SUBCELLULAR TRANSLOCATION OF MOLECULES ASSOCIATED WITH SYNAPSE SPECIFIC PLASTICITY

KARA M. MCNAIR

A thesis presented for the degree of PhD

Division of Neuroscience and Biomedical Systems
Institute of Biomedical and Life Sciences
University of Glasgow
G12 8QQ

June 2005

ABSTRACT

Activity-dependent remodelling of synaptic connections is a fundamental process by which information is acquired (learning) and stored for subsequent retrieval (memory). Remodelling of glutamatergic synapses displays distinct temporal components with an initial modification of pre-existing synaptic proteins, for example by phosphorylation, followed by transcription- and translation-dependent late phases. Using a proteomics (differential gel electrophoresis (DIGE))-based approach, we have investigated global changes in protein expression in response to pharmacological, synaptic and behavioural stimulation of glutamatergic synapses in area CA1 of the rodent hippocampus.

In initial experiments, mouse hippocampal slices were subjected to pharmacological activation of glutamate receptors and protein extracts from cornu ammonis (CA)1 subfields were harvested at 4hrs following incubation. The entire hippocampal proteome was subsequently subjected to 2 dimensional (2D) gel electrophoresis to separate component proteins and enable the quantification of individual protein spot abundance levels compared with control tissues.

Of the 2946 protein spots resolved, 79 showed significantly altered abundance levels following activation of glutamate receptors when compared to vehicle controls (35 increased / 44 decreased abundance (–1.91 to 1.9 fold change), all $p<0.05$). Inclusion of a treatment group incorporating the N-methyl-D-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonovaleric acid (D-AP5) revealed that for the majority of spots (58 of 79) altered abundance was sensitive to NMDA receptor blockade consistent with previous reports of NMDA receptor activation being linked to the regulation of multiple plasticity-related genes. Following in-gel tryptic digestion and matrix assisted laser desorption/ ionisation – time of flight (Maldi-tof) / Q-star mass spectrometry, database searching revealed the identity of 39 protein spots displaying glutamate receptor activation-dependent modulation of expression. Identified proteins belonged to a diverse variety of functional classes including those associated with glutamate receptor cycling (e.g. n-ethylmaleimide sensitive fusion protein (NSF)), cytoskeleton-associated proteins, proteins involved in vesicle

trafficking and metabolic and ubiquitination proteins as well as proteins with as yet no known association with either glutamate receptors or plasticity mechanisms (e.g. DJ-1).

Pharmacological stimulation of glutamate receptors was also investigated in isolated dendritic fields as an attempt to identify those proteins which that are regulated locally at glutamatergic synapses of area CA1 in the rat hippocampus. Isolated dendritic fractions of CA1 hippocampal neurons were treated with glutamate (100μM, 4hrs) alone and in the presence of the NMDA receptor blocker D-AP5 (50μM, 4hrs). A total of seven proteins were identified as significantly differentially expressed ($p<0.05$) as a result of glutamate treatment, and all regulation was blocked by D-AP5. Very small fold changes in expression were observed, with only one protein increasing in expression (heat shock protein (HSP) 8, average fold change of 1.18) and the remaining 6 decreasing in expression (-1.08 to –1.13 fold change). From the seven proteins highlighted 2 have been identified as beta actin and HSP8.

In further experiments to assess possible differential changes in protein expression associated with long term potentiation (LTP), bulk high frequency afferent stimulation was used to evoke a robust synaptic potentiation of glutamatergic synapses in hippocampal area CA1. Potentiated and basal stimulated neuropil were subsequently harvested at 10 and 240 minutes and protein extracts subjected to DIGE. Of the 71 proteins spots displaying differential abundance in potentiated tissues at 10 minutes (50 down-regulated, 21 up-regulated (-1.79 to 1.51 fold change) and 51 proteins spots displaying differential abundance at 240 minutes (30 down regulated, 21 up-regulated (-1.43 to 2.0 fold change)), only 3 protein spots displayed altered expression at both time points. Again, mass spectrometric/ bioinformatic identification revealed altered expression across a diverse range of protein classes with Fatigo gene ontology analysis showing discrete patterns of functionally related proteins to be differentially regulated at specific time points post stimulation. In particular, a major proportion of the proteins exhibiting altered abundance at 10 minutes following LTP induction relate to energy and metabolism. However, at 4 hours post LTP induction the proportion of altered proteins involved in energy metabolism declined whereas the principal component of differentially regulated identified proteins relate to cytoplasmic organisation and biogenesis (e.g.

cytoskeletal and associated proteins). This finding is consistent with rapid onset but transient activity-dependent synaptic processes being followed by later onset structural reconfiguration and reconsolidation. The majority of proteins identified are known to be expressed in neurons and indeed many have previously been linked to plasticity processes including LTP (e.g. calcineurin, voltage dependent anion channel 1 (VDAC1), mitogen activated protein kinase kinase 1 (MEK1), growth associated protein 43 (GAP43)). Moreover, the altered abundance of individual separated protein spots in this study reflects both post-translational modifications to existing proteins as well as alterations in protein biosynthesis and degradation pathways. Although many of the identified proteins are known to be expressed at somatic and dendritic loci, several modulated proteins occur at pre-synaptic terminals where they govern transmitter release and may be important regulators of plasticity (e.g. synapsin 2, syntaxin 1A, copine-6). The inclusive nature of the tissue sampling additionally permitted analysis of the parallel involvement of the supporting glial cells. Indeed, altered expression of glial fibrillary associated protein (GFAP) was evident at 240 minutes post LTP induction, an effect very much delayed in relation to the transient glial membrane response to glutamate seen following LTP in area CA1.

The field of learning and memory encompasses research not only into the molecular mechanisms involved in cognitive decline associated with, for example Alzheimer's disease (AD), but also cognitive enhancement such as that demonstrated following exposure of animals to an enriched environment. Therefore, in a final set of experiments differential protein expression was investigated in two animal models representative of cognitive enhancement (environmental enrichment, EE) and cognitive impairment in the TAS10 transgenic mouse model of AD. Exposure of rats to an enriched environment (including toys, activity wheels etc.) for 15 hours per day, 5 days per week for a total experimental period of 6 weeks identified 32 protein spots from isolated dendritic regions and 42 protein spots from isolated somatic regions to be significantly differentially expressed ($p<0.05$). Many of the identified proteins are known plasticity-related proteins (cyclin dependent kinase 5 (CDK5), Rho-associated coiled-coil forming kinase 1 (ROCK1)), but with no prior association to enrichment studies of this kind. Cluster analysis using the Fatigo data-mining tool again highlighted the importance of energy metabolism and cytoplasmic organisation processes following environmental enrichment. It also suggested a relatively large

degree of signal transduction in the isolated dendritic fractions of CA1 hippocampal neurons.

Finally, analysis of hippocampus and somatosensory cortex from the brains of TAS10 transgenic mice was carried out by DiGE. The transgenic mouse line TAS10 over expresses the 695-amino acid isoform of the human amyloid precursor protein which itself contains the Swedish double familial AD mutation. These mice show an age dependent increase in the deposition of beta amyloid in the brain in addition to spatial learning and working memory deficits. Previous reports that the pathophysiology in these mice was greater in the cortex region than the hippocampal formation is further suggested by the fact that more (32 compared to 23) protein spots demonstrated significant changes in expression levels in the cortex region of these mice compared to the hippocampus.

In summary, the novel proteomics based approach adopted for this study was successfully used to highlight the regulation of proteins involved in plasticity-related processes in the mammalian brain, including synapse specific forms of plasticity. In addition, the identification of many of these regulated protein molecules provides interesting and novel insights into the molecular events occurring in plasticity-related processes including glutamatergic afferent stimulation and long term potentiation.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor Dr. Stuart Cobb for giving me the opportunity to undertake a fascinating and challenging project, and for providing me with his help and advice throughout the course of my studies. In addition, I would like to thank him for his careful scrutiny of this thesis. I would also like to thank Dr Ceri Davies, for his advice in many aspects of this PhD and for his critical evaluation of this thesis.

I am also grateful to all the staff at the Sir Henry Wellcome Functional Genomics Facility for their technical assistance, in particular to Drs Richard Burchmore and Andy Pitt for their help and frequent discussions on many aspects of this work.

I have been fortunate to work with some fantastic people over the past few years and thank them for their encouragement, help and friendship. Special thanks to Leanne Ferrigan, Melissa McBride, Shiva Roshan-Milani and Helene Widmer for making my studies so enjoyable.

To my parents Alex and Maureen, love and thanks go to them for their continual belief, encouragement and support, which has always helped me to achieve my goals.

And finally, a special thanks and much love goes to my family - David, Isaac and Max. Couldn't have done it without you!

ABBREVIATIONS

2D	2 dimensional
Abeta	beta amyloid
ACN	acetonitrile
ACPD	aminocyclopentane-*trans*-1,3-dicarboxylic acid
ASCF	artificial cerebrospinal fluid
AD	Alzheimer's disease
AmBic	ammonium bicarbonate
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
APP	amyloid precursor protein
APS	ammonium persulphate
ASB14	amidosulfobetaine-14
AT	amino acid transport
ATP	adenosine tri phosphate
BACE1	beta secretase protein
BDNF	brain derived neurotrophic factor
BVA	biological variance analysis
CA (1, 2, 3 or 4)	cornu ammonis
Ca2+	calcium
CaMKII	calcium/ calmodulin dependent protein kinase II
CaMKIV	calcium/ calmodulin dependent protein kinase IV
cAMP	cyclic adenosine monophosphate
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
CIB	calcium ion binding
CNS	central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
CO&B	cytoplasmic organisation and biogenesis
CREB	c-AMP responsive element binding protein
CRMP	collapsing response mediator protein
Da	Daltons

D-AP5	D-2-amino-5-phosphonovaleric acid
DIA	difference in-gel analysis
DiGE	Difference gel electrophoresis
DTT	dithiothreitol
EE	environmental enrichment
EM	energy metabolism
EPSP	excitatory post synaptic potential
ERK	extracellular regulated kinase
EST	expressed sequence tags
GFAP	glial fibrillary acidic protein
HFS	high frequency stimulation
hnRNPK	heterogeneous nuclear ribonucleotide protein K
Hrs	hours
HS ACSF	high sucrose artificial cerebrospinal fluid
Hsc	heat shock cognate
Hsp	heat shock protein
Hz	hertz
ICAT	isotope coded affinity tag
i.p.	intra peritoneally
IPG	immobilised pH gradient
Kif	kinesin superfamily protein
LC	liquid chromatography
LTD	long-term depression
LTP	long-term potentiation
L-LTP	late phase long-term potentiation
Maldi-tof	matrix assisted laser desorption/ ionisation – time of flight
MAP	microtubule associated protein
MAPK	microtubule associated protein kinase
MCPG	Methyl-4-carboxyphenylglycine
μg	microgram
mGluR	metabotropic glutamate receptor
μl	micro litre
μm	micro metre
μM	micro molar

mA	milli ampere
mls	millilitres
ms	milli seconds
MS	mass spectrometry
MS/ MS	tandem mass spectrometry
MW	molecular weight
NCBI	National Centre for Biotechnology Information
NF(h. l. m)	neurofilament (heavy, light medium)
NG	neurogenesis
NGF	nerve growth factor
NL	non linear
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NSF	N-ethyl maleimide sensitive fusion protein
NT	neurotransmitter production and release
p	probability
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
PKA	protein kinase A
PKC	protein kinase C
PMF	peptide mass fingerprint
PM&T	protein modification and transport
PPF	paired pulse facilitation
PS	population spike
PS1	presenilin 1
PSD	post synaptic density
PTKs	protein tyrosine kinases
PTM	post translational modification
RTS	response to stress
S.D.	standard deviation
SDS	sodium dodecyl sulphate
s.e.m.	standard error of the mean
SIG	signal transduction
ST	synaptic transmission

STP	short term potentiation
TBS	theta burst stimulation
TFA	trifluoroacetic acid
Thr	threonine
TG	transgenic
T/T	transcription and translation
V	volts
VDAC	voltage dependent anion channel
VDCC	voltage dependent calcium channel
Vhrs	volt hours
VMT	vesicle mediated transport
WT	wild type

CONTENTS **PAGE NUMBER**

LIST OF FIGURES AND TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LIST OF FIGURES AND TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 1

Introduction

1.1 General Introduction

This thesis investigates the molecular changes occurring as a result of neuronal plasticity within the hippocampus. Neuronal plasticity occurs during development, following neuronal injury and is largely experience dependent. Whilst a Medline search reveals more than 13,000 citations for plasticity within the entire central nervous system, and over 4000 citations for plasticity in the hippocampus alone, we are still some way from elucidating the exact changes that are occurring as a result of, or indeed resulting in, cellular plasticity in the brain. The requirement at present seems to be for an increasingly global approach whereby we can identify the changes occurring as a direct result of plasticity related events thus providing a platform with which to further investigate these changes in relation to their location and timing. Hopefully such an investigation will elucidate the molecular events that precede or accompany cognitive processes such as learning and memory.

The hippocampal formation is the brain structure that has been the main focus for neuronal plasticity studies over the past 30 years. Its central role in activity dependent forms of plasticity and plasticity arising from neuronal damage and development will be discussed in more detail in the following text. LTP in the hippocampus, a synapse specific form of plasticity thought to provide a cellular correlate for learning and memory processes, will also be discussed at length, including the vital role of protein synthesis and modification in the induction and maintenance of this plasticity model.

Finally, the implementation of genomics and proteomics into the neurosciences and their potential as powerful tools in the study of the molecular mechanisms involved in synapse specific plasticity events in the hippocampus will end this chapter.

1.2 The Hippocampus

The hippocampus generates a large degree of scientific interest not only because of its highly organised structure allowing for ease of experimental manipulation but also due to its known relevance in cognitive functions such as learning and memory (Milner & Penfield, 1955; Penfield & Milner, 1958). The hippocampus is one of a

group of structures forming the limbic system and is a part of the hippocampal formation, including the dentate gyrus, subiculum, and entorhinal cortex (Amaral, 1999).

1.2.1 Structure of the hippocampus

The hippocampus lies along the bottom edge of the temporal neocortex. Cross sections of the hippocampus illustrate a sea horse like shape. It is conventionally divided into 4 subfields (Lorente de No, 1934) – cornu ammonis (CA) 1, CA2, CA3 and CA4, of which the CA1 and CA3 fields form the majority (figure 1.1). In addition to these four regions, the hilar region, or hilus, can be found between the dentate gyrus and the stratum granulosum of the CA3 subfield. The hippocampus has 3 distinct layers. A single layer of pyramidal cells, the stratum pyrimidale, has afferent fibres running transversely above (stratum oriens) and below (stratum radiatum and stratum lacunosum-moleculare) them. The dentate gyrus also consists of three layers - a polymorphic layer (the hilus), a granular layer (stratum granulosum) and a molecular layer (stratum moleculare).

Figure 1.1 The hippocampal formation

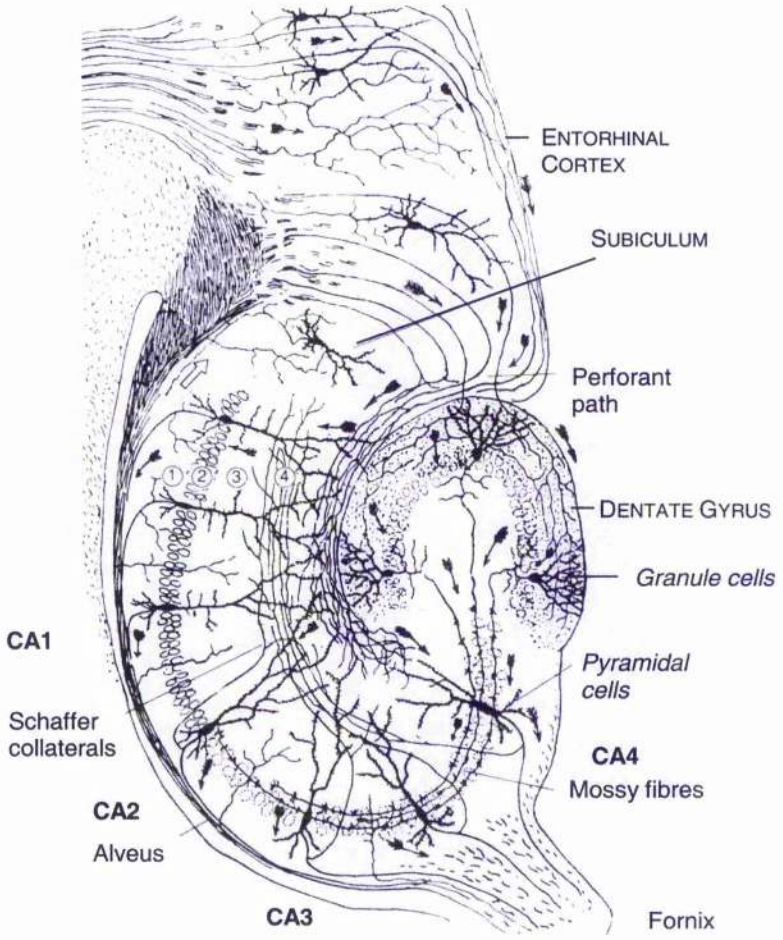


Diagram of the hippocampal formation. Each region of the hippocampal formation is indicated: dentate gyrus, entorhinal cortex, subiculum with the hippocampus proper separated into CA1-CA4 regions. Principal cell types are illustrated and the direction of the arrows represents the flow of information throughout the hippocampal formation. The circled numbers 1-4 indicate the different strata of the hippocampus; 1-*stratum oriens*, 2-*stratum pyramidale*, 3-*stratum radiatum*, 4- *stratum lacunosum-moleculare. Adapted from* Brown, TH and Zador, AM 1990: Hippocampus. In GM Shepherd (ed.), The Synaptic Organization of the Brain, 3rd edn. Oxford University Press. (Original drawing by Ramon y Cajal).

1.2.2 Inter-hippocampal pathways

There are three main inputs to the hippocampus: the entorhinal cortex, the septum and a third input from the contralateral hippocampus. Several smaller inputs from the brainstem, hypothalamus, thalamus and amygdala also exist.

The entorhinal cortex (EC) is the origin of the major afferent input to the hippocampus - the perforant pathway. This pathway can be further segregated into lateral and medial sections dependent on its original location. The axons of the perforant path arise principally in layers II and III of the entorhinal cortex, with minor contributions from layers IV and V. Axons from layers II/IV project to the granule cells of the dentate gyrus and pyramidal cells of the CA3 region, while those from layers III/V project to the pyramidal cells of the CA1 and the subiculum. In addition, the EC provides an interface between the hippocampus and the rest of the neocortex.

The septum provides the main cholinergic input to the hippocampus via the fimbria, the dorsal fornix, the supracallosal striae and the amygdaloid complex. Additionally this input, which originates in the medial septal nucleus and the nucleus of the diagonal band, provides GABAergic fibres (Costa *et al.*, 1983).

Input from the contralateral hippocampus comes from the commissural fibres. Commissural fibres are derived from axons that project from the ipsilateral/ contralateral CA3 region of the hippocampus to the ipsilateral CA1 region. These fibres enter the fimbria then collect in the fornix (Knowles, 1992).

1.2.3 Intra-hippocampal pathways

Within the hippocampal formation resides multiple pathways, the major being the mossy fibre pathway where unmyelinated axons from the granule cells of the dentate gyrus synapse onto CA3 neurones and the Schaeffer collateral pathway where axons from CA3 neurones synapse onto CA1 pyramidal neurones. These synapses are regarded as part of a trisynaptic pathway. The first set of synapses in the pathway being the connection between the perforant path and the dentate gyrus. Even within

individual subfields there are synaptic connections, most notably the recurrent system in the CA3 hippocampal region. This system is formed by axon collaterals of the pyramidal neurones projecting back to their neighbouring cells. Additionally, pyramidal neurones of the CA3 region also receive powerful synaptic inhibition (Brown & Johnston, 1983; Griffith *et al.*, 1986; Miles & Wong, 1984, 1987). There are thought to be at least 15 types of inhibitory interneurones innervating the pyramidal cells of this subfield. Recurrent excitation is thought to be responsible for a predisposition of hippocampal circuits to the generation of epileptiform activity brought about by disinhibition (Johnston & Brown, 1984, 1986; Traub *et al.*, 1987). The axons of the CA1 pyramidal cells synapse with the subiculum and to some degree the entorhinal cortex forming a 'closed loop' of information.

This description of a trisynaptic circuit regulating hippocampal information processing however is somewhat oversimplifying the situation. Instead of one region purely innervating the next region in the pathway, innervation of projected regions also occurs. The only exception is the CA3 neurones, which solely innervate the CA1 region. In contrast, the perforant path projections from the entorhinal cortex not only synapse onto dentate granule neurones but also onto CA3 pyramidal cells and, to a lesser extent, CA1 pyramidal cells (Deadwyler *et al.*, 1987). In addition, there is extensive connectivity along the septo-temporal axis of the hippocampus.

1.2.4 Cell types

The main neurones of the hippocampal CA3 and CA1 subfields are termed pyramidal neurones of which there are approximately 210,000 and 320,000 cells respectively in Wistar rat hippocampi (Boss *et al.*, 1987). Pyramidal cells are mainly located in stratum pyramidale. These multipolar cells project apical dendrites through stratum radiatum to the stratum lacunosum-moleculare and basal dendrites through stratum oriens. The morphology of hippocampal pyramidal cells varies from the CA3 region through to the CA1 region. For example, the cell bodies of CA3 principal cells are smaller in size, whilst the apical dendrites are longer, thinner and have a more regular branching pattern. The principal cells of the dentate gyrus of which there are an estimated 700,000 in the rat hippocampus are called the granule cells

which are in fact smaller in diameter than hippocampal pyramidal neurones (Seress & Pokorny, 1981; Boss *et al.*, 1985, 1987).

Interneurones are inhibitory neurones that appear widely distributed throughout the hippocampus and can be found in stratum oriens, pyramidale and radiatum. Several types of these neurones exist including basket cells, oriens/ alveus interneurones and lacunosum-moleculare interneurones. These types of inhibitory interneurones comprise nearly 10% of the total neuronal population. Interneurones were initially thought to solely innervate pyramidal cells, however, some hippocampal interneurones have been shown to synapse onto other interneurones allowing for disinhibition to occur (Acsady *et al.*, 1996; Gulyas *et al.*, 1996; Freund & Buzsaki, 1996).

1.2.5 Neurotransmitters in the hippocampus

The two main neurotransmitters in the hippocampus are glutamate, the major excitatory neurotransmitter and gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter. Several neuromodulators of the hippocampus are known to exist including noradrenaline and acetylcholine.

The trisynaptic circuit previously described is responsible for excitatory neurotransmission throughout the hippocampal formation. Glutamate, synthesised from glutamine is the major excitatory neurotransmitter in the brain and its role as a neurotransmitter was first described back in 1959 by Van Harreveld and Mendelson at the crustacean neuromuscular junction. The vital role of glutamate in neurotransmission in CA1 hippocampal neurones was later elucidated by Collingridge and colleagues (Collingridge *et al.*, 1983a, b). It is both stored and released in a Ca^{2+} dependent manner. Glutamate acts on two major types of ionotropic receptors - AMPA receptors (quisqualate and kainate) and NMDA receptors. In fact, the hippocampus is one of the most NMDA receptor dense regions of the brain. Additionally glutamate acts on metabotropic glutamate receptors (mGluRs) (Nicoletti *et al.*, 1986; Sugiyama *et al.*, 1987).

AMPA channels are both Na^+ and K^+ permeable (McLennan, 1983). Therefore activating these receptors at normal, negative membrane potentials causes a rapid depolarisation (excitation) of the cell. NMDA receptors also allow the transport of Na^+ through its channel, but are additionally permeable to Ca^{2+} (Pumain *et al.*, 1987; Mayer & Westbrook, 1987). One other important feature of this ion channel is its voltage dependency (Dingledine, 1983; Nowak *et al.*, 1984). At normal resting membrane potentials this ion channel is blocked by a single Mg^{2+} ion which prevents the transport of ions into and out of the cell. When the membrane is depolarised (usually by activation of AMPA receptors), this Mg^{2+} block is released. Hence, both agonist activation and depolarisation are required simultaneously for activation of this receptor. One important feature of glutamate is that, as well as being an essential neurotransmitter in the mammalian brain, it also has the capability to cause excitotoxicity and subsequent neuronal cell death, mainly through its involvement in Ca^{2+} entry into neuronal cells (Choi, 1985; Abele *et al.*, 1990). Metabotropic receptors are G-protein coupled receptors that can be further subdivided into three groups dependent on their sequence, pharmacology and intracellular signalling mechanisms. For example, group I, constituting mGluR 1 and 5, are coupled, via Gq to PLC activation and intracellular calcium signalling whilst group II (mGlu2, 3) are coupled via Gi/o to inhibition of adenylate cyclase as are the group III (mGlu4, 6, 7 and 8) receptors. Although mGluRs do not directly participate in fast synaptic transmission, they still remain important modulators of brain excitability and plasticity (Bashir *et al.*, 1993; Bortolotto *et al.*, 1994; Thuault *et al.*, 2002).

Inhibitory neurotransmission is mediated via GABA receptors of which there are three receptor subtypes - A, B & C. $GABA_A$ and $GABA_C$ are both ionotropic receptors, whereas $GABA_B$ is a metabotropic receptor. GABA's role as a neurotransmitter was initially discovered in the crayfish in 1959 and reported by Van Der Kloot & Robbins. Although undoubtedly an inhibitory neurotransmitter in the mature hippocampus, GABA exerts fast excitatory neurotransmitter effects in early post natal life (Ben-Ari *et al.*, 1994; Leinekugel *et al.*, 1999). It is synthesised by glutamic acid decarboxylase (GAD) from glutamate and metabolised inside the presynaptic terminals of neurones and in glial cells by GABA transaminase. Hence, GAD is commonly used as a marker for GABAergic neurones (Crochemore *et al.*, 2005; Brackman *et al.*, 2004; Storm-Mathisen *et al.*, 1983). The $GABA_A$ ion channel

was cloned and sequenced nearly 20 years previously by Schofield *et al* (1987). Activation of this receptor by agonists such as GABA results in the flow of Cl^- ions through the channel which usually hyperpolarizes the cell membrane thereby reducing neuronal excitability. The increased membrane conductance also provides a shunting form of inhibition. Activation of $GABA_B$ receptors results in the opening of K^+ channels through a G-protein coupled messenger system and, in addition, reduces presynaptic levels of Ca^{2+}. Virtually all hippocampal interneurones are GABAergic (Freund & Buzsaki, 1996) and with $GABA_A$ exerting its effect preferentially on neuronal dendrites. A deficit of GABA inhibition is hypothesised to underlie most forms of epilepsy. For example, there are reported losses of GABAergic neurones in human cortical epileptic foci (Ribak, 1983, 1985) which is also mimicked in the kindled rat model of epilepsy (Callahan *et al.*, 1991; Loscher & Schwark, 1987). Additionally, enhancing GABA levels in the brain is an effective anticonvulsant, as are GABA receptor modulators such as benzodiazepines (Meldrum and Chapman, 1986).

1.2.6 Synapses

Chemical neurotransmission by glutamate and GABA neurotransmitters takes place at synapses in the central nervous system. Synapses tend to fall into one of two categories previously defined by Gray in 1959 – type 1 synapses or type 2 synapses. Pre and post synaptic structures of type 1 synapses are typically asymmetric, whereas type 2 synapses are symmetric in nature. Asymmetric synapses are thought to exist due to the presence of large electron dense structures, which occur postsynaptically e.g. the postsynaptic density (PSD). Asymmetric synapses are commonly associated with excitatory neurotransmission in contrast to symmetric synapses that are commonly associated with inhibitory neurotransmission.

Pyramidal cell dendrites receive virtually all synaptic input from pre synaptic axon terminals (axodendritic) although synaptic connections onto cell bodies, (axosomatic – common for GABAergic inputs), and in rare cases axons, (axoaxonic), are also known to occur. Pre and postsynaptic membranes are separated by a synaptic cleft typically 20-30nm in size.

The pre-synaptic membrane comprises a number of different proteins that have several distinct functions. Pre synaptic terminals contain synaptic vesicles (which themselves contain neurotransmitters such as GABA, glutamate and glycine), mitochondria, endosomes and coated vesicles. In addition, large dense-core vesicles containing peptide neurotransmitter are also found in pre synaptic terminals. Neurotransmitter release from pre synaptic terminals takes place from active zones, and synaptic vesicles are found adjacent to these sites of neurotransmitter release. Synaptic vesicles are associated with proteins such as synapsin which bind both synaptic vesicles and the cytoskeletal protein actin - possibly creating an anchor and controlling mechanism for the release of synaptic vesicles (Bloom *et al.*, 2003; Hilfiker *et al.*, 2005). Other related synaptic vesicle proteins include synaptobrevin (Baumert *et al.*, 1989), Rab3 (Fischer von Mollard *et al.*, 1990), synaptophysin (Wiedenmann & Franke, 1985) and synaptotagmin (Surkova & Grishin, 1991). Ca^{2+} channels are found within the pre synaptic membrane. Entry of calcium into the terminal via voltage dependent calcium channels (VDCCs) resulting from an action potential, in turn modulates neurotransmitter release via exocytosis (Terrian *et al.*, 1990; Takahashi & Momiyama, 1993; Wu & Saggau, 1994).

The major feature of the post-synaptic site is the postsynaptic density, which lines the cytoplasmic face of the postsynaptic membrane. Additionally the postsynaptic membrane contains neurotransmitter receptors such as the NMDA (Petralia *et al.*, 1994a, b), AMPA (Blackstone *et al.*, 1992) and mGlu (Martin *et al.*, 1992) receptors. The PSD contains microtubules and neurofilaments and their associated proteins. Proteolytic enzymes, protein kinases, phosphoproteins and protein phosphatases are all found to reside in or near the PSD. Most post-synaptic terminals located on pyramidal cell dendrites are found on specialised structures called dendritic spines.

1.2.7 Dendritic spines

Perhaps the most common synaptic specialization of dendrites is that which Spanish anatomist Ramon y Cajal referred to as "espinas", since they resembled the thorns on a flower stem. The vast majority of excitatory and a proportion of inhibitory synaptic connections are made onto dendritic spines. In addition to baseline synaptic

transmission, dendritic spines are considered to be the locus for synaptic plasticity. Dendritic spines are small protrusions from the main shaft of dendrites typically less than 1 μm in length. Contained within spines is the postsynaptic density. Compartmentalisation of the PSD as well as various signalling molecules is one of the main functions of the dendritic spine. For example Ca^{2+} accumulation is limited to the individual activated spine (Sabatini & Svoboda, 2000), due to the constricting nature of the spine neck, preventing the escape of Ca^{2+} into the dendritic shaft.

Despite their modest size, some dendritic spines contain a specialized type of smooth endoplasmic reticulum and referred to as "spine apparatus" (Gray, 1982). Ribosomes too are found in the spines, and it is considered that this constitutes a mechanism for the localised targeting of newly synthesized proteins to the postsynaptic density within dendritic spines (Steward & Reeves, 1988).

Spines have been classified by shape as thin, stubby, mushroom- and cup-shaped (Spacek & Hartmann, 1983). However, spine morphology is not static; spines can change in size and shape over time. These structural changes are known to be mediated in part through changes in the actin cytoskeleton (Fifkova & Delay, 1982) as spines are themselves enriched in actin (Kaech *et al.*, 1997). It has long been hypothesised that such changes may be the basis of memory itself (Bailey & Kandel, 1993; Bailey *et al*, 1996; Engert & Bonhoeffer, 1999; Yuste & Bonhoeffer, 2001) as associative memory formation increases the observation of dendritic spines in the hippocampus (Leuner *et al.*, 2003).

Figure 1.2 Dendrites and spines of a hippocampal pyramidal cell

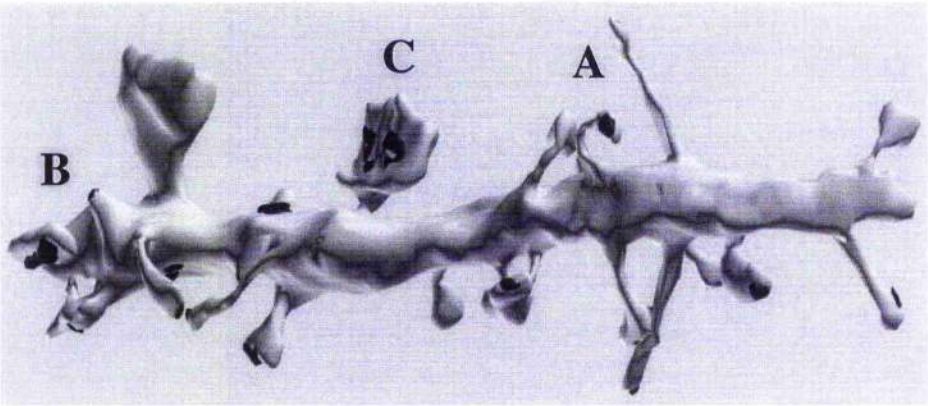


Diagram illustrating a segment of pyramidal cell dendrite from stratum radiatum (CA1) with thin (A), stubby (B), and mushroom-shaped (C) spines. Spine synapses coloured in red, stem (or shaft) synapses are coloured in blue. Taken from Harris *et al.*, (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: Implications for the maturation of synaptic physiology and long-term potentiation. *J.Neurosci.* **12**:2685-2705.

1.2.8 Learning and memory

Different components of the limbic system have been shown to play a critical role in various aspects of emotions, fear and other forms of learning and memory (Geinisman *et al.* 2000; McMillan *et al.* 1987; Cardinal *et al.* 2002).

