effect as it significantly reduced immobility time in the forced swim test, while immobility time following UBP ECS did not differ significantly from sham ECS. This may be reflected in some recent clinical studies of various forms of UBP ECT for depression that reported very low remission rates at only 6–13% (Loo et al., 2007; Quante et al., 2011).

A potential benefit of UBP ECS – and UBP ECT for treating depression – is a reduction in the cognitive side effects associated with ECS treatment (Sackeim et al., 2008). In the present study there was a non-significant trend for similar decreases in performance in both the UBP and BP groups compared to the sham control group on hippocampal-dependant reference memory in the water plus maze probe trial. However, we did not find any difference between the effects of UBP and BP ECS even though it induced less cellular, molecular and behavioural changes than BP ECS. These cognitive findings should be interpreted with some caution because the sample sizes in our study may have been too small to detect a relatively reduced impact of UBP ECS on memory. Additionally, the interval of 10 days between end of treatment and cognitive assessment may have led to early post treatment differences between UBP and BP groups no longer being apparent, reflecting recovery of hippocampal cognitive function over time following ECS as it occurs in patients treated with ECT (Semkovska and McLoughlin, 2010; Semkovska et al., 2011). Interestingly, some recent studies attempting to cause neuronal loss using ECS have found that repeated brief seizures can cause specific neuronal death within hippocampal structures under certain conditions (Cardoso et al., 2011). However, these same studies also report that cell loss does not occur when ECS is administered on a 24–48 hour schedule (Cardoso et al., 2008). The

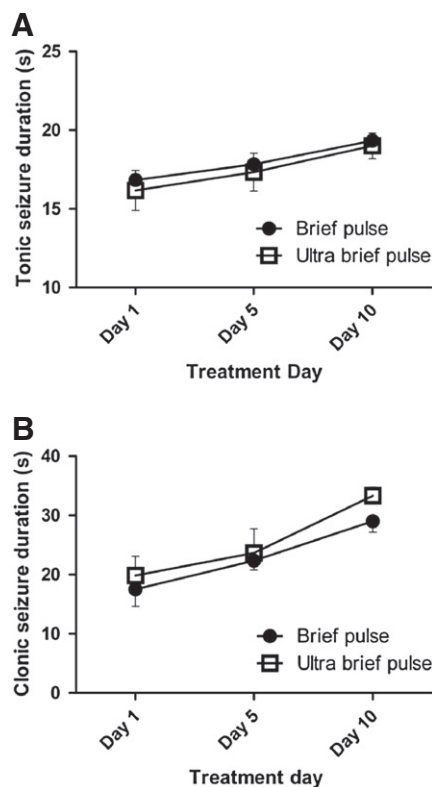


Fig. 2. Seizure durations. (A) Tonic and (B) clonic seizure durations are shown on the first, fifth and tenth treatment days of the randomly allocated ECS course. Durations of both tonic ($p = 0.038$) and clonic ($p < 0.0001$) seizure phases increased significantly over time in both BP and UBP groups but there was no significant difference between treatment groups or significant Treatment $\times$ Time interactions. $n = 6$ per group.

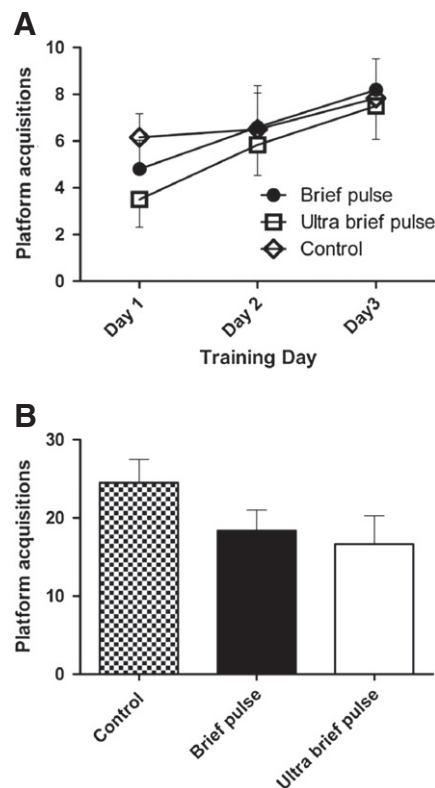


Fig. 3. Water plus maze. (A) After completing the randomly allocated course of ECS, animals obtained the platform more frequently over successive training days 1–3 ($p = 0.024$). Treatment type had no effect on number of platform acquisitions. $n = 5/6$ per group. (B) The number of platform acquisitions on probe test day 4 did not differ significantly between groups. BP and UBP groups obtained the platform less frequently than the sham group although this difference was not significant.

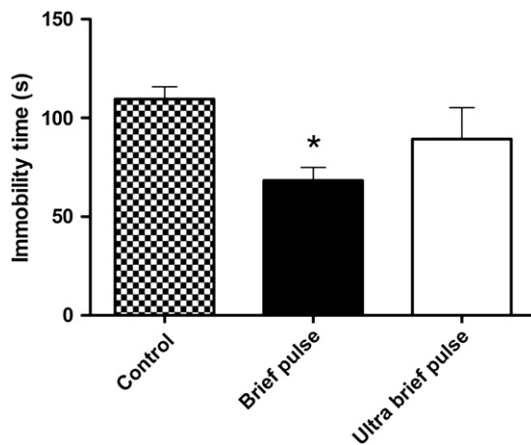


Fig. 4. Forced swim test following ECS. BP animals spent significantly less time immobile than UBP or control animals. The decrease in immobility times in the UBP group was not significantly lower than in controls. $n = 6$ per group, * $p < 0.05$ compared to control group.

interval between the final seizures in the programme must be short (2 h) to cause the morphological and behavioural changes observed, possibly resulting from neuronal vulnerability during the post ictal period. Of note, analyses of rodent brain following conventionally spaced BP ECS, i.e. not designed to deliberately induce neuronal death, have not found evidence of neuronal loss (Cardoso et al., 2008; Dalby et al., 1996; Gombos et al., 1999; Jinno and Kosaka, 2009; Vaidya et al., 1999). Based upon the findings of the present study, we would predict the same for UBP ECS.

For experimental purposes we altered only one parameter in the treating ECS stimulus – the pulse width. Therefore, one possible explanation for the relatively small antidepressant-related effects we found with UBP ECS is that the total stimulus charge per session administered was only 60% that for BP ECS, i.e. 1.575 mC compared to 2.625 mC. Increasing the frequency of UBP stimuli or the duration of UBP ECS administration might result in greater anti-depressant effects. However, despite the lower total charge for UBP ECS used in the present study, both forms of ECS induced motor seizures that were indistinguishable. Even though we found large and meaningful differences between UBP and BP ECS on several different measures, some caution is warranted as noted above because of the relatively small sample sizes and the risk of type 1 error. Our findings therefore require further exploration and replication, possibly examining reported differences at other time-points. Finally, the effects of UBP ECS on animal models of depression, in addition to normal rats, need to be studied. For example, BP ECS has

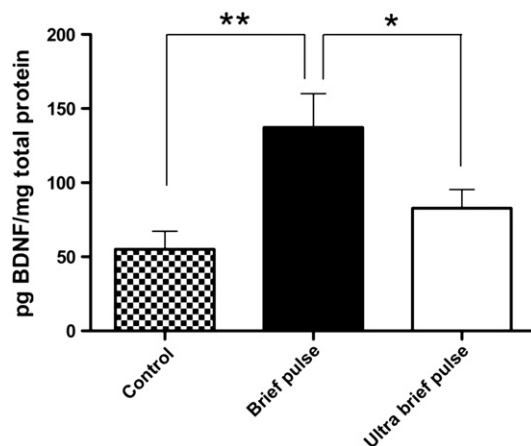


Fig. 5. BDNF protein levels in the hippocampus following ECS. BDNF was significantly increased in the BP group compared to the control and UBP ECS groups. The increase seen in BDNF levels in the UBP group was not significantly higher than in the control group. $n = 6$ per group, * $p < 0.05$, ** $p < 0.01$ compared to control and UBP groups.