The initial insights on the role of the hippocampus came from studies of amnesia in human patients following removal of the hippocampus plus neighbouring medial temporal structures (Scoville and Milner, 1957). The most famous example is of patient HM, who, following complete (bilateral) removal of his medial temporal lobes, including hippocampus, for the treatment of epilepsy, suffered from severe amnesia. Hippocampal damage or loss such as that suffered by patient HM results in anterograde amnesia - the loss of the ability to form new memories with retention of old memories. In addition, the memory skills required to learn new tasks or habits can be retained even in the absence of the hippocampus. Several other reports offer extensive evidence implicating the hippocampus and related structures in the formation of episodic memories in humans (Reilly 2001; Aggleton and Brown 1999) and in consolidating information into long-term declarative memory (Mumby *et al.* 1999).

1.3 Neuronal plasticity

Plasticity refers to the changes in the structure and/ or function of neurones, synapses or neural networks and occurs during neuronal development, as a result of neuronal injury (stroke, trauma), experience and during cognitive processes such as learning and memory. Indeed, the main focus of this thesis relates to synaptic plasticity events, more specifically long-term potentiation of synaptic responses in glutamatergic afferents of the CA1 region of the rodent hippocampus. LTP has been observed in several structures of the CNS including the hippocampus, which itself is central to cognitive processes. Moreover, LTP is thought to provide researchers with a molecular correlate to learning and memory processes.

For the purposes of this section however, plasticity refers not only to synaptic plasticity, but also refers to altered neuronal function which occurs as a result of changes in neurone number and or/ molecular events occurring within neuronal cells.

The brain loci in which plastic changes occur depend on the type of experience that occurs. For example, in the nucleus accumbens (NA) and the ventral tegmental area (VTA) - 2 major components of the mesolimbic dopamine pathway involved in the control of motivational behaviour - plasticity of excitatory synapses was observed following the administration of drugs of abuse such as cocaine (Thomas & Malenka, 2003). There is also a large degree of both functional neuronal plasticity in terms of corticomotor output in the cerebral cortex following stroke when subjects were exposed to appropriate rehabilitation methods (Koski *et al.*, 2004).

Plasticity is not only confined to brain regions of the CNS. Plastic changes in the spinal cord are also well documented (Boal & Gillette, 2004). For example, following complete spinal cord injury (SCI) a high level of functional recovery represented by standing and stepping can be achieved in adult mammals. This level of recovery is dependent on the level and types of motor training or experience following injury (Edgerton *et al.*, 2001; Wernig *et al.*, 1995).

Perhaps the greatest example of plasticity comes from neural stem cells from which all 3 types of CNS cells are derived (neurones, astrocytes and oligodendrocytes). Even after the development of these 'stable' forms of cells there remains three putative progenitor cell populations. One population can be found in the subgranular zone of the dentate gyrus of the hippocampus (Kaplan & Hinds, 1977) and the two other populations exist in and near the anterior lateral ventricular wall of the cerebral cortex - the subventricular zone astrocytes and the ventricular ependymal cells (Dvorak & Feit, 1978). Such neurogenesis will be discussed in fuller terms in relation to experience dependent plasticity, where new neurone formation in the dentate gyrus region of the hippocampal formation is well-documented (Kempermann G & Gage FH, 1999).

1.3.1 Developmental neuronal plasticity

Plasticity is optimal during development, although the adult brain retains a capacity for functional and structural organisation. In fact, the high degree of plasticity observed in the juvenile brain not only refers to normal developmental changes, but also to plasticity associated with neuronal injury (Neville & Bavelier, 2002). Developmental plasticity can be defined in three phases. In the initial phase future neurones proliferate; in the second phase they migrate to their place of differentiation which takes place in the third phase where their final size, length of processes and organisation of input and output circuits is determined.

Developmental plasticity in the CNS is also thought to involve N-methyl-D-aspartate (NMDA) receptors which are transiently enhanced early in life (Hestrin, 1992). The developmental regulation of NMDA receptors may in part explain the differences seen post-natally in the degree of long term potentiation, a form of brain plasticity thought to provide researchers with a cellular correlate for learning and memory. In young rats following an early handling regime the amplitude of LTP was greater (Wilson *et al.*, 1986). Furthermore, a study by Shankar *et al* in 1998 investigating the degree of long term potentiation in the CA1 region of the hippocampus from young and aged animals identified that NMDAR-dependent LTP was significantly reduced in magnitude in aged animals.

Previous investigations have compared the process of synaptogenesis in young and old animals in the hippocampal dentate gyrus and have demonstrated that the aged brain has a diminished capacity for re-innervation following massive denervation of a specific area. Further by Anderson *et al* in 1986 focused on the lesion-induced plasticity of the CA1 hippocampal region in young adult and aged rats. While both age groups were able to restore synaptic density to pre-lesion levels, younger animals returned to these levels much quicker than their older counterparts. Other studies have also shown that although brain repair following injury is certainly reduced in older animals it is still apparent, although slower to initiate (Cotman & Hoff, 1983; McWilliams & Lynch, 1983, Anderson *et al.*, 1986) possibly due to the reduced ability generate new neurones.

1.3.2 Neuronal injury

The brain can reorganise extensively following neuronal damage. It is thought that whilst the neuronal plasticity that accompanies neuronal injury provides a functional improvement, it can also result in unwanted effects such as spasticity and epilepsy.

Hippocampal plasticity resulting from neuronal injury was initially described by Nadler and colleagues (Nadler *et al.*, 1974, 1977) who detailed the role of neuronal plasticity in the functional recovery commonly observed following neuronal lesioning. A common example of neuronal lesioning comes from studies of the entorhinal cortex and its associated afferent projections. For example Fagan and Gage (1994) discovered sprouting of cholinergic afferents following lesioning of one of the major inputs to the hippocampal formation, the perforant path. Hoff (1986) identified that lesioning of the entorhinal cortex not only resulted in decreased input to the dentate gyrus region of the hippocampus, but additionally resulted in transneuronal plasticity, affecting input to other regions of the hippocampus along the trisynaptic pathway of the hippocampus. In support of this initial discovery Poduri *et al* in 1995 also identified a much reduced CA3 neuronal population, but in contrast to the study by Hoff did not identify such changes in any other subfield.

It is also apparent that lesions, particularly of the CA3 subfield of the hippocampus can result in the generation of epileptic discharges in the CA1 region of the hippocampus (Franck & Schwartzkroin, 1985). In fact, unilateral lesion of the hippocampus is a commonly used model of increased seizure susceptibility (Feldblum *et al.*, 1990; Bundman *et al.*, 1994). Other models include kainic acid – induced or pilocarpine – induced epilepsy as well as the kindling model of epilepsy which involves periodic electrical stimulation of the brain. Indeed, this kindling model of epilepsy is in fact a plasticity model where repeated physiological stimulation results in an enhanced neuronal response. A report by Represa and colleagues in 1989 demonstrated that kindling of the amygdala and entorhinal cortex resulted in sprouting of the dentate gyrus and CA3 regions respectively. In fact, morphological changes are frequently observed following the induction of abnormal excitatory neurotransmission such as epilepsy. Petit *et al* (1989) reported an observed increase in overall synaptic density almost immediately following synaptic activation

in the CA3 and CA1 regions of the hippocampus, only to ultimately decrease with prolonged hyper-excitability. Kindling has been shown to involve the increased participation of NMDA receptors in excitatory synaptic transmission (Yeh *et al.*, 1989). Finally, the generation of epileptic seizures is known to induce protein synthesis – possibly to mediate all of the previously identified morphological changes (Pico and Gall, 1989).

In addition to generic changes in neuronal numbers, more specific morphological changes are frequently observed following neuronal injury. Bundman and colleagues (1994a, b) identified an increase in the number of somatic spines and spine synapses on dentate gyrus granule cells following periods of seizure activity. Indeed, there appears to be some requirement for dendritic spines in neuroprotection as loss of dendritic spines leads to a cognitive decline in certain neurodegenerative diseases. Segal (1995) proposed that by isolating the synapse from the dendrite the spine is able to protect neurones by biochemically isolating rises in intracellular calcium commonly associated with toxic insults. The structure of dendritic processes in temporal brain regions was examined in human tissue from patients who had suffered brain trauma, tumours or congenital malformations (Castejon *et al.*, 1995). Dendrites appeared swollen and fragmented, and had associated changes in their ultrastructure such as ER, mitochondria and spine apparatus.

Plastic changes down to the molecular level have also been the subject of interest. For example, Kwak and Matus (1988) identified changes in the cellular distribution of both tubulin and microtubule associated protein 2 (MAP2) in the dentate gyrus of rat hippocampus following the unilateral lesion of the entorhinal cortex, whilst Ulas and colleagues (1990) used *in vitro* autoradiography to determine the density of NMDA receptors following the same type of neuronal insult. In the CA1 region of the hippocampus, molecular changes down to the level of gene transcription have been investigated (Pazman *et al.*, 1997). Neurotrophic factors such as BDNF and NT-3 have also been shown to be regulated as a result of neuronal injury (Hicks *et al.*, 1997a).

1.3.3 Experience dependent plasticity

Environmental enrichment, the exposure of animals to complex inanimate stimulation, and increased social stimulation can result in biochemical, electrophysiological and behavioural changes. Such experience and environmental enrichment can also modify the structure (Globus *et al.*, 1973; Greenough and Volkman, 1973) and growth (Kempermann *et al.*, 1997) of mammalian neurones and their synaptic connections such as enlarged synaptic boutons, increased dendritic arborisations and spine numbers as well as an increased neurone to glial ratio (Globus *et al.*, 1973; Volmar & Greenough, 1972; van Praag *et al.*, 2000). Exposure of animals to a more complex environment also results in increased dendritic branching from the occipital, frontolateral and temporal cortices (Volkmar and Greenough, 1972; Greenough *et al.*, 1973) and cerebellum (Flocter and Greenough, 1979; Greenough *et al.*, 1986). Synaptogenesis (density of synapses per unit length of dendrite) increases in the visual cortex (Turner and Greenough, 1985), motor cortex (Turner *et al.*, 2003), corpus striatum (Comery *et al.*, 1995) and hippocampus (Rampon *et al.*, 2000). Such morphological changes may be responsible for the enhancement of medial perforant path synaptic strength observed in rats exposed to an enriched environment (Foster *et al.*, 1996).

Several researchers have demonstrated increased neurogenesis (new neurone formation) following environmental enrichment (Kempermann *et al.*, 1997; Nilsson *et al.*, 1999). In fact, adult hippocampal neurogenesis in mice living in an enriched environment increases as much as 5 fold compared to control animals (Kempermann *et al*, 2002). Neurogenesis in the dentate gyrus region of the hippocampus as is seen following environmental enrichment (EE) can also be achieved as a result of a voluntarily increased amounts of physical activity (van Praag *et al.*, 1999) mimicking the thoughts of Spurzheim in 1815 who suggested that the brain was capable of increasing in size following exercise (Spurzheim, 1815).

Enrichment of this kind not only induces neurogenesis, but also up regulates gliogenesis in the hippocampus (Steiner, 2004). The main difference with gliogenesis as compared to the formation of new neurones is that the expression of new astrocytes is transient in nature whereas newly generated neurones have been shown

to remain in the granule cell layer for nearly a year (Kempermann *et al.*, 2003). Additionally, the functional interaction between neurones and glial cells is also increased.

EE induces a number of changes at the molecular level. Neurogenesis following EE is accompanied by increased hippocampal levels of BDNF, NGF and receptors (Falkenberg *et al.*, 1992; Pham *et al.*, 1999; Ickes *et al.*, 2000; Torasdotter *et al.*, 1998). Furthermore, an increase in AMPA receptor binding in the hippocampus was observed by Foster *et al* in 1996. Interestingly, both neonatal handling and exposure to an enriched environment leads to the increased expression of genes encoding the glucocorticoid receptor suggesting a role of such stress receptors in promoting neuronal protection (Mohammed *et al.*, 1993; Olsson *et al.*, 1994).

Exploration of spatially complex or enriched environments elicits patterns of electrical activity in hippocampal neurones of area CA1 that are similar to patterns of electrical stimulation used to induce long term potentiation in hippocampal slices (O'Keefe, 1979; Otto *et al.*, 1991). Duffy and colleagues in 2001 demonstrated a significant enhancement of LTP in the CA1 region of the hippocampus following 8 weeks of exposure to an enriched environment possibly through a reduced threshold for LTP induction. Increased CREB immunoreactivity in the hippocampus was observed (Williams *et al.*, 2001). CREB is involved in the transcriptional control required not only for the maintenance of LTP but additionally has been identified as modulated in long term memory (Yin *et al.*, 1994; Bourtchuladze *et al.*, 1994). Indeed, environmental enrichment can also enhance learning and memory in rodents (Escorihuela *et al.*, 1995; van Praag *et al.*, 1999; Rampon *et al.*, 2000). Bruel-Jungerman and colleagues reported on enhanced long-term memory following environmental enrichment. This increase in long term memory was associated with the formation of new neurones in the dentate gyrus as demonstrated by the use of an antimitotic agent to reduce the neurogenesis (2005).

Exposure to an enriched environment was also shown to improve recovery following neuronal injury such as cerebral ischemic events (Ohlsson & Johansson, 1995; Johansson & Ohlsson, 1996) and CNS lesioning (van Rijzingen *et al.*, 1997). Additionally an enriched environment has been shown to reduce neuronal apoptosis

in the rat hippocampus by 45%, with additional protection against kainic acid induced seizures and excitotoxic injury (Young *et al.*, 1999). It is possible that the enrichment is not assisting in the recovery of the damaged neurones, but in fact compensates for loss of function associated with neuronal damage. Intriguingly, in Huntingdon's disease (HD) the characteristic motor symptoms and drop in body weight associated with this neurodegenerative disorder are alleviated following environmental enrichment (Spires *et al.*, 2004).

Very little information on plasticity within the CA1 region of the hippocampus has been obtained with respect to environmental enrichment. The study by Duffy and colleagues in 2001 is one of the few. The studies by Olsson and colleagues (1994) and Rasmussen and colleagues (1998) on glucocorticoid and 5HT receptor expression also focus on the CA1 region of the hippocampus. Faherty *et al* (2003) demonstrated that animals raised in an enriched environment had significant morphological alterations (increased dendritic branching, increased neuronal volume, and increased dendritic length) in both the CA1 and dentate gyrus regions of the hippocampus. Remarkably, the changes in the CA1 region were more prominent than the alterations in the dentate gyrus.

1.4 Long term potentiation (LTP)

Plasticity often manifests itself as changes in synaptic connections – 'synaptic plasticity'. In 1949, Donald Hebb proposed that synaptic change underlies behavioural and cognitive plasticity. Among the first neuroscientists to suggest that learning was not the product of new cell growth was the Spanish anatomist Santiago Ramon y Cajal. In 1894 he proposed that memories might be formed by strengthening the connections between existing neurones to improve the effectiveness of their communication. Hebbian theory introduced by Donald Hebb in 1949, echoed Ramón y Cajal's ideas, and further proposed that cells may grow new connections between each other to enhance their ability to communicate:

> *Let us assume that the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability.... When*

an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased. (Hebb, The organization of behavior)

Similarly, memories may be forgotten through the weakening or loss of connections.

Long-term potentiation is the most studied and perhaps the best-understood example of a synapse that obeys Hebb's Postulate. Long-lasting changes of synaptic efficacies in the rabbit hippocampus were found experimentally by Bliss and Lomo (1973). The result obtained was consistent with Hebb's postulate because the joint activation of pre- and postsynaptic units led to a strengthening of synaptic efficacy.

Since its original discovery LTP has been observed in a variety of other neural structures, including the cerebral cortex, cerebellum, amygdala, and many others. The underlying mechanisms of LTP are generally conserved across these different regions, but subtle differences in the precise molecular machinery do appear to exist between sites. Although the hippocampus receives the majority of the research focus concerning LTP, studies carried out by Kandel's group examining habituation, sensitisation and conditioning in the Californian sea slug *Aplysia* are thought to represent simple forms of learning and memory. For the purposes of this thesis however, the following section will focus purely on LTP in the CA1 region of the hippocampus, bearing in mind that mechanisms in the CA1 region are sometimes not conserved amongst other hippocampal regions and brain structures.

LTP at glutamatergic synapses can be induced experimentally by applying patterned high frequency stimulation to the connection between two neurones. This stimulation causes the release of glutamate from the presynaptic terminal, allowing it to bind to neurotransmitter receptors (NMDA, AMPA, mGluR) embedded in the postsynaptic membrane. Though a single presentation of the stimulus is usually not sufficient to induce LTP, repeated presentations cause the postsynaptic cell to be progressively depolarized. In NMDAR-dependent synapses, this progressive depolarization relieves the magnesium blockade of the NMDA receptor (Herron *et al.*, 1986). When

the next stimulus is applied, glutamate binds the NMDA receptor and calcium floods the postsynaptic cell, rapidly increasing the intracellular concentration of calcium. It is this rapid rise in calcium concentration that induces LTP (Harvey & Collingridge, 1992). NMDAR-dependent LTP has been demonstrated in the hippocampus particularly in the Schaffer collaterals and perforant pathway. Activation of the voltage dependent NMDA receptor is required for the induction and maintenance of LTP in the CA1 region of rat hippocampus both *in vivo* and *in vitro* (Harris *et al.*, 1984; Wigstrom and Gustafsson, 1986; Collingridge *et al.*, 1988; Reymann *et al.*, 1989). Bath application of NMDA resulted in the potentiation of the synaptic response (Thibault *et al.*, 1989), an effect blocked by NMDA receptor antagonists. Short term potentiation is also partially mediated through NMDA receptors (Anwyl *et al.*, 1989). NMDA receptors mediate a postsynaptic calcium influx. The calcium permeability of the NMDA receptor is controlled by its voltage dependent Mg^{2+} block. Therefore the calcium permeability of the NMDA receptor and the subsequent calcium influx is postulated as the biochemical trigger for the induction of LTP. There are several types of LTP that do not depend on NMDA receptors. NMDAR-independent LTP has been observed, for example, in the amygdala, where it depends instead on voltage-gated calcium channels (Weisskopf *et al.*, 1999).

NMDA receptor-dependent LTP classically exhibits four main properties making it a suitable candidate for a cellular model of learning and memory. LTP can be rapidly induced (1) by either strong tetanic stimulation of a single pathway, or cooperatively (2) via the weaker stimulation of many. Similar to this cooperative nature is the observation that when weak stimulation of a single pathway is insufficient for LTP induction, simultaneous strong stimulation of another pathway will induce LTP at both pathways through association (3).Once induced, LTP at the activated synapse is not propagated to adjacent, unactivated synapses as LTP is input specific (4).

LTP is often divided into three phases, an early, protein synthesis-independent phase (E-LTP) that lasts between one and three hours which results from covalent modifications of pre-existing protein molecules, an intermediate translational-dependent phase occurring two to three hours following induction and a protein synthesis-dependent phase (L-LTP) that lasts from about 4hours and can be maintained for weeks *in vivo* (Morris, 2004).

1.4.1 LTP induction

Post-synaptically, the early phase of LTP is expressed primarily through the enhancement of AMPA receptor sensitivity. The first demonstration of their role in LTP was detailed by Davies *et al* in 1989. They showed that the sensitivity of CA1 neurones in hippocampal slices to AMPA ligands increased following LTP induction. This was confirmed some years later by Fedorov and colleagues (1997). Responses to AMPA exhibited a fast increase (2-3 min) following LTP induction. A similar study by Kessler and colleagues however did not detect any such changes in AMPA agonist binding following LTP (Kessler *et al.*, 1991) and neither did Rammes *et al* (1999). Nayak *et al.*, 1998 reported that synthesis of AMPA receptor subunits is increased three hours after LTP induction an effect blocked by inhibitors of PKA and transcription. AMPA receptor antagonists were only effective in blocking LTP at high concentrations (Kapus *et al.*, 2000). Perhaps the most convincing evidence for a role of AMPA receptors in LTP is that in adult GluR-A knock-out mice, associative LTP was absent in the CA1 region of hippocampal slices (Zamanillo *et al.*, 1999). Increased responsiveness or expression of AMPA receptors is thought to occur via protein kinases, increasing single-channel conductance of existing functional AMPA-Rs or by recruiting new high-conductance-state AMPA-Rs (Derkach *et al.*, 1999). A vital role for AMPA receptor trafficking in LTP has been extensively discussed in a review by Malinow and Malenka in 2002 and again by Rob Malinow in 2003.

The concept of 'silent synapses' was introduced by Liao and colleagues in 1995. Silent synapses are thought to be previously non-functional synapses at normal resting potential which subsequently become active following the induction of LTP. This was confirmed by John Isaac's group around the same time. They were also able to demonstrate an NMDA receptor dependency for this mechanism (Isaac *et al.*, 1995). Insertion of AMPA receptors into these previously silent, post synaptic membranes is one possibility. Increased insertion of AMPARs appears to be crucial for CA1 LTP via activation of the AMPAR-associated PI3K (Man *et al.*, 2003) although Grosshans *et al* (2002) reported that this was specific for neonatal tissue and LTP in the adult rat does not alter membrane association of AMPARs.

Metabotropic glutamate receptors (mGluRs) are the third group of glutamate receptors associated with LTP. In 1991, Aronica *et al* demonstrated an enhanced sensitivity of these receptors following LTP induction in the CA1 region of rat hippocampus. Agonists of these receptors, such as trans-ACPD, were also shown to enhance both short term potentiation and long term potentiation in the hippocampal slice (McGuiness *et al.*, 1991; Collins *et al.*, 1995). Activation of mGluRs by 1S, 3R-ACPD can induce the same stable form of LTP, but without the STP component (Bortolotto and Collingridge, 1993). Musgrave *et al* in 1993 demonstrate a requirement for activation of mGluRs as well as NMDA receptors in the induction process of LTP.

MGluRs appear to play a role in the coincidental increase in AMPA receptor sensitivity seen with the induction of LTP as using MCPG an antagonist of these receptors prevents LTP and the associated increase in AMPA receptor sensitivity (Sergueeva *et al.*, 1993). This finding, however, was contradicted by Manzoni *et al* in 1994 who found no effect of this antagonist on LTP. This is possibly an effect of drug concentration as was reported by Bortolotto *et al* (1995). MCPG was also shown by Little *et al* in 1995 to block both NMDA receptor dependent and independent forms of LTP.

Bortolotto and Collingridge (1995) went on to investigate the induction of ACPD-induced LTP and its relationship with AMPA receptors. LTP induced by ACPD was blocked by the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX). ACPD-induced LTP was associated with a parallel increase in the sensitivity of CA1 neurones to AMPA. In addition, ACPD has been shown to facilitate the induction of LTP by weak, not strong theta burst stimulation (Cohen & Abraham, 1996). Surprisingly, Breakwell and colleagues have previously shown a block of LTP by mGluR *agonists* such as ACPD and the generic antagonist MCPG (1998).

mGluR mutant mice in which there is a deletion in the gene encoding metabotropic glutamate receptor 1 show substantially reduced LTP and impaired context specific associative learning (Aiba *et al.*, 1994). Mutant mice expressing no mGluR5 had a decreased response of mutant CA1 neurones to low concentrations of ACPD

suggesting that mGluR5 may be the primary high affinity ACPD receptor in these neurones. LTP in mGluR5 mutants was significantly reduced in the NMDA receptor (NMDAR)-dependent pathways such as the CA1 region and dentate gyrus of the hippocampus, whereas LTP remained intact in the mossy fibre synapses on the CA3 region, an NMDAR-independent pathway. These mutant mice were also impaired in the acquisition and use of spatial information in both the Morris water maze and contextual information in the fear-conditioning test (Jia *et al.*, 1998; Lu *et al.*, 1997). Use of a specific mGluR5 antagonist LY344545 however, did not result in the prevention of LTP in the CA1 region of rat hippocampal slices (Doherty *et al.*, 2000).

Group I metabotropic glutamate receptors (mGluR), which are linked to heterotrimeric G-proteins of the G (q) family (G (q) and G (11)), have been reported to facilitate hippocampal LTP. However, Kleppisch *et al* in 2001 demonstrate that G (alpha) q-deficient mice show normal long-term potentiation in the hippocampal CA1 region. On the other hand, weak theta burst stimulation was insufficient to induce LTP in Galpha q and Galpha11 mutant mice. In wild-type mice, LTP induced by weak theta burst stimulation (TBS) was abolished by inhibiting group I mGluRs (Miura *et al.*, 2002).

Calcium is a fundamental requirement for the induction of LTP. The main source for this calcium is influx through the NMDA receptor ionophore, although other potential sources include voltage-dependent calcium channels and release from intracellular stores. Dunwiddie and Lynch first investigated the role of calcium in long term potentiation in 1979. Using *in vitro* hippocampal slices, a lowering of calcium prevented LTP but basic synaptic transmission and other forms of potentiation such as paired pulse facilitation and short term (post tetanic) potentiation remained unaffected, suggesting differing calcium dependencies. Similarly, Mulkeen and colleagues in 1988 demonstrated that increasing the external calcium concentration resulted in an increase in LTP in the CA1 region of the rat hippocampus. In 1981 after the initial study by Dunwiddie and Lynch, Baimbridge and Miller demonstrated that tetanic stimulation of the Schaffer-commissural input produces a significant increase in the uptake and retention of calcium. Further evidence for the involvement of Ca^{2+} in LTP of CA1 pyramidal neurones was

provided by Kuhnt *et al.*, (1985). They induced LTP by tetanic stimulation of the stratum radiatum of hippocampal slices from guinea pigs and visualised Ca^{2+} binding sites by electron microscopy. Electron-dense, Ca^{2+}-containing deposits were found in low numbers in unstimulated slices on pre- and postsynaptic sites. In the stratum radiatum of tetanized slices the overall number of deposits as well as the number of deposits in dendrites was shown to be increased. The same group went on to demonstrate an increase in presynaptic calcium levels following LTP (Agoston & Kuhnt, 1986) although a study by Muller and Lynch in 1989 proposed that such changes in presynaptic Ca^{2+} were not in fact responsible for LTP, later confirmed by Wu and Saggau in 1994. An increase in extracellular calcium was shown to cause a long-lasting (8hrs) enhancement of the synaptic response in CA1 pyramidal neurones. It was thought that both tetanus- and Ca^{2+}-induced LTP share some common mechanisms, as additional tetanization after Ca^{2+} induced LTP was not followed by an additional potentiation of the response (Reymann *et al.*, 1986; Melchers *et al.*, 1987).

Calcium binding proteins and calcium ion channel agonists and antagonists have been shown to affect LTP in area CA1 of the hippocampus. Anti-S-100, a calcium binding protein prevents LTP induction (Lewis & Teyler, 1986). An elegant study by Rob Malenka's group in 1988 used three different experimental approaches to investigate the role of Ca^{2+} in LTP one of which was to use nitr-5, a photolabile calcium chelator, which releases calcium in response to ultraviolet light. Photolysis of nitr-5 on CA1 pyramidal cells resulted in a large enhancement of synaptic transmission. Pharmacological blockade of intraneuronal calcium release by dantrium (which inhibits calcium release from the sarcoplasmic reticulum and diminishes the rise in the intraneuronal calcium ion concentrations elicited by NMDA receptor activation) completely blocked the induction of LTP in the CA1 region of the rat hippocampal slice. Malenka also demonstrated a temporal requirement for Ca^{2+} in the induction of LTP. Tetanus-induced rises in postsynaptic, intracellular Ca^{2+} lasting at most 2-2.5 s was found to be sufficient to generate LTP. Smaller increases or shorter duration rises in intracellular Ca^{2+} are thought to result in a short term potentiation of the synaptic response. Harvey & Collingridge demonstrated that thapsigargin, which depletes most intracellular Ca^{2+} pools by blocking ATP-dependent Ca^{2+} uptake into intracellular compartments, blocked the

induction but not the expression of LTP and had no effect on synaptic transmission or on responses mediated by N-methyl-D-aspartate (NMDA) receptor activation (1992).

Dendritic spines, the sites of synaptic contact, may function to isolate and amplify synaptically mediated increases in postsynaptic calcium. In 1990, Regehr and Tank illustrated postsynaptic calcium accumulation using microfluorometric analysis in individual CA1 pyramidal cells during stimulus trains that induce LTP. They report a transient increase in Ca^{2+} localised near activated synapses, an increase blocked by D-AP5. Sabatini *et al* (2002) demonstrated a definitive requirement for local rises in dendritic spine Ca^{2+} for the induction of synaptic plasticity. They identified that spines are specialized structures with low endogenous Ca^{2+} buffer capacity that allows large and extremely rapid Ca^{2+} changes and that, under physiological conditions, Ca^{2+} diffusion across the spine neck is negligible, and the spine head functions as a separate compartment on long time scales, allowing localized Ca^{2+} build-up during trains of synaptic stimuli.

Kullman *et al* (1992) studied the role of Ca^{2+} entry via voltage-sensitive Ca^{2+} channels in long-term potentiation (LTP) in the CA1 region of the hippocampus. A depolarization-induced potentiation of the synaptic response was augmented in raised extracellular Ca^{2+} and was blocked by intracellular BAPTA, a Ca^{2+} chelator, or by nifedipine, a Ca^{2+} channel antagonist, indicating that the effect was mediated by Ca^{2+} entry via voltage-sensitive Ca^{2+} channels. NMDAR and voltage-dependent calcium channel (VDCC) antagonists applied independently have been shown to reduce the magnitude of long-term potentiation (LTP) in area CA1 of the hippocampal slice preparation. When used in combination, the antagonists completely block the induction of LTP *in vivo* (Morgan & Teyler, 1999). Freir & Herron in 2003 further investigated the role of L-type VDCCs in LTP *in vivo*. Two structurally different, L-type VDCC blockers, verapamil and diltiazem depressed the induction of LTP in a dose-dependent manner. The effect of the VDCC agonist BAY K8644 on LTP in the stratum radiatum of the CA1 region of the hippocampus *in vitro* was investigated by Mulkeen *et al.* in 1987. They discovered that BAY K8644 enhanced LTP but, interestingly, the Ca^{2+} channel antagonist verapamil did not alter LTP, but did inhibit the action of BAY K8644 on LTP indicating a mode of action of BAY K8644

through voltage dependent calcium channels, and that these calcium channels do not directly mediate LTP.

1.4.2 Protein kinases

Substantial evidence for protein regulation in the form of post translational modification by protein kinases throughout the entire process of long term potentiation has been provided by numerous groups, indicating their crucial role in the plasticity process. Some of the major contributors and their involvement in LTP are detailed below.

The binding of calcium to calmodulin directly activates CaMKII. Calcium/calmodulin-dependent protein kinase II (CaMKII) is necessary for LTP induction, is persistently activated by stimuli that elicit LTP, and can, by itself, enhance the efficacy of synaptic transmission. Evidence comes from studies showing that inhibitors of CaMKII prevent the induction of LTP (Malinow *et al.*, 1989) and high frequency stimulation of CA1 afferents in rat hippocampus results in LTP induction with an associated NMDA receptor dependent long-lasting increase in Ca^{2+}-independent and total activities of CaMKII (Miyamoto & Fukunaga, 1996).

A requirement for CaMKII in hippocampal LTP and spatial learning has been demonstrated by several groups using a variety of methods including the use of transgenic mouse models. Silva *et al* (1992a, b) demonstrated that mice with no alpha-CaMKII exhibited specific learning impairments and in a separate study in the same year they report a deficit in hippocampal LTP. Additionally, in 1998, Giese *et al* introduced a point mutation into the alpha CaMKII gene that blocked the autophosphorylation of threonine at position 286 (Thr286) of this kinase without affecting its CaM-dependent activity. The mutant mice had no NMDA receptor-dependent LTP in the hippocampal CA1 area and showed no spatial learning in the Morris water maze. Such auto-phosphorylation converts CaMKII from the Ca^{2+}-dependent form to the Ca^{2+}-independent form. The autophosphorylation of CaMKII was investigated by Pettit *et al* in 1994 who demonstrated that a constitutively active form of CaMKII introduced into neurones of hippocampal slices with a recombinant vaccinia virus potentiated the synaptic response occluded synaptically induced LTP.

CaMKII activity is thought continuously auto-phosphorylated on Threonine 286 following the initial LTP induction stimulus. Autophosphorylation and thus constitutive activity suggests a role for CaMKII not only in LTP induction but also in the maintenance phase, most likely through its actions on AMPA receptors (Malinow *et al.*, 1988; Derkach *et al.*, 1999). Transgenic mice that express a mutated Ca^{2+}-independent form of CaMKII have been shown to have normal long-term potentiation (LTP) at 100 Hz, but they lack LTP in response to stimulation at 5-10 Hz and are impaired on spatial memory tasks (Rotenberg *et al.*, 1996). This suggests that role for the autophosphorylated form of CaMKII may differ from the initial phosphorylated form expressed soon after LTP induction.

In addition to CaMKII, CaMKIV, which is phosphorylated by CaMK kinase and localized predominantly in neuronal nuclei, and its functional role as a cyclic AMP-responsive element-binding protein (CREB) kinase in high frequency stimulation (HFS)-induced LTP in the rat hippocampal CA1 region was investigated by Kasahara *et al* in 2001. CaMKIV was found to be transiently activated in neuronal nuclei after HFS. Phosphorylation of CREB, which is a CaMKIV substrate, and expression of c-Fos protein, which is regulated by CREB, increased during LTP. This increase was inhibited mainly by CaMK inhibitors and suggests that CaMKIV functions as a CREB kinase and controls CREB-regulated gene expression during HFS-induced LTP in the rat hippocampal CA1 region (Kasahara *et al.*, 2001).

Protein kinase A (PKA) or cAMP dependent protein kinase serves a role similar to that of CaMKII, but PKA's effects are broader. LTP-inducing tetanic stimulation in rat hippocampal area CA1 elicits an NMDA receptor dependent increase in the levels of cAMP (Chetkovich *et al.*, 1991) subsequently determined to act via CaMKII activation of adenylyl cyclase (Chetkovich & Sweatt, 1993). cAMP was shown to increase the amplitude of the population spike in the CA1 region of the hippocampus (Slack & Pockett, 1991) and the slope of the EPSP, subsequently occluding HFS-induced LTP (Slack & Walsh, 1995). The role of the cAMP pathway in LTP was studied in the CA1 region of hippocampus by Blitzer *et al* (1995). Widely spaced trains of high frequency stimulation generated cAMP postsynaptically via NMDA receptors and calmodulin. This is consistent with the Ca^{2+}/calmodulin-mediated stimulation of postsynaptic adenylyl cyclase. The early phase of LTP produced by

the same pattern of high frequency stimulation was dependent on postsynaptic cAMP. However, synaptic transmission was not increased by postsynaptic application of cAMP as was seen by Slack & Walsh.

PKA's activity is enhanced during LTP induction by these previously observed elevated levels of cAMP as a result of calcium's activation of adenylate cyclase. Matthies and Reymann first demonstrated a requirement for cAMP dependent protein kinase in LTP in 1993 using PKA inhibitors. In the same year Frey and colleagues also showed that inhibitors of PKA blocked L-LTP, and also that analogs of cAMP induced a potentiation that blocked naturally induced L-LTP, a protein synthesis dependent effect suggesting a role in the late phase of LTP for PKA. Several other studies using PKA inhibitors confirm a central role for PKA in LTP. Bath application of the PKA inhibitor H89 suppresses the early LTP induced by a single tetanus (Otmakhov *et al.*, 2000). In 2002 Otmakhov & Lisman showed that the postsynaptic application of a PKA inhibitor reversed the LTP in CA1 pyramidal cells. The decrease of LTP produced by these inhibitors was evident immediately after induction. These results indicate that PKA is important in the *early* phase of LTP (contrary to the work carried out by Frey and colleagues in 1993) and that its locus of action is postsynaptic. Indeed, postsynaptic application of PKA inhibitors in the presence of high frequency stimulation to CA1 pyramidal cells prevented a persistent form of LTP (Duffy & Nguyen, 2003). In a very elegant study by Matsushita *et al* in 2001 a PKA inhibitory peptide was delivered exclusively into the nuclear compartment of neurones in the brain which subsequently blocked both cAMP response element binding (CREB) protein phosphorylation and long lasting long term potentiation (LTP) induction, but not early LTP. It has also been proposed that cAMP response element (CRE)-mediated gene expression is stimulated by signals that induce long-term potentiation (LTP). In 1996, Impey *et al* found that CRE-mediated gene expression was greatly increased in the late stages of LTP. In addition, inhibitors of PKA blocked L-LTP and the associated increases in CRE-mediated gene expression.

Confusion certainly surrounds the timing of PKA activity as in yet another set of experiments PKA was found to be transiently activated immediately after LTP induction in area CA1 of the hippocampus but was not found in the early or late

stages of LTP suggesting, contrary to other reports, that PKA activity was required for the induction and not the maintenance of LTP. This was an NMDA receptor dependent effect (Roberson & Sweatt, 1996).

Once again transgenic mice have been used to study the role of PKA in LTP. Mice that express an inhibitory form of the regulatory subunit of PKA, R (AB), not only had reduced hippocampal PKA activity but also had a significantly decreased level of L-LTP in area CA1, with no effect on basal synaptic transmission or the early phase of LTP. Moreover, the L-LTP deficit was paralleled by behavioural deficits in spatial memory and in long-term memory for contextual fear conditioning (Abel *et al.*, 1997). R (AB) transgenic mice were also used by Woo and colleagues to once again show significantly reduced LTP in area CA1 of hippocampal slices as compared with slices from wild-type mice (Woo *et al.*, 2000). This impairment in LTP was found to be rescued by the acute application of two inhibitors of protein phosphatase-1 and protein phosphatase-2A (Woo *et al.*, 2002).

As with studies looking at other mediators of synapse specific plasticity such as LTP, the pattern of activity used to induce plasticity determines the outcome of the studies in a large and varied way and should be a major consideration when designing experiments relating to LTP. An in-depth study into the patterning of synaptic stimulation was carried out in PKA mutant mice by Woo and colleagues in 2003. They identified that PKA-dependent LTP is selectively recruited by temporally spaced, multiburst synaptic stimulation. Impairment of LTP expression was evident when LTP was induced by applying repeated, temporally spaced stimulation (4 1-s bursts of 100-Hz applied once every 5 min). In contrast, LTP induced by applying just 60 pulses in a theta-burst pattern was normal in slices from R (AB) mice as compared with slices from wild-type mice. Also, Rp-cAMPS a generic inhibitor of PKA blocked the expression of LTP induced by both stimulation patterns suggesting a subunit specific role of PKA in different forms of LTP.

In the mammalian brain protein kinase C (PKC) is present in high concentrations and has been shown to phosphorylate several substrate phospho-proteins which may be involved in the generation of LTP. In fact, much of the information pertaining to PKC involvement in LTP comes from its kinase activity on other proteins such as B-

50 (also known as GAP-43 and protein F1). For example, shortly after the induction of LTP in the intact hippocampal formation there was an increase in the phosphorylation of Protein F1 *in vivo*. It had already been demonstrated that PKC had the ability to phosphorylate Protein F1 *in vitro*. Not only was this found immediately after LTP induction, but it was subsequently found 1hr and even 3days following LTP (Lovinger *et al.*, 1986). Furthermore, the amplitude of synaptic plasticity was also found to be directly related to the extent of protein F1 phosphorylation. Protein F1 in the hippocampus was also altered in its phosphorylation after an experience involving memory of a spatial environment. Interestingly, PKC activity was shown to translocate to neural membranes following the induction of LTP (Akers *et al.*, 1986). Following up on this study Akers and Routtenberg (1987) showed that following treatment of hippocampal synaptosomes with Ca^{2+}, protein kinase C activity in the synaptic membrane and protein F1 *in vitro* phosphorylation were elevated in a dose-dependent manner. Successful NMDA receptor blockade by AP-5 prevented the phosphorylation of protein F1 induced by LTP (Linden *et al.*, 1988). PK C is itself selectively activated by phorbol esters. Phorbol esters were shown by Malenka *et al* in 1986 to potentiate excitatory synaptic transmission in the hippocampus and in doing so to further occlude synaptically induced LTP. Finally, Hu *et al* in 1987 studied the effect of intracellular injection of PKC in CA1 pyramidal neurones in hippocampal slices. Injection of this active enzyme elicited a long-lasting enhancement of synaptic transmission, similar to LTP.

Lovinger and colleagues (1987) then went on to show the effect certain PKC inhibitors (mellitin, polymyxin B, H-7) on LTP. When inhibitors were given 15 min before LTP, initial potentiation was unaffected, yet responses decayed to baseline levels by 60 min after the onset of potentiation. PKC inhibitor treatment 10 min after LTP onset induced decay of responses to pre-LTP baseline levels within 50 min of ejection. Inhibitor applied 60 min after LTP onset induced substantial decay but not to baseline levels. Potentiation was unaffected by inhibitor treatment 4 h after the induction of LTP. Measurement of PKC subcellular distribution revealed that inhibitor significantly reduced the proportion of PKC associated with the membrane. In slight contrast to the Lovinger study Reymann *et al* in 1988 went on to show that another inhibitor of PKC, polymyxin B, prevents the late phase (>6h) of electrically induced LTP of synaptic transmission to CA1 neurones. In agreement with the

findings of Lovinger and colleagues they also found a decrease in the potentiation of the synaptic response in the earlier phases (30mins, 2hrs). Reymann *et al* (1988) additionally investigated the effect of a different PKC inhibitor K-252b. Post-tetanic potentiation and initial LTP was found to be expressed normally, thereafter potentiation declined back to baseline within 60 min. Although the majority of studies demonstrated that PKC is involved in the maintenance phase of LTP Wang & Feng in 1992 report that using three PKC inhibitors: polymyxin B (PMB), PKC-(19-31), and H7 30 min before the HFS, PMB in adequate dosage or a combination of PMB and PKC-(19-31), each at a low dosage, could block the development of LTP completely including its initial induction phase. With the delivery beginning at the time of the tetanus, PKC-(19-31) or H7 slowly caused the established LTP to decline to the baseline; this decline was greatly accelerated when PMB and PKC-(19-31) or PMB and H7 were given together. PMB and PKC-(19-31) given together 75-90 min or even 3 h after the tetanus caused a decline of the maintained LTP similar to the decline observed when both inhibitors were given at the time of the tetanus. These results show that postsynaptic PKC is essentially involved in both the initial induction and the subsequent maintenance of LTP, contrary to other studies.

However, conflicting evidence for the role of PKC in LTP has been reported. More specifically, Muller *et al* in 1990 used a phorbol ester induced synaptic potentiation and the PKC inhibitor H-7. As opposed to affecting LTP directly, Muller and colleagues propose a role for PKC in modulating the NMDA receptor component of the synaptic potentiation. This was again demonstrated in 1993 by Lopez-Molina and colleagues who studied the effects of calphostin C, an antagonist of the regulatory subunit of protein kinase C, on the induction and expression of long-term potentiation (LTP) and on responses mediated by activation of NMDA receptors in rat hippocampal slices. No effect of calphostin C was observed on pre-established LTP. In contrast, the drug was found to prevent LTP induction. Calphostin C did not alter synaptic transmission mediated by activation of AMPA receptors but did considerably interfere with the function of NMDA receptors.

Behavioural studies have shown that the hippocampal levels of PKC correlates with spatial learning ability i.e. reduced performance in the Morris water maze was found in mice with reduced levels of hippocampal PKC (Wehner *et al.*, 1990). Transgenic

mice (PKC gamma mutants) showed a greatly reduced LTP of synaptic transmission in the hippocampus. Additionally these PKC gamma-mutant mice show a slight deficit in hippocampal dependent learning tasks (Abeliovich *et al.*, 1993a, b).

In 1991 O'Dell *et al* showed that blockade of another set of protein kinases - the tyrosine kinases - selectively blocked the induction of LTP when applied in the bath or injected into the postsynaptic cell. By contrast, the inhibitors had no effect on the established LTP or on normal synaptic transmission. In 1992, the same group utilised mice with mutations in four nonreceptor tyrosine kinase genes, fyn, src, yes, and abl all expressed in the hippocampus. Mutations in src, yes, and abl did not affect the induction or maintenance of LTP. However, in fyn mutants, LTP was found to be impaired. In addition, fyn mutants showed impaired spatial learning (Grant *et al.*, 1992). In 1999, Lu and colleagues revealed enhanced synaptic transmission and a reduced threshold for LTP induction in fyn-transgenic mice, somewhat contrary to the study by O'Dell in 1991.

Although the expression of a mutant form of the tyrosine kinase src was ineffective at preventing or impairing LTP in the study by O'Dell, a subsequent investigation by Lu *et al* in 1998 demonstrated that induction of LTP in CA1 pyramidal cells was prevented by blocking the tyrosine kinase Src, and that Src activity was increased by the induction of LTP. Moreover, direct activation of Src in the postsynaptic neurone enhanced excitatory synaptic responses, thereby occluding synaptically induced LTP. It is thought that one possible involvement of Src in LTP is via its up-regulation of the function of NMDA receptors. EPQ (pY) EEIPIA, an activator of the Src family of PTKs, produced a gradual and robust increase in the synaptic response which occluded LTP suggesting that Src family kinases play an important role in LTP induction at Schaffer collateral-CA1 synapses (Huang & Hsu, 1999). Finally, Henderson *et al* (2001) showed that mice lacking the entire EphB2 tyrosine kinase receptor had reduced LTP at hippocampal CA1 synapses. EphB2 was also up regulated in stimulated hippocampal pyramidal neurones *in vitro* and *in vivo*.

1.4.3 Late phase LTP

Late LTP can be experimentally induced by a series of trains of tetanic stimulation. Unlike early LTP, late LTP requires gene transcription and protein synthesis, making it an attractive candidate for the molecular analog of long-term memory.

One of the first demonstrations of a requirement for protein synthesis in the late phase of LTP was by Stanton and Sarvey in 1984. Using emetine, cyclohexamide, or puromycin decreased the frequency of occurrence of LTP in field CA1 elicited by repetitive stimulation of the Schaffer collaterals. In contrast, Stanton and Sarvey found that the protein synthesis inhibitor anisomycin was unable to block LTP. Anismoycin was used however by Frey and colleagues in 1988. A 3-h treatment with anisomycin immediately following tetanization resulted in a gradual decline in the potentiation of the excitatory postsynaptic potential (EPSP) and population spike (PS). These conflicting results on the use of anisomycin were investigated further by Mochida *et al* in 2001 who applied optical recording techniques to rat hippocampal slices using a voltage-sensitive oxonol dye. In the presence of anisomycin, the potentiation of the EPSP lasted about 2-3 h, followed by a gradual decline in the signal amplitude. The reversible inhibitor cycloheximide was also used by Deadwyler and colleagues in 1987. Stimulation-induced LTP of the population spike was reduced in slices incubated in cyclohexamide for 15 min and completely blocked if incubated for 30 min.

A requirement for *de novo* protein synthesis has been shown for the maintenance of a late phase of LTP by Frey *et al* in 1989. In this study apical dendrites were separated from their cell bodies. In doing so they were only able to induce a relatively short form of potentiation (3 hrs) compared to that in intact slices (8-10 hrs). Therefore, transcription of new mRNA in the soma of neurones is not thought to be required for up to 3 hrs following LTP induction, but is required for longer periods of potentiation. A similar study by Nayak *et al* (1998) show that late-phase LTP can be obtained in rat hippocampal CA1 region in which the cell bodies of *presynaptic* Schaffer collateral/commissural fibres were removed. Thus, transcription of presynaptic genes is not necessary to support maintenance of late-phase LTP.

Transcription and translation products have both been investigated in terms of their relationship with LTP in the CA1 region of the hippocampus. In 1996 Frey *et al* used the RNA synthesis inhibitor actinomycin D to demonstrate that the maintenance of hippocampal LTP in the CA1 region *in vitro* was influenced by actinomycin D around the 4 hr time point. *In vivo* actinomycin D prevented a late stage of LTP in the dentate gyrus.

As well as the involvement of *de novo* protein synthesis in the establishment of a late phase of LTP there is also a wealth of evidence for local protein synthesis in dendrites and dendritic spines of hippocampal CA1 pyramidal neurones. The first such evidence came from a study by Wenzel *et al* in 1993. This report showed that just sixty min after LTP-inducing, high-frequency stimulation, the total number of ribosomes, polysomes, and the number of membrane-bound ribosomes increased significantly. More recently, Bradshaw *et al* (2003) showed that when the protein synthesis inhibitor emetine is applied locally to the apical dendritic field of CA1 pyramidal cells in the murine hippocampus, late LTP is impaired at apical but not at basal dendrites, and conversely when emetine is applied locally to basal dendrites, late LTP is impaired only at basal dendrites. Thus, local protein synthesis modulates the expression of tetanically induced late LTP at Schaffer-commissural synapses on CA1 pyramidal cells.

The most recent study investigating protein expression was by Behnisch *et al* in 2004. Here we characterized the dynamics of protein synthesis associated with the induction of L-LTP using a transgenic mouse model in which a cAMP responsive element (CRE)-regulated promoter drives production of an enhanced yellow fluorescent protein (eYFP). They found that eYFP fluorescence increased after less than 30 min following LTP induction. Application of transcription and translation suppressors and the NMDA receptor antagonist D-AP5 inhibited the L-LTP and prevented the rise in eYFP levels. The gene expression and protein synthesis that mediate the long-term changes of LTP generally take place in the cell body, but LTP is synapse-specific; LTP induced at one synapse does not propagate to adjacent inactive synapses. Therefore, the cell is posed with the difficult problem of synthesizing plasticity-related products in the nucleus and cell body, but ensuring they only reach synapses that have received LTP-inducing stimuli.

1.4.4 Synaptic tagging

The synthesis of a "synaptic tag" at a given synapse after LTP-inducing stimuli was proposed by Frey and Morris in 1997. It is thought that by tagging an activated synapse this may serve to capture plasticity-related proteins shipped cell-wide from the cell body. Given two widely separated synapses, an LTP-inducing stimulus at one synapse initiates several signalling cascades that promote gene expression in the cell nucleus. At the activated synapse, a short-lived synaptic tag is produced, possibly resulting from the products of local protein synthesis. The products of gene expression are then transported globally throughout the cell, but are only captured by synapses that express the synaptic tag. Thus only the activated synapse is potentiated, demonstrating LTP's input-specificity (Frey & Morris, 1997).

Synaptic tagging may also explain LTP's associative nature. For example, if one synapse undergoes LTP induction by high frequency stimulation and a separate synapse is only weakly stimulated by a stimulus not normally suited to LTP induction, both synapses will undergo LTP. It is thought that whilst weak stimuli are unable to induce gene expression in the cell body, they do in fact promote local protein synthesis and hence the synthesis of a synaptic tag. Alternatively, covalent modifications to existing protein molecules could also represent such a tag. Simultaneous high frequency stimulation of a separate pathway induces gene transcription products which are shipped throughout the cell. With both synapses expressing the synaptic tag, both are then able to capture the protein products leading to the induction of LTP in both pathways (Frey & Morris, 1998).

Finally, the synaptic tag hypothesis may also explain LTP's cooperative nature. While weak stimulation of a single pathway is insufficient to induce LTP, the simultaneous weak stimulation of two pathways is sufficient. As noted previously, weak stimulation promotes local protein synthesis/ modification and hence the generation of a synaptic tag. However, weak stimulation is insufficient to trigger late LTP due to its inability to mediate the induction of gene expression products. It was demonstrated by Barco *et al* in 2002 that simultaneous weak stimulation of two pathways results in the convergence of kinases that sufficiently activate the transcription factor CREB-1 thereby inducing the synthesis of plasticity-related

proteins, which are distributed throughout the cell as described previously. The identification of the synaptic tag or tags is an important feature of neuroscience research to date. It is possible that kinases such as CaMKII and AMPA receptor insertion into the postsynaptic membrane of activated synapses could play a role as this marker for synaptic activity, however, no definitive answer has yet been provided.

1.4.5 Presynaptic vs. postsynaptic

Much controversy surrounds the cellular location responsible for the induction and/ or maintenance of LTP. Whilst the majority of evidence suggests a post synaptic location, for example Kauer *et al* in 1988 demonstrated a postsynaptic requirement by an increased sensitivity of the post synaptic neurone to synaptically released glutamate; some evidence has been provided suggesting a presynaptic location. In 1990 Rob Malinow and Joe Tsien used whole-cell voltage clamp techniques to study synaptic transmission in hippocampal slices and showed that transmitter release was significantly enhanced. Meshul and Hopkins (1990) provided further evidence for a presynaptic location of LTP. They identified that induction of LTP was associated with a significant decrease (30%) in the percentage of synaptic vesicles containing calcium deposits. These changes persisted for at least one hour following the induction of LTP and were not observed in slices that received high-frequency stimulation in the presence of the NMDA receptor antagonist, APV, which blocked LTP. These data suggest that LTP may be accompanied by long-term changes in intraterminal calcium homeostasis and the number of synaptic vesicles. These effects may be related to the reported increase in transmitter release following the induction of LTP.

The roles of a selection of presynaptic proteins in LTP have also been investigated. Takahashi *et al* in 1999 used mice deficient in the SNARE complex associated protein complexin-II. These mice had expected levels of synaptic transmission and paired-pulse facilitation. However, long-term potentiation (LTP) in both CA1 and CA3 regions was impaired, suggesting that certain presynaptic proteins do have a role in the establishment of hippocampal LTP.

Quantal analysis remains the backbone of evidence for presynaptic loci in the induction and maintenance of LTP. Sokolov *et al* (2002) investigated quantal release in the early and later phases of hippocampal CA1 LTP. At these time points they discovered an increase in the quantal content, but not in the quantal size, indicating possible presynaptic modulation. The coefficient of variation of the response amplitude and the number of failures decreased during both post-tetanic periods suggesting both temporal phases are maintained by presynaptic changes, including increases in release probabilities and the number of effective release sites. Liao *et al* (1992) showed that a larger proportion of LTP was attributable to a change in presynaptic mechanisms. They found an increase both in the number of quanta released and in quantal amplitude, consistent with combined pre- and postsynaptic modifications. This was confirmed by a study by Kullman and Nicoll in the same year.

Zakharenko and colleagues (2001) were able to visualise an enhancement in presynaptic activity resulting from short and long term potentiation of the synaptic response in area CA1 of the hippocampus using a fluorescent marker FM 1-43. Following activation of presynaptic terminals, FM 1-43 is taken up into synaptic vesicles in an activity dependent manner as a result of endocytosis. Subsequent activity resulting in exocytosis leads to a release of dye from presynaptic terminals, and therefore correlates with presynaptic activity. Such an increase in activity was observed following the induction of LTP.

Leahy and colleagues (1993) showed an increase in the presynaptic protein kinase C-dependent growth-associated protein-43 phosphorylation, 10 min after high-frequency, afferent stimulation. This was in agreement with a previous study by Gianotti and colleagues in 1992.

Another way to determine if LTP expression includes a presynaptic locus is to investigate a presynaptically mediated form of potentiation such as paired-pulse facilitation (PPF), which is an increase in a second population excitatory postsynaptic potential when it is elicited shortly after a first. Previous authors have found no change in PPF in association with LTP (Asztely *et al.*, 1996). This hypothesis was re-examined by Schulz *et al* in 1994. In the CA1 region of the rat

hippocampal slice preparation PPF both increased and decreased significantly in association with LTP such that the average PPF of all slices did not change, in agreement with previous studies (Schulz *et al.*, 1994).

One hypothesis of presynaptic facilitation is that enhanced CaMKII activity during early LTP gives rise to CaMKII autophosphorylation and constitutive activation. Persistent CaMKII activity then stimulates NO synthase, leading to the enhanced production of the putative retrograde messenger, NO. As NO is a diffusible gas, it freely crosses the synaptic cleft to the presynaptic cell leading to a chain of molecular events that facilitate the presynaptic response to subsequent stimuli (Schuman & Madison, 1991).

1.4.6 LTP and behavioural memory

Richard Morris provided early evidence that LTP was indeed related to long term memory. He tested the spatial memory of rats treated with the NMDA receptor blocker D-AP5. Treated and control groups were then subjected to the Morris water maze, in which rats were placed into a pool of cloudy water and tested on how quickly they could locate a platform hidden beneath the water's surface. It was discovered that D-AP5 significantly impaired the ability of rats to locate the hidden platform. Moreover LTP was easily induced in control animals, but could not be induced in the brains of APV-treated rats. Similarly, Susumu Tonegawa in 1996 demonstrated that the CA1 region of the hippocampus is crucial to the formation of spatial memories. Place cells located in the CA1 region are responsible for creating "place fields" – markers of the rat's environment. Tonegawa found that by impairing the NMDA receptor, specifically by genetically removing the NMDAR1 subunit in the CA1 region, the place fields generated were substantially less specific than those of controls and as a result these rats performed very poorly on spatial tasks compared to controls providing more support to the notion that LTP is the underlying mechanism of spatial learning. Enhanced NMDA receptor activity in the hippocampus has also been shown to produce enhanced LTP and an overall improvement in spatial learning. Philpot and colleagues (2001) used mice with over-expressed levels of the NMDA receptor subunit NR2B. These mice displayed more

pronounced long-term potentiation and were better performers in spatial learning tasks.

In addition to a relationship between the NMDA receptor expression and LTP, mGluR1 mutants show impaired context specific associative learning as well as reduced LTP (Aiba *et al.*, 1994). MGluR5 mutant mice were also impaired in the acquisition and use of spatial information in both the Morris water maze and contextual information in the fear-conditioning test (Lu *et al.*, 1997). Kinases also play a coincidental role in LTP and cognition. For example, alpha CaMKII mutant mice displayed no NMDA-receptor dependent LTP in the hippocampal CA1 area and in addition showed no spatial learning in the Morris water maze. The PKA mutant mice previously described (R (AB) mice), as well as having a significant L-LTP deficit had paralleled behavioural deficits in spatial memory and in long term memory for contextual fear conditioning (Abel *et al.*, 1997). Behavioural studies have also shown that the levels of PKC in the hippocampus correlates with spatial learning ability i.e. reduced performance in the water maze was found in mice with reduced levels of hippocampal PKC (Wehner *et al.*, 1990). As well as showing a deficit in synaptic potentiation, PKC gamma-mutant mice show a slight deficit in hippocampal dependent learning tasks (Abeliovich *et al.*, 1993 a, b). Hence, the correlation between LTP and long term memory is well documented, allowing this model to be used to study the underlying molecular mechanisms of cognitive processes such as learning and memory.

1.5 Alzheimer's Disease (AD)

One of the most prominent progressive neurological disorders characterised by severe cognitive impairment is AD - a progressive neurological disorder characterised by severe cognitive impairment. Post mortem studies of brains from AD patients revealed the presence of senile plaques that contain the 40-42-amino acid peptide beta amyloid (Abeta). Abeta is generated by the sequential cleavage of amyloid precursor protein (APP) by a beta secretase protein (BACE1) (Hussain *et al.*, 1999; Vassar *et al.*, 1999) and also the gamma secretase complex, which contains presenilin-1 and nicastrin (De Strooper *et al.*, 1998; Xia *et al.*, 1998a; Yu *et al.*, 2000).

Subsets of AD patients harbour genetic mutations to the APP protein, resulting in an inherited familial form of AD. One of the most extensively studied mutations is the so-called Swedish mutation, which results in early-onset AD (Mullan *et al.*, 1992). This mutant form of APP contains two mutations located close to the beta secretase cleavage site and are postulated to increase cleavage of APP by BACE1, resulting in an over expression of Abeta (Citron *et al.*, 1992).

Exogenously applied Abeta causes deficits in long-term potentiation, a cellular model of learning and memory, *in vitro* and *in vivo* (Freir *et al.*, 2001; Itoh *et al.*, 1999; Sun & Alkon, 2002; Walsh *et al.*, 2002; Wang *et al.*, 2002).

Several transgenic mice models over-expressing the various mutant forms of human APP have been generated and have been shown to exhibit severe amyloid plaque pathology, deficits in behaviour and memory tasks (Chapman *et al.*, 1999; Games *et al.*, 1995; Hsiao *et al.*, 1996), and altered neuronal morphology (Richardson *et al.*, 2003). The effects of mutant APP and thus Abeta over expression remain controversial. A number of studies have described changes in LTP in the CA1 and dentate gyrus in transgenic APP mice both *in vivo* and *in vitro* (Chapman *et al.*, 1999; Jolas *et al.*, 2002; Larson *et al.*, 1999). In contrast, a number of other groups have observed no changes in LTP, despite finding significant deficits in basal hippocampal glutamatergic synaptic transmission in various strains of mice with AD related hallmarks (Fitzjohn *et al.*, 2001; Hsia *et al.*, 1999; Roder *et al.*, 2003).

The TAS10 mice line over expresses human APP, under the control of Thy-1, a neuronal promoter that is restricted to brain regions affected by AD, i.e. cortex and hippocampus (Richardson *et al.*, 2003). By 12months of age these mice show marked deficits in spatial learning and memory in the Morris water maze in addition to the appearance of Abeta containing plaques.

An increased understanding of the molecular aspects of LTP may be used to develop strategies to reverse cognitive decline associated with AD.

1.6 Genomics and proteomics in the neurosciences

The discovery of the numerous molecules in the previous text not only points towards a greater amount of research in this field but also highlights the advent of new technology highly suited to neuroscience research. From the genomics era of microarrays and the human genome comes the post genomic era of proteomics, protein chips and analyses of protein-protein interactions.

Large scale brain genomics has been termed neurogenomics (Boguski & Jones, 2004). Of the 30,000 genes expressed in the human genome, over half are also found to be expressed in the brain, hence its complex nature (Sandberg *et al.*, 2000). Neurogenomics encompasses not only genomic research involving gene expression and function using expressed sequence tags, cDNA libraries and transgenic mice, but also involves proteomics which itself includes protein separation and bioinformatics identification. Given the large number of techniques made available to researchers it is perhaps not surprising that neurogenomics has been and will continue to be responsible for many advances in neuroscience.

1.6.1 Genomics

Expressed sequence tags were first introduced to neuroscience in 1992 by Adams *et al.* Using this technique they identified nearly 2,500 genes expressed in the human brain. ESTs have subsequently been widely utilised in microarray experiments. Microarray technology requires the complimentary hybridisation between nucleic acids. Pieces of known DNA sequences are immobilised onto a microarray 'chip' and fluorescently labelled targets from the sample in question hybridise onto their complimentary targets. The degree of hybridisation and subsequent fluorescence is thought to be directly proportional to the target abundance. Investigation into changes in gene expression can be carried out using multiple arrays and each array can subsequently be standardised and compared. Microarrays now have the capability to identify many thousands of genes in a single sample and a wealth of information concerning gene expression in the hippocampus has been gleaned over the past five or so years. Such genomic studies have been utilised to highlight the region specificity of particular genes across different regions of the brain (Zirlinger

et al., 2001). Identification of such subfield-specific neuronal markers provides researchers with tools for investigating the neuronal function of particular areas in the brain. Very recently micorarray analysis was used to identify gene expression profiles in different sub regions within a structure (Datson *et al.*, 2004). Over 700 genes were found to differ in expression levels between the CA3 and dentate gyrus region of the hippocampus, highlighting that even within a single structure, neuronal populations can differ considerably. A more detailed study of this nature was in fact carried out 3 years previous by Zhao *et al* (2001). They not only investigated differences in gene profile between the CA3 and dentate gyrus regions but additionally investigated the CA1 region of the hippocampus and, rather strangely, the spinal cord. Again, they discovered different expression profiles between the different subfields. This is perhaps not surprising given that research in the past has proven that physiological properties between hippocampal subfields can vary considerably. Amazingly even within a single subfield differences in gene expression can be found. Not only between pyramidal cells and interneurones of the hippocampus but also between pyramidal neurones indicating that even single cell populations are not homogeneous (Kamme *et al.*, 2003). In addition, cells express a combination of genes that is in part dictated by its unique environmental requirements and experiences.

Microarrays have proved invaluable to investigate gene expression profiles resulting from hippocampal plasticity. This includes studies concerning hippocampal development, injury, disease and the resultant gene expression profiles following periods of learning and enrichment. A substantial report was published by Mody and colleagues in 2001 concerning gene expression profiles in the developing mouse hippocampus. They investigated 11,000 genes using microarray chip technology and gene cluster analysis and subsequently identified a number of groups such as neuronal proliferation, differentiation and synapse formation which were prominent in their expression profiles in the developing mouse hippocampus. In addition this group submitted their data to a gene profile database. Microarray studies have also been used to provide a link between different forms of neuronal plasticity. For example, Elliot and colleagues in 2003 demonstrated that genes found to be regulated in the dentate gyrus during development were similarly regulated during epilepsy for

example genes found to be involved in cell morphology and axon outgrowth or cellular proliferation and fate determination.

Injury induced gene expression has been studied by Jin *et al* (2001) (cerebral ischaemia), French *et al* (2001), (seizure-induced), Matzilevich *et al* (2002) (lateral cortical impact), Long *et al.*, (2003), Li *et al* (2004) (traumatic brain injury) and Gilbert *et al* (2003) (hypoxia-ischaemia). Perhaps the most extensive use of microarrays with respect to neuronal injury relates to the investigation of gene expression profiles in neurodegenerative diseases such as Alzheimer's. Several groups have carried out research of this nature. Some utilised human hippocampus containing neurofibrillary tangle-lesions (Hata *et al.*, 2001) and compared to regions of the brain free from such lesions, whilst others investigated specific regions of the hippocampus – the CA1 region for example (Colangelo *et al.*, 2002). Additionally, mouse models of AD have been used by some researchers to examine changes in gene profiles. Reddy *et al* (2004) used a transgenic model over expressing APP, whereas Mirnics *et al* (2005) used mice with a conditional knock out of presenilin-1.

The importance of identifying the molecular components of the hippocampus responsible for its major role in learning and memory has led to the emergence of several microarray studies in this field. Long-term memory consolidation in rabbits was studies by Cavallaro *et al.*, in 2001. The same group later investigated gene expression following spatial learning in rats (Cavallaro *et al.*, 2002). A similar study was performed by Leil *et al* in 2003. A further interesting study by the same group in 2002 used two different strains of mice that differ in their performance in the Morris water maze. 27 genes differed between the poor and the accomplished performers, most of which were of unknown function, but all of which provided a basis for genes involved in behavioural aspects of learning and memory. Learning and memory studies could in theory encompass enrichment studies such as environmental enrichment and exercise based tasks. Indeed Tong and colleagues in 2001 investigated the effects of exercise on gene expression in the rat. In this study they identified a number of genes related to neuronal plasticity such as BDNF, NGF and MAP kinase phosphatases. A similar study by Molteni *et al* in 2002 demonstrated similar expression profiles with changes in the abundance of synapsin I, CaMKII, CREB and the NMDA receptor again suggesting a role for exercise induced

hippocampal plasticity. Aging studies have also benefited from microarray analysis (Blalock *et al.* 2003). LTP has been investigated in terms of gene expression, but only in the CA3 region *in vivo* (Thompson *et al.*, 2003). As yet, no such complimentary studies in the CA1 region have been reported.

1.6.2 Proteomics

Proteomics is the next step in the 'omic' chain. Proteomics is defined as *'the qualitative and quantitative comparison of proteomes under different conditions to further unravel biological processes'* in the **Expert Protein Analysis System** (ExPASy) website. Proteomics has the advantage over microarray and other genomic type studies in that certain issues pertaining to post translational modifications, alternative splicing are taken into account. But rather than being thought of as superior to array studies, proteomics is complimentary to such genomic approaches. Proteomics has been used to profile certain organism's proteomes or to examine expressional differences. Advances in neuroproteomics have been limited to the last 5 years. Very recently a project to map the entire proteome of the human brain was initiated. 2D gel electrophoresis combined with mass spectrometry is currently the mainstay of proteomics although recent technological advances in this field include the use of protein microarrays and with the advent of larger protein arrays such as the surface-enhanced laser desorption/ ionization time of flight (SELDI-TOF)-based protein chip system. This system has been widely used already and if proved successful may provide researchers with a high throughput protein screen.