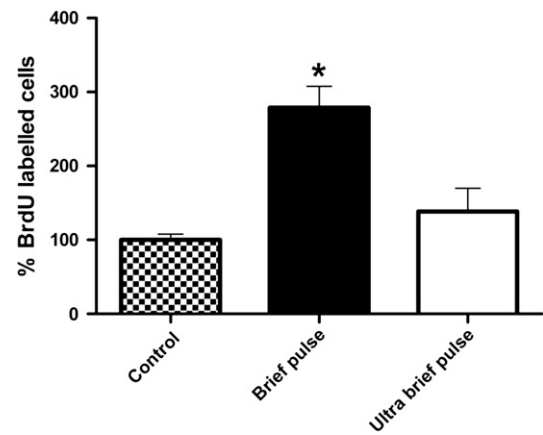


Fig. 6. Cell proliferation following ECS. BP treatment significantly increased the relative number of BrdU-labelled cells in the dentate gyrus compared to sham-treated control animals ($p < 0.05$) (percentage of control). The increase in BrdU-labelled cells was not significantly higher in the UBP group than in controls. $n = 4$ per group, * $p < 0.05$.

been reported to reduce forced swim test immobility time in Flinders Sensitive Line rats (Jimenez-Vasquez et al., 2007), normalise both impaired sucrose preference (a measure for anhedonia) and reduced hippocampal BDNF levels induced by chronic mild stress (Gersner et al., 2010), and reverse corticosterone-mediated inhibition of neurogenesis (Hellsten et al., 2002). It would therefore be important to study the therapeutic effects of UBP ECS in similar models of depression.

5. Conclusions

We found UBP ECS to be much less efficient than BP ECS in normal rats for inducing a range of cellular, molecular and behavioural changes that are believed to be important for antidepressant mechanisms. Comparing the effects of different stimulus parameters of ECS in animals allows us to better understand this antidepressant therapy and determine whether modifying the existing parameters of ECT may be of benefit for patients with depression.

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References

- Cardoso A, Assuncao M, Andrade JP, Pereira PA, Madeira MD, Paula-Barbosa MM, et al. Loss of synapses in the entorhinal–dentate gyrus pathway following repeated induction of electroshock seizures in the rat. *J Neurosci Res* 2008;86:71–83.
- Cardoso A, Lukoyanova EA, Madeira MD, Lukoyanov NV. Seizure-induced structural and functional changes in the rat hippocampal formation: comparison between brief seizures and status epilepticus. *Behav Brain Res* 2011;225:538–46.
- Castren E, Rantamaki T. The role of BDNF and its receptors in depression and antidepressant drug action: reactivation of developmental plasticity. *Dev Neurobiol* 2010;70:289–97.
- Dalby NO, Tonder N, Wolby DPD, West M, Finsen B, Bolwig TG. No loss of hippocampal hilar somatostatinergic neurons after repeated electroconvulsive shock: a combined stereological and in situ hybridization study. *Biol Psychiatry* 1996;40:54–60.
- Diamond DM, Park CR, Heman KL, Rose GM. Exposing rats to a predator impairs spatial working memory in the radial arm water maze. *Hippocampus* 1999;9:542–52.
- Dunne R, McLoughlin DM. Systematic review and meta-analysis of bifrontal electroconvulsive therapy versus bilateral and unilateral electroconvulsive therapy in depression. *World J Biol Psychiatry* 2011. [Nov 18, Epub ahead of print].
- Eranti S, Mogg A, Pluck G, Landau S, Purvis R, Brown RG, et al. A randomized, controlled trial with 6-month follow-up of repetitive transcranial magnetic stimulation and electroconvulsive therapy for severe depression. *Am J Psychiatry* 2007;164:73–81.
- Gersner R, Toth E, Isserles M, Zangen A. Site-specific antidepressant effects of repeated subconvulsive electrical stimulation: potential role of brain-derived neurotrophic factor. *Biol Psychiatry* 2010;67:125–32.
- Gombos Z, Spiller A, Cottrell GA, Racine RJ, Burnham WM. Mossy fiber sprouting induced by repeated electroconvulsive shock seizures. *Brain Res* 1999;844:28–33.

- Hellsten J, Wennstrom M, Mohapel P, Ekdahl CT, Bengzon J, Tingstrom A. Electroconvulsive seizures increase hippocampal neurogenesis after chronic corticosterone treatment. *Eur J Neurosci* 2002;16:283–90.
- Jansson L, Wennstrom M, Johanson A, Tingstrom A. Glial cell activation in response to electroconvulsive seizures. *Prog Neuropsychopharmacol Biol Psychiatry* 2009;33:1119–28.
- Jimenez-Vasquez PA, Diaz-Cabiale Z, Caberlotto L, Bellido I, Overstreet D, Fuxe K, et al. Electroconvulsive stimuli selectively affect behavior and neuropeptide Y (NPY) and NPY1 receptor gene expressions in hippocampus and hypothalamus of Flinders Sensitive line rat model of depression. *Eur Neuropsychopharmacol* 2007;17:298–308.
- Jinno S, Kosaka T. Neuronal circuit-dependent alterations in expression of two isoforms of glutamic acid decarboxylase in the hippocampus following electroconvulsive shock: a stereology-based study. *Hippocampus* 2009;19:1130–41.
- Kellner CH, Knapp R, Husain MM, Rasmussen K, Sampson S, Cullum M, et al. Bifrontal, bitemporal and right unilateral electrode placement in ECT: randomised trial. *Br J Psychiatry* 2010;196:226–34.
- Kesavapany S, Banner SJ, Lau KF, Shaw CE, Miller CCJ, Cooper JD, et al. Expression of the Fe65 adapter protein in adult and developing mouse brain. *Neuroscience* 2002;115:951–60.
- Loo C, Sheehan P, Pigot M, Lyndon W. A report on mood and cognitive outcomes with right unilateral ultrabrief pulsewidth (0.3 ms) ECT and retrospective comparison with standard pulsewidth right unilateral ECT. *J Affect Disord* 2007;103:277–81.
- Loo CK, Sainsbury K, Sheehan P, Lyndon B. A comparison of RUL ultrabrief pulse (0.3 ms) ECT and standard RUL ECT. *Int J Neuropsychopharmacol* 2008;11:883–90.
- Madsen TM, Treschow A, Bengzon J, Bolwig TG, Lindvall O, Tingstrom A. Increased neurogenesis in a model of electroconvulsive therapy. *Biol Psychiatry* 2000;47:1043–9.
- Malberg JE, Eisch AJ, Nestler EJ, Duman RS. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* 2000;20:9104–10.
- McCall WV, Reboussin DM, Weiner RD, Sackeim HA. Titrated moderately suprathreshold vs fixed high-dose right unilateral electroconvulsive therapy — acute antidepressant and cognitive effects. *Arch Gen Psychiatry* 2000;57:438–44.
- Newton SS, Collier EF, Hunsberger J, Adams D, Terwilliger R, Selvanayagam E, et al. Gene profile of electroconvulsive seizures: Induction of neurotrophic and angiogenic factors. *J Neurosci* 2003;23:10841–51.
- Niemantsverdriet L, Birkenhager TK, van den Broek WW. The efficacy of ultrabrief-pulse (0.25 millisecond) versus brief-pulse (0.50 millisecond) bilateral electroconvulsive therapy in major depression. *J ECT* 2011;27:55–8.
- Perera TD, Dwork AJ, Keegan KA, Thirumangalakudi L, Lipira CM, Joyce N, et al. Necessity of hippocampal neurogenesis for the therapeutic action of antidepressants in adult nonhuman primates. *PLoS One* 2011;6(4):e17600.
- Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatment. *Nature* 1977;266:730–2.
- Quante A, Luborzewski A, Brakemeier EL, Merkl A, Danker-Hopfe H, Bajbouj M. Effects of 3 different stimulus intensities of ultrabrief stimuli in right unilateral electroconvulsive therapy in major depression: a randomized, double-blind pilot study. *J Psychiatr Res* 2011;45:174–8.
- Sackeim HA. Convulsant and anticonvulsant properties of electroconvulsive therapy: towards a focal form of brain stimulation. *Clin Neurosci Res* 2004;4:39–57.
- Sackeim HA, Prudic J, Devanand DP, Kiersky JE, Fitzsimons L, Moody BJ, et al. Effects of stimulus-intensity and electrode placement on the efficacy and cognitive effects of electroconvulsive-therapy. *N Engl J Med* 1993;328:839–46.
- Sackeim HA, Prudic J, Nobler MS, Fitzsimons L, Lisanby SH, Payne N, et al. Effects of pulse width and electrode placement on the efficacy and cognitive effects of electroconvulsive therapy. *Brain Stim* 2008;1:71–83.
- Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, et al. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 2003;301:805–9.
- Scott BW, Wojtowicz JM, Burnham VM. Neurogenesis in the dentate gyrus of the rat following electroconvulsive shock seizures. *Exp Neurol* 2000;165:231–6.
- Segi-Nishida E, Warner-Schmidt JL, Duman RS. Electroconvulsive seizure and VEGF increase the proliferation of neural stem-like cells in rat hippocampus. *Proc Natl Acad Sci U S A* 2008;105:11352–7.
- Semkovska M, McLoughlin DM. Objective cognitive performance associated with electroconvulsive therapy for depression: a systematic review and meta-analysis. *Biol Psychiatry* 2010;68:568–77.
- Semkovska M, Keane D, Babalola O, McLoughlin DM. Unilateral brief-pulse electroconvulsive therapy and cognition: effects of electrode placement, stimulus dosage and time. *J Psychiatr Res* 2011;45:770–80.
- Sienaert P, Vansteelandt K, Demyttenaere K, Peuskens J. Randomized comparison of ultra-brief bifrontal and unilateral electroconvulsive therapy for major depression: clinical efficacy. *J Affect Disord* 2009;116:106–12.
- U.K. ECT Review Group. Efficacy and safety of electroconvulsive therapy in depressive disorders: a systematic review and meta-analysis. *Lancet* 2003;361:799–808.
- Vaidya VA, Siuciak JA, Du F, Duman RS. Hippocampal mossy fiber sprouting induced by chronic electroconvulsive seizures. *Neuroscience* 1999;89:157–66.