Mass spectrometry can not only identify proteins, but can determine structural information, for example post translational modifications. In addition it has the capability to quantify relative and absolute amounts of protein. More commonly mass spectrometric techniques such as peptide mass fingerprinting and tandem mass MS sequencing are used in conjunction with 2D gel electrophoresis which alone has the means to resolve thousands of proteins from a single organism's proteome. 2D gel electrophoresis is conventionally thought of as problematic for several reasons. Firstly, poor reproducibility of 2D gels results in questionable results particularly for studies where expression differences are being investigated. Secondly, in the past it has been difficult to quantify changes in protein abundance not only due to

reproducibility problems, but also due to the analysis of such changes. Software was not made available to researchers to allow comparison across different gels, and therefore analysis proved extremely subjective. Finally, and perhaps most importantly, several protein species are not represented on 2D gels due to their hydrophobicity, for example membrane proteins. Understandably, their absence from neuroscience based studies poses a problem for research groups. The recent emergence of technological advances is helping combat these issues. Several 'gel-free' alternatives have been developed. These entail the use of nanoscale liquid chromatography interfaced with mass spectrometry (LC-MS and LC-MS/ MS). Such approaches have been used to analyse complex proteomes of yeast and malaria. A second, gel free, approach uses isotope-coded affinity tags (ICAT). Using ICAT two populations are tagged with isotope coded affinity tags which differ in mass by 8Da. The two ICAT protein populations are pooled, digested and MS/ MS is subsequently used to quantify and identify pairs of individual peptides (Griffin *et al.*, 2001).

1.6.3 Difference gel electrophoresis (DiGE)

Gel based advances have also been made. Gels for both 1st and 2nd dimensions were routinely cast in the laboratory further contributing to reproducibility issues. Now though, it is possible to buy both 1st and 2nd dimension high quality pre cast gels, circumventing this problem. One of the most useful developments appears to be the introduction of Difference Gel Electrophoresis (DiGE). DiGE was first described back in 1997 by Unlu and colleagues (although further validated by von Eggeling *et al* and Tonge *et al* in 2001) and has several distinct advantages over conventional 2D gel electrophoretic methods. This 2D gel based technique utilises the availability of 3 spectrally resolvable CyDyes to allow three different samples to be run and resolved on a single gel. Two broad classes of CyDyes are available. One minimally labels the proteins in a sample, the other involves saturation labelling of the proteins. The principal behind the two kinds is essentially the same. Minimal dyes have an NHS-ester reactive group, and covalently attach to the epsilon amino group of lysine of proteins via an amide linkage. These dyes label approximately 1-2% of the available proteins and then only on a single lysine per protein i.e. "minimal labelling". The lysine amino acid in proteins carries a single positive charge at neutral or acidic pH. CyDye DIGE Fluor minimal dyes also carry a single positive charge which ensuring

that the pI of the labelled protein does not significantly alter compared to the same unlabelled protein. Saturation labelling is typically used for scarce samples although at present only two dyes (Cy3 and Cy5) are available (Shaw *et al.*, 2003; Greengauz-Roberts *et al.*, 2005). CyDye DIGE Fluor dyes from the Scarce Sample Labelling Kit have a maleimide reactive group, and are designed to covalently bond to the thiol group of cysteine residues of proteins via a thioether linkage. To achieve maximal labelling of cysteine residues the protocol uses a high fluor to protein labelling ratio. This type of labelling method labels all available cysteines on each protein under the conditions used resulting in the majority of cysteine groups in a protein from a sample, being labelled. For this reason, this method has been called *saturation* labelling. The CyDye DIGE Fluor saturation dyes have a net neutral charge and therefore do not alter the charge on a protein after labelling. They are also matched for molecular weight ensuring that the same protein from a standard and test sample labelled with the different dyes will overlay when separated on a single gel.

In addition, DiGE utilises an internal standard, which allows accurate cross gel analysis, not previously obtainable. The internal standard is in fact a pool of a proportion of all the biological replicates in a DiGE experiment. This way, all proteins from all samples are represented within the standard. By using the internal standard a group of gels containing the same standard can be normalised to account for gel to gel variation, and individual proteins can subsequently be standardised to their own reference in the internal standard. The first use of the internal standard was reported by Alban *et al* in 2003, previous groups did not incorporate its use into their analysis. Finally, software was developed for specific use with DiGE, namely the DeCyder software package, which allows accurate and quantifiable analysis of changes in protein expression with minimal user intervention. The software was first used by Gharbi *et al* and Zhou *et al* in 2002. The software automatically co-detects protein spots on a single gel taking into account background subtraction. This co-detection algorithm further reduces gel-gel variation as no intra gel matching is required. It can then match these spots across a number of separate gels and subsequently normalises across images, and standardises each protein spot. Statistical analysis (T-test, ANOVA) is then carried out on individual protein spots. Differences <10% can be detected by >95% confidence.

The use of DiGE to date has been minimal presumably due to costing issues. From the initial study in 1997 (Unlu *et al*, 1997) only around 50 reports have been published incorporating protein profiling (Gade *et al.*, 2003) and protein expression studies (Zhou *et al.*, 2002). The majority of these studies investigated protein profiles and expression differences in cancer (Zhou *et al.*, 2002; Somiari *et al.*, 2003, 2005; Friedman *et al.*, 2004; Wang *et al.*, 2004; Seike *et al.*, 2004). Only a few studies have used DiGE in combination with the investigation of protein expression in the CNS. Swatton *et al.*, 2004 investigated post mortem human brain for protein expression profiles and Prabakaran *et al* in the same year utilised DiGE (amongst several other genomic techniques) to identify a number of mitochondrial associated proteins involved in schizophrenia. Van den Bergh *et al* (2003a) identified age-related protein expression differences for the primary visual cortex of kitten and adult cat using DiGE. As this is a relatively new technology, it is perhaps expected that such studies will be more commonplace in the future when research laboratories will gain access to the equipment necessary for carrying out such experiments. As a result many of the published reports detailing protein expression in the CNS come from standard 2D gel electrophoresis studies.

1.6.4 Neuronal proteomics

Lubec *et al.*, 1999 investigated protein expression in samples obtained from downs syndrome and AD brains. In fact, a substantial amount of work has been undertaken to examine protein expression related to Downs' syndrome (Cheon *et al.*, 2001; Bajo *et al.*, 2002). AD has also been the focus of much proteomic investigation. Tilleman *et al.*, (2002a, b) have used several mouse models of AD to examine protein expression related to certain pathologies associated with AD. Expression of oxidised proteins in ApoE knock out mice was investigated by Choi *et al* in 2004. Expression of a double mutation of APP and PS1 results in the change in expression of several hippocampal proteins some of which have previous reference to learning and memory (Vercauteren *et al.*, 2004).

Glycosylation, nitrosylation and oxidation of proteins associated with AD has been undertaken by proteomic investigation (Kanninen *et al.*, 2004; Castegna *et al.*, 2003).

It has also been possible to examine tissue from human AD sufferers. Much of this work has led to the discovery of many proteins potentially involved in the neurodegeneration associated with AD (Castegna *et al.*, 2002a, b). Aging research has also benefited from 2D gel electrophoresis and mass spectrometric analysis. The first such study by Tsugita *et al* in 2000 isolated brain proteins from the cerebellum, striatum, cerebral cortex, hippocampus and cervical spinal cord from mice ranging from 2months old to 24 months old. 2D gel electrophoresis allowed the identification of several proteins differentially expressed over the brain regions, and a proportion of proteins (17) to vary with age. A small number of differentially regulated proteins were identified when comparing young and old brain tissue (Chen *et al.*, 2003). Poon *et al* in 2004 investigated protein oxidation in the senescence-accelerated mouse (SAM) model. SAMP8 is known to exhibit age-dependent learning and memory deficits and differential protein expression was evident in aged mice brains compared with young mice.

Much work has focussed on neuropsychiatric disorders such as schizophrenia and bipolar disorder in the frontal cortex, cerebral spinal fluid and hippocampus (Johnston-Wilson *et al.*, 2000; Jiang *et al.*, 2003; Edgar *et al.*, 1999a, 2000).

Neuronal injury has also been investigated in terms of associated protein expression. Krapfenbauer and colleagues investigated changes in low abundance brain proteins following kainic acid administration in rats using 2D gel electrophoresis and Maldi-tof mass spectrometric analysis on cytosolic and mitochondrial proteins whose expression level differed (Krapfenbauer *et al.*, 2001a, b). Traumatic brain injury has also been subject to proteomic investigation (Jenkins *et al.*, 2002; Siman *et al.*, 2004). Surprisingly, not many studies have focussed on protein regulation in epilepsy. Of those that have Eun *et al* (2004) studied differential protein expression in human cerebral cortex from epileptic subjects. A second study by Junker in 2005 used a global proteomics approach to identify protein expression in cerebral tissue previously subjected to kindling.

Protein profiling of different brain regions has been carried out by some researchers. For example, Beranova-Giorgianni and colleagues in 2002 identified 30 prominent

proteins from the mouse cerebellum by 2D gel electrophoresis, Maldi-tof and LC/MS/MS mass spectrometry. The proteome of brain endothelium has even been reported by Hasseloff *et al* in 2003. Edgar and colleagues have also produced a proteomic map of the human hippocampus (1999b). Developmental protein expression in the cerebellum of foetal brain was addressed by Fountoulakis and colleagues in 2002.

Furthermore, analysis of more defined neuronal structures has been carried out. Jimenez *et al* in 2002 identified newly synthesised proteins in the synaptosomes of squid optic lobes by the incorporation of ^{35}S methionine into synaptosomes, autoradiography of newly synthesised proteins and mass spectrometric analysis of these proteins. The squid optic lobe preparation was used as it is thought to provide a purer sample free from contamination from other cell types. They discovered that approximately 80 proteins were newly synthesised.

Synaptic plasticity per se has not been addressed using proteomic technology. A study by Paulsen and colleagues in 2003 and later in 2004 investigated protein expression following blockade of the NMDA receptor. They used MK-801 administration to the cerebral cortex and thalamus as a model of schizophrenia. The NMDA receptor however, has received a degree of attention particularly by Holger Husi and colleagues. They isolated NMDAR complexes (NRC) from rat and mouse brain and identified 77 proteins within this complex. They later went on to isolate and characterise NMDAR--PSD-95 complexes (Husi & Grant, 2001). The proteomic profile of the post synaptic density however has received a substantial amount of attention. Li *et al* (2004a) used 2D gel electrophoresis in combination with mass spectrometry as well as ICAT to determine the protein profile of the rat PSD. The identified proteins included neurotransmitter receptors, proteins for maintenance and modulation of synaptic structure, sorting and trafficking of membrane proteins, generation of anaerobic energy, scaffolding and signalling, local protein synthesis, and correct protein folding and breakdown of synaptic proteins. Yoshimura and colleagues in 2004 analysed the protein constituents of the postsynaptic density (PSD) using a liquid chromatography (LC)-based protein identification system with electrospray ionization (ESI) tandem mass spectrometry (MS/MS). Using this

technique they were able to identify 492 proteins belonging to signalling transduction pathways, including receptors, ion channel proteins, protein kinases and phosphatases, G-protein and related proteins, scaffold proteins, and adaptor proteins. Structural proteins, including membrane proteins involved in cell adhesion and cell-cell interaction, proteins involved in endocytosis, motor proteins, and cytoskeletal proteins were also identified. Jordan *et al* in 2004 described the identification of 452 proteins isolated from biochemically purified PSD fractions of rat and mouse brains using nanoflow HPLC coupled to electrospray tandem mass spectrometry. In addition to identifying some previously described PSD components they were also able to detect proteins involved in cell signalling as well as regulators of ubiquitination, RNA trafficking, and protein translation.

Similarly, proteins of the rat forebrain synaptic plasma membranes were isolated and this particular study involved several approaches using one-dimensional (1D) (SDS)-PAGE, strong cation-exchange (SCX) chromatography and capillary reversed-phase high performance liquid chromatography (HPLC) techniques (Stevens *et al.*, 2003). In addition to these gel and HPLC separation stages, complementary information was obtained by using both matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectrometry. Many of the functional protein classes identified by Li *et al* (receptors, ion channels) were also identified here by Stevens *et al.*, 2003. Synaptosomal preparations have been used to look more specifically at the identification of voltage dependent anion channels in guinea pig hypoxic and normal brains (Liberatori *et al.*, 2004).

Proteomic approaches also include the use of immunoprecipitation in combination with 1D SDS PAGE and mass spectrometric analysis. The literature on this topic is by far too numerous to include in this introduction, but suffice to say it is a way to identify proteins that for interactions and complexes.

A few studies have investigated protein interactions with the NMDA receptor using proteomic based technology. Murata *et al* (2005) identified a novel proline rich synaptic protein in the synaptic membrane of rat forebrain. This protein PRR7 was

found to co-localise with PSD-95 and co-immunoprecipitate with NR1 and NR2B subunits of the NMDA receptor.

One of the major problems with 2D gel electrophoresis especially in the brain is the shear complexity of the sample. One way to overcome such problems is to prefractionate the sample to provide a purer protein sample. Several methods of prefractionation are available to the researcher, both before and after protein sample collection. For example, Mouledous and colleagues (2003) utilised laser-capture microdissection to investigated protein expression changes in specific cell types. This technique has been utilised by several other groups. Successful subfractionation of brain homogenates in cytosolic, mitochondrial and microsomal by ultracentrifugation was performed by Krapfenbauer and colleagues in 2003. Subcellular proteomics such as this is an alternative to more global approaches taken by other groups, but may well be seen as a trade off. This approach was also adopted to investigate the protein components of clathrin coated vesicles from rat brain (Ritter *et al.*, 2004).

The second major problem is the absence of membrane proteins from such analysis. This has very recently been tackled by Nielsen *et al* (2005) by using an approach that depletes non-membrane molecules from entire tissue samples and membranous samples enriched and digested prior to LC MS/MS. This proved a successful approach with 862 proteins being identified, more than 60% of them membrane proteins.

The hippocampus has itself been the focus of a large degree of proteomic investigation. Hypoxia in the CA1 and CA3 regions of the hippocampus was investigated in terms of protein expression differences in these regions (Klein *et al.*, 2003). They found a single hypothetical protein showing strong up regulation in the CA1 region compared to the CA3 subfield with possible valuable implications in intermittent hypoxia and sleep apnoea. Hippocampal protein profiling was carried out to possibly link protein derangement to impaired cognitive function (Kirchner *et al.*, 2004) in mice lacking nNOS. These mice had impaired ability in the Morris water maze, and subsequently displayed differential protein expression for a number of proteins including glycolytic enzymes, glucose regulated proteins and mRNA binding proteins such as HNRNPH.

Proteomic research in the neurosciences, as well as taking the form of global 2D gel analysis as has been described, is also useful for the analysis of new protein synthesis. Changes in protein spot abundance indicate a number of possibilities. As well as indicating changes in protein composition (i.e. PTMs, protein cleavage) it is also indicative of increased or decreased protein biosynthesis. It is impossible, however, to determine the exact sequence of events occurring without further analysis. One was to determine whether or not protein biosynthesis is occurring is to incorporate radiolabelled methionine into hippocampal slices, and to use autoradiography to isolate those proteins which have been newly synthesised. This is possible due to the incorporation of radiolabelled methionine into the new protein molecule. This approach has successfully been adopted by a few groups. Fazeli and colleagues (1993) investigated *de novo* protein synthesis following the induction of long term potentiation in the dentate gyrus *in vivo*. As is consistent with the current train of thought concerning protein synthesis in long term potentiation they identified a single spot changing in expression 1hr after LTP induction, but by 3hrs, thought to be a protein synthesis dependent phase, 11 spots were identified. This approach not only identified increases in expression but also identified decreases. Similar studies were carried out in 1986 and then again in 1991. Protein synthesis in the neocortex, caudate-putamen, and the hippocampus following transient forebrain ischemia was investigated in rats at 3 and 18 hrs. Newly synthesized (^{35}S-labeled) and constitutive (unlabeled) proteins were analyzed by two-dimensional gel electrophoresis and fluorography. In all three brain regions, specific proteins underwent preferential synthesis while others showed decreased synthesis (Kiessling *et al.*, 1986). Changes in the pattern of newly synthesized polypeptides were investigated in the *in vitro* hippocampal slice following exposure to repetitive stimulation with and without the induction of long-term potentiation. Using ^{35}S methionine labelling of polypeptides and two-dimensional gel electrophoresis, we detected an NMDA receptor dependent increase in the rate of synthesis of two polypeptides in CA1 in response to repetitive stimulation of the Schaffer collaterals (Uenishi *et al.*, 1991).

One of the earliest proteomic CNS studies was carried out in 1983 by Heydorn *et al.* A spatial map and the concentration of several proteins were established utilizing 2D PAGE. The microdissected regions included cortical areas as well as nuclei from the hypothalamus, amygdala, thalamus, forebrain, and hindbrain and proteins were

visualized using silver staining and quantitated by computerized scanning densitometry.

One of the most impressive proteomic investigations was carried out by Oguri *et al* in 2002. Due to the concern over protein spot separation when using 2D gel electrophoresis, this group optimised the 2D gel process in terms of protein spot representation by using seven 1st dimension gels over several narrow pH ranges to increase protein spot resolution in the across the pI scale, and then to separate proteins by their molecular weight on 14 polyacrylamide gels. In doing this, detectable spot number increased from 853 to an impressive 6677 protein spots. This study highlights one of the many concerns with 2D gel electrophoresis and provides a possible feasible solution to the problem.

1.7 Summary

In summary, research investigating the molecular mechanisms of LTP in the past 30 years has identified a large number of molecules central and in some cases fundamental to the induction and maintenance processes of long term potentiation. Even so, the list of molecules is by no means exhaustive, and there remains a significant gap in our knowledge, with particular reference to the later stages of this synapse specific form of plasticity. The development of novel techniques out-with the neuroscience bracket has provided researchers with the ability to take their research several steps further than was previously obtainable. Proteomics is one such technique newly adopted by the neuroscience community which has allowed a more generic approach to CNS research thereby providing a platform for further investigation.

This thesis describes the use of such an approach to aid in the identification of molecules involved in synaptic plasticity in the hippocampus. Initial development of the technique precedes the investigation of protein regulation following receptor stimulation and finally, behavioural models related to cognition. The results reported in this thesis will hopefully lay the groundwork for other similar investigations and more importantly elucidate some of the molecular workings involved not only in basic neuronal signalling, but also in cognitive processes in the mammalian brain.

1.8 Study aims

The main aims of this study were as follows:

- To develop a global proteomics approach suited to the identification of protein regulation occurring in the rodent brain, in particular the hippocampal formation.

- To investigate protein modulation following glutamate receptor activation in area CA1 of the mouse hippocampus.

- To investigate the subcellular localisation of proteins regulated by pharmacological stimulation of glutamate receptors on isolated dendritic layers from area CA1 of rat hippocampus.

- To investigate protein regulation associated with high frequency activation of glutamatergic afferents and potentiation of the synaptic response (LTP) at early and late time points following the induction of LTP.

- And finally, to investigate protein expression profiles in the hippocampus of an animal model representative of neurogenesis, increased learning capacity and increased neuronal plasticity namely environmental enrichment, and an animal model representative of neurodegeneration and associated cognitive decline, the TAS10 transgenic mouse model of AD.

Chapter 2

Materials and general methods

2.1 *In vitro* hippocampal slice experiments

2.1.1 Hippocampal slice preparation

2.1.1.1 Electrophysiology

Animals were supplied by Harlan UK Ltd. and housed in group cages under controlled environmental conditions (temperature 19-23°C, 12hr light/ dark cycles). Animals had *ad libitum* access to food and water.

Male ICR mice or male Wistar rats (see individual results chapters for details) (4-8weeks) were terminally anaesthetised in accordance with UK Home Office guidelines under the authority of the UK Animals (Scientific Procedures) Act, 1986, with 1500mg/kg *i.p.* of urethane. Following unconsciousness and lack of paw pinch pain reflex the chest was opened to expose the heart and a 19-½ gauge needle was inserted into the left ventricle of the heart with a small incision made in the right atrium. Approximately 20-60mls of ice cold (4°C), oxygenated (95% O_2, 5% CO_2), high sucrose artificial cerebrospinal fluid (HSACSF) (Kuenzi *et al.*, 2000) was perfused transcardially to rapidly cool the brain. Perfusion was judged to be complete when extremities such as tail, paws etc. were emptied of blood. A scalpel was used to make an incision down the middle of the head to reveal the scull. Sharp dissecting scissors then cut the skull down its midline from back to front and the bone folded to each side to expose the brain. The brain was gently removed from the skull using a small spatula and transferred into ice-cold high sucrose ACSF. The cerebellum was removed and the brain hemisected in the sagital plane down the midline. The surface parallel to the desired orientation, in this case horizontal, was trimmed and glued (using a cyoacrylate adhesive) to the stage of a vibrating blade microtome (Leica VT1000, Milton Keynes, UK). For the orientation of the horizontal slice preparation see figure 2.1. Slices were cut to a thickness of 400μM and then transferred to a nylon mesh with a submerged holding chamber where they were maintained at 37°C. The high sucrose solution was gradually changed over to a normal ACSF solution (15mls every 15 minutes over a period of 1hour) and maintained at room temperature before the slices were ready for use.

2.1.1.2 Pharmacological stimulation

Hippocampal slices used for pharmacological stimulation were prepared by cervical dislocation of the rodent following anaesthesia (1500mg/kg *i.p.* urethane). Again, a scalpel was used to make an incision down the middle of the head to reveal the scull. Sharp dissecting scissors were then used to cut the skull down its midline from back to front and the bone folded to each side to expose the brain. The brain was gently removed from the skull using a small spatula and transferred into ice cold (4°C) normal ACSF. The cerebellum was removed and the brain hemisected. Horizontal slices were prepared as described in section 2.1.1.1 of this chapter and transferred to an interface style holding chamber where they were kept in a humidified, oxygen enriched environment for 1hr.

Figure 2.1 Orientation of rodent brain for horizontal slice preparation

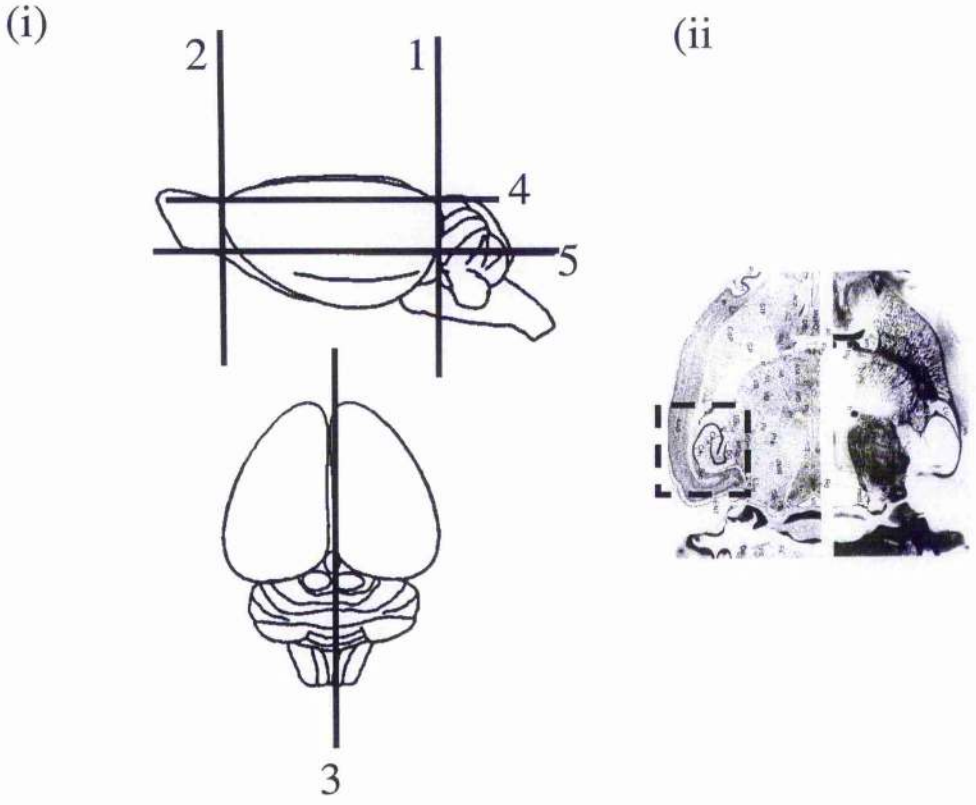


Diagram illustrating the dissection of rodent brain for the production of horizontal slices. (i) The cerebellum was removed (1) followed by a small portion of the rostral (R) section of the brain. The brain was then hemisected (3). These central portions of brain were placed on their side and a small part of the dorsal section was removed to allow this side to be glued to the stage of the vibrating blade microtome (4). Whole brain slices were cut on the horizontal plain (5) and hippocampi removed from surrounding tissue (dotted square) (ii)

2.1.2 Equipment

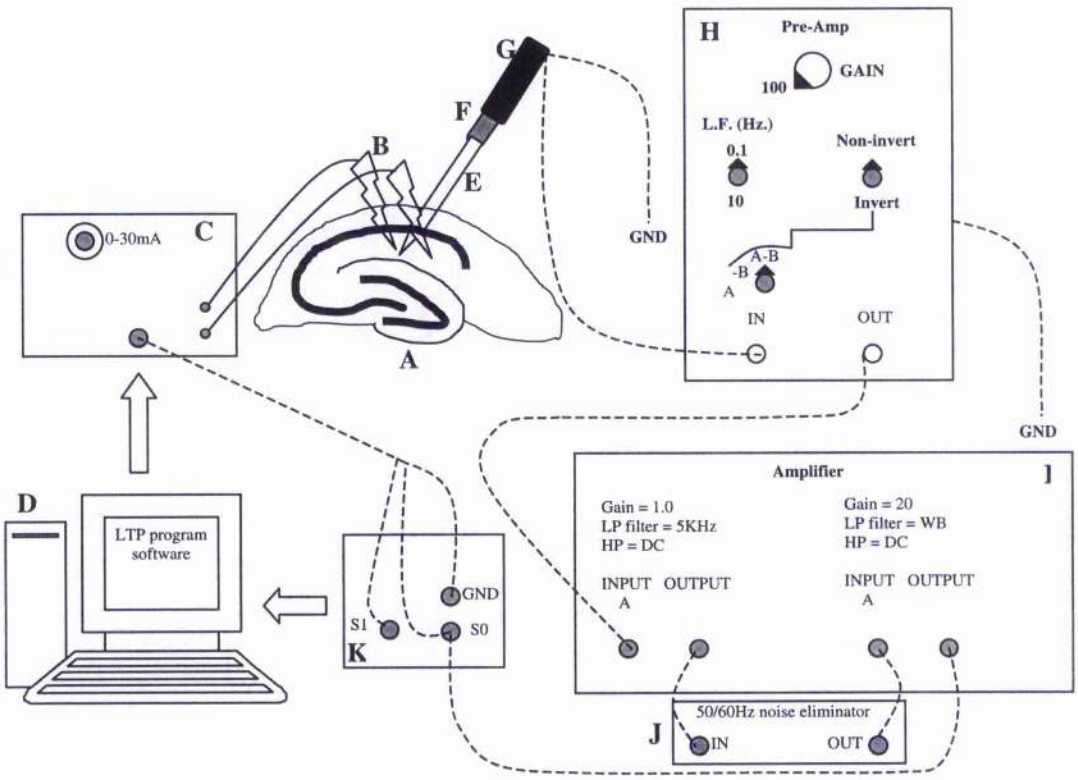
Stimulating electrodes were made from two lengths of formvar coated nickel 80% / chromium 20% wire of 0.05mm thickness (Advent Research Materials Ltd., UK) wound together and soldered onto gold pins (World precision instruments, Florida, USA). The sections of wire soldered to the gold pins had their insulation removed with a scalpel blade to allow conductivity from the stimulator to the electrodes. Stimulating current was applied through the electrodes using an isolated constant current stimulator (Model D52A, Digitimer Ltd., UK). The current ranged from 0-30mA.

Recording electrodes were made from standard walled (1.2mm O.D. x 0.69mm I.D.) borosilicate glass capillaries (Clark Electromedical instruments, UK - check) on a Flaming/ Brown micropipette puller (Model P-87, Sutter Instrument Co., USA).

The electrodes were filled with ACSF solution and were connected to a headstage (nl100, Neurolog, UK) via a silver chloride pellet containing electrode holder.

Pre-amplification (x100) was carried out by a Neurolog system pre-amplifier (Digitimer, UK). Further amplification (x20) was carried out by a Brownlee instrumentation amplifier (Model 440, Brownlee Precision, USA) giving a total amplification of x2000. A Hum Bug device (Quest Scientific sold via Digitimer, UK) was used to eliminate 50Hz line frequency noise. Data were captured by an ADC42 data acquisition board (Digitisation rate = Pico Technology Data Acquisition, University of Glasgow, UK) and transferred directly to the hard-disc of a P.C. (Genie P3 1GHz, Viglen, UK).

Figure 2.2 Electrophysiology recording set up.



Schematic of the experimental set up used for electrophysiological recordings in hippocampal slices (A). Stimulating current through bipolar electrodes (B) was delivered by an isolated stimulator (C) connected to a PC (D). The stimulation pattern was triggered by the LTP software program (www.ltp-program.com). Recording electrodes (E) anchored by electrode holders (F) were placed within the stratum radiatum of the CA1 region of the slice. Recordings were sent through a headstage (G) to a pre amplifier (H), which initially increased the response 100 fold. This gain was further increased 20 fold by an instrumentation amplifier (I). At this stage the response was also filtered. Filter settings allowed the low pass of 5KHz signals and a Humbug (J) was used to remove further 50/ 60Hz noise. The signal was then sent to an ADC42 data acquisition board with a 2-pathway capability (S0 and S1) (K) where it was captured and sent back to the PC for further analysis.

2.1.3 Data capture and analysis

Data were generated and captured with the aid of the LTP program. This free and custom designed LTP software was downloaded from www.ltp-program.com (Anderson & Collingridge, 2001) and was operational in MS-DOS mode. The LTP software was used to drive the experiments including triggering the isolated stimulator, writing the data sweeps to hard disc and calculating EPSP amplitude and slope measurements. Data was subsequently exported to Microsoft ® Excel (2003) and the Origin ® graph package (Version 6.1, OriginLab Corporation, USA) for further of-line analyses. Each Origin or Excel data point represents the pool of 3 individual excitatory post-synaptic potential (EPSP) values and each combined data point was expressed as a percentage of the mean baseline EPSP response. Statistics (Students t-test) were performed on normalised data due to the variability in EPSP amplitude across individual experiments. Error bars represent the standard error of the mean (s.e.m.).

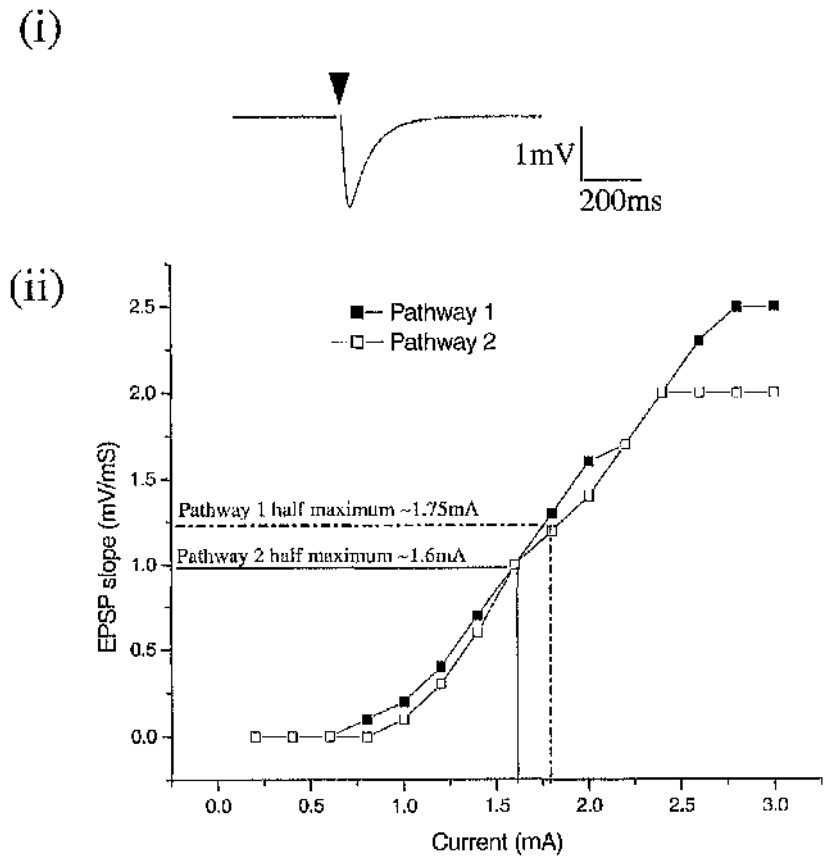
2.1.4 Electrophysiology recordings

2.1.4.1 Electrophysiology recordings – LTP induction (recording only – no tissue collection)

Following complete exchange of ACSF (high sucrose to normal) slices were transferred to a submerged recording chamber (Figure 2.3) and perfused at a rate of 1-2ml/minute with oxygenated ACSF heated to 28°C before entering the bath by an in-line temperature controller (TC-20, npi, UK) one hour prior to recording. At this time a single recording electrode was placed in the stratum radiatum of the CA1 region of the hippocampal slice. Two stimulating electrodes were placed either side of the recording electrode to stimulate two independent pathways. Electrodes were positioned 1hr prior to recordings to allow the slice to fully equilibrate. Small amounts of stimulating current (0-30mA) were administered to the slice to test the EPSP responses (Figure 2.3a). Once slice viability had been ascertained electrodes were positioned, input/ output (I/O) curves were constructed for both pathways (Figure 2.3b). This entailed stimulating both pathways alternately every 30 seconds

with gradual increases in current intensity and measuring the EPSP output. The current that produced half the maximum response for each pathway in the I/O curves was then used for the remainder of the experiment. A 30 minute stable (<10% change in amplitude) baseline recording (one stimulation every 30seconds) on both pathways was required before commencing with induction of LTP. To induce LTP, three high frequency tetani (100Hz stimulation for 1s, 10 minute inter-tetani interval) were administered to one of the stimulated pathways in the slice. Pathway independence was determined by lack of cross over effect of tetanisation in the control pathway. Recordings were then followed for up to 4 hours post tetanus (a single stimulation every 30 seconds per pathway).