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Facing challenges in Proteomics today and in the coming decade: Report of Roundtable Discussions at the 4th EuPA Scientific Meeting, Portugal, Estoril 2010

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1. Introduction

Roundtable Discussion sessions were conceived for the first time at the 4th Edition of the EuPA Annual Scientific Meeting that took place in Estoril, Portugal, from the 23rd to 27th of October, 2010. Under the theme “2010: a proteomics odyssey towards the next decades”, young and senior scientists had the opportunity to discuss particular issues of different proteomic areas distributed by eleven luncheon roundtables moderated by recognized experts in the field. During two hours, the roundtable discussions proceeded in a friendly environment, in which the participants (about 8–10 per roundtable) were encouraged to ask questions and express their opinions without restraint (Fig. 1). The topics covered a range of recurring issues such as the capabilities and limitations of several proteomic methodologies and suggestions on how researchers may better overcome them. The major goal devised by roundtable leaders was to provide independent advice, describe limitations, give practical tips, help set realistic expectations and answer questions from the participants. Participants were also encouraged to be ambitious in generating new solutions or strategies for the future.

Topics included themes such as sample collection/preparation/biobanking and experimental design in disease biomarkers discovery, labeling vs label-free quantitative proteomics, imaging MS on cryo vs FFPE preserved tissues, protein

post-translational modifications, peptidomics and bioinformatic tools, databases, data management and data mining, interactomes, networks and pathways analysis, SRM/MRM challenging, translational proteomics: from the lab to the field.

The summary results of these roundtables are presented below by their leaders with the collaboration of some of its participants.

2. RT1 — experimental design in disease biomarkers discovery (leader: Peter James)

The starting point for the discussion was the recent change in the guidelines for authors that the four major Proteomics journals have announced regarding experimental design. The advances in mass spectrometry in recent years have made the acquisition of large datasets of identified proteins fairly routine. Many laboratories have access to instruments that can generate multiple MS/MS spectra at high quality per second. This has led to situations where experiments can be performed that generate thousands of identified proteins.

2.1. More is not better

The first problem that this has led to is the confidence level that can be assigned to identification of a protein. Guidelines



Fig. 1 – Glimpses of the Roundtable Discussions at the 4th EuPA Annual Scientific Meeting, Estoril, 23–27 October, 2010.

have now been discussed and a probability based cut-off level and an estimate of the false discovery rate used in identification is required by all Journals. However this is not a simple problem. Many identifications are now discarded if only one peptide is used for identification, the so-called 'one hit wonders' which can account for 50% of all IDs in an experiment. This can lead to odd situations in which a protein ID from a very high quality spectrum is eliminated whilst another coming from two weak spectra is accepted. The two-hit requirement would also rule out many studies of post-translational modifications in which an affinity purification step is used to enrich phosphopeptides leaving only 1.4 peptides per protein on average.

2.2. *Less is even worse*

The second problem that the new instruments and techniques have created is the $n=1$, 'one-experiment wonder'. Two factors contribute to this, the relatively long instrument analysis time required for a two-dimensional experiment (usually SDS-PAGE, slice, RP-HPLC-MS or SCX-RP-HPLC) which is in the order of a day, and the high costs involved (be it isotope labeled amino acids in SILAC or reagents such as TMT or iTRAQ). All of the major Journals have now implemented guidelines to try and counteract this trend and several also ask for independent confirmation of major conclusions through the use of orthogonal data such as qPCR, immuno-histochemistry, Western blotting or more controversially, by Molecular Reaction Monitoring. The approach that is then favored is a low power discovery phase followed by a larger scale and confidence application of these techniques as verification. What is missing in many cases, especially in the biomarker discovery field is an appreciation of the true number of replicates and independent samples (in this particular case, samples mean patients) that are required to give an accurate estimate of the validity of an experiment. In a simple example, if 50 samples from 'sick' patients are compared to an equal number of matched (sex, age etc.) controls, and a single variable such as the level on one biomarker is used, a fairly accurate estimate of the experiment's power can be made. If however 2000 variables are being measured (e.g. spots on a 2D gel) the result will be much harder to evaluate. There will always be a set of variables that will generate an apparently high discriminatory power when combined but in reality are just due random effects. Low sample numbers and high number of variables must always be analyzed in terms of statistical power, a term that very few understand and even less apply.

2.3. *No solutions, just more problems*

The most commonly asked question in most experiments is — "How many samples do I need?" To which the correct answer is "What is your question?" One must define what is an acceptable failure rate and this is justified in terms of expense and outcome. A basic biological problem solving exercise will require a completely different set of parameters than a phase II clinical study. One obvious solution is to use multiplexing to increase the number of samples being analyzed within a fixed window of instrument time. Isotope labeling by SILAC can be extended to six-plex and iTRAQ even to 8-plex. However

the equation is not so easy. The increase in sample number in SILAC is accompanied by a large increase in the difficulty in analyzing the data and the increased problems with peptide overlap. Similarly increasing multiplexing from 2- to 4- to 8-fold using iTRAQ suffers from a loss in accuracy in quantification since the parent ion intensity remains constant (assuming that a maximum loading is being used) whilst the ion current is being divided amongst more samples lowering the signal/noise ratio. If a 2D-PAGE approach is used together with DIGE labeling, the problem of assignment becomes difficult. Very often a pool is used with a higher loading for protein identification and spots are cut from this. However most spots contain multiple proteins and unless one cuts proteins from two preparatory gels, one control and one experiment, it will be hard to define which protein in the spot was changing its expression level. The concept of pooling in general as a method to increase sampling is also a difficult one. The creation of a pool increases the number of samples that can be run however the biological variation within a group is lost entirely and hence the significance of an observation is greatly reduced since one cannot rule out that an outlier is influencing the whole group. In addition the creation of a pool implies that an assumption has been made i.e. all the samples within a pool have been treated the same way. If this assumption proves to be wrong, for example, one tumor was accidentally thawed and had undergone extensive proteolysis, the entire pool would be worthless.

2.4. *Conclusions*

The take home message from the discussion was quite simple: get a statistician involved before the experiment is done. Design the experiment to fulfill the goal of answering the biological question and of meeting the Journal requirements. This will save a lot of wasted time and money and contribute to improving the credibility of proteomics as a whole.

3. **RT2 — sample collection/preparation/ biobanking for biomarker discovery (leaders: Jean-Charles Sanchez & Pier Giorgio Righetti)**

It was not in the best mood that we started this RT (round table) considering that during the meeting we had been alerted to a paper by Mitchell [1] harshly criticizing a decade of proteomic research dedicated to biomarker discovery, to the tune of: "These 10 years of work and billions of dollars have been largely unsuccessful"; "Biomarkers have been the biggest disappointment of the decade; they turned out to be so non-specific as to be next to useless, far from the "Holy Grail" envisioned"; "I am sick of seeing papers proving that a known biomarker is a marker for yet another disease"; "Biomarkers discovered so far are mainly the same proteins that pop up in all kinds of diseases, indicating that the organism is under some kind of stress"; ">1250 presumptive biomarkers have been listed so far in the literature, but none has yet reached clinical trials or has been approved". Not bad for a start!

What went wrong? We started focusing on sample collection: long ago, clinicians used to store biopsies and

body fluids at -20°C . Later on, it was understood that even in the frozen state such samples would degrade, so the storage strategy was shifted to deep freezers, at -80°C . Even this latter storage conditions came under criticism, so that today the collection is done by immediately dumping biological specimens in liquid nitrogen (-170°C). So our technicians are seen humbly tiptoeing in surgery theaters with a small Dewar in their hands, ready to plunge the precious sample in liquid nitrogen, with the understanding that there would simply be no time for biological degradation via, e.g., activation of proteases etc. Well, one of the participants of the RT alerted us that even this sample collection condition might not be proper: the best way for collection would be to “boil” the biological specimens. We tried to figure out how our technicians would feel by showing up in the surgery room with a frying pan in their hands. But a smart participant (probably a fugitive from the Iron Curtain) had noted a booth in the meeting of a small Swedish company who had developed a gadget able to immediately bring the specimen to 95°C while dehydrating it, so as to fully inactivate proteases. According to them, this is even better than dumping in liquid nitrogen (but perhaps it is too early to assess that). Another alternative was also suggested, namely tissue lyophilisation. It is a low-cost and user-friendly substitute for the storage and shipping of samples prior to protein extraction. The efficiency of lyophilisation is crucial as the sample should be free of any moisture content. Some researchers are afraid to use this strategy being concerned about the potential cross-linking of proteins via amide bonds.

Another important issue here, for biomarker discovery, was that the sample should be categorized (and matched) on the basis of patient's sex, age, weight, ethnicity and life-style factors such as smoking and alcohol use (abuse?). Luckily, nobody insisted on categorizing also in terms of sex habits!

Others argued that perhaps we are witnessing the crepuscule of freelance biomarker discovery (as done by all of us in universities with our meager resources). Shall we switch to Big Science, thus leaving biomarker discovery to large research networks, involving industry, experts in genetics, analytical chemistry and molecular biology, as well as computational wizards, epidemiologists, statisticians and, why not, magicians able to chase evil spirits? There goes academic freedom!

The other strong issue was sample pre-fractionation (especially in biological fluids) for digging deep into the low to very-low abundance proteome. Starting from the year 2003, the trends have been to remove the first 7 or 14 or even 20 or more of the population of high-abundance proteins, a subtraction method based on immuno-depletion. Plenty of kits are commercially available for such a task. Even though this has been by far the most popular approach, the death-bell for this methodology was rung by Tu et al. [2] who stated: “Either top 7 or top 14 immuno-depletion resulted in a 25% increase in identified proteins compared to unfractionated plasma”; “Although 23 low-abundance ($<10\text{ ng mL}^{-1}$) plasma proteins were detected, they accounted for only 5–6% of total protein identifications in immuno-depleted plasma”; “In both unfractionated and immuno-depleted plasma, the 50 most abundant plasma proteins accounted for 90% of cumulative spectral counts and precursor ion intensities, leaving little capacity to sample lower abundance proteins”. They were generous, in

a way, considering that Zhi et al. [3] candidly admitted that, whereas in neat serum they could detect 568 proteins, in MARS-14 depleted serum they could only identify 497 proteins, i.e. 10% less! A dead-end street, then. That would then leave only the combinatorial peptide ligand library technology (CPLL) as the viable alternative for digging deep into biological fluids proteomes, considering that this methods permit discovering not just that meager 25% more (or 10% less) but typically from 300 to 600% more. There ensued a hot debate, as some scientists argued that recent reports on this technique too [4–6] had also stated that CPLLs could just be as good (or bad) as immuno-subtraction.

An important part of sample quality at hand that was discussed was the way that clinical samples are handled in a study setting. As protein expression analysis activities are intimately linked to collaborative efforts with colleagues from many different research fields, communication and cross-functional optimization is mandatory. In this respect the discussions we had was the actual interface and hand-over of clinical samples from hospital environments to the laboratory. Detailed and well worked out protocols that ensure optimal sampling and sample handling procedures are crucial. In addition, the consistency whereby protocols are executed is essential in order to achieve reproducibility within a given patient cohort and in-between sampling occasions. In real-life studies, this is of particular importance since a study can run over a longer time period, the sample processing cycles in some cases can be as long as several years [7].

We also touched on the exciting HPP (Human Proteome Project) initiative that was recently launched, approved and started in Sydney at the 9th HUPO World Congress, on the 23rd September 2010. This Global initiative will certainly have extant experience on applying sample preparation protocols and sample handling procedures on patient samples from many biobanks around the World [8].

Having apparently no viable instrument left for biomarker discovery, the RT discussion left all of us with a feeling of destitution and despair, and a sour taste in our mouths. No wonder the participants assaulted the food table and razed it clean of any cakes and sweets left, up to the last crumb!

4. RT3 — labeling vs label-free quantitative proteomics (leader: Juergen Cox)

The prior experience of the group was diverse in that many quantification strategies had been used by several participants in their quantitative proteomics projects. Quantitative proteomics workflows can be distinguished into three main categories based on the labeling strategy: metabolic labeling, chemical labeling and label-free approaches. Fortunately prior experience with all of them was represented in the discussion group. The general perception was that in terms of quantitative precision there exists a hierarchy of these methods with metabolic labeling being the most precise technology, followed by chemical labeling and label-free approaches being the least precise. While this is the generic ranking of the approaches in terms of precision there is some

degree of uncertainty in the community about the actual degree of precision gain from more elaborate labeling technologies. In our experience we usually see a five-fold gain in precision when performing SILAC labeling as compared to a similar experiment with comparable measurement times performed without labeling. For the comparison of diverse chemical labeling methods with label-free approaches, several opinions existed in the discussion group and also in the community. Some researchers report no significant improvements by chemical labeling while others see clear advantages. A thorough side-by-side evaluation of the performance of these methods on realistic complex proteomics samples would be desirable.

Methods based on labeling can be further subdivided into those where the quantification is performed in the MS/MS spectra based on characteristic reporter ions or those with quantitative information in the MS spectra. Experience on the comparison of those methods was mixed. Reporter ion-based methods like iTRAQ have the potential advantage that they can be highly multiplexed, allowing to analyze several samples at once. Their main disadvantage is that often several peptides are selected together for fragmentation, and therefore a mix of abundances for several peptides is often recorded. To avoid this, sample complexity needs to be reduced which sometimes (over-) compensates the gain achieved from multiplexing. Here intelligent software would potentially help distinguishing those peptides where the quantitative information can safely be used from those that are contaminated by other peptides.

The software plays a big role in the precision of the final quantification results. While this is the case for all labeling methods, it is label-free quantification that benefits most from good software and algorithms. The simplest data analysis of label-free quantification data is the MS/MS spectral count which consists of counting the number MS/MS spectra that the instrument acquired for each identified peptide and protein. Obviously this is an approximate method but surprisingly it works to some extent and it can give rough estimates for the quantification of the abundant proteins. For low mass resolution MS data, this might often turn out to be the best way to quantify label-free data, simply because the MS level quantitative information for the different peptides is strongly overlapping and hard to disentangle. However, nowadays high-resolution mass spectrometers are readily available and on these kinds of instruments quantification based on the MS level signals is highly superior to counting MS/MS spectra because of its higher information content.

While quantification based on the counts of MS/MS spectra per protein work to some degree and result in rough quantitative estimates for abundant proteins, a tremendous improvement can be obtained by using the extracted ion current on the MS level as the basic signals for the label-free quantification. There is a big potential here for computational scientists to improve the MS based quantitative label-free data by applying advanced correction and normalization algorithms. We speculated what quantitative proteomics workflow will look like in ten years from now. One interesting perspective for the future was that by this time the algorithmic part of quantitative proteomics might have advanced that far that no kind of labeling will be needed at all.

5. RT4 — imaging MS on cryo vs FFPE preserved tissues (leader: Ron M.A. Heeren)

5.1. FFPE versus cryopreserved tissue analysis

Imaging mass spectrometry is rapidly finding its way in many applied proteomics areas. The technology has matured to a level that instrument manufacturers offer complete molecular imaging solutions based on mass spectrometry. Sample preparation is key to the success of the method. A substantial time of the discussion focused on various aspects of the different sample preparation methods available. Of particular interest was the comparison between MS imaging results obtained from Formalin Fixed Paraffin Embedded (FFPE) and cryopreserved tissue. Clearly the advantage of working with FFPE tissue is the large-scale availability of tissue samples in various biobanks that exist throughout the world. One of the issues experienced by some of the participants was the limited sensitivity from this type of samples. A potential cause was discussed to be the extensive fixation and cross-linking in the formalin fixation step. Even though protocols using proteolytic digestion have been developed that provide a way around this issue the results obtained from cryopreserved tissue sample were perceived as superior. A particular difficulty in the interpretation of the data obtained from FFPE tissue surfaces after on tissue digestion is the reduced number of identifiable tryptic peptides. This in part is due to the lack of database searching software that is capable of dealing with formaldehyde modification and cross-links. It was suggested to evaluate if existing cross-link interpretation software might be helpful in alleviating this problem. Extensive a-specific cross-linking might hamper this solution. Part of this discussion on practical aspects of on tissue digestion for both tissue types focused on enzyme deposition method. In particular, the reproducibility was questioned and discussed. Automated droplet deposition techniques were compared and contrasted with spray technologies. The consensus was that the spray technologies are more practical as they suffer less from variations in temperature and humidity whereas when using micro droplets these environmental variables need to be handled carefully.