Figure 2.3 Glutamatergic synaptic transmission in the CA1 region of mouse hippocampus



(i) Example of an excitatory post synaptic potential response recorded from the stratum radiatum of the CA1 region of mouse hippocampus. Black triangle represents time of stimulation (ii) An example of two I/O curves of two separate pathways in a hippocampal slice. Pathway 1 required approximately 1.75mA to produce a half maximal response (dotted line), whereas pathway 2 required approximately 1.6mA to produce a half maximal response (solid line).

2.1.4.2 *Electrophysiology recordings – LTP induction for tissue collection*

In order to optimise the number of potentiated synapses for subsequent proteomic analysis, the experimental setup was as is detailed in section 2.1.4.1 with the following modifications. Both pathways within the Stratum Radiatum were subjected to simultaneous high frequency stimulation (HFS) (as described previously) to ensure maximum stimulation of Schaffer collateral afferents. Recordings were made for either 10 or 240minutes following the final high frequency tetanus. At the end of these experiments, tissue was collected, prepared and frozen as described in section 2.3.1. Control slices were subjected to baseline stimulation (0.033Hz) for the duration of the experiment (either 60 or 290 minutes).

2.1.4.3 *Electrophysiology recordings – NMDA receptor blockade*

Methods were similar to those described in section 2.1.4.1 with the following modifications. The slices were perfused with the NMDA receptor antagonist D-AP5 (50μM) 20 minutes prior to, during and 60 minutes after 3 HFS. Recordings were made for 1 hour after the final high frequency tetanus. No control pathway stimulation was in place for these experiments.

2.1.4.4 *Electrophysiology recordings – Protein synthesis inhibition*

Methods were similar to those described in section 2.1.4.1 with the following modifications. Slices were perfused with the protein synthesis inhibitor emetine (20μM) 30minutes prior to, during and 60 minutes after HFS. Recordings of this pathway were made for a further 60 minutes. Emetine was then washed out for 60minutes before the second pathway was subjected to 3 HFS, 10 minutes apart. Recordings were continued for up to 4 hours following the third tetanisation.

2.1.5 Non electrophysiology hippocampal slice experiments

2.1.5.1 Pharmacological manipulation of hippocampal tissue

Whole hippocampal slices or isolated dendritic fractions (see section 2.1.5.2) from the stratum radiatum of the hippocampal CA1 region were transferred to petri dishes lined with ACSF saturated filter paper contained within a humidified holding chamber (37°C, 95%O_2/ 5% CO_2). The slices were bathed in 2mls of, (1) NACSF, (2) glutamate (50μM), (3) glutamate (50μM) + D-AP5 (50μM) for 4 hours. At the end of these experiments, tissue was collected, prepared and frozen as described in section 2.3.

2.1.5.2 Isolation of dendrites from the stratum radiatum of the CA1 region of rodent hippocampus

Certain experiments in this thesis utilised isolated apical dendrites harvested from acute hippocampal slices. Following preparation, slices were placed on ice cold glass plates, within ice cold ACSF where the neurons of the hippocampal CA1 region were viewed at low power under a dissecting microscope. Using an ice-cold scalpel blade dendrites were carefully separated from their cell bodies by making a transaction line within stratum radiatum paralleled with and close to stratum pyrimidale. Isolated dendritic fields were then placed in petri dishes on filter paper as described in section 2.1.5.1, or immediately harvested and snap frozen as described in section 2.3, for further proteomic analysis.

2.2 *In vivo* experiments

2.2.1 *In vivo* experiments – TAS10 transgenic mouse model

Hippocampus and cortex (400μm thick acute slices) were supplied courtesy of Dr Ceri Davies, GSK, Harlow, UK. These slices were obtained from transgenic (TG) TAS10 and wild type (WT) male and female mice, 12-14 months of age. The mice were euthanised by cervical dislocation and decapitated in accordance with UK Home Office guidelines. Horizontal slices were prepared from the whole brain minus

cerebellum using a Vibroslicer (Campden Instruments, Loughborough, UK). The hippocampus and cortex were dissected free from the surrounding tissue and the snap frozen in liquid nitrogen for storage at –70°C prior to proteomic analysis.

2.2.2 *In vivo* experiments – environmental enrichment

All animals used in this study were freshly weaned (4-6 week old) Lister Hooded rats obtained from Harlan, UK. Housing conditions were 4-6 per cage on 12h light/dark cycles. Animals were assigned to three groups: 1) Enriched placed in box with lots of toys 2) Non-enriched- placed in a box with no toys 3) Controls (kept in home cage). Animals spent approx 15 hours per day (5days/ week) in these conditions (overnight from 5pm till 8am) and experiments lasted for 6 weeks in total. Animals were sacrificed using an overdose of anaesthetic (5ml). Anaesthetic used was Avertin (Tri-bromo-ethanol) and it was injected intraperitoneally. Following unconsciousness and lack of paw pinch pain reflex a scalpel was used to make an incision down the middle of the head to reveal the scull. Sharp dissecting scissors then cut the skull down its midline from back to front and the bone folded to each side to expose the brain. The brain was gently removed from the skull using a small spatula and transferred into ice-cold normal ACSF. The cerebellum was removed and the brain hemisected in the sagital plane down the midline. For the orientation of the horizontal slice preparation see Figure 2.1. Slices were cut to a thickness of 400μM on a tissue chopper and the hippocampus dissected free from surrounding tissue. Dendrites were isolated from stratum pyramidale as described in section 2.5.1.2 and dendritic and somatic fractions snap frozen in liquid nitrogen prior to storage at -70°c and proteomic analysis.

2.3 Tissue collection

At the end of all experiments, slices were placed on a clean, ice cold, microscope slide and the CA1 regions of the hippocampal slices micro-dissected. The entire hippocampus and cortex was collected from TAS10 transgenic and wild type mice. All micro-dissected regions were then placed in ice-cold eppendorfs, snap frozen in liquid nitrogen and stored at -70°C until further use.

In the case of the slices from the environmental enrichment studies, apical dendrites were isolated from cell bodies in the CA1 region as described in section 2.1.5.2 of this chapter and stored as previously described.

2.4 Proteomics studies

2.4.1 Comparative 2D gel analysis

2.4.1.1 Sample lysis

Samples were lysed in 100μl of a CHAPS based lysis buffer by grinding in a 1.5ml centrifuge tube with a plastic pestle (Sigma, UK). Samples were then freeze/ thawed 5 times by placing them in liquid nitrogen and thawing in a water bath heated to no more than 30°C. Samples were then left at room temperature for 1 hour to aid protein solubilisation before being sonicated for 16 minutes in 4 cycles of 4 minutes with a 1min interval between cycles to prevent overheating and crystallization of urea. To remove cell debris samples were centrifuged at 13000 rpm for 15 minutes on a bench top centrifuge (MSE microcentaur, Scotlab, UK). The supernatant was transferred to a fresh eppendorf and a small portion (10μl) of the supernatant was then used for protein concentration determination.

2.4.1.2 Protein concentration determination

Protein concentration was measured using the Pierce BCA (bicinchoninic acid) protein assay reagent kit (Pierce, USA). Due to the small amount of protein in our samples the microplate protocol was used and a standard curve was prepared using bovine serum albumin (BSA - concentrate from assay kit). The working range for the standard curve was 20-2000μg/ml. Standards were prepared using the following dilutions:

Table 2.1 Composition of standard protein concentration assay solutions

Tube	Volume of lysis buffer (μl)	Volume and source of BSA	Final BSA concentration
A	0	300μl of stock	2000μg/ml
B	125	375μl of stock	1500μg/ml
C	325	325μl of stock	1000μg/ml
D	175	175μl of vial B dilution	750μg/ml
E	325	325μl of vial C dilution	500μg/ml
F	325	325μl of vial E dilution	250μg/ml
G	325	325μl of vial F dilution	125μg/ml
H	400	100μl of vial G dilution	25μg/ml
I	400	0	0μg/ml (Blank)

Table listing the amounts of lysis buffer and standard BSA required making all the concentrations required for a standard curve.

Next, the BCA working reagent (WR) was prepared. To determine the total volume of WR required the following equation was used:

(# Standards + # unknowns) (# Replicates) (Volume of WR per sample) = total volume WR required.

Typically 3 replicates per sample were used. Each sample was a 1:10 dilution (10μl of sample: 90μl of lysis buffer), and this was taken into account when the final readings were calculated. The volume of WR required for this microplate procedure was 200μl and it was prepared fresh by the addition of 50 parts of reagent A to 1 part of reagent B and mixed well by vortexing. 25μl of each standard and unknown sample were pipetted into individual wells of a 96 well microplate. Then, 200μl of the WR was added to each well and mixed thoroughly on a shaker for 30 seconds. The plate was protected from light, incubated at 37°C for 30minutes and allowed to cool to room temperature before the absorbance of each well was measured. The absorbance was measured with an Opsys MR plate reader (Dynex Technologies,

UK) using Revelation Quicklink software (Version 4.03, Dynex Technologies, UK) at a wavelength of 562nm. The software subtracted the average reading of the blank replicates from the remaining standards, plotted the standard curve using a quadratic curve fit and calculated the concentration of each unknown sample using this curve.

2.4.1.3 1st dimension

Protein mixtures were initially separated according to their charge using isoelectric focusing (Gelfi & Righetti, 1983) on Amersham's Ettan ™ IPGphor ™ system. Samples were run on immobilised pH gradient (IPG) Dry Strip gels that contained a pre-formed pH gradient immobilised within homogenous polyacrylamide gels (Görg *et al.*, 1988). These 24cm IPG strips were routinely rehydrated using the rehydration loading method as reported by Rabilloud *et al.*, (1994) (see individual results sections for pH gradients used). However, in one instance the samples were loaded onto the first dimension using the cup loading method. This method has been found to improve the resolution of proteins at the basic end of the 2D gel. IPG strips were rehydrated in 450μl of rehydration buffer in the absence of sample in an Immobiline DryStrip re-swelling tray. The IPG strip was placed gel side down onto the rehydration buffer, being careful not to introduce any bubbles that would result in uneven re-swelling of the gel. Silicone oil (3mls) was laid over the IPG strip to prevent evaporation and urea crystallisation. The lid was replaced on the reswelling tray and the strips were left to rehydrate at room temperature over night. The maximum concentration suitable for cup loading was 100μg/ 100μl. The total volume the sample cups could hold was 100μl. Positioning of the sample cup was dependent on the nature of the proteins from the sample. It was decided for the experiments within this thesis that protein samples would be positioned within the central region of the gel as we were unaware of whether or not our sample was best suited to acidic or basic application. The rehydrated IPG strips were removed from the re-swelling tray and placed in IPG ceramic cup loading strip holders. The sample cup was pushed into place where it sat just on top of the gel. To ensure correct positioning of the cups 100μl of silicon oil was pipetted into them and if no leakage occurred the cups were deemed to be placed appropriately. The silicon oil was removed before addition of sample. Up to 100μl of sample was added into the cup and the lid replaced on the ceramic coffin. For rehydration loading, sample volume

equating to 150μg of protein was added to rehydration buffer to a total volume of 450μl. Dithiothreitol (DTT) and the IPG buffer appropriate for the pH range of the IPG strips used were added fresh to the rehydration buffer prior to the 1st dimension run. This mixture was then spread evenly along the base of a ceramic IPG strip holder and the IPG strip placed gel side down onto the sample mixture, being careful not to get any air bubbles trapped between solution and strip. The anodic end of the strip was positioned at the pointed end of the coffin. For both cup loading and rehydration loading 900μl of silicone oil (Dry Strip cover fluid, Amersham Biosciences, UK) was pipetted over the top of the IPG strip to prevent it drying out and the lid of the holder replaced. It was important to ensure that the IPG strip touched both electrodes in the holder and that the holder itself made contact with the anode and cathode of the IPGphor. Samples were run using a preset program:

Rehydration loading:

Step 1	Step – n – hold	30V	14:00Hrs or 420Vhrs
Step 2	Step – n – hold	500V	1Hr or 500Vhrs
Step 3	Step – n – hold	1000V	1Hr or 1000Vhrs
Step 4	Gradient	8000V	0.53Hrs or 3975Vhrs
Step 5	Step – n – hold	8000V	10Hrs or 80000Vhrs

Cup loading:

Step 1	Gradient	500V	0.01Hrs or 10Vhrs
Step 2	Gradient	4000V	1:30 or 3400Vhrs
Step 3	Step – n – hold	8000V	6:30Hrs or 56600Vhrs

at 20°C, at amperage of 50mA per gel.

Successful 1st dimension runs resulted in greater than 60000 total Vhrs (cup loading) and 75000 total Vhrs (rehydration loading) on the IPGphor.

If for any reason it was not possible to continue with the 2nd dimension immediately strips could be stored at –70°C for approximately 3 months.

2.4.1.4 IPG strip equilibration

IPG strip equilibration was carried out immediately after the 1st dimension run or immediately prior to 2nd dimension if the strips were frozen. Strips were placed in a plastic tube containing equilibration buffer and placed on a shaker set at slow speed. Strips were equilibrated in 10mls of equilibration buffer 1 for 13minutes then equilibrated in 10mls of equilibration buffer 2, again for 13minutes. Buffer 1, containing dithiothreitol preserved the fully reduced state of the denatured and un-alkylated proteins. Buffer 2, which contained iodoacetamide, alkylated thiol groups preventing reoxidation during the second dimension and gel distortion. This second equilibration step was also used to prevent unwanted reactions of cysteine residues that may affect subsequent mass spectrometric analysis.

2.4.1.5 2nd dimension

IPG strips containing equilibrated protein samples were then run on a 2nd dimension to further separate the proteins by their molecular weight using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE). Large format (24cm) pre-cast plastic backed polyacrylamide gels (Amersham Biosciences, UK) were used for comparative 2D experiments only. Pre-cast gels were inserted into gel cassettes. IPG strips were dipped in electrophoresis buffer to lubricate them and aid their transfer to the top of the 2nd dimension gels. The strip was pushed down firmly but gently onto the top of the polyacrylamide gel to ensure no gaps or bubbles would appear that might cause distortion of the final 2D gel pattern. The acidic end of the strip was positioned to the left hand side of the gel. Finally, strips were sealed into position using agarose-sealing solution. This solution contained a dye (bromophenol blue) to aid in tracking the progress of the 2nd dimension run. These large format gels were run using the Ettan Dalt II system (GE Healthcare, UK). Gels were slotted between plastic seals separating anodic and cathodic buffers. When using pre-cast gels, buffer kits were used (GE Healthcare, UK). Gels were run at a constant voltage of 100V at 20°C overnight or until the dye front had run off the bottom of the gel.

2.4.1.6 Sypro ™ orange staining

Comparative gels were visualised using the fluorescent stain, Sypro orange (Molecular Probes, UK). On completion of the second dimension, proteins in the gels were fixed into position using a 10% methanol/ 7.5% acetic acid mixture. Gels were bathed (500mls/ gel) in this fixative overnight. Proteins were then probed with 0.05% SDS solution (500mls, 1hr/ gel) before being stained with Sypro orange (1:5000 dilution) (500mls/ gel, overnight). For best results, it was important to carry out this staining procedure in a clean polyethylene tray and to protect it from light. Before imaging, the gels were rinsed briefly in distilled water to remove any background stain.

Figure 2.4 Positioning of reference markers on 2D gels

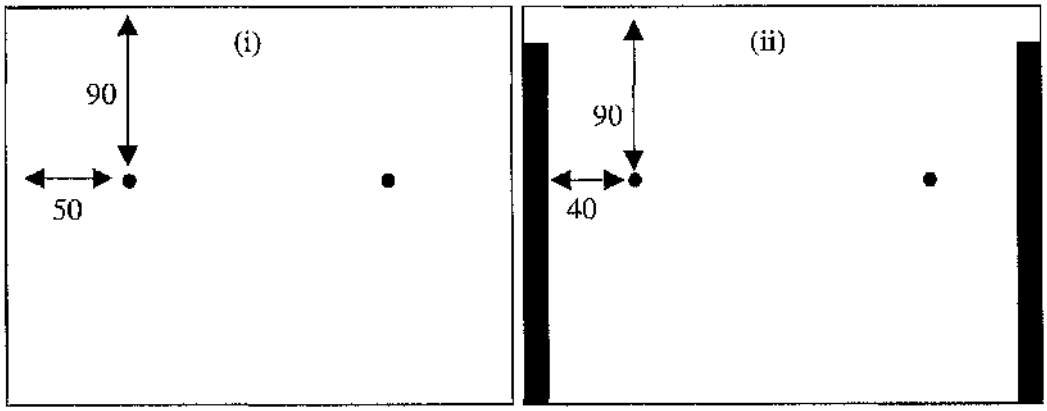


Diagram of a pre cast gel (i) and low fluorescence gel plate (ii) with reference markers attached. Dimensions (in mm) are labelled on each gel image. It is worth noting that positioning the markers too far into the centre of the gel may interfere with the gel spot pattern.

2.4.1.7 Scanning

Prior to scanning 2 fluorescent reference markers (GE Healthcare, UK) were placed on the plastic backing of each gel (see Figure 2.4). These were used to provide coordinates for selected protein spots. Gels were scanned using a Typhoon 9400 variable mode imager (GE Healthcare, UK). A small amount of distilled water was placed on the glass platen of the scanner to ensure no air bubbles were trapped between the gel and the scanner plate. Gels were placed plastic side down. The settings used for scanning Sypro orange stained gels were as follows:

Emission wavelength : 580nm
Excitation wavelength: 532nm
Laser : Green

Gels were initially scanned at a low resolution (1000microns) in order to determine the strength of the laser (PMT voltage). The PMT voltage most commonly ranged from 450 to 650. Once the ideal PMT voltage had been established, final scans were made using a higher (100microns) resolution. On completion of the scan images were saved as .gel files for further analysis. Gels were stored in fix (10% methanol/ 7.5% acetic acid) solution until further use.

2.4.1.8 Analysis

Basic visual comparison was made across gels to determine changes in protein expression between treatment groups. Due to the nature of this analysis, only protein spots which were perhaps present in one treatment and absent in another were deemed to be differential and therefore investigated further. There was no quantification other the than presence / absence of these spots.

2.4.1.9 Picklist generation

The 2D gel analysis program ImageMaster ™ 2D Elite (Amersham Biosciences, UK) was used to highlight and assign coordinates to each protein spot of interest. To

do this gel images were inserted into the program, reference markers were identified and the protein spots that were to be picked were highlighted. In doing this coordinates were assigned to each protein spot and a pick list was generated. The format of the pick list was one that was recognised by the spot handling workstation. It was important to ensure that reference markers were added in the correct order i.e. left marker becomes internal reference 1 (IR1) and right becomes internal reference 2 (IR2). The pick list was transferred to the spot handling workstation.

2.4.1.10 Ettan ™ Spot Handling Workstation

The workstation comprised a fully automated spot picker, digester and spotter. Although fully automated a description of the procedure carried out by the workstation follows. Gels to be picked from were covered in distilled water placed in specialised bar coded gel cassettes and inserted into the workstation. Picking takes place under liquid firstly to prevent the gel drying out but also to maintain a vacuum required for accurate gel cutting. Also placed into the workstation were 96 well plates (for gel plugs as well as peptides) and Voyager (Maldi-tof) target plates (as many as was required for the number of spots to be picked). A 1.4mm pick head was used to pick out gel plugs that were inserted into a 96 well plate. They were then incubated in 300μl of 50mM ammonium bicarbonate (AmBic) in 50% methanol for 30minutes. Gel plugs were subsequently dried for approximately 20minutes. Prior to trypsin addition, the gel plugs were primed with 20mM AmBic, followed by addition of enzyme (10μl of Trypsin, 0.2μg Porcine, Promega, UK) in 20mM AmBic)). Proteins contained within the gel plugs were digested for 4 hrs at 37°C. To extract the peptides, gel plugs were incubated for 20 minutes in 120μl of 50% acetonitrile (ACN) /0.1% trifluoroacetic acid (TFA) and 80μl of this peptide mixture was transferred to a second microplate where the peptides were dried down for 90min. Once dried the peptides were reconstituted in 3μl of 50% ACN/ 0.5% TFA. 0.3μl of peptide was mixed with 0.3μl of matrix, • -cyano-4-hydroxycinnamic acid (CHCA), and spotted onto a Voyager target plate for mass spectrometric analysis.

2.4.1.11 Mass spectrometry

2.4.1.11.1 Maldi-tof mass spectrometric analysis

Peptides were initially analysed using matrix assisted laser desorption ionization–time of flight (Maldi-tof) mass spectrometry on a Voyager DE™ – PRO (Perceptive Biosystems, CA, USA). Analysis was carried out in positive reflector mode using a laser setting around 2350. The low mass gate setting was set at 750 Da, and the spectra were acquired over a range of 750 – 3500 Da. The accelerating voltage was 20 KV, the grid voltage set to 76 % and the guide wire voltage set to 0.006 %, with an extraction delay time of 160 nsec. 100 shots per spectrum were collected, with a combination of 3 spectra per spot.

Spectra were then exported to Data Explorer ™ (Version 4, Applied Biosystems, UK) where they were internally calibrated using the 842.5099 and 2211.1046 trypsin peaks, processed to reduce background noise, and a monoisotopic peak list generated and entered into the Mascot PMF database. Data was searched using the NCBInr database.

1) Advanced baseline correction
2) Noise filtering/ smoothing
3) Peak de-isotoping
4) Calibration (842.5099 and 2211.1046 trypsin peaks)
5) Peaks identified and list generated

Those peptide mixtures that were unidentified by Maldi-tof analysis were transferred within the Sir Henry Wellcome Functional Genomics Facility (www.gla.ac.uk/functionalgenomics/) for Electrospray (LC/ MS/ MS) analysis which enabled identification of proteins from their amino acid sequence.

2.4.2.11.2 Electrospray MS/MS Mass Spectrometry

1.4 mm gel plugs were excised from and subjected to in-gel trypsin digest in an Ettan Spot Handing Workstation (GE Healthcare), following standard protocols. The

resulting tryptic peptides were solubilized in 0.5 % formic acid and fractionated by nanoflow HPLC on a C18 reverse phase column, eluting with a continuous linear gradient to 40 % acetonitrile over 20 minutes. Eluate was analysed by online electrospray tandem mass spectrometry using a Qstar Pulsar (Applied Biosystems).

Mass spectrometric analysis was performed in IDA mode (Analyst QS software, Applied Biosystems), selecting the four most intense ions for MSMS analysis. A survey scan of 400–1500 Da was collected for 3 s followed by 5 sec MSMS scans of 50–2000 Da using the standard rolling collision energy settings. Masses were then added to the exclusion list for 3 min. Peaks were extracted using the Mascot script (BioAnalyst, Applied Biosystems) and automatically exported to the Mascot (Matrix Science) search engine.

2.4.1.12 Mascot database searching

Access to this database was obtained through www.matrixscience.com. Glasgow University itself has two in-house licences for this facility. Peptide mass fingerprints (PMFs) were searched within the NCBInr protein database under the Mus musculus taxonomy. One missed cleavage was permitted when searching. Carbamydomethylation of cysteine was highlighted as the fixed modification and oxidation of methionine highlighted as the variable modification. Peptide tolerance was initially set at 70ppm, although was sometimes subject to change.

The peptide sequences identified from the MS/ MS mass spectrometry were also matched against the NCBInr Mus musculus database with a peptide tolerance of 1.5 Da and a MSMS ion tolerance of 0.5 Da allowing for two missed cleaves and variable methionine oxidation.

For the PMF search and the MS/ MS search proteins are matched with the identified peptides and each protein is assigned a mascot score, which is a probability-based Mowse score (Perkins *et al.*, 1999). The Mascot score is equal to -10 log (P), where P is the estimated probability that the observed match is a random event. For example, a score of 100 represents a 1 in 10^{10} chance that the protein match is a

random event providing a high degree of certainty to the identity of the protein in question. An objective measure of the significance of the result is then given by a threshold which represents a point at which the identification would be expected to occur at random with a frequency of less than 5%. In the case of PMF this threshold was 62, and in the case of MS/ MS this threshold was 36.

2.4.2 DiGE analysis

2.4.2.1 Sample Lysis

Methods were similar to those already described in section 2.4.1.1 with the following modifications. The lysis buffer used here was an ASB14 buffer which had been reported to aid in the solubilisation of membrane bound proteins (Henningsen *et al.*, 2002.). 120μl of this buffer was added to the protein sample.

2.4.2.2 Precipitation

In order to obtain an optimal CyDye ^{TM} labelling reaction any contaminants such as lipids and salts present in the protein sample were removed. To do this the protein sample underwent an ammonium acetate precipitation. 600μl (5 x sample volume) of 0.1M ammonium acetate in 100% methanol was added to the sample, mixed vigorously and stored overnight at 20°C. The precipitate (protein) was spun down in a refrigerated (4°c) centrifuge (Harrier 18/80, Sanyo Gallenkamp, UK) for 10 minutes at 13000rpm. The supernatant was discarded and the resulting protein pellet washed with 80% 0.1M ammonium acetate, centrifuged at 13000rpm, this time for 3minutes. This was repeated once more. The pellet was then washed in 80% acetone and centrifuged at 13000rpm for 3 minutes. This process was repeated a further 2 times. It was important that when carrying out these washing steps the pellets were fully disturbed, as salt remaining in the sample would interfere with the running of the first dimension. After the final wash the supernatant was once again removed and the sample allowed to air dry for 10 minutes. Drying for any longer than this resulted in re-solubilisation problems and loss of protein. Dried protein pellets were resuspended in 70μl of ASB14 lysis buffer by vortexing (5 minutes) and sonication (5 minutes).

2.4.2.3 Experimental design

It was important to determine the design of the experiment before labelling protein samples. Exact details will follow in the individual results chapters.

Tables 2.2 & 2.3 Examples of DiGE experimental design

Example 1. Control vs. treatment A

Gel Number	Cy 2	Cy 3	Cy 5
1	Standard	Control	Treatment A
2	Standard	Control	Treatment A
3	Standard	Treatment A	Control
4	Standard	Treatment A	Control

Example 2. Control vs. treatment A over two time points

Gel Number	Cy 2	Cy 3	Cy 5
1	Standard	Control	Treatment A (Time 1)
2	Standard	Control	Treatment A (Time 1)
3	Standard	Treatment A (Time 1)	Treatment A (Time 2)
4	Standard	Treatment A (Time 1)	Treatment A (Time 2)
5	Standard	Treatment A (Time 2)	Control
6	Standard	Treatment A (Time 2)	Control

Tables showing possible experimental design for 2 different types of DiGE experiments. Randomisation and zero labelling bias were required in the experimental design. It was also recommended that biological replicates were used (minimum n=4) for increased statistical confidence.

2.4.2.4 CyDye labelling and labelled sample preparation

Three Cy Dyes ™ were used in each experiment. Cy 2 labelled the internal standard, with Cy 3 and 5 labelling the experimental groups. The internal standard comprised a portion of every other sample in the experiment. For example, in comparing A vs. B (minimum 4 replicates of A & B). Each internal standard was required to total 50μg per gel resulting in a total of 200μg for the entire experiment. Therefore 25μg (200/8) of each sample was pooled to make the internal standard. This pool was divided equally between all the gels. For successful labelling protein concentration was required to be greater than 1mg/ml, and the pH of the sample lay between 8 and 9. It was most important however, that the concentration or pH was consistent across all samples.

CyDyes were reconstituted in high quality dimethylformamide (DMF) to a concentration of 1nmol/μl. Each CyDye fluor (Cy2, 3 and 5) was warmed to room temperature for 5 minutes prior to addition to the DMF and the reconstituted CyDyes vortexed well for 30 seconds. The fluors were then centrifuged for 30 seconds at 12000g. Reconstituted dyes were further diluted to a working stock solution by adding 2μl of the fluor stock solution to 3μl of DMF. 5μl contained 2000pmol of fluor therefore 1μl contained 400pmol. 1μl of working dye solution was added to 50μg of protein in a microfuge tube. The mixture was vortexed well, centrifuged briefly and vortexed once again. Labelling was carried out in the dark for 30 minutes. Other protocols for this reaction suggest that the reaction should be carried out on ice. This was not appropriate here as the ASB14 detergent used in the buffer solidifies on ice and the labelling would be affected. The labelling reaction was stopped by the addition of 1μl of 10mM lysine. The mixture was again vortexed, centrifuged and vortexed again before being left in the dark for 10 minutes. If not used immediately the labelled samples were stored at -70°C until subsequently required.

2.4.2.5 1st dimension of DiGE

Labelled samples were then ready to run on the first dimension. All the labelled samples to be run on a single gel (Cy2, 3 and 5) were placed into the one microfuge

tube and mixed well. Where there was a large difference in volume between labelled samples, this was compensated for by the addition of lysis buffer. For example if the Cy 2 labelled sample was 12μl, the Cy 3 labelled sample 20μl and the Cy 5 labelled sample 25μl, 13μl of buffer was added to the Cy 2 sample and 5μl to the Cy 3 sample. Ideally this was avoided in the first instance by keeping protein concentration similar between samples. Once the final volume of all three samples was determined, and equal volume of 2x sample buffer was added. This mixture was then left for 10 minutes in the dark.

In all DiGE experiments, the IPG strips were again rehydrated in the presence of protein sample as was previously described. For all DiGE runs, the Ettan IPGphor was covered to protect the samples from light and to prevent any photo-bleaching occurring.

2.4.2.6 Polyacrylamide gel formation

For DiGE to be successful the 2nd dimension gels had to be laboratory made, using low fluorescent glass plates. Plastic backed pre cast gels were found to be unsuitable due to the high fluorescence of the plastic backing.

Either a 6 or 14 gel caster was used to pour the gels.

2.4.2.6.1 Preparation of gel plates

It was important that gel plates were dust and scratch free, as both interfered with gel analysis. To help minimise dust on the plates they were cleaned with distilled water and ethanol in a fume cupboard, and the assembly of the casters was also performed within a fume cupboard. Once cleaned the plates were treated with bind silane solution. Bind silane immobilised the polyacrylamide gel onto one glass plate making it suitable for spot picking in the later stages. 1.5mls of Bind Silane ^{TM} was pipetted onto the back glass plate (with spacers) and wiped evenly across the plate with a lint free tissue. All bind silane treated plates were left for a minimum of 1.5hrs in the fume cupboard. Once dried reference markers were placed onto gel back plates (see Figure 2.4).

2.4.2.6.2 *Preparation of gel solution*

For the 6 gel caster 450mls of gel solution was required, 900mls for the 14 gel caster. All components of the gel solution with the exception of TEMED and ammonium persulphate were placed in a 1 litre flask and mixed well. The solution was then filtered (0.22μm filter) into presterilised stericups (Millipore, UK) using a vacuum pump. The filtered solution was then ready for the addition of TEMED (125μl of a 10% solution) and ammonium persulphate (APS, 9mls of 10% solution). Polymerisation normally commenced 10-15 minutes after the addition of TEMED and APS.

2.4.2.6.3 *Gel caster assembly - Ettan Dalt II caster*

The front plate of the gel caster was removed to allow easy access. Firstly a thick separator sheet was placed at the back of the caster. This was followed by a back plate (with spacers) and the top plate of the gel. These were pressed firmly together to ensure a good seal between them. A thinner separator sheet was then added followed once again by the gel plates in the same manner and so on, until all gel plates were added. A small gap still remained after addition of all plates and separators. This was filled with more separator sheets. A very small (approx 1mm) gap was left at the top to prevent over packing of the gels, which would result in distortion of the images. A foam gasket was placed into the groove of the front plate. The front plate was then replaced and screwed back into position.

When using the 6 gel caster no further preparation was required and the gel solution (containing 'TEMED and APS) was poured at a steady rate into the caster. A displacement fluid was required however when using the 14 gel caster. A plastic tube with funnel was inserted into the grommet of the gel caster. 100mls of displacing fluid was added to the hydrostatic balance chamber. Gel solution (containing TEMED and APS) was mixed well and poured into the funnel of the caster at a steady rate being careful not to introduce too many bubbles. The solution was poured until it reached 1-2cm below the desired height. The plastic tube was removed from the grommet immediately afterwards to displace the gel to its final height. 1.5mls of water saturated iso-propanol was immediately pipetted over the top of the gels to

ensure a level surface. The top of the caster was loosely covered with cellophane and the gels were then left to polymerise overnight. Once polymerised, the gels were removed from the caster, cleaned of all excess polymerised gel and the top surface rinsed with distilled H_2O. Gels were generally used the day after they were made, but were suitable for use up to 2 weeks if stored in gel storage solution at 4°C.