5.2. Multimodal imaging

A tendency towards multimodal imaging was observed in the scientific program. This invoked a question on what the proper protocols are that allow these different (MS based and non-MS based) molecular imaging modalities to be confidently combined. An example that combines Secondary Ion Mass Spectrometry at high spatial resolution for metabolites with a MALDI imaging study was discussed. It was pointed out that protocols for these types of studies exist in literature. Briefly, the SIMS analysis precedes MALDI as SIMS can be performed without any sample preparation. After matrix deposition a matrix enhanced SIMS analysis can be performed. ME-SIMS experiments in the static mode examine less than 1% of the surface area to a depth of approximately 50–100 nm. The limited amount of surface material removal leaves the same

sample useable for MALDI imaging experiments. When the MALDI experiments are performed at lower spatial resolution leaving unanalyzed areas in between laser probe locations it is possible to acquire interlaced MALDI-MSI datasets. This could for example result in a MALDI-FTICR-MS and a MALDI-TOF dataset from the same tissue section. After depletion of the matrix layer the tissue can be washed to remove any residual matrix molecules. Depending on the stability of the tissue this would allow subsequent H&E staining for conventional histology analysis; information needed for annotation of the imaging MS datasets. With this protocol it would be possible to obtain 5 analytically relevant datasets utilizing different imaging modalities of one and the same tissue section. As the different modalities provide different spatial and spectral resolution and coverage, the co-registration is still challenging and currently occurs mainly manually. Some groups use optical images taken during the (cryo-) sectioning procedure for this purpose, others are developing strategies utilizing MS compatible fiducially markers. In particular, for 3 dimensional imaging and reconstruction this strategy becomes important.

5.3. Dataset size and resolution

Concern was expressed by the participants about the increasing size of the datasets resulting from imaging mass spectrometry. The instruments have matured and have been automated to a larger extent but the data analysis protocols did not undergo a similar maturation process. As a result, the analysis of imaging MS data is perceived as the biggest bottleneck in the analytical protocol. Individual groups are successfully developing innovative data reduction and classification protocols with a focus on MS based tissue classification. The dissemination of these methods is however limited. It was remarked that there is a definite need for a computational resource for imaging mass spectrometry. Suggestions ranged from databases with freely available software (some of which exists at www.maldi-msi.org) to concerted European programs on computational research for imaging MS. This is in part believed to be directly linked to the improvements in the imaging MS systems that have increased capabilities, both in spatial resolution as well as the increased use of high mass resolution for MS imaging studies. The possibility to observe more detail on similarly large areas will increase the number of spectra per square micrometer, which will quadratically increase the dataset size. This in turn will put larger constraints on the computational infrastructure needed in an imaging MS laboratory.

It has been questioned if increased spatial resolution will result in enhanced information without improving the sensitivity of the mass spectrometer. At some point, the pixels will become so small that only a limited amount of molecules are accessible for ionization. In that case the ionization efficiency has increased dramatically, approaching almost 100%. This seems unlikely to happen in the very near future, which means that the spatial resolution is ultimately limited by the sensitivity of the mass spectrometer. One other aspect of this debate around the pros and cons of high resolution analysis is matrix deposition. High spatial resolution MS image acquisition with matrix assisted desorption and ionization techniques will ultimately reveal the crystal surface. The size of the matrix

crystals needs to be smaller than the spatial required for analysis. Cellular details in tissue hence require matrix crystals that are substantially smaller than 1 μm . Unfortunately, small crystals also lead to sensitivity losses, which limit the applicability of this method. A good compromise between spatial resolution and sensitivity is a crystal size around 10–20 μm , which is suitable for most MALDI imaging approaches.

6. RT5 — protein post-translational modifications (leader: Nicholas Morrice)

This roundtable discussion had seven participants from a mixture of industry and academia. The general topics covered were a) digestion strategies for modified proteins to obtain high sequence coverage, b) reducing carryover in autosamplers, c) PTMs in fusion proteins especially in linker regions, d) sequence coverage of proteins from plasma and serum, e) in gel deglycosylation of proteins using PNGase F, f) sample preparation to preserve PTMs and g) venom peptides from South American Green Frog.

- a) Digestion strategies covered were in solution and in gel digestions with enzymes other than trypsin, such as chymotrypsin, Asp-N, elastase etc., and the problems often encountered with database searching using non-specific cleavage. Long search times with Mascot when no enzyme plus PTMs are selected.
- b) Sample carryover in autosamplers was discussed and a solution using trifluoroethanol as described by Karl Mechtler.
- c) Fusion proteins are often phosphorylated in the linker region and the chair pointed out the thrombin cleavage site in GST fusion proteins, the precision protease site, myc tags and HA tags are almost always phosphorylated in vivo.
- d) Sequence coverage maps of proteins from serum and plasma often require no enzyme searches.
- e) A protocol for in gel deglycosylation with PNGase F was described for removing N glycans and the use of trimethylamine for removing O-glycans was also discussed.
- f) Sample preparation is so important and the composition of cell/tissue lysis buffers is critical if PTMs are to be preserved. The use of phosphatase inhibitors to inhibit a broad range of phosphatases, the use of iodoacetamide or NEM to inhibit deubiquitination enzymes (DUBs) and the merits of cell lysis in boiling SDS were all discussed.
- g) The identification of oxidized cysteines, D-amino acids and O-linked peptides in frog venom proteins were all discussed. It was agreed these particular proteins do present a serious challenge to modern proteomic methods.

It was concluded that many of the problems with PTM analysis can be addressed with better bioinformatics tools, but sample preparation is still key to a successful outcome. The participants all agreed that there were new techniques that they had heard about either at the roundtable or in the main meeting that they could apply to their particular PTM analyses.

7. RT6 — peptidomics and bioinformatic tools (leader: Eugene Kolker)

7.1. What is the state of proteomics at the moment?

Proteomics is undergoing an incredible growth as we learn more and better ways to prepare samples, analyze samples, and get the most from the experimental data. One of the biggest improvements that is long over-due is the realization and application of standardization. Both on the lab side and on the analysis side we have been working within our own labs, without enough thought to how we could best work together. When we look beyond our own walls, we realize that in order to realize the full potential of our work, we must combine our efforts. However, without common data points in the samples, and a shared platform for analysis, we cannot combine our experimental data without incurring unacceptable uncertainties.

There are many ways to prepare a sample, run a proteomics experiment, and analyze the raw data. Each of these steps can introduce uncertainties or biases that can disrupt comparisons between or even within an experiment.

7.2. What specifically can we do to standardize in the lab?

Well, first we need to have experimental standards, mixtures that can be spiked into experimental samples so that they can serve as points of reference within every sample. That would help make comparisons across samples easier to accomplish. These reference mixtures could also be used to tune experimental techniques. The mixtures could cover a range of concentrations and proteomic properties so they could serve as a test for a given lab. If the low-range concentrations within the mixture are being detected reliably then the system is functioning optimally. Kolker's team is working on mixtures that we hope to make available to other labs to aid in the standardization efforts.

7.3. Is it possible to also standardize our analyses?

Analysis is very problematic right now. All too often the researcher sends their sample away to a black box analysis, and gets back results without really knowing what happened. It does not have to be that way. What is needed is an easily accessible, customizable and free analysis site where researchers can upload their data files and quickly run analyses. Kolker's team has built SPIRE (Systematic Protein Investigative Research Environment), a web-based pipeline for mass spectrometry data analysis (Kolker et al., 2011, Journal of Proteomics, submitted). It can be accessed at <https://www.proteinspire.org>. They also built a SPIRE community website, <http://spireanalysis.org/>, where the proteomics community, analysis experts, and computational scientists can come together to discuss analysis questions, SPIRE usage, issues and solutions. Kolker hopes that SPIRE can become as widely known by the proteomics community as possible. SPIRE allows a researcher to sit in her/his own lab and upload their raw data files to the website. The analysis parameters can be set through the website, and then the analysis will run without any need for oversight, until done. When finished, results files can be examined through

the website and/or downloaded for local use. SPIRE currently provides a complete proteomics data analysis, starting with raw MS data and finishing with protein identifications and quantitation organized by a user supplied experimental design. In future SPIRE is aimed at the integration of and the choice between the best tools for analysis. For example, SPIRE currently offers the choice between multiple database search algorithms and spectral library searches, as well as a novel approach to combining the results from these searches. Another example is the integration of two protein identification approaches, one generated in their own lab, and the other generated in the Ruedi Aebersold's lab to create an Integrated Protein Model (IPM, Higdon et al., 2011, Journal of Proteomics, submitted).

Large proteomics data sets are becoming a data-management issue. Storing, transfer, and analyzing them can be a time-consuming and inefficient process. Is there anything we can do about proteomics data handling issues? In conjunction with these efforts Kolker lab has been working with NSF to tackle the issues associated with the transition of proteomics from a lab-by-lab science to a data-intensive science.

Proteomics has been a hypothesis driven science — get an idea, run an experiment to prove or disprove the idea, and then go from there. The new approach that has gained acceptance and an enthusiastic following, is one in which data are gathered, in a rigorous manner, and then questions are asked of it. The strength of this approach can only be realized if a great deal of data is gathered. Unfortunately, at the moment our cyber infrastructure is not prepared to handle all these data, and many of our researchers do not know how to take full advantage of the approach. In fact, even the funding agencies are rethinking their strategies as they realize that data management resources and costs may need new funding approaches. The need for new approaches does not stop there. Education of new scientists needs to have a component that supports development of data management knowledge and skills. Scientists need to know how to get the most from collaborations, whether formal or informal, and how to best use the already available data sets to avoid duplication of effort. On the other side of the coin, scientists need further instruction in people management skills. Science is no longer in an ivory, isolated tower. Highly collaborative scientific efforts can be effective if the collaborators are well aligned. Research scientists will be talking to others in their fields as well as outside their fields, and progress will only be made if cross-field communication is clear.

Proteomics is changing, and change can be good. We must be ready with new skills, new approaches and a readiness to make the most of the opportunities the changes will bring.

8. RT7 — databases, data management and data mining (leader: Fredrik Levander)

The first part of the discussion mainly concerned public database resources of interest for proteomics. The amount of resources for proteomics data has grown tremendously, and there are now several parallel online repositories for the storage of results from proteomics experiments. Integration of data between repositories and other protein and genome

databases is the next step in the pipeline. There are now for example links from UniProt to PRIDE, but several challenges remain before the resources can be considered integrated. A major challenge for integration of data between different databases is especially how to handle quality control; which cutoffs in terms of false discovery rates to use, for example. The informatic treatment of differentially modified forms of the same protein is another issue. Information about specific modified forms of proteins are difficult to transfer between databases, since there is most often one gene–one accession number, and different ways to annotate modified proteins.

Furthermore, an important, but challenging, task is to add quantitative proteomics data to the repositories, which is especially difficult due to the numerous workflows available for proteomics. The upcoming mzQuantML format, developed by the Proteomics Standards Initiative will be an important component for sharing and integration of quantitative proteomics data. Once quantitative proteomics data becomes available in repositories in standard formats, new possibilities for functional analyses of combined omics datasets will emerge.

After this general database discussion, the focus turned to practical data management and data mining in the proteomics laboratory. For local data management there are numerous tools for data analysis and data management available. The largest issue seems not to be the availability of tools, but rather how to navigate between all these tools, and choose the most appropriate solution for the lab. While there exist rather complete data management solutions, and numerous data analysis tools; both open source free solutions and commercial solutions — most of these remain installed in just a few labs. There is an apparent gap between producers and consumers of proteomics software considering that new algorithms are not adapted by other labs until absolutely necessary. Difficulties in installing and using public software seem to be one of the bottlenecks. Many people will drop software that does not work out of the box if the software does not provide indispensable tools. The discussion thus focused much on how to overcome this situation, but unfortunately no obvious solution could be found. Finally, the discussion went more into specific tools to handle local data management and to analyze data from specific workflows.

9. RT8 — interactomes, networks and pathways analysis (leader: Roman Zubarev)

During the roundtable discussion, several issues have been raised relevant to the usage of expression proteomics data in pathway analysis. Why are we interested in pathway analysis in the first place? To learn more about cell signaling, discover new signaling pathways and to apply existing knowledge if signaling information for identification and quantification of signaling pathways.

Today, in post-genomic era, researchers measure how proteins change their abundance, modifications and interaction partners in time and in different cellular processes, including pathological ones. The measurement results on thousands of proteins are best explained by pathway analysis.

Unlike metabolic pathways that are controlled by flow (flux) of chemical reactions, following conservation law, signaling pathways involve amplification of the downstream signal. This allows one, starting from available proteomics data, to perform upstream analysis and determine the key element (nod) of the pathway, even though that element itself is not part of the proteomics analysis.

Among other topics discussed were: how does the cell regulate proteins (low abundant proteins are regulated more, or the other way around)? Is there such a thing as household proteins? Do pathways even exist (aren't they just kinetically favored routes in protein networks, which easily change their path if the network changes)? Up-regulation vs down-regulation — should one treat them equally? Can expression proteomics yield sufficient pathway information to render the much more challenging phosphoproteomics largely redundant (except for very short time scales)? The consensus was that too little is known to definitely answer many of these questions, however basic they might appear. Therefore, more experiments, and many more discussions, are needed for pathway analysis to become firmly rooted on the proteomics soil.

10. RT9 — SRM/MRM challenging (leader: Paola Picotti)

The focus of the 9th round table discussion was the development and application of targeted proteomic workflows based on selected reaction monitoring (SRM, also called MRM). The essence of the approach is the generation of specific, quantitative mass spectrometric assays for each protein of interest and their subsequent application to the quantification of the proteins through multiple samples [9]. For each protein, representative peptides are selected (proteotypic peptides (PTPs) [10], or signature peptides [11]) and, for each PTP, an SRM assay is established to quantify the peptide and, by inference, the corresponding protein.

SRM exploits the unique targeting capabilities of triple quadrupole (QQQ) mass spectrometers. The first and the third quadrupoles of a QQQ instrument act as filters, to isolate ions of predefined m/z values corresponding to a PTP and one of its specific fragments, while the second quadrupole serves as collision cell. Several such precursor/fragment ion pairs (also called SRM transitions) can be monitored within the same analysis, yielding chromatographic peaks that allow for the relative or, if suitable heavy-labeled standards are used, absolute quantification of the corresponding peptides. The non-scanning nature of this mode of MS operation translates into a high sensitivity and a linear response over a wide dynamic range. This can facilitate the detection of low-abundance proteins in highly complex mixtures, which is crucial for systematic quantitative studies [12]. The technique is also reproducible [13] and accurate [14], attributes that are critical for the generation of consistent quantitative data sets from multiple samples.

During the round table discussion, the optimal design of an SRM experiment was discussed, which involves four major steps: 1) selection of a set of target proteins; 2) selection of suitable PTPs; 3) generation of the corresponding SRM assays;

4) application of the assays to the quantitation of the proteins. The participants to the discussion agreed that the advantages of SRM become particularly striking in hypothesis-driven experiments, where specific sets of proteins need to be consistently quantified from different samples. Protein sets typically targeted in SRM experiments include biologically related proteins (e.g. those involved in a specific biochemical or signaling network), sets of candidate biomarkers, or sets of proteins identified as responders to a given perturbation in large-scale discovery experiments. Once the target protein set is defined, the subsequent step involves the selection of suitable, detectable PTPs for each protein. Such step is typically guided by prior information extracted from existing proteomic datasets, acquired in the same laboratory or stored in publicly accessible proteomic data repositories [15]. PTPs for proteins previously undetected can also be predicted by computational tools [10,11]. However, such tools can still be error-prone when estimating the detectability ranking of a set of peptides and their improvement was identified as a major requirement for the future. The step subsequent to peptide selection is typically the development of the SRM assay for the target peptides, which includes the identification of intense peptide fragments to be monitored by SRM, their relative intensities and the peptide elution time. The participants to the discussion concurred that this step used to be a major bottleneck of the workflow, but is now accelerated by the use of synthetic analogs of the target peptides (e.g. purified or in the form of crude synthetic peptide libraries [16], measured in batches on QQQ instruments, to derive the final SRM assay coordinates. The importance of depositing the extracted SRM assay coordinates to publicly accessible databases [17] was emphasized, to enable their archivation and dissemination. The further development and enrichment of such databases of SRM assay coordinates (ideally to coverage of complete proteomes) is identified as a direction for the future, to eliminate the redundant extraction of SRM coordinates for the same sets of peptides in different laboratories and promote their sharing within the scientific community. Last, in a typical SRM experiment the SRM assays are applied to the quantitation of the set of target proteins in different samples. A discussed challenge in this step was the number of peptides that can be simultaneously monitored within the same chromatographic analysis, without compromising on the sensitivity of the measurements. An accurate scheduling of SRM transition acquisition over the chromatographic time [14] or the use of new instrument control software based on data-dependent SRM acquisition [18] (SRM-triggered SRM) were suggested as future directions towards an increased multiplexing of SRM. Finally, the need for computational tools that enable the automatic analysis of SRM data and the statistical evaluation of their false discovery rate was emphasized.