2.4.2.7 2nd dimension

Equilibrated 1st dimension IPG strips (see section 2.4.1.4) were dipped in electrophoresis buffer and pushed gently but firmly onto the surface of the gel. 1ml of agarose gel solution was pipetted on top of the strip to hold it in place. Once the agarose had set, gels were placed in the Ettan Dalt II unit by slotting between the plastic seals. Any unused slots were filled with blank cassettes. 1 x tris/ glycine electrophoresis buffer was added to the lower chamber and once all slots were filled, 2 x electrophoresis buffers was added to the sealed upper chamber to the level indicated on the unit. The lid of the unit was lowered, making sure the electrodes were submerged in the buffer, and the unit started. The gels were run at 20°C overnight. The initial voltage was 40V for 20 minutes for gentle entry of the sample into the polyacrylamide gel. The remainder of the 2nd dimension was run at 100V overnight, or until the dye front had run off the bottom of the gel.

2.4.2.8 Scanning/ imaging

Gels were scanned immediately after the second dimension, before diffusion of proteins, although we have found that even 24hrs after the second dimension gel images still showed focussed protein spots. Gels were scanned on a Typhoon 9400 variable mode imager using DiGE settings. In order to correctly orientate the gels, a metal frame was placed on the surface of the typhoon and the gels were slotted into this plate. It was possible to scan 2 gels at the same time. The settings for scanning DiGE gels are described below. Initial scans (on one gel only) were made at low resolution (1000microns) for each CyDye image. Individual gel images were saved in suitable DiGE format as .gel files and all three images (Cy2, 3 and 5) were saved in a data set file (.ds). After scanning the files were opened in ImageQuant ™ software (Version 5.2, Amersham Biosciences, UK) and the pixel density of the

darkest spot measured. A box was drawn around the darkest spot on each gel image. From the *Analysis* pull down menu *Volume Review* was selected. This provided the maximum value (in pixels) of the spot. Pixel density values were required to lie within 50,000 – 80,000. Gels with values higher then this were subsequently rescanned at a lower PMT voltage. Once the optimum PMT voltage had been established for all 3 CyDye images (Cy2, 3 and 5) this voltage remained constant for all images in all gels. Final gel images were scanned at high resolution (100microns). When all scans were completed, those gels required for further staining and spot picking were fixed in a methanol/ acetic acid fixing solution overnight, or until further required. The top plate of the gel was removed before fixing by inserting a plate spreader between the plates and gently easing them apart.

Figure 2.5. Settings used for scanning DiGE gels

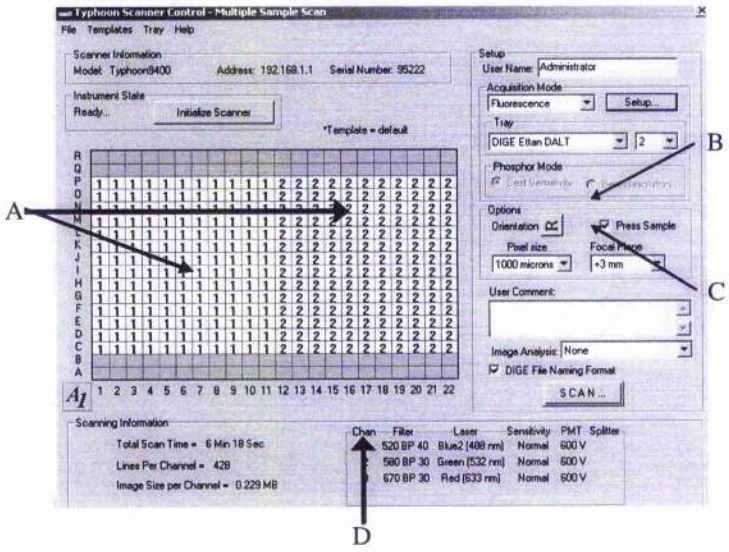


Image of typhoon scanner control panel detailing requirements for DiGE gel scanning. As gels were anchored by a metal plate gel positions remained constant (A). To prevent bubbles appearing between gel plates or between the typhoon platen and the glass plates the sample was pressed (B). The focal plain was set at +3mm due to the thickness of the glass plates (C). Emission wavelengths and excitation wavelengths as well as laser type and strength was set in the typhoon scanner control (D).

2.4.2.9 *Preparative gel preparation*

In order to identify the regulated protein it was important to have sufficient protein content within the spots for successful mass spectrometric analysis. Therefore, alongside our DiGE gels, preparative gels were run containing approximately double the amount of protein. Two methods were used to do this. Method 1 used an entirely separate gel which protein spots were picked from. The second method used one of the DiGE analytical gels loaded with excess protein as a preparative gel.

For separate gel preparation, sample preparation and 1st dimension was performed as described for comparative gels. Approximately 300μg of protein was loaded onto the 2D gel. As this protein sample was unlabelled Sypro orange staining was used to visualise the image (see section 2.4.1.6)

When using the analytical gel as a preparative gel, once the DiGE sample for this gel had been labelled, and the labelling reaction stopped, 150μg excess protein was added to the sample. This gave a total of 300μg of protein. In this gel the volume of 2x sample buffer was equivalent to the volume of labelled protein and excess protein. For example:

Volume of total labelled protein (x) + volume of total excess protein (y) = volume of 2 x sample buffer

The process for running this gel is the same as for the other DiGE gels. After scanning this gel to obtain Cy2, 3 and 5 DiGE images the gel was then stained with Sypro orange (see section 2.4.1.6) and rescanned to obtain a preparative gel image.

2.4.2.10 *Processing gel images for use in DeCyder™ software.*

For successful analysis in DeCyder all images from a single gel were required to be the same size. The overlay image containing all three CyDye gel images (available in dataset folder) was opened within ImageQuant ™ tools and cropped to remove extraneous detail from the edges of the gel. The resulting cropped image was then resaved as a dataset file and it was this file that was inserted into the DeCyder

software. It was beneficial to try and crop each gel to the same size to make analysis of gels easier and more accurate.

2.4.2.11 DeCyder

2D gel images of protein samples were initially investigated in the differential in-gel analysis (DIA) module, followed by batch processing and finally in the biological variation analysis module.

2.4.2.11.1 DIA module

The DeCyder DIA icon was double clicked and a new workspace created by clicking File: Create Workspace. In the earlier version of DeCyder (Version 4) only 2 gel images could be processed at the same time. DeCyder 5 however allowed the processing of all three gel images. In this section I will be discussing the use of DeCyder 5, as there are more steps to follow in this version, making use of DeCyder 4 fairly straightforward. The triple detection option is chosen when processing DiGE analytical gels, whereas the double detection option is used when processing single gel experiments and preparative gels.

This mode was used solely for the purposes of comparing between 2 samples and for processing comparative gels prior to insertion into the BVA module. In the window image that appeared the first image file (Cy3) was clicked on, followed by the 2nd (Cy5) in the case of the two gel comparison. In the case of the preparative gel the same image was inserted twice i.e. as both primary and secondary image, then gels were processed by selecting process: process gel images. The algorithm selection was the co-detection algorithm. A window appeared requesting an estimated number of spots. Typically 2500-3000 spots were estimated (see individual results section) and OK was clicked to commence the processing. When processing was complete the number of estimated spots was amended if required. Thus, gel images with protein spots not picked out by the software had too few protein spots estimated and the estimated number was subsequently increased. Conversely, protein spots split into more than one by the software had too many protein spots estimated and the

number was decreased. The resulting .xml file from processing of the gel images was exported into BVA if required (preparative gels only.)

2.4.2.11.2 Batch Processing

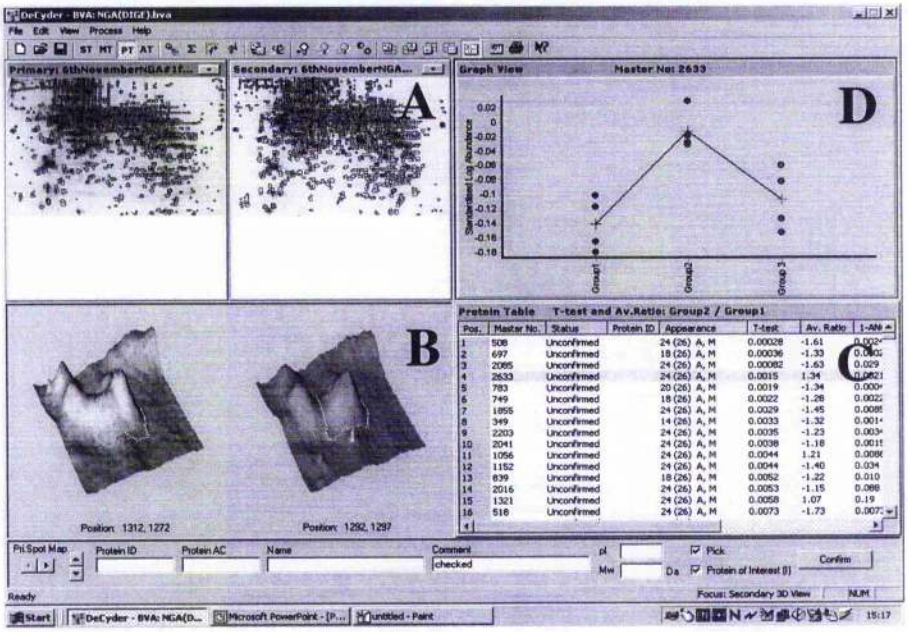
For a large number of gels, the DIA process was time consuming and labour intensive. Therefore, the DeCyder batch processor performed the gel processing automatically. Batch processing resulted in the generation of both .DIA and .BVA files.

2.4.2.11.3 Biological Variation Analysis (BVA)

The BVA file generated in the Batch processor was opened and standard (Cy2) gels were matched to a master gel (also standard Cy2). The master gel was selected by the Batch Processor and was most commonly the gel with the largest number of protein spots. It was possible to assign any Cy2 gel image as the master in situations where other gel images would result in a greater ease of matching (nicer spot pattern, less streaking etc.). To match the gel images a few (approximately 30) proteins were manually matched between the master standard (Cy2) image and the remaining Cy2 images then the automatic matching process was carried out by the software. Once the gels were matched, statistical analysis was undertaken, providing ratios and t-test values for each individual protein spot. Spots with t-test values $p<0.05$ were investigated further by checking that they were matched accurately across every gel. Once a definitive list of protein spots of interest had been compiled the appropriate preparative gel was imported into BVA. This gel was also matched to the master gel. Confirmed spots were assigned to be picked. The preparative gel was assigned as a pick gel and reference markers on this gel were identified. A picklist was generated from this gel image and exported as a text file in a format suitable for the Ettan spot handling workstation.

Spot picking, use of the Ettan spot handling workstation, mass spectrometry and database searching were all performed as previously described in sections 2.4.1.10 through 2.4.1.11.

Figure 2.6 BVA using DeCyder software



Screen view showing components of BVA analysis using DeCyder software. From gel images (A) individual contour plots are generated (B) representing individual protein spots in every gel. Abundance of these protein spots is present not only in tabular form (C) but also in graphical representation (D).

2.5 Drugs, solutions and buffers

2.5.1 Electrophysiology

(i) Drugs

Drug (MW)	Concentration (μM)	Quantity	Supplier
Urethane (89.09)	25%	25g in 100ml	Sigma, UK
D-AP5 (197.13)	50	5mg in 10ml*	Tocris, UK
Emetine (553.6)	20	11mg in 10ml*	Sigma, UK
Glutamate (183.6)	50	9.2mg in 10ml*	Sigma, UK

*Further diluted 1:10 in normal ACSF

(ii) Slice ACSF solutions

	Normal ACSF		**High sucrose ACSF**		
Salt	**Concentration (mM)**	**Quantity * (g/L)**	**Concentration (mM)**	**Quantity ** (g/L)**	**Supplier**
NaCl	124	72.47	87	5.084	BDH, UK
$NaHCO_3$	26	21.84	25	2.1	Fisher Scientific, UK
KCl	3	2.24	2.5	0.186	BDH, UK
$MgSO_4$	1	2.46	1	0.1725	BDH, UK
NaH_2PO_4	1.25	1.95	1.25	0.15	BDH, UK
Glucose	10	18.02	25	4.954	Fisher Scientific, UK
Sucrose	-	-	75	25.67	Fisher Scientific, UK
$CaCl_2$	2	2mls	0.5	0.5mls	BDH, UK

* Dissolved in 1L to make 10x stock solution. Stored at 4°C

** Dissolved in 1L to make 1x solution. Stored at 4°C

2.5.2 Proteomics

(i) Lysis buffer

	CHAPS buffer		ASB14 buffer		
	Concentration	Quantity (g/10ml)	Concentration	Quantity (g/10ml)	Supplier
Urea	8M	4.85	8M	4.85	BDH, UK
Tris	40mM	0.048	10mM	0.012	Sigma, UK
Magnesium acetate	-	-	5mM	0.011	Sigma, UK
CHAPS	4%	0.4	-	-	Sigma
ASB14	-	-	2%	0.2	Calbiochem, UK

Dissolved in double distilled (dd) H_2O. 1ml aliquots stored at -20°C

(ii) Ammonium acetate precipitation

	Concentration	Quantity	Supplier
Ammonium acetate*	0.1M	0.77g in 100ml	Fisher Scientific, UK
Ammonium acetate **	80% (w/v)	80mls 0.1M stock	
Acetone**	80% (w/v)	80mls stock	Riedel-De Hahn

* Dissolved in methanol. Stored at -20°C

** Diluted to 100mls in ddH_2O. Stored at -20°C

(iii) CyDye labelling

	Final Concentration	Quantity	Supplier
CyDyes (Reconstituted)*	1nmol/μl	25nmol added to 25μl DMF	Amersham Biosciences, UK
Working CyDye solution**	400pmol/μl	2μl reconstituted stock + 3μl DMF***	
Dimethylformamide (DMF) (>99.8% pure. <0.05% H2O)	-	-	GE Healthcare, UK
Lysine (182.6)****	10mM	0.018g in 10ml ddH_2O	Sigma, UK

* Stored at -70°C

** Stored at -70°c for 3 months

*** 5µl contains 2000pmol of fluor

**** Stored at 4°C

(iv) Sample buffer (2x)

	Final concentration	**Quantity**	**Supplier**
Urea	8M	4.85g in 10ml ddH2O	
ASB14	2%	0.2g in 10ml ddH2O	
DTT (154.2)*	20mg/ml	20mg in 1ml	Sigma, UK
IPG buffer*	2%	20µl in 1ml	GE Healthcare,UK

* Added fresh to stock 2 x sample buffer. 1ml aliquots stored at -20°C

(v) Rehydration buffer

	CHAPS buffer		**ASB14 buffer**		
	Concentration	Quantity	Concentration	Quantity	Supplier
Urea	8M	4.85g	8M	4.85g	
CHAPS	2%	0.2g	-	-	
ASB14	-	-	2%	0.2g	
Bromophenol blue	-	Few grains	-	Few grains	Sigma, UK
DTT*	5mg/ml	5mg to 1ml	5mg/ml	5mg to 1ml	
IPG buffer*	0.5%	0.5µl to 1ml	0.5%	0.5µl to 1ml	

1ml aliquots stored at -20°C

* Added fresh to 1ml aliquots

(vi) Equilibration buffer

	Equilibration buffer 1		Equilibration buffer 2		
	Concentration	**Quantity**	**Concentration**	**Quantity**	**Supplier**
Urea	6M	72.07g	6M	72.07g	
Tris (1.5M pH8.8)	50mM	6.7mls	50mM	6.7mls	
Glycerol (92.09)	30%	69mls	30%	69mls	Riedel – de Haen
SDS	2%	4g	2%	4g	BDH, UK
Bromophenol blue	-	Few grains	-	Few grains	
DTT*	10mg/ml	100mg in 10ml	-	-	
Iodoacetamide*	-	-	25mg/ml	250mg in 10ml	Calbiochem, UK

Stored in 10ml aliquots at -20°C

*Added fresh to 10mls of equilibration buffer

(vii) Bind silane solution

	Final concentration	**Quantity**	**Supplier**
Ethanol	80%	8mls	Fisher Scientific, UK
Glacial acetic acid	1.9%	200μl	BDH, UK
Bind Silane	0.1%	10μl	Amersham Biosciences, UK

Prepared fresh and made up to 10mls with ddH_2O

(viii) 12.5% homogenous gel solution

	Amount required (ml)	Supplier
Acrylamide-Bis(37.5:1)	375	Bio-Rad
1.5M Tris-Cl pH8.8	225	
ddH_2O	281	
10% SDS	9	
10% APS*	9	
10% TEMED*	1.25	Amersham Biosciences, UK

*Added fresh

Gel solution (made fresh) based on Laemmli, UK Nature 227, 680-685 (1970)

(viiii) Gel storage solution

	Final concentration	Quantity	Supplier
Tris-Cl (1.5M pH8.8)	0.375M	50ml	
10% SDS	0.1% (w/v)	2ml	

Made up to 200mls with ddH_2O. Stored at 4°C

(v) Displacing fluid

	Final concentration	Quantity	Supplier
Tris-Cl	0.375M		
Glycerol	50%	50ml	
Bromophenol blue	-	Few grains	

Prepared fresh. Made up to 100mls with ddH_2O

(vi) Agarose sealing solution

	Final concentration	Quantity	Supplier
SDS electrophoresis buffer	-	100mls	-
Agarose	0.5%	0.5g	Sigma, UK
Bromophenol blue	-	Few grains	

All ingredients were placed in a 250ml flask and mixed well. Mixture was then heated gently in a microwave until the agarose had completely dissolved. Stored at 4°C.

(vii) Tris/ glycine electrophoresis buffer

	Final concentration	Quantity	Supplier
Tris (121.14)	25mM	30.25g	Sigma, UK
Glycine (75.07)	192mM	144g	Fisher Scientific, UK
SDS (288.38)	0.1% (w/v)	10g	BDH, UK

Made up to 1L with ddH_2O for 10x stock solution. Stored at room temperature.

(viii) Fixing solution

	Final Concentration	Quantity	Supplier
Methanol (32.04)	10%	100mls	BDH, UK
Acetic Acid (60.05)	7.5%	75mls	BDH, UK

Made up to 1L with ddH_2O. Stored at room temperature.

(viiii) Sypro orange staining solutions

	Final concentration	Quantity	Supplier
Sypro orange*	1/5000	200μl	Molecular Probes, UK
0.05% SDS**	0.05%	5mls of 10% SDS	

* Made up to 1L in 7.5% acetic acid/ ddH_2O solution. Stored at 4°C in a light tight container

** Made up to 1L with ddH_2O

(x) Spot handling workstation solutions

	Final concentration	Quantity	Supplier
AmBic/ Methanol	50mM in 50%		BDH, UK
AmBic	20mM		BDH, UK
Trypsin			Promega
ACN/ TFA	50% in 0.1%		BDH, UK
ACN/ TFA	50% in 0.5%		BDH, UK
CHCA			Sigma, UK

(xi) Miscellaneous solutions

10% SDS: 100g in 1L ddH_2O stored at room temperature.

10% APS: 1g in 10ml ddH_2O prepared fresh.

10% TEMED: 500μl in 4.5mls of ddH_2O prepared fresh.

1.5M Tris-Cl: 181.7g in 1L ddH2O (Using 6M HCl to obtain pH 8.8) Stored at 4°C.

Chapter 3

Development of a global proteomics approach for the identification of proteins regulated by synaptic plasticity in the rodent hippocampus.

3.1 INTRODUCTION

The role of proteins and their regulation in synaptic plasticity, including synapse specific forms of plasticity such as LTP, has been subject to a great deal of research in many laboratories for over thirty years. In this time considerable advances have been made, not only in the knowledge associated with this field of research, but also in the tools that have been made available to the researchers.

One approach adopted by some researchers is to use cDNA microarrays to examine differential gene expression following plasticity or cognitive enhancement. For example, Thompson and colleagues in 2003 used microarrays to examine gene expression changes following the induction of LTP *in vivo* in the mossy fibre – CA3 pathway. More recently Hong *et al* (2004) investigated the induction of late response genes by NMDA receptor activation and identified those genes using gene chip technology. This approach has been used to look for changes in transcription in many other regions of the CNS out with the hippocampus for example in the spinal cord, prefrontal and visual cortices and auditory systems (DiGiovanni *et al.*, 2005; Kaiser *et al.*, 2004; Li *et al.*, 2004b; Lachance & Chaudhuri, 2004; Lomax *et al.*, 2000). It has also been used to investigate the effect of brain trauma (Li *et al.*, 2004b), drug treatment (Kaiser *et al.*, 2004; Yao *et al.*, 2004), spinal cord injury (DiGiovanni *et al.*, 2005) neuropsychiatric disorders (Lehrmann *et al.*, 2003), AD (Dickey *et al.*, 2003) aging (Blalock *et al.*, 2003; Prolla, 2002) and epilepsy (Elliott *et al.*, 2003) in terms of alterations in gene expression profiles.

Microarray techniques have enabled the global characterisation of multiple regions in the diseased brain (Katsel *et al.*, 2005), gene expression profiles in microdissected neurons from human hippocampal sub regions (Torres-Munoz *et al.*, 2004) and genes regulated by learning in the hippocampus (Leil *et al.*, 2003). However, microarray technology also presents certain limitations and challenges. In particular, the interpretation of microarray studies is complicated by the occurrence of alternative splicing and post-translational modifications, studies that focus on the expression of functional protein molecules provide complementary information to the array studies. In fact, combined approaches have been adopted by several research groups

(Paulson *et al.*, 2003; Yoshida *et al.*, 2001; Le Naour *et al.*, 2001; Kuo *et al.*, 2005) to increase the power of their observations.

Proteomics, the qualitative and quantitative comparison of proteomes, has been used to study protein expression within the CNS. Klein *et al* (2003) have explored protein regulation in the CA1 and CA3 regions of the hippocampus following hypoxia and an investigation by Khawaja *et al* (2004) used 2D gel electrophoresis to identify protein changes resulting from chronic antidepressant treatment. Many studies have used 2D gel electrophoresis followed by mass spectrometric analysis to investigate protein expression changes in mouse models of AD (Castegna *et al.*, 2002, 2003; Choe *et al.*, 2002; Kanninen *et al.*, 2004; Choi *et al.*, 2004). NMDA receptor blockade by MK801 using the aforementioned combination of cDNA microarrays and 2D gel electrophoresis (Paulson *et al.*, 2004) has also been investigated. The postsynaptic density, which has a vital role in the regulation of synaptic plasticity by integrating post-synaptic signalling as well as acting as a scaffold for the formation and maintenance of neuronal synapses, has also been the focus of attention with regard to proteomics. Yamauchi *et al* in 2002 investigated the molecular components and phosphorylation dependent regulation of the PSD and Husi *et al* back in 2000 detailed the many proteins associated with the NMDA receptor within this complex.

In relation to synaptic plasticity few proteomics studies exist to date. As previously mentioned Paulson *et al* investigated the effect of MK801 on protein expression in the thalamus, an approach used as a hypo-glutamatergic model of schizophrenia. Its relationship to plasticity in terms of the role of the NMDA receptor however is unclear since this study focussed on blocking NMDA activity rather than NMDA receptor activation. In contrast, the study by Husi *et al* (2000) into the molecules complexed with the NMDA receptor and highlighted a number of proteins that have previously been linked to synaptic plasticity and cognitive processes including PSD-95, protein kinase C and CaMKII (Migaud *et al.*, 1998; Malenka *et al.*, 1986; Malinow *et al.*, 1989).

Most of the previous studies such as those by Paulson and colleagues (2004) and Klein *et al* (2003) use standard 2D gel electrophoresis methods involving protein detection methods such as Coomassie blue and silver staining combined with

analyses that are unable to account for differences in gel to gel running. 2D gel methods such as these are, in some biological systems, not optimal where subtle changes in protein expression are expected. Within the last 10 years a new powerful proteomics tool, difference gel electrophoresis (DiGE) has been developed which counteracts many of the common problems associated with 2D gel electrophoresis, such as inconsistencies in gel running and subjective issues associated with the image analysis (Unlu *et al.*, 1997; Tonge *et al.*, 2001; Alban *et al.*, 2003). DiGE has so far been utilised by a relatively few research groups to examine differential protein expression in the CNS. In the visual cortex and striate cortex of the cat, proteins differentially expressed as a result of aging were identified (Van den Bergh *et al.*, 2003a, b). Cancer-specific protein markers have also been highlighted using this technique (Zhou *et al.*, 2002). In combination with transcriptomics and metabolomics (possible the next step in the 'omic' chain), DiGE was used to identify several components associated with mitochondrial dysfunction and oxidative stress which were found to be differentially expressed in brain tissue from schizophrenic subjects (Prabakaran *et al.*, 2004).

As yet, however, this progressive proteomics tool has not been adapted to investigate protein changes associated with synapse specific plasticity. Indeed, only a single study by Fazeli and colleagues (1993) has utilised 2D gel electrophoresis to investigate protein regulation, more specifically *de novo* protein synthesis following the induction of synaptic plasticity events such as LTP. This study, although elegant and progressive at its time of publication, suffered from certain limitations – namely it was unable to identify the proteins that were observed to have changed in expression. I have therefore chosen to use a comprehensive approach of 2D gel electrophoresis, DiGE and bioinformatics analysis which not only investigates protein regulation and expression relating to synaptic plasticity in the hippocampus but also allows the identification of such modulated protein molecules.

3.2 STUDY AIMS

To investigate protein regulation associated with synaptic plasticity-related events in the rodent hippocampus, the development of a global proteomic approach was required. The specific aims of the experiments detailed in this chapter were thus:

1. To develop an optimal protein extraction method for use with hippocampal tissue.

2. To purify hippocampal protein extracts including the removal of interfering substances and to subsequently quantify protein extract concentration from hippocampal tissue.

3. To resolve the proteins which form part of the hippocampal proteome using 2D gel electrophoresis.

4. To develop a statistically robust and quantifiable 2D gel electrophoresis approach (DiGE) and optimise it for use with the hippocampal slice preparation extracts.

5. To validate the DiGE proteomic method by the comparison of multiple replicates of the same sample and to compare protein expression levels across the somato-dendritic axis.

3.3 METHODS

Detailed methodology is provided in Chapter 2 of this thesis. All experimental tissue used in this chapter was obtained from male ICR mice (aged 4-8 weeks). Standard 2D gel electrophoresis optimisation was performed on hippocampal protein extracts run on pH 4-7 IPG 1st dimension gels as were initial DiGE optimisation experiments and sub fractionation experiments. DiGE validation (same sample) experiments were run on pH 3-10NL gradient gels.

Table 3.1 Experimental design used for DiGE preferential labelling experiments

Gel	Cy2	Cy3	Cy5
1	Standard	Standard	Standard
2	Standard	Standard	Standard
3	Standard	Standard	Standard
4	Standard	Standard	Standard

Table detailing the experimental design for investigating the possibility of preferential labelling of particular proteins from hippocampal CA1 regions using DiGE, DeCyder DIA and DeCyder BVA. Standard samples (pooled tissue divided into 12) were labelled with Cy2 and in this instance Cy3 and Cy5.

Table 3.2 Experimental design used for hippocampal pyramidal cell sub-fractionation experiment.

Gel	Cy2	Cy3	Cy5
1	Standard	Dendritic (n=1)	Dendritic (n=4)
2	Standard	Dendritic (n=2)	Dendritic (n=3)
3	Standard	Somatic (n=3)	Somatic (n=2)
4	Standard	Somatic (n=4)	Somatic (n=1)

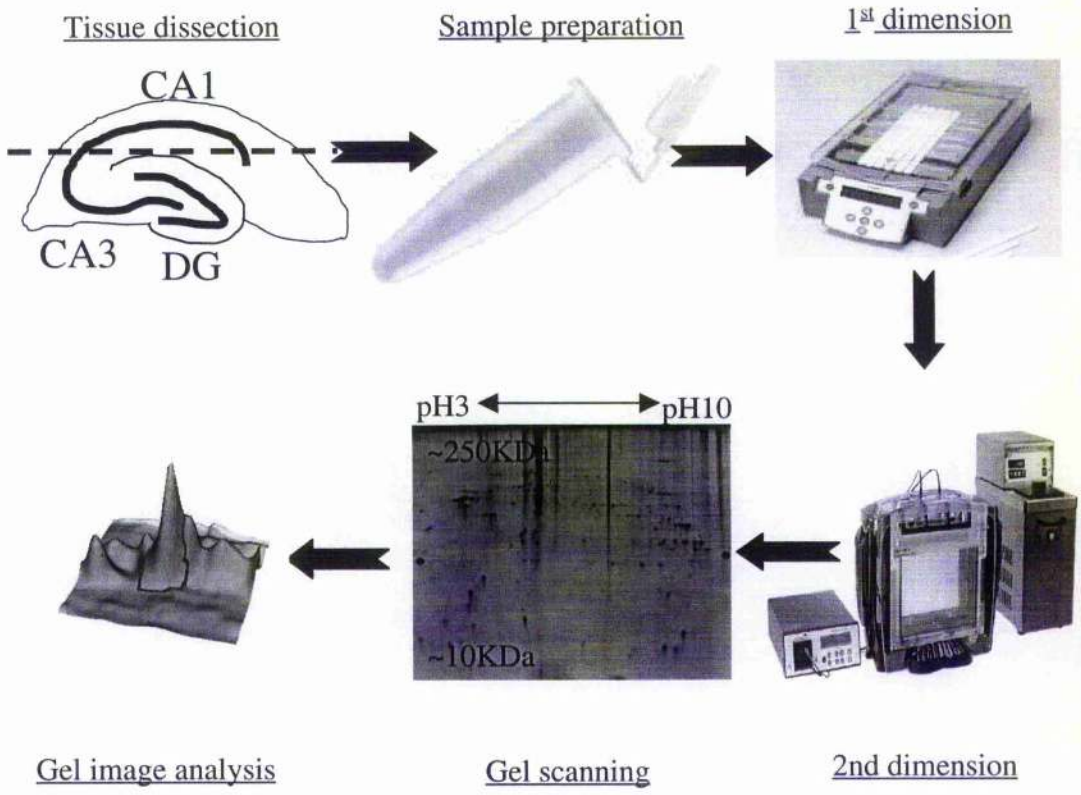
Table detailing the experimental design for comparison of the protein content of the dendrites to the cell bodies of hippocampal CA1 pyramidal cells using DiGE, DeCyder DIA and DeCyder BVA. Standard samples were labelled with Cy2 and in this instance dendritic samples were labelled with Cy3 and somatic samples with Cy5. A 'dye flip' was used to eliminate any bias that may occur in labelling.

3.4 RESULTS

3.4.1 Optimisation of standard 2D gel electrophoresis for use with hippocampal slices

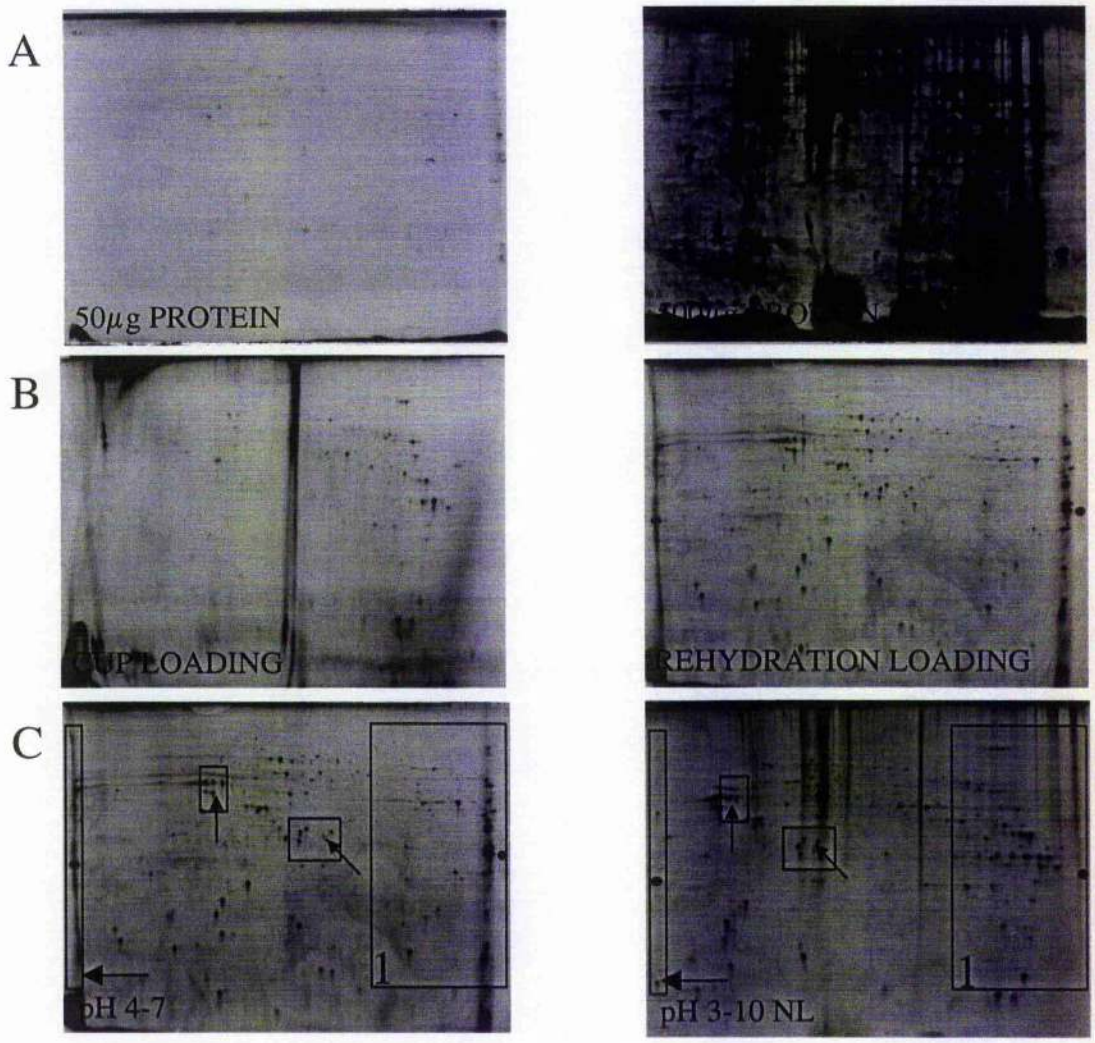
Following on from the electrophysiology experiments a proteomic analysis model was developed to evaluate the proteome of the rodent hippocampal CA1 region before and after synaptic plasticity events such as LTP or glutamate receptor activation. CA1 regions dissected free from surrounding hippocampal tissue were lysed with buffer to extract protein and this sample was run on a 2D gel to separate the proteins according to their pH in the first instance and subsequently by separation on the basis of their molecular weight. Figure 3.1 illustrates this procedure to help visualise the entire process from slice to gel. Although a fairly straightforward process, a large degree of optimisation was required to perfect the process and to obtain 2D gels suitable for analysis. Initial optimisation concentrated on the amount of protein required to run a successful 2D gel. It was found that protein amounts less than 100μg resulted in little or no protein on the 2D gel image whereas concentrations around 500μg caused overloading of the gel and many proteins precipitated from the gel (Figure 3.2a). A protein level of 150μg was found to be optimal.

Figure 3.1 Proteomic analysis of hippocampal proteins using 2D gel electrophoresis



Schematic diagram illustrating the process by which hippocampal slices undergo 2D gel electrophoresis. CA1 regions from 400μM thick hippocampal slices are dissected free, (dotted line), and treated with buffers to separate out and purify proteins. Protein samples then undergo initial separation according to their charge (1st dimension), followed by further separation according to their molecular weight (2nd dimension). Gels are stained with fluorescent dye and scanned. Resulting gels contain thousands of separated protein spots covering a wide pH and MW range.

Figure 3.2 Optimisation of the proteomic analysis of hippocampal CA1 regions using 2D gel electrophoresis.



Optimisation of the proteomic analysis of the CA1 region of rodent hippocampal slices. Protein samples were run on large format polyacrylamide gels. A. The appropriate amount of protein to be loaded onto each gel was investigated. Too little (50μg) resulted in no protein spots, too much (500μg) resulted in over saturation of the 2D gel. B. Gel images resulting from rehydration and cup loading of 150μg of protein sample. Cup loading resulted in a precipitation of the protein sample and little separation of protein in the area where the cup was placed. Rehydration loading on the other hand produced a clear, well separated proteomic map of hippocampal proteins. C. Samples could be separated along a pH 4-7 or pH 3-10 (NL) gradient. pH 4-7 produced greater separation in the more central areas of the 2D gel, but resulted in the precipitation of acidic and basic proteins. pH 3-10 (NL) did not separate the protein samples sufficiently in the central gel region, but did allow the visualisation of proteins at the extreme ends of the 2D gel.

Two methods are commonly used in 2D gel electrophoresis to load protein samples into the 1st dimension gel – cup loading and rehydration loading. Depending on your sample type and composition one method would generally be preferential over the other. Thus, cup loading is preferential for samples with an abundance of basic proteins or samples containing high amounts of DNA. Cup loading our CNS derived protein sample in the central region of the 1st dimension IPG strip resulted in precipitation of the sample from the cup as can be seen by the black streak through the middle of the gel in Figure 3.2b. Rehydration loading on the other hand resulted in clear and even gel images, best suited to accurate and efficient proteomic analysis.

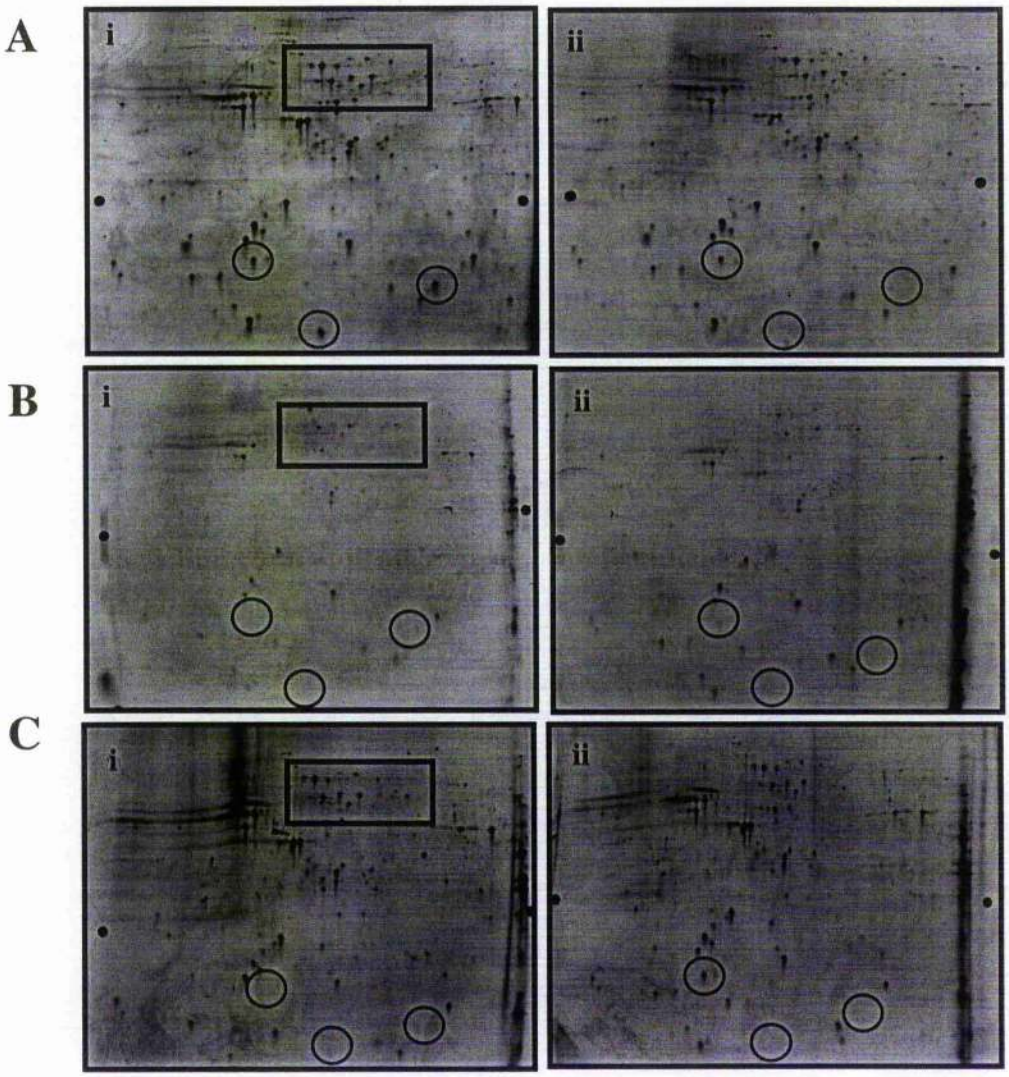
The pH range over which the proteins are separated can be also be varied due to the availability of several different IPG strips and corresponding IPG ampholytic buffers covering a range of pH gradients. Running brain samples over a narrow pH range (pH 4-7), whilst separating out the proteins more efficiently in the central region of the gel resulted in the loss of very basic and acidic proteins as is highlighted by the rectangle on the left hand side of the gels in Figure 3.2c (i). This precipitation of protein at the extreme ends of the gel also caused disruption to adjacent proteins. Samples run on a wider pH gradient, pH 3-10 (NL) however, did not display this loss (Figure 3.2c (ii)), but did result in a lesser degree of resolution in the central region of the gel (Figure 3.2c (iii)) thus a trade off between adequate separation and the restricted analysis of sub-fractions of proteins present, an issue which complicates differential expression analysis, mass spectrometry and protein identification.

3.4.2 Development of DiGE for investigation of differential protein expression in the rodent hippocampus following synaptic plasticity

Initial proteomics experiments examined the effect of glutamate treatment of CA1 rat hippocampal protein expression using standard 2D gel electrophoresis methods. Analysis was made using pair wise comparisons between sets of gels. The example in Figure 3.3 illustrates the inconsistencies associated with this type of analysis. Protein expression between glutamate treated (Ai, Bi, Ci) and control (ACSF) tissue (Aii, Bii, Cii) was compared in three sets of gels. The first and third set of gels (Figure 3.3Ai, ii) show an abundant expression of protein over the entire gel region in contrast to the second set (Figure 3.3Bi, ii) which appear to have a much reduced

protein level. Although the samples loaded were of different origin for each gel, the amount of total protein was exactly the same. Not only do large regions of gels differ in their protein expression (Figure 3.3, black box) but individual protein spots also differ. Thus, individual protein spots were either present in both gels within a set (Figure 3.3a, black circles), in one gel of a set (Figure 3.3b, black circles) or were absent in both gels of a set (Figure 3.3c, black circles). In some cases particular proteins were only present in a single gel (Figure 3.3, white circles). The question of modest changes in protein expression was not addressed using this method.

Figure 3.3 Comparison of protein expression from glutamate and control (ACSF) treated hippocampal CA1 regions using standard 2D gel electrophoresis analysis.

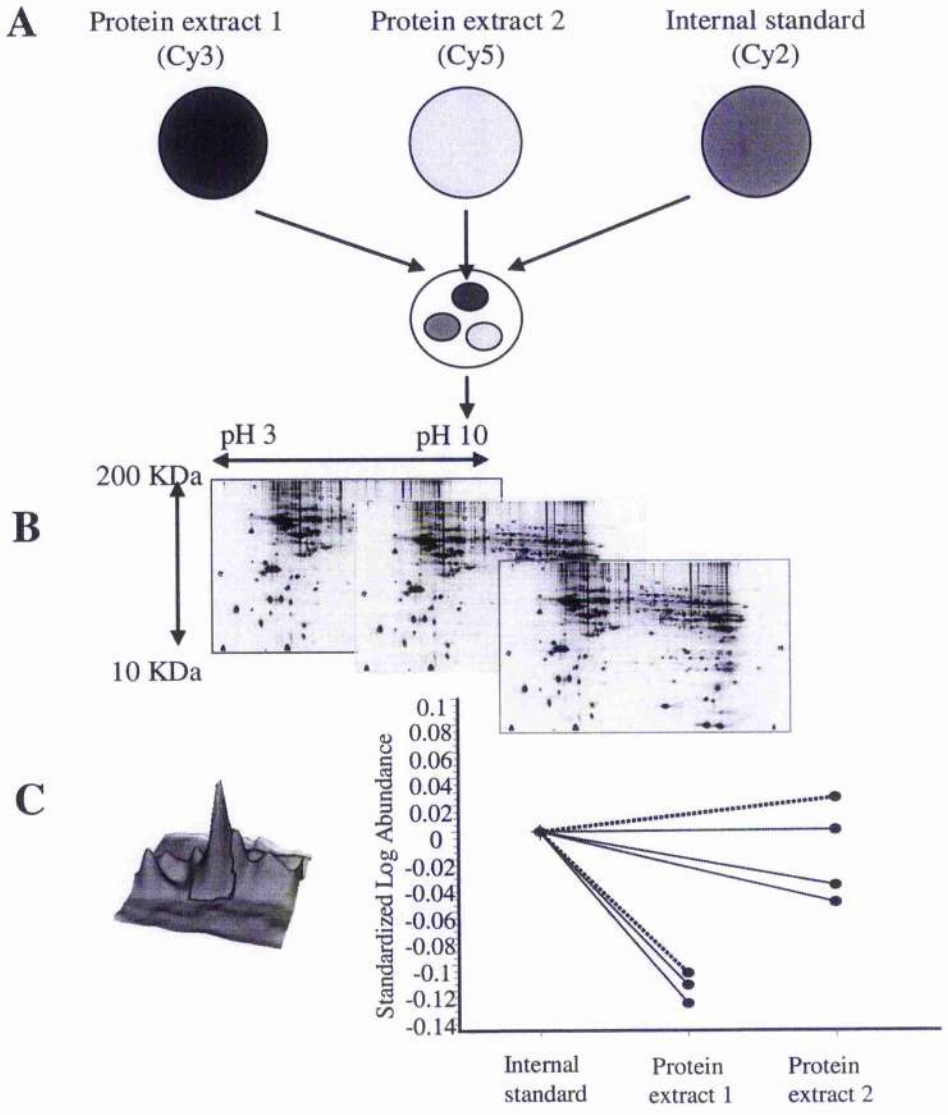


Comparison of glutamate treated hippocampal CA1 regions with control (ACSF) CA1 regions. Gel images of control (Ai, Bi & Ci) and glutamate (Aii, Bii & Cii) treated tissue show very different expression profiles not only between treatments (Ai versus Bi) but also across gel pairs (A versus B versus C). Protein spots can be found in one pair of gels, but absent in all the others (white circles), whilst other protein spots are present in either both gels of the pair, neither gels of the pair or in one gel from a pair (black circles). Not only does the expression of individual spots vary, but entire gel regions are either devoid of protein or show extremely low protein expression (black box).

To allow for a more accurate investigation into changes in protein expression DiGE was employed in which different treatment groups of hippocampal protein samples could be labelled and separated on a common gel (Figure 3.4). Standard, control and treated extracts were labelled with 3 spectrally resolvable CyDyes (Cy2, 3 or 5) and pooled together prior to 2D-gel electrophoresis separation. Using a typhoon imager, individual gels were scanned at three different wavelengths and 3 separate gel images were generated. These images were then analysed using DeCyder software, which detected the total number of spots on each gel and calculated the abundance and the corresponding fold change between treated and control protein extracts. Contaminants such as salts and lipids that would interfere with CyDye labelling of certain proteins were removed. Prior to this clean up process 1320 spots were detected on a single gel. Following purification of the protein extract using an ammonium acetate precipitation protocol an average of 2838 spots were detected by DeCyder software indicating an improved labelling process (Figure 3.5a(i)).

Polyacrylamide gel quality was also an important factor in the overall success of the DiGE process. The over compression of gels whilst casting resulted in the distortion of the gels, which is visible on the bottom right hand corner of the gel in Figure 3.5a (i). Releasing the pressure in the caster prevented this problem from occurring. Measures were also taken to reduce the amount of dust present in a gel. Protein spots contaminated by dust particles contained within the gel solution and on the gel plates were excluded from the analysis by DeCyder. A reduction in contamination from dust and dirt particles increased the number of protein spots available for differential analysis (Figure 3.5a (ii)).

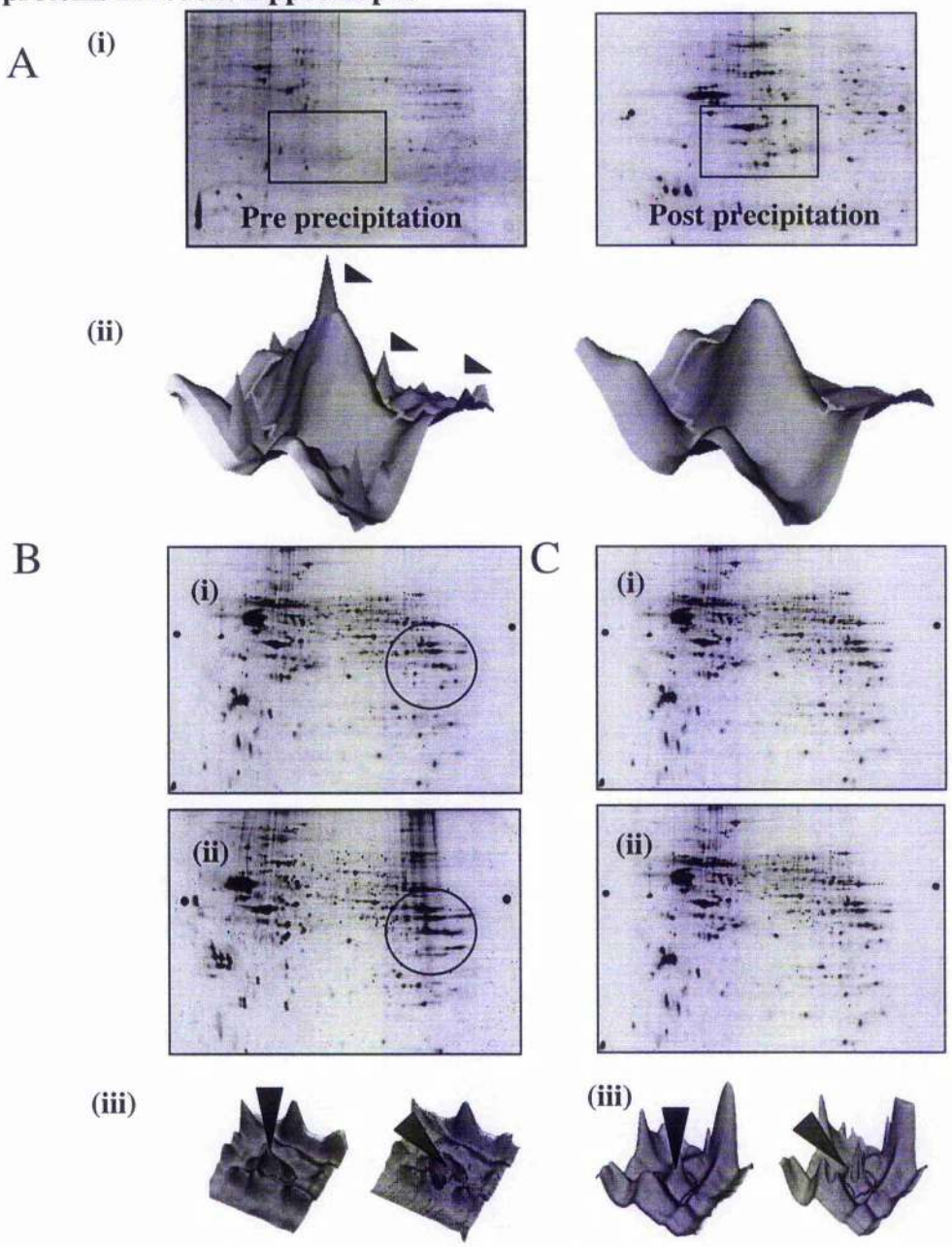
Figure 3.4 DiGE methodology



Schematic diagram of the DiGE process. A. Individual protein extracts are labelled with either Cy3 or Cy5 and a pooled representation of all experimental samples (standard) labelled with Cy2. B. All labelled extracts/ standards are pooled and separated on a single 2D gel and scanned to reveal three separate images. C. Scanned images are transferred to DeCyder software analysis programme for the generation of 3D images and graphical representations of differential protein expression levels.

Spots identified as differentially expressed following DeCyder analysis were picked from the 2D gel by the spot handling workstation and analysed by mass spectrometry. The type of gel from which these spots were picked was important in determining the accuracy and success rate of protein identification. Thus, using a separate preparative gel, loaded with up to 500μg of protein and over stained with Sypro orange made the gel matching process and subsequent spot picking accuracy inefficient (Figure 3.5b). In contrast, use of a DiGE analytical gel loaded with excess unlabelled protein not only provided sufficient protein content for mass spectrometric analysis but also simplified the gel matching process due to the near identical spot patterns, resulting in the accurate picking of selected protein spots from a 2D gel (Figure 3.5c).

Figure 3.5 Development of DiGE for identification of differentially expressed proteins in rodent hippocampus

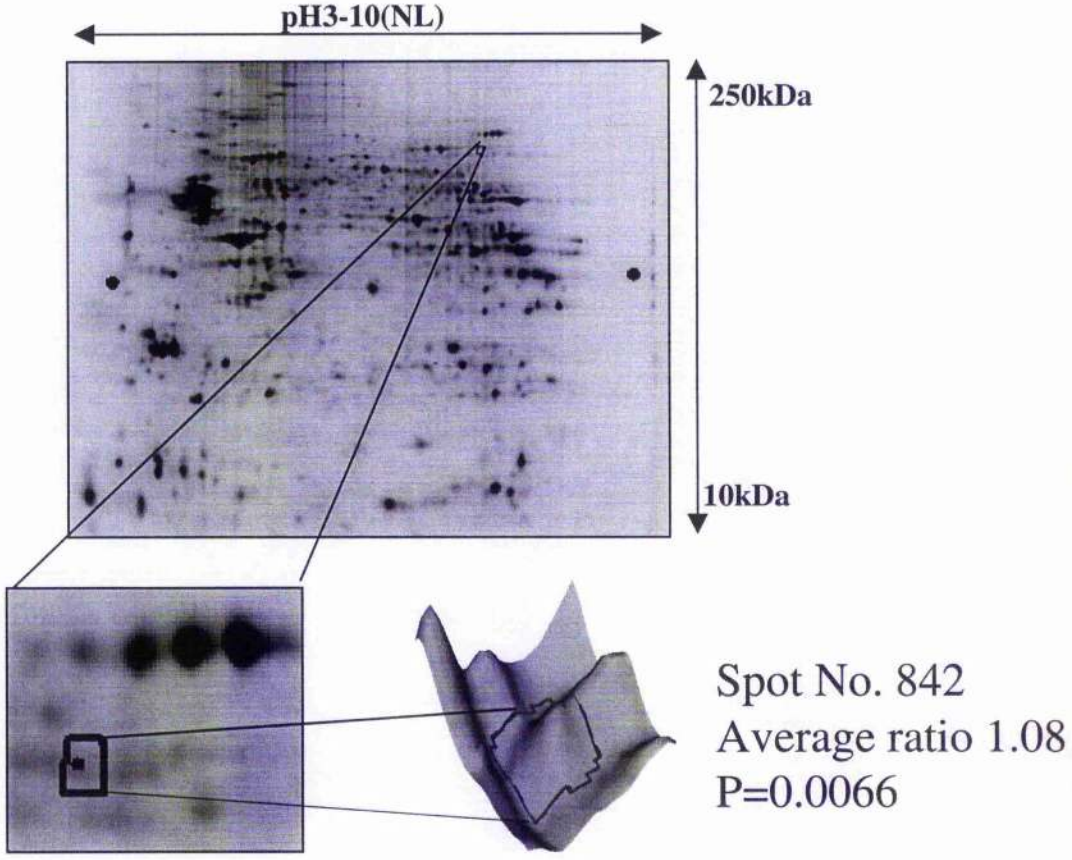


(A) Increased protein labelling was achieved following sample clean up using an ammonium acetate precipitation (i). Improved gel quality (Ai, black rectangle) was achieved by not over compressing the gel caster and by keeping the gel formation process as clean as possible (Aii) to remove dust particles (arrow heads) that result in the elimination of protein spots from analysis. (B) Separate preparative gels were often difficult to match to the original analysis gel (black circle) (Bi) resulting in poor gel matching and poor spot picking accuracy (pick spot outline in pink in 3D view). DiGE analytical gels loaded with excess unlabelled protein were easily matched due to the near identical spot patterns (Bii) and allowed for accurate spot picking (pick spot outline in pink). Black arrowhead indicates protein spot requiring picking. Pink red arrowhead indicates protein spot actually picked.

3.4.3 Preferential labelling of particular hippocampal proteins

Preferential labelling of certain proteins by either Cy3 or Cy5 would lead to the generation of false positive results. To determine whether any labelling bias occurs when labelling protein extracts from hippocampal tissue a 'same sample' experiment was performed. A protein sample prepared from CA1 regions of hippocampal slices was divided into 12 aliquots. 4 of these were labelled with Cy2, 4 with Cy3 and the remaining 4 with Cy5. Following 2D gel electrophoresis, gel scanning and image analysis using DeCyder software, only one from the 2300 protein spots detected, emerged as being significantly ($p=0.0066$) differentially expressed (Spot number 842, fold change 1.08) (figure 3.6).

Figure 3.6 Investigation into possible preferential labelling of hippocampal proteins using DiGE

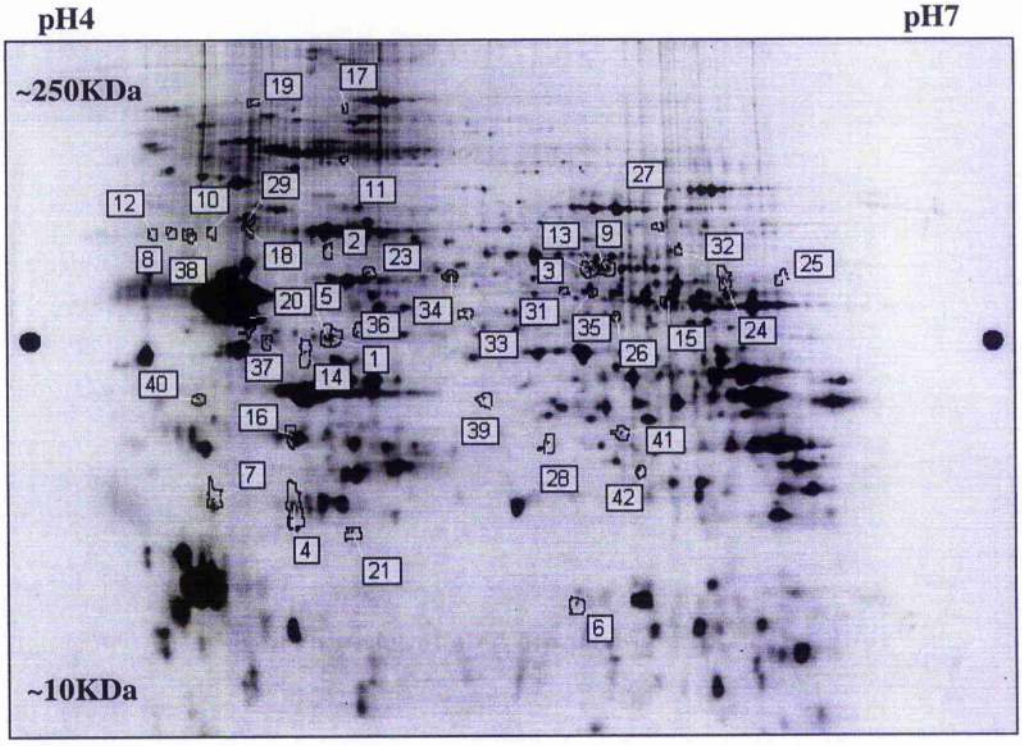


Same sample analysis to detect any CyDye labelling bias in hippocampal protein samples. From the 2300 ± 102 protein spots labelled and detected by DeCyder software, only one (spot number 842) showed a significant (p=0.0066) difference in expression (Average ratio 1.08) indicating that preferential labelling is at its minimum and the generation of false positives using hippocampal protein extracts is not a significant problem.

3.4.4 Sub-fractionation of hippocampal CA1 pyramidal cells

Dendritic regions from CA1 pyramidal cells were dissected free from their corresponding cell bodies, and the two separate fractions were subjected to DiGE and DeCyder gel analysis. It was hoped that this crude sub-fractionation would go some way to reducing the complexity of the protein sample, as well as provide a method which could subsequently be used to determine the locality of differential protein expression in different plasticity systems. There was no significant difference between the relative numbers of protein spots between the two different fractions (dendritic: 2745, Somatic: 2759). That said DiGE analysis highlighted 42 protein spots that were significantly different between dendritic and somatic regions. Approximately half of these, (22), showed decreased dendritic expression compared to the cell body with fold changes ranging from –2.01 to –1.11 (average fold change –1.35 ± 0.04). The remaining 21 were increased in their dendritic expression levels ranging from fold changes of 1.18 to 2.16 (average fold change 1.48 ± 0.05). Of these 42, 18 have been identified by Maldi-tof mass spectrometric analysis (Table 3.3).

Figure 3.7 Analysis of subfractionated hippocampal tissue by DiGE



2D gel image of dendritic hippocampal proteins separated according to their pI in the first instance (pH4-7) followed by separation by their molecular weight (approximately 250,000Da – 10,000Da). Annotations represent regulated protein spots as identified by DiGE and DeCyder image analysis.

Table 3.3 List of protein spots differentially expressed between dendritic and somatic regions of hippocampal CA1 pyramidal cells.

Spot # [1]	Master # [2]	Protein I.d. [3]	T-test [4]	Fold change [5]	Mass (Da) [6]	NCBInr Acc # [7]	Mowse Score [8]
1	1980		0.0034	-2.01			
2	1558		0.0057	-1.62			
3	1600		2.30E-05	-1.47			
4	2464	Syntaxin 1A	0.0078	-1.47	33205	gi 15011853	141
5	1977	Guanine deaminase	0.0043	-1.46	51494	gi 6753960	182
6	2595		0.0057	-1.44			
7	2460		0.0097	-1.44			
8	1432		0.0042	-1.42			
9	1602	CRMP2	4.10E-05	-1.4	62531	gi 3122040	95
10	1438		0.0075	-1.4			
11	949		0.00031	-1.35			
12	1417		0.0018	-1.35			
13	1587	Matricin	0.00058	-1.33	61021	gi631730	73
14	2039		0.0019	-1.25			
15	1779	GAD-65	0.0046	-1.21	66037	gi 1352217	65
16	2291	Isocitrate dehydrogenase 3	0.009	-1.21	42453	gi 18700024	100
17	598		0.0086	-1.2			
18	1442	Hsc 70	0.00065	-1.18	73701	gi 1072476	82
19	483		0.0011	-1.17			
20	1898	ATP synthase (A)	0.0031	-1.14	68567	gi1718086	163
21	2502	Prohibitin	0.0076	-1.12	29804	gi 6679299	82
22	1257		0.0026	-1.11			
23	1642	TCP 1, theta subunit	0.0041	1.18	60088	gi1174621	122
24	1645		0.0087	1.2			
25	1659		0.0076	1.21			
26	1853	tryptophanyl/ tRNA synthase	0.0026	1.24	77072	gi 12856926	154
27	1346		0.0094	1.29			
28	2316		0.0048	1.31			
29	1362	Hsc70	0.00092	1.33	73701	gi 1072476	102
30	1455		0.0033	1.39			
31	1715	Synapsin 2	0.00094	1.4	52818	gi 8567410	69
32	1513		0.002	1.42			
33	1852	hnRNPK	0.00092	1.45	51230	gi 3384620	97
34	1640	Protein disulphide isomerase	0.00015	1.46	57894	gi 56872	88
35	1702	Synapsin 2	0.002	1.46	52818	gi 8567410	152
36	1949	GFAP	0.007	1.49	49935	gi 319914	126
37	531		0.00052	1.52			
38	1986	calcineurin	0.0035	1.53	34261	gi26338027	70
39	1467		0.006	1.54			
40	2173		0.0018	1.64			
41	2198		0.00043	1.87			
42	2271	Sirtuin 2	0.0022	1.99	40155	gi12851673	85
43	2376		5.70E-06	2.16			

Table listing the protein spots differentially expressed between somatic and dendritic regions of area CA1 pyramidal cells, following in-depth biological variance analysis. [1] Spot number corresponds to the number annotated on the gel image in figure 3.7 [2]

Master number is allocated to each individual protein spot by DeCyder BVA software. Those protein spots significantly differentially expressed [4 & 5], $p<0.01$, were subjected to mass spectrometric analysis for protein identification [3] by Maldi - tof mass spectrometry. Mascot database searching generated protein identities, mass [6] and NCBI accession numbers [7], [8] Mowse scores greater than 61 for PMF were thought to be significant hits.

3.5 DISCUSSION

The amalgamation of LTP and proteomics is one not previously undertaken, although several other researchers have employed variations on this theme (Fazeli *et al.*, 1993; Thompson *et al.*, 2003). The individual technical components however are well established and it was on this basis that this combination of approaches was chosen to investigate protein regulation associated with synaptic plasticity in the rodent hippocampus.

Proteomic investigation initially required the optimisation of standard 2D gel electrophoresis methods for use with hippocampal tissue. The main issue associated with the application of proteomics to hippocampal tissues that was perhaps not clear from the results section of this chapter was the limited availability of tissues for analysis. Hippocampal slice experiments such as those previously described yield small amounts of tissue and even smaller amounts of purified protein (approximately 0.5mg/ml of protein per CA1 subfield). This proved to be a limiting factor in these studies. To maximise the relative proportion of potentiated tissue area, two stimulation electrodes were used to target as many afferent fibres as possible. Nevertheless protein concentration was still a limiting factor and pooling of 3 or 4 slices was required to produce sufficient protein for proteomic analysis using 2D gel electrophoresis. In this respect the concentration of the protein sample is important for several reasons. Firstly, the simplest reason is visualisation on the 2D gel (Figure 3.2a). The volume of sample that can be loaded onto the first dimension is limited and if a sample is too dilute then the total amount of protein loaded onto a gel is greatly reduced and proteins are not visible on the gel. Overloading of a gel is clearly a problem, but one that can be easily remedied. Secondly, mass spectrometric analysis requires a certain amount of protein to be present within individual spots for successful protein identification. A minimum of 300μg of protein is generally required for the identification of relatively abundant protein spots, which initially requires a fairly high concentration of sample (close to 5mg/ml). For those proteins of low copy number in a cell, loading higher amounts of total protein does not necessarily correlate with an increased probability of identification. Indeed, increased protein loading will result in precipitation of many proteins from the sample in the 1st dimension. Finally, when labelling protein samples with fluors such as the CyDyes

used for DiGE, dilute samples can result in poor labelling as the NHS ester moiety is degraded.