In summary, as attested by the number of talks at the meeting involving SRM and by the interest of the participants to the round table discussion, SRM was identified as a powerful technology for protein quantification and a major direction for targeted proteomic workflows. The technology is however still in its early times for protein analysis and challenges like the ones discussed above will have to be addressed in the near future, to increase its widespread application in non-specialized laboratories.

11. RT10 — translational proteomics: from the lab to the clinic (leader: Christoph W Turck)

11.1. Biomarker discovery and validation

There are great expectations for translating proteomics research into clinical applications. As was evident from a number of presentations at the 4th EuPA Meeting in Estoril, Portugal, proteomics scientists have moved beyond the development of sophisticated equipment and methodologies and are applying their technology to biomarker discovery and validation projects. An example is the recent FDA approval of a protein panel for the diagnosis of ovarian cancer that was discovered by the SELDI TOF technology. Furthermore, diagnostic methods based on mass spectrometry are increasingly used for the detection of metabolic diseases during new-born screening.

However, there are a number of issues that still must be resolved before proteomics technologies can be translated into clinical laboratories on a routine basis. First, the analytical procedures used in the biochemistry laboratory are complex and require personnel experienced with the operation of mass spectrometry and other sophisticated equipment. Therefore, the development of simplified procedures and equipment that can be adapted in the clinical laboratory has to be a major focus. These include mass spectrometers for an optimized measurement of specific analytes or, alternatively, the implementation of traditional immunochemical methods including multiplexed protein array based assays. Second, the identification of novel biomarkers and therapeutic targets as well as their validation heavily depend on the availability of high quality human specimens. Biomarker validation is one of the major hurdles in this regard and requires the analysis of large patient cohorts and controls that are in many cases difficult to obtain. Major efforts in biobanking will be critical for future success in this area.

11.2. Biomarker assay implementation

Finding an industrial partner for the implementation of novel technologies is often required. A recent success in this area is the joint development of a prototype protein array chip for the diagnosis of Systemic Lupus Erythematosus, an autoimmune disease. However, development of a biomarker assay does not end here. A major step is getting the assay onto the market. Clinical laboratories often have certain preferences when it comes to hardware, software, reagents and other things relevant for daily routine use. It is often a challenge to convince users to implement new technologies like multiplex detection systems that require different sample handling and data interpretation. It will be critical that the different stakeholders including the development laboratory, industrial partner, the clinical laboratory personnel and clinicians work closely together.

11.3. The importance of high quality specimens

Asthma and chronic obstructive pulmonary disease (COPD) are the most common non-infectious disorders of the airways. Despite years of extensive research prior to proteomics

Table 1 – Translational plant proteomics areas according to the objectives of the research. In this table papers dealing with plant developmental processes and responses to environmental factors are not included, being it covered in very recent published reviews (Hashiguchi et al. 2010).

Areas	Objectives	Examples	Some references
Food and beverages technology/trazeability	Characterize raw materials, processing, storing conditions and the final product. Safety issues.	Changes in the protein profile in response to storage conditions of fruit and vegetables (e.g. cold treatment).	Pedreschi et al. (2010)
Nutrigenomics		Changes in response to postharvest diseases. Proteomics of cereal seeds and derived products. Protein presence and content in beverages.	Kussmann et al. (2010)
Substantial equivalence in transgenic crops	Characterize unintended, pleiotropic effects. Nutritional and safety issues	Comparative proteomic profiling of transgenic plants and its wild counterpart.	Barros et al. (2010)
Allergen identification and characterization	Development of accurate, high-throughput approaches for protein allergen identification and quantification	Proteomic analysis of pollen, nut foods, latex derived products.	D'Amato et al. (2010)
Diversity (biodiversity)	Proteotyping*	The protein species profile of biological units, from protein structural and functional families to ecosystems, through organules, cells, tissues, organs, individuals, genotypes, mutants, transgenics, populations, species. It depends on the developmental stage and its affected by external physico-chemical and biological factors	Park et al. (2009)
Others	Biofuels	Identifying new targets for biofuel crop engineering	Lorito et al. (2010)
	Agronomic characteristics and practices	Apomixis, crop protection techniques	
	Fiber industry	Fiber proteomics analysis	
	Biomedicine	Protein targets of bioactive compounds, drugs	

* Understood as: the protein species profile of biological units, from protein structural and functional families to ecosystems, through organules, cells, tissues, organs, individuals, genotypes, mutants, transgenics, populations, and species. It depends on the developmental stage and it's affected by external physico-chemical and biological factors.

attempting to identify and validate relevant diagnostic biomarkers, as well as developing novel therapeutic agents, the number of FDA approved treatments is limited and there is still a lack of effective diagnostic and prognostic markers. Clinical samples available to accomplish this include, but are not limited to, plasma, induced sputum, bronchoalveolar lavage fluid and lung biopsies. The challenge in clinical proteomics is obtaining sufficient, clinically relevant, well characterized patient material for proteomics analysis and validation. From the researchers' point of view, it is absolutely essential to use clinically relevant samples from well characterized patients to further understand the mechanisms of disease and to identify biomarkers. Proteomics is still a science in its infancy. The first publications on asthma or COPD proteomics efforts appeared in 2001. Since then, 79 and 36 reports related to the proteomics of asthma and COPD, respectively, were published. With the continued exponential growth of the proteomics field it is expected that fully validated biomarkers for the diagnosis and prognosis of asthma and COPD will become available in the near future.

11.4. Animal models

Animal models are used for biomarker discovery in many disease areas. Homogeneous genetic backgrounds and standardized housing conditions allow for a controlled interrogation of specific aspects of a particular phenotype including anxiety and depression that are faithfully represented in the animal [19]. At the same time, findings from animal models are often of limited practical use until moved into the clinic.

Major depression is a serious illness that affects approximately 121 million people worldwide. By 2020, it will be the world's second leading cause of 'disability adjusted life years' according to the World Health Organization. Electroconvulsive therapy (ECT) is a very effective treatment available for depression. It is associated with a swift response and high rates of remission among patients. ECT is also of use in treating cases of drug resistant depression [20]. In an animal model of ECT, electroconvulsive stimulation (ECS) allows the effects of this treatment to be studied in the brain. Using 2D DIGE and mass spectrometric techniques, protein changes induced by ECS in

brain and plasma samples can be identified. Elucidating the molecular mechanisms of action of ECS will lead to an increased understanding of this treatment as well as the neurobiology of depression itself.

Plasma samples collected from patients treated with ECT in a concurrently running clinical trial allow one to compare and contrast candidate protein biomarkers detected experimentally. Combining laboratory and clinical experimental approaches brings us closer to real-world application of experimental findings and to potential benefits for patients.

12. RT11 — translational plant proteomics: from the lab to the field/table (leader: Jesus Jorin Novo)

The origin of the word protein comes from the Greek language and can be translated with “the first” or “fundamental”. This reflects the key role of proteins in the living system. By using a proteomics approach, we do pretend to answer “how”, “where”, “when”, and “what for” are the several hundred thousand of individual protein species produced in a living organism, how do they interact with one another and with other molecules to constitute the dynamic cellular building, how do they work in order to control and regulate programmed growth and development, and to interact with their biotic and abiotic environment. During the last 15 years, and in order to approach the above questions experimentally, continuous improvements in techniques and protocols have being made at all workflow stages, starting from the laboratory (tissue and cell fractionation, protein extraction, depletion, purification, separation, MS analysis) and ending at the computer (algorithms for protein identification and bioinformatics tools for data analysis, databases and repositories).

In the case of plants, the full potential of proteomics is far from being fully exploited in biological research, if compared with other organisms (e.g. humans and *S. cerevisiae*). Even so, the approach is making important contributions to the knowledge of the biology of these organisms, at least for the model species *Arabidopsis thaliana* or the crop plant rice [21,22], and is starting to contribute to the study of other orphan food and fiber providing species [23]. Mass spectrometry-based proteomics is used to gain insight into different processes, from development to response to environmental physico-chemical or biological conditions, from perception to gene expression through signaling, from genotype to phenotype [24].