The 1st dimension of 2D-gel electrophoresis has many factors that also required optimisation. Sample loading can be carried out by two methods – cup loading and rehydration loading. Cup loading is known to be preferential for separating samples with high DNA content, and for better resolution of basic proteins. When using this method for hippocampus-derived proteins it appeared to be unsuccessful in that a large proportion of the protein spots normally visible in a 2D gel were not present following cup loading of our protein sample. In addition, precipitation of proteins resulted in a large degree of streaking across the 2D gel. There are several reasons for this. As previously mentioned the sample being used here was dilute and therefore requires a large volume to be loaded into the sample cup and onto the first dimension. Also, with cup loading the nature of the proteins within a sample determines the positioning of the sample cup, whether it is at the basic or acidic end, or both. When using cup loading in these experiments the sample cup was placed in the central region of the gel (acidic), this resulted in the precipitation of the sample (Figure 3.2b). Further investigation using a more concentrated sample and varying the positioning of the sample cup may well have generated clearer and more agreeable gel images. However because the rehydration loading method proved to be successful, further investigation was not thought to be necessary. That said, for in depth analysis of basic proteins, which appear highly abundant in our sample, cup loading would perhaps be the best approach.

The nature of the electrophysiology experiments also meant that only a sub population of afferents were stimulated and hence only a proportion of the hippocampal neurons potentiated. Isolation of these cells was not possible, although other groups have used techniques such as laser capture micro-dissection to combat problems such as this (Banks *et al.*, 1999) although again this has limitations, namely the amount of cells required to obtain sufficient protein for analysis. Another possible solution would be to bulk stimulate the hippocampus as is sometimes done *in vivo* when investigating LTP. This would result not only in the stimulation of a larger proportion of synaptic connections, but would also provide a greater abundance of hippocampal tissue.

Another issue of substantial importance is the degree of separation of the individual proteins on a 2D gel. This is important in that many of the regulated proteins may be 'hidden' beneath larger, more abundant proteins. Also, individual proteins do not always resolve sufficiently causing problems in subsequent gel and mass spectrometric analysis. For example, a particular protein spot may appear to increase in abundance, but if that spot contains multiple proteins then the question arises as to which one or ones are responsible for the change? Secondly, identification of all of the proteins contained within a particular spot generates a large number of false positives, none of which can be discounted until validation studies are performed. One way to overcome this problem is to use different pH gradients in the 1st dimension. Gradient gels exist from wide ranging (pH3 – 10) to 'zoom' gels (down to individual pH ranges) to intermediate gradients (pH 4 – 7). As well as increasing the resolution of proteins within a sample narrower pH gradients allow an increased amount of protein to be loaded onto a gel, thereby increasing the protein content of spots which facilitates successful mass spectrometric analysis. Hippocampal protein samples were run on both pH3-10 (non-linear) and pH4-7 gradients. Whereas pH3-10 displayed the widest range of proteins, resolution, particularly in the central region of the gel was not as high as when samples were run on pH4-7 strips. Initial 2D studies carried out before the implementation of DiGE used pH4-7 to help resolve proteins in the central region of the gel. Subsequent studies however used pH3-10 strips since this provided a more global representation of proteins (including the basic proteins that precipitate when using pH4-7, see figure 3.2c) and to maximise the availability of proteins for analysis. As well as resulting in the loss (precipitation) of very acidic and a large proportion of basic proteins when samples were run on pH4-7 IPG strips, the precipitation of these proteins also causes a distortion of the gels in adjacent regions causing further difficulties in gel matching and analysis. Resolution problems could be further addressed in the future by using the zoom gels previously mentioned to focus in on particular spots. Other options include the use of gradient gels to increase the separation in the 2nd dimension or running different gradient concentrations of homogeneous gels – a procedure that would be time consuming, and in terms of running DiGE experiments, costly.

Finally, in order to visualise the separated proteins 2D gels had to be stained with either a Coomassie blue, fluorescent or silver stain. Coomassie staining (including

the more sensitive colloidal Coomassie) was fairly unsuccessful for our samples as the amount of protein typically loaded onto a gel (150μg) was insufficient for successful Coomassie staining. Typically this type of stain would only result in the visualisation of the most abundant proteins (approximately 10-20 protein spots, data not shown). Fluorescent stains such as Sypro orange and Sypro ruby however were sensitive enough to detect much larger numbers of proteins and presumably protein entities at much lower abundance across the 2D gel.

Initial investigations into protein regulation resulting from or in synaptic plasticity in the hippocampus were performed using the optimised 2D gel methods previously discussed. Glutamate treatment of hippocampal slices was used as a method to stimulate neurons en masse. Proteomes of samples extracted from glutamate treated hippocampal slices and control (ACSF) treated slices were compared to determine any changes in expression. It was only possible to identify proteins that either appeared (or disappeared) as a result of treatment as gel running, staining and imaging differences made the investigation of subtle changes in expression difficult and meaningless. The example shown in figure 3.3 of the results section highlights these inconsistencies. Not only were entire regions different, but individual protein spots also varied greatly. As such, even the detection of new or degraded proteins appeared to be unreliable. If this method were to be used efficiently it would require a very large number of replicates to be confident in the results. Even so, this method is still commonly used and is more suited to systems where large fold changes in protein expression are expected or where the system under investigation is less complex. Thus, a more robust and reliable model was required to identify protein changes thought to occur as a result of synaptic plasticity in the hippocampus and as a result, protein expression analysis progressed to using DiGE.

The pre electrophoretic labelling of samples and the running of these multiple samples within a single gel along with the inclusion of an internal standard in conjunction with powerful gel analysis software provides a robust and powerful proteomics system with the ability to quantify changes in protein expression with statistical confidence (Illustrated in figure 3.4). DiGE is a relatively new proteomics tool that helps to combat many of the issues that hamper proteomics research such as running difference, time consuming analysis and lack of standardisation. In order to

use this methodology for our studies additional optimisation was required over and above that needed for the standard 2D gel methods. Initial labelling of hippocampal protein extracts was poor (fewer protein spots detected, large degree of gel distortion) as a result of the high lipid content of this type of tissue (1320 Spots detected when normally around 2800 detected, Figure 3.5a). To combat this, contaminants such as lipids and salts were removed using an ammonium acetate precipitation method. Other more common precipitation methods such as TCA, acetone and Amersham's 2D clean up kit were less well tolerated and all resulted in a severe loss of total protein (typically more than half) from our sample. As concentration was already a concern any further loss of protein was unacceptable. Thus, ammonium acetate precipitation was deemed the most suitable method for this type of preparation resulting in very modest (10-20%) protein losses.

Another factor in the overall success of the DiGE process is the quality of the polyacrylamide gels. As can be seen in figure 3.5b distorted gel images make for difficult gel matching and analysis. With careful preparation these problems can be avoided although such efforts are extremely time consuming and laborious. Until pre-cast low fluorescence gels are made available this process will remain an arduous but necessary task.

The generation of false positive results due to a labelling bias for particular proteins is a contentious issue associated with DiGE. To address this concern an experiment was designed in which the same sample was split into 8 individual samples and protein expression was compared between them. As a result of this 'same sample' experiment only one protein spot out of approximately 2300 spots detected was identified as significantly different and hence preferentially labelled following DiGE suggesting that labelling bias was not a major problem in terms of hippocampal protein samples. In addition, even this protein spot shows a very modest change.

Spot resolution was briefly touched upon when discussing 2D gel optimisation. As well as the methods previously discussed another possible solution to this problem was addressed using DiGE. Sample pre-fractionation and sub-fractionation (for example the extraction of cell components such as mitochondria by centrifugation in a high sucrose density gradient), is commonly used as a method of simplifying

complex protein samples (Cordwell *et al.*, 2000; Yuan & Desiderio, 2005; Krapfenbauer *et al.*, 2003). In relation to this, one of the major issues associated with synaptic plasticity in general and more specifically LTP in the hippocampus is the cellular location of proteins that may be regulated in the plasticity process. Evidence for localised (dendritic) and somatic protein synthesis exists. In an attempt to elucidate the locality of these changes whilst at the same time reducing the complexity of the protein sample, dendritic regions were isolated from neuronal cell body regions and DiGE performed on the isolated fractions. DiGE of these two neuronal sub regions yielded 42 proteins spots that exhibited significant ($p<0.01$, Student's t-test) differentiation between the 2 different regions indicating a certain degree of sub fractionation. The proteins identified included an enrichment of synaptic proteins such as synapsin c (increase of 1.46 fold, $p=0.002$), but there was also evidence of contributions from other cell types as was evident from the identification of glial fibrillary acidic protein (average increase of 1.49 fold, $p=0.007$). Although limitations such as contamination from interneurones, astrocytes and even pyramidal cell bodies still exist, this kind of simple micro-dissection goes some way to simplifying the complex protein profile of a sample such as this. Other preparations such as synaptosomal fractions would be ideal for examining changes in synaptic proteins following synapse specific plasticity such as LTP. Other groups have used this approach. For example a very recent study used rat synaptosomes to examine the effect of acrylamide intoxication on neurotransmission in central and peripheral synapses (Barber & LoPachin, 2004) by running out the proteins complexed with acrylamide in the synapses of rat brain on a 2D gel. In this instance the whole brain was used. Other studies have examined the whole hippocampus (Terrian *et al.*, 1988) or more purified preparations such as the squid optic lobe (Jimenez *et al.*, 2002). The use of synaptosomal fractions would certainly be a step forward in terms of focussing upon synaptic proteins such as glutamate receptors (NMDA, AMPA and mGlu) and presynaptic proteins (synapsin, GAP-43) which play a central role in synaptic plasticity in the hippocampus (Anwyl *et al.*, 1989; Muller *et al.*, 1988; Otani *et al.*, 1993; Sergueeva *et al.*, 1993; Spillane *et al.*, 1995; Routtenberg & Lovinger, 1985). Moreover, such an approach might reveal novel, low abundance proteins involved in the plasticity process. Finally and as previously mentioned, localised protein synthesis could be further investigated.

In many of the subsequent chapters a major challenge has been the mass spectrometric identification of differentially expressed proteins. Firstly, as the CyDyes used for DiGE are extremely sensitive and given that the software can detect protein spots that are not visible to the naked eye, not surprisingly many spots have insufficient amounts of protein contained within them rendering them inadequate for mass spectrometric analysis and protein identification. Even some visible spots do not contain sufficient amounts of protein for successful protein identification as the total amount of labelled protein run on a single gel amounts to only 150μg. There are three methods available as potential solutions to this problem. The first method uses a separate 'preparative' gel containing 300-500μg of protein. Protein spots requiring identification in the DiGE gels were matched up to their corresponding spots in the preparative gel. These corresponding protein spots were processed through the spot handling workstation and subjected to mass spectrometric analysis. The disadvantage with this process is that separate gels are notoriously difficult to match up and the possibility of mismatching is greatly increased with this method. One advantage, however, is that by using a preparative gel you can have several attempts at identifying spots and can keep returning to the experiment in question. The second method uses the DiGE analytical gel post stained with Sypro orange as a 'pick' gel. As previously mentioned, only 150μg of protein is loaded onto these gels, and therefore only the most abundant proteins can be identified. In its favour however, this method ensures accurate spot picking. The final method was found to be the most successful in the present study and combines the advantages of the previous two methods. DiGE analytical gels were loaded with excess 'invisible' protein i.e. unlabelled protein. Following analysis the gel with excess protein was post stained with Sypro orange and matched up to the original analytical image. As the spot pattern is identical (with the exception of a small 500Da shift in the protein due to the weight of the CyDyes) and the protein content is sufficient (300μg) for mass spectrometric analysis this method appears to be the most successful.

In summary, a combined electrophysiology and proteomics approach has been developed providing us with a powerful tool with which to investigate global changes in protein expression not only in entire regions of rodent hippocampus, but also within sub-regions such as dendrites and cell bodies. Standard 2D gel electrophoresis methods were optimised for hippocampal tissue and further

optimisation was carried out for DiGE - a robust proteomics system allowing accurate quantification of protein changes with statistical confidence. Every aspect of the process from sample preparation, 2D protein separation and mass spectrometric analysis was optimised resulting in a successful and reliable approach to address the question of differential protein expression in the hippocampus in relation to synaptic plasticity processes.

Chapter 4

Changes in global protein expression in area CA1 of rodent hippocampus resulting from pharmacological activation of glutamate receptors.

4.1 INTRODUCTION

Glutamate, acting through ionotropic (N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)), kainate and metabotropic (mGlu) receptors, is the major excitatory neurotransmitter in the brain. Activation of these receptors mediates fast synaptic transmission. There is also a definitive role for glutamate in long-term changes in synaptic strength associated with cognitive processes in the brain (Bliss & Collingridge 1993). Excessive stimulation of glutamate receptors in anoxic conditions is also thought to mediate 'excitotoxicity' of hippocampal pyramidal neurons (Olney *et al.*, 1971, Abele *et al.*, 1990). Therefore, any molecular changes occurring as a result of glutamate receptor activation are not only central to the mechanics of basal synaptic transmission, but also to neuronal processing of information in learning and memory and to pathologic conditions such as stroke, seizures and neurodegenerative disorders such as AD.

Glutamate receptors are ubiquitously expressed and found on all neurons of the brain including hippocampal pyramidal cells. Stimulation of post-synaptic glutamate receptors on these cells, particularly NMDA type glutamate receptors, induces membrane depolarisation followed by increase an in cytosolic Ca^{2+}. This increase in Ca^{2+} triggers the activation of protein kinases such as CaMKII, PKA and tyrosine kinases such as Fyn and Src that ultimately result in increases in synaptic efficiency (Tan *et al.*, 1994; Greengard *et al.*, 1991; Roberson & Sweatt 1996; O'Dell *et al.*, 1991). These signalling molecules act in the short term to phosphorylate and modify existing protein molecules which are in close proximity to the post-synaptic membrane, whilst in the longer term triggering of transcriptional factors such as CREB to initiate the *de novo* transcription of genes and the translation of mRNAs that act to maintain these increases in the strength of synaptic transmission (Ghosh *et al.*, 1994; Schurov *et al.*, 1999; Mabuchi *et al.*, 2001).

The discovery of these late phase protein molecules is therefore of great interest as it may provide us with an increased understanding into complex neuronal processes such as cognition and neurodegeneration. A recent study by Hong and colleagues (2004) investigated the induction of late response genes following NMDA receptor activation using microarray chip technology. In their own words this study provides

groundwork for more in depth investigations into molecules regulated in plasticity and cognitive related mechanisms. Indeed, they identified a number of late phase genes previously implicated in NMDA receptor mediated synaptic transmission (Clathrin, MAPK) as well as over 150 unknown molecules thought to be novel modulators. Whilst providing a wealth of information, a complementary proteomics approach would identify changes in functional protein molecules as well as taking into account any alternative splicing or post translational modifications occurring.

Proteomic studies, however, are not without their limitations. Complexity of sample and reduced representation of subtypes of proteins, specifically membrane bound proteins, are certainly issues. The use of lysis buffers with optimal ingredients for better representation of hydrophobic proteins and rigorous lysis methods (sonication, freeze thawing) aid in the discovery of membrane bound proteins. Sub fractionation of sample prior to 2D gel electrophoresis goes some way to reducing the complexity of certain protein samples.

Using a combination of the *in vitro* hippocampal slice preparation and a powerful proteomics tool, difference gel electrophoresis (DiGE), we have investigated the differential protein expression resulting from glutamate receptor activation in area CA1 of the rodent hippocampus. In addition, the role of the NMDA receptor in these changes in protein expression was also scrutinised. To identify which proteins are differentially expressed in or near the post synaptic membrane and to simplify the complex nature of tissue samples of this kind, proteins localised in the dendrites of CA1 subfields of hippocampus were isolated and modulation of these proteins was investigated following pharmacological stimulation of post-synaptic glutamate receptors.

4.2 Study Aims

Glutamate receptor stimulation may result in an influx of Ca^{2+} into hippocampal neurones and subsequent triggering of numerous signalling cascades ultimately inducing transcription and translation products that may be important for the maintenance of glutamate receptor mediated increases in synaptic strength. The main aims of this chapter were to:

1. Investigate global protein expression and regulation in the CA1 region of mouse hippocampus in response to pharmacological stimulation of glutamate receptors.

2. Investigate the possible contribution of NMDA subtype of glutamate receptors in the glutamate-induced modulation of hippocampal protein expression using the NMDA receptor antagonist D-AP5.

3. Investigate the regulation of protein expression in response to pharmacological activation of glutamate receptors within somatic and dendritic layers of the hippocampal area CA1.

4.3 METHODS

Methods are as described in chapter 2 of this thesis with the following additional information. Hippocampal slices prepared from male ICR mice (4-8 weeks) were used for glutamate receptor activation experiments. Male Wistar rats (4-8 weeks) were used for isolated dendritic fraction experiments. Protein samples obtained from glutamate and control treated slices (Aim 1) were run on pH4-7 linear gradients strips. Protein samples obtained from vehicle control, glutamate and glutamate & D-AP5 treated slices (Aim 2) and isolated dendritic fractions were run on pH 3-10 non linear gradient strips. Mass spectrometry was performed on spots picked from analytical DiGE gels, separate Sypro Orange stained gels and analytical Sypro Orange stained preparative gels as is described in further detail in chapter 2 of this thesis.

Table 4.1 Experimental design for BVA of control tissue vs. glutamate (100 μM, 4 hrs)

Gel	Cy2	Cy3	Cy5
1	Standard	Glutamate	Control
2	Standard	Glutamate	Control
3	Standard	Glutamate	Control

Table detailing the experimental design employed to examine the effect of 2 treatment groups (glutamate, 100μM, 4hrs, n=3; vehicle control, ACSF, n=3), on protein regulation in CA1 hippocampal tissue using DiGE, DeCyder DIA and DeCyder BVA. Standard samples containing an equal portion of all the experimental samples were labelled with Cy2 and in this instance glutamate samples were labelled with Cy3 and control samples with Cy5.

Table 4.2 Experimental design for BVA of control CA1 tissue vs. either glutamate (100μM, 4hrs) or glutamate (100μM, 4hrs) + D-AP5 (50μM, 4hrs)

Gel	Cy2	Cy3	Cy5
1	Standard	Control	Control
2	Standard	Control	Control
3	Standard	Glutamate	Glutamate
4	Standard	Glutamate	Glutamate
5	Standard	D-AP5	D-AP5
6	Standard	D-AP5	D-AP5

Table illustrating the experimental design employed to examine the effect of 3 treatment groups (vehicle control, ACSF, (n=4), glutamate, 100μM, 4hrs (n=4), glutamate 100μM, 4hrs (n=4) + D-AP5 50μM, 4hrs (n=4)) using incorporation of an internal standard containing equal portions of all the experimental samples all labelled with Cy2 and the labelling of the three treatment groups with either Cy3 or Cy5.

Table 4.3 Experimental design for BVA of control (ACSF) treated isolated dendritic tissue vs. glutamate (100μM, 4hrs) or glutamate (100μM, 4hrs) + D-AP5 (50μM, 4hrs) treated samples

Gel	Cy2	Cy3	Cy5
1	Standard	Glutamate	D-AP5
2	Standard	Glutamate	Control
3	Standard	D-AP5	Control
4	Standard	D-AP5	Glutamate
5	Standard	Control	D-AP5
6	Standard	Control	Glutamate

Table illustrating the experimental design employed to examine the effect of 3 treatment groups (control (n=4), glutamate (n=4), glutamate + D-AP5 (n=4)) on isolated dendrites of CA1 hippocampal neurons using incorporation of an internal standard all labelled with Cy2 and the randomised labelling of the three treatment groups with either Cy3 or Cy5.

4.4 RESULTS

4.4.1 Glutamate treatment of the CA1 region of mouse hippocampus

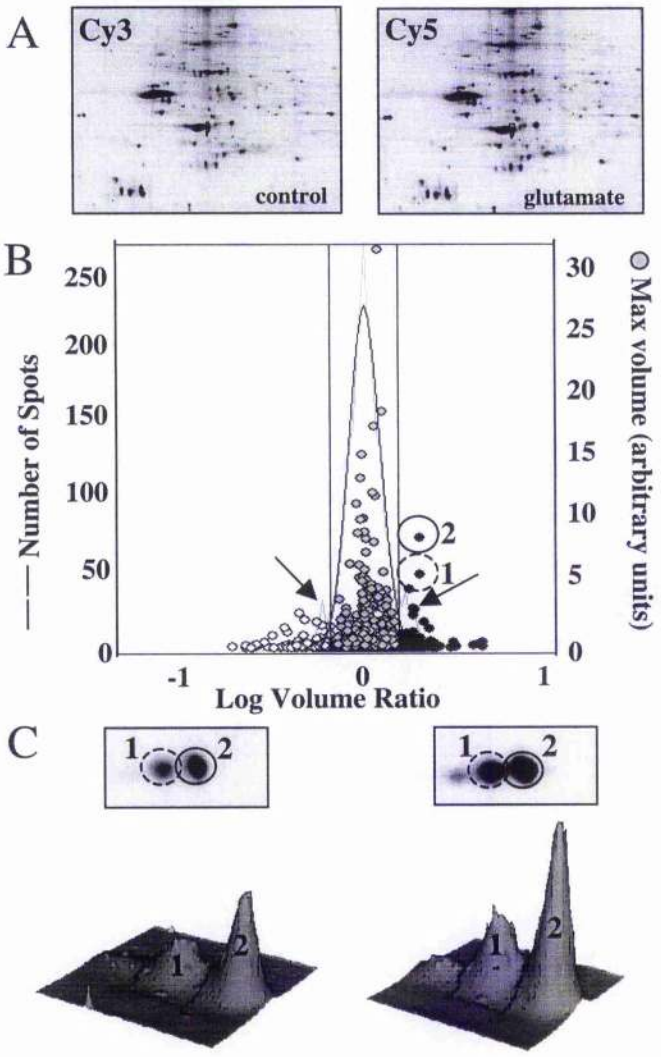
Initial experiments assessed protein regulation resulting from pharmacological stimulation of glutamatergic afferents in the CA1 region of mouse hippocampus by the treatment of acute hippocampal slices with either glutamate (100μM, 4hrs) or vehicle control (ACSF). These investigations into the effect of glutamate treatment on differential protein expression in the CA1 region of the hippocampus utilised protein extracts from area CA1 that were subsequently labelled, resolved by 2D gel electrophoresis and analysed in DIA mode of DeCyder software. DIA compared two gel images (vehicle control (ACSF) (Cy3) versus glutamate (100μM) (Cy5)) to assess the relative changes in spot abundance associated with pharmacological stimulation of this kind. Output from DIA consists of gel images, a histogram detailing spot frequency, volume and the log of the volume ratio and 3D contour plots of selected protein spots (Figure 4.1). The light grey curve on the histogram in figure 4.1b represents the frequency distribution of the log volume ratios. In addition to the main peak around 0, there appear to be two smaller peaks (Figure 1B, arrows) possibly representing discrete populations of protein spots with increased differential expression. Threshold settings could be varied and as a result of an increased threshold a decrease in the number of modulated proteins was observed (1.5 fold: 347 proteins, 4 fold: 5 proteins). At a threshold value of 1.5 fold a large number of proteins appear to be both up and down regulated following glutamate receptor stimulation (Table 4.4). When using a cut off of 2 standard deviations, which is recommended for obtaining statistically significant results ($p<0.05$) for normally distributed data, 121 protein spots showed an increase in expression and 145 showed a decrease in expression.

Table 4.4 Relative distribution of protein expression dependent on threshold set in DIA

	Number of spots		
Threshold	**Decrease**	**Similar**	**Increase**
1.5 fold	153	2249	194
2.0 fold	49	2513	34
2.5 fold	19	2570	7
3.0 fold	13	2579	4
4.0 fold	3	2591	2

Table illustrating the number of spots with decreased, increased or similar abundance levels as a result of glutamate treatment (100μM, 4hrs) of hippocampal CA1 regions. This was calculated over a wide range of threshold values (1.5 to 4.0 fold). These numbers are generated from a single gel pair (Control (Cy3) versus glutamate (Cy5)). Increasing the threshold in DIA reduces the number of regulated protein spots and very few demonstrate more than a 2.5 fold change in expression following glutamate receptor stimulation.

Figure 4.1. Preliminary differential in-gel analysis reveals regulated expression of hippocampal proteins following pharmacological activation of glutamate receptors.



A. Representative Cy3 (vehicle control) and Cy5 (100μM glutamate treated) gel images obtained from a single DiGE gel. Note that running the two samples on the same gel allows for identical running conditions and near identical spot patterns. B. Histogram showing log volume ratios (x-axis) of individual spots (circles) calculated from the Cy3 (vehicle control) and Cy5 (glutamate) gel images from the single DiGE gel shown in A. The left Y-axis corresponds to the spot frequency, whilst the right Y-axis represents the maximum volume of the spot. Individual proteins spots show a decrease (white circles), no change (grey circles) and increase (black circles) in log volume ratio. Horizontal black lines represent the threshold values set in DIA (set here as 1.5 fold). The grey curve is representative of the frequency distribution of the log volume ratios, whereas the black curve is the normalized modal frequency fitted to the spot ratios so that the modal peak is zero. The arrows represent small, distinct populations of protein spots displaying differences in log volume ratio. C. Magnified gel view and contour plots of an illustrative protein spot displaying an increase in the log volume ratio for this protein at 4 hours following glutamate application.

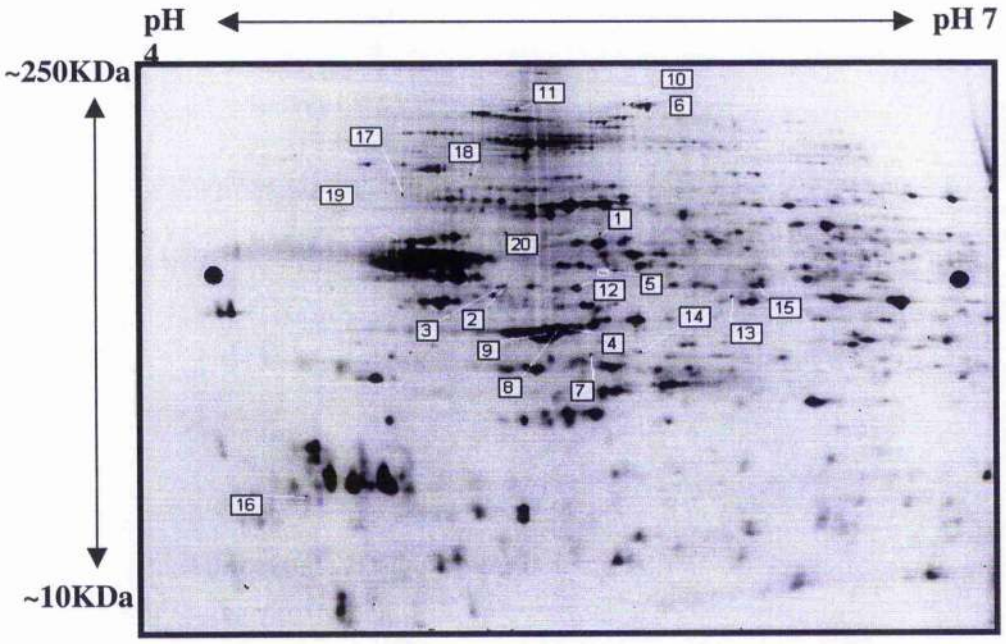
To obtain accurate and quantifiable proteomics data, an increased number of gels representing biological replicates were compared. Therefore, using the Biological Variance Analysis (BVA) mode of DeCyder software which allows the comparison across many gels due to the incorporation of an internal standard, differential protein expression in the CA1 region of the hippocampus was again compared this time using 3 biological replicates (3 groups, 3-4 slices per replicate, total of 12 slices/group) for control and treated groups. At a significance threshold of $p<0.01$, 20 protein spots were found to change in abundance following glutamate treatment (figure 4.2, table 4.5). Of these 20, the fold change in abundance of 14 protein spots increased (1.12 to 1.3 fold change), whilst that of the remaining 6 decreased (-1.13 to −1.33 fold change). Those protein spots with the same identity were found to lie adjacent to each other along the first dimension axis on the 2D gel, for example spot numbers 2 & 3 which were both subsequently identified as dynactin-2 (figure 4.2, table 4.5), suggesting possible modulation of charge trains. This is further illustrated in figure 4.3. Beta actin, found in spots 4, 8 and 9 on the 2D gel, showed a significant increase in expression following pharmacological stimulation of glutamate receptors. This increase was uniform across all beta actin spots in the charge train (fold changes of 1.26, 1.18 & 1.16) and the internal standard for this experiment, appropriately, lies halfway between control and treated samples.

Table 4.5 Regulation of CA1 hippocampal proteins by glutamate

Spot number[1]	Master number[2]	Protein ID [3]	T-test value [4]	Fold change [5]	Mass (Da) [6]	Accession number [7]	Mowse Score [8]	Biological process [9]
1	1331		0.008	1.3				
2	1793	Dynactin-2	0.01	1.29	44204	gi 28076935	80	CO &B
3	1806	Dynactin-2	0.00075	1.28	44204	gi 28076935		CO &B
4	2037	Beta actin	9.10E-05	1.26	39446	gi 49868	98	CO &B
5	1669	CRMP 2	0.0075	1.22	62531	gi 6753676	98	NG
6	219	Clathrin	0.00091	1.2	193202	gi 25072051		VMT
7	2173		0.0014	1.2				
8	2043	Beta actin	0.00079	1.18	39446	gi 49868	98	CO &B
9	2075	Beta actin	0.0055	1.16	39446	gi 49868	70	CO &B
10	216	Clathrin	0.0059	1.14	193202	gi 25072051		PM&T
11	260		0.0048	1.12				
12	1804	Guanine deaminase	0.0029	1.12	51494	gi 6753960		NG
13	1842	Proteosome26s	0.0065	1.12	53289	gi 28503436		PM&T
14	2158		0.0079	1.12				
15	1791		0.0043	-1.13				
16	2627		0.0012	-1.14				
17	1064		0.0099	-1.19				
18	899		0.00048	-1.23				
19	1398		0.0039	-1.28				
20	1345		0.0028	-1.33				

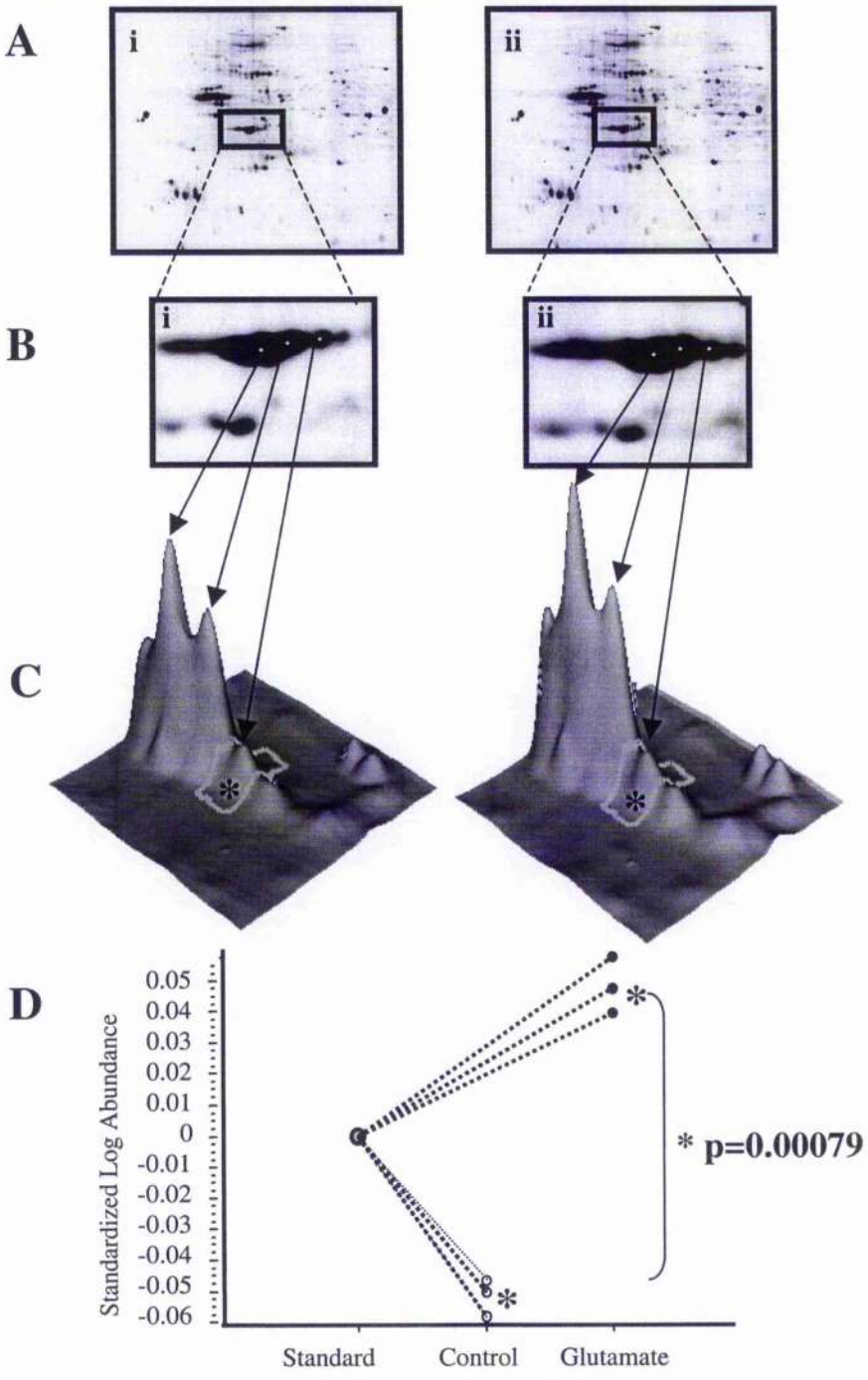
Table listing the protein spots differentially expressed as a result of glutamate treatment (100μM, 4hrs) of mouse hippocampal CA1 regions. [1] Spot number corresponds to the annotation on figure 4.2. [2] Master number was allocated to the individual protein spot by DeCyder software. Only 20 protein spots showing significance values of $p<0.01$ were sent for mass spectrometric analysis by Maldi-Tof and Mascot PMF database searching, from which 10 were identified [3]. The corresponding values for protein