During the last 15 years of intensive research in proteomics, in general, and in plant proteomics, in particular, we have realized and learnt that the proteins, as molecular entities, are more complex and difficult to be handled than ever imagined (e.g. How many protein species per gene? What is the biological role of each posttranslational modification? How many interactions?). To totally decipher the whole proteome of just one single species and its biological meaning, requires multinational collaborative efforts, the use of complementary approaches of first (mainly based on 2-DE), second (those of quantitative, either label or label-free, shotgun proteomics), or third (hypothesis driven, such as MRM) generation technologies, and the validation by other complementary omics — (transcriptomics, metabolomics) or classical biochemistry and cellular biology approaches. In this direction different

international initiatives have been established or just initiated, such as The Multinational Arabidopsis Steering Subcommittee (http://www.masc-proteomics.org/mascp/index.php/Main_Page), The “Plant Proteomics in Europe” COST FA 0603 (<http://www.costfa0603.org/>), the INPPO (<http://www.inppo.com/>), and the Asia Oceania Agricultural Proteomics Organization-AOAO (Prof. S. Komatsu, personal communication).

However, science goes beyond just knowledge and research should be translated in order to improve human health and life quality [25]. Thus, the generated knowledge and developed analytical tools should be exploited in favor of a more sustainable, productive (both from a qualitative and quantitative point of view), and environmental-friendly agriculture and food industry. This is recognized by the scientific community dealing with plant biology research, in general, and plant proteomics, in particular. In the last two years a number of projects and publications within this area of translational proteomics in agriculture and food industry have appeared (Table 1). In summary, the final goal is to increase feed, food and fuel production as basis of our existence here on earth, both qualitative and quantitatively, with less inputs, preserve biodiversity and the environment, characterize natural biodiversity and select elite genotypes to get into classical breeding or biotechnology.

13. Summary and acknowledgements

At least three main achievements were obtained with these Roundtable Discussions. First, important issues in proteomics field were arisen from a live discussion between senior and young scientists. Second, a number of practical recommendations for handling thorny issues were provided. Third, realistic expectations were established for the field, such as biological and analytical needs/demands as driving force in the constant improvement of new instrumental techniques and methods. In general, the thoughts provided here will certainly be a useful information for those handling common situations in proteomics research, putting also in place new questions and challenges for the next decades.

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REFERENCES

- [1] Mitchell P. Proteomics retrenches. *Nat Biotechnol* 2010;28:665–70.
- [2] Tu C, Rudnick PA, Martinez MY, Cheek KL, Stein SE, Slebos RJ, et al. Depletion of abundant plasma proteins and limitations of plasma proteomics. *J Proteome Res* 2010;9:4982–91.
- [3] Zhi W, Purohit S, Carey C, Wang M, She JX. Proteomic technologies for the discovery of type 1 diabetes biomarkers. *J Diabetes Sci Technol* 2010;4:993–1002.
- [4] Bandow JE. Comparison of protein enrichment strategies for proteome analysis of plasma. *Proteomics* 2010;10:1416–25.
- [5] Dwivedi RC, Krokhn OV, Cortens JP, Wilkins JA. Assessment of the reproducibility of random hexapeptide peptide library-based protein normalization. *J Proteome Res* 2010;9:1144–9.
- [6] Ernoul E, Bourreau A, Gamelin E, Guette C. A proteomic approach for plasma biomarker discovery with iTRAQ labelling and OFFGEL fractionation. *J Biomed Biotechnol* 2010;2010:927917.
- [7] Marko-Varga G, Ogiwara A, Nishimura T, Kawamura T, Fujii K, Kawakami T, et al. Personalized medicine and proteomics: lessons from non-small cell lung cancer. *J Proteome Res* 2007;6:2925–35.
- [8] Hancock W, Omenn G, Legrain P, Paik YK. Proteomics, human proteome project, and chromosomes. *J Proteome Res* 2011;10:210.
- [9] Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol Cell Proteomics* 2006;5:573–88.
- [10] Mallick P, Schirle M, Chen SS, Flory MR, Lee H, Martin D, et al. Computational prediction of proteotypic peptides for quantitative proteomics. *Nat Biotechnol* 2007;25:125–31.
- [11] Fusaro VA, Mani DR, Mesirov JP, Carr SA. Prediction of high-responding peptides for targeted protein assays by mass spectrometry. *Nat Biotechnol* 2009;27:190–8.
- [12] Picotti P, Bodenmiller B, Mueller LN, Domon B, Aebersold R. Full dynamic range proteome analysis of *S. cerevisiae* by targeted proteomics. *Cell* 2009;138:795–806.
- [13] Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol* 2009;27:633–41.
- [14] Stahl-Zeng J, Lange V, Ossola R, Eckhardt K, Krek W, Aebersold R, et al. High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. *Mol Cell Proteomics* 2007;6:1809–17.
- [15] Vizcaino JA, Côté R, Reisinger F, Barsnes H, Foster JM, Rameseder J, et al. The proteomics identifications database: 2010 update. *Nucleic Acids Res* 2010;38:D736–42.
- [16] Picotti P, Rinner O, Stallmach R, Dautel F, Farrah T, Domon B, et al. High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. *Nat Methods* 2010;7:43–6.
- [17] Picotti P, Lam H, Campbell D, Deutsch EW, Mirzaei H, Ranish J, et al. A database of mass spectrometric assays for the yeast proteome. *Nat Methods* 2008;5:913–4.
- [18] Kiyonami R, Schoen A, Prakash A, Peterman S, Zabrouskov V, Picotti P, et al. Increased selectivity, analytical precision, and throughput in targeted proteomics. *Mol Cell Proteomics* 2011;10 M110.002931.
- [19] Ditzel C, Varadarajulu J, Czibere L, Gonik M, Targosz BS, Hambach B, et al. Proteomic-based genotyping in a mouse model of trait anxiety exposes disease-relevant pathways. *Mol Psychiatry* 2010;15:702–11.
- [20] Group UER. Efficacy and safety of electroconvulsive therapy in depressive disorders: a systematic review and meta-analysis. *Lancet* 2003;361:799–808.
- [21] Jorrín-Novo JV, Maldonado AM, Echevarría-Zomeño S, Villedor L, Castillejo MA, Curto M, et al. Plant proteomics update (2007–2008): second-generation proteomic techniques, an appropriate experimental design, and data analysis to fulfill MIAPE standards, increase plant proteome coverage and expand biological knowledge. *J Proteomics* 2009;72:285–314.
- [22] Wienkoop S, Baginsky S, Weckwerth W. *Arabidopsis thaliana* as a model organism for plant proteome research. *J Proteomics* 2010;73:2239–48.
- [23] Abril N, Gion JM, Kerner R, Müller-Starck G, Cerrillo RM, Plomion C, et al. Proteomics research on forest trees, the most recalcitrant and orphan plant species. *Phytochemistry* 2011 (PubMed PMID code: 21353265).
- [24] Kaufmann K, Smaczniak C, de Vries S, Angenent GC, Karlova R. Proteomics insights into plant signaling and development. *Proteomics* 2011;11:744–55.
- [25] Greenland A, Leach J. Translational research for developing and sustainable agriculture. *Curr Opin Plant Biol* 2008;11:163–5.

-
- [26] Hashiguchi A, Ahsan N, Komatsu S. Proteomics application of crops in the context of climatic changes. *Food Research International* 2010;43:1803–13.
- [27] Pedreschi R, Hertog M, Lilley KS, Nicolai B. Proteomics for the food industry: opportunities and challenges. *Critical Reviews in Food Science and Nutrition* 2010;50:680–92.
- [28] Kussmann M, Panchaud A, Affolter M. Proteomics in nutrition: status quo and outlook for biomarkers and bioactives. *Journal of Proteome Research* 2010;9:4876–87.
- [29] Barros E, Lezar S, Anttonen MJ, Van Dijk P, Röhlig RM, Kok EJ, Engel K-H. Comparison of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. *Plant Biotechnology Journal* 2010;8:436–51.
- [30] D'Amato A, Bachi A, Fasoli E, Boschetti E, Peltre G, Sénéchal H, Sutrad JP, Citterio A, Righetti PG. In-depth exploration of *Hevea brasiliensis* latex proteome and “hidden allergens” via combinatorial peptide ligand libraries. *Journal of Proteomics* 2010;73:1368–80.
- [31] Park KM, Choi YH, Park WM, Na HJ, Na YJ, Lee DH, Chung WI, Kim DS. Proteomic pattern-based identification of molecular phenotypes from *Arabidopsis thaliana* ecotypes. *Plant Science* 2009;177:7–18.
- [32] Lorito M, Woo SL, Harman GE, Monte E. Translational research on trichoderma: from 'Omics to the Field. *Annual Review of Phytopathology* 2010;48:395–417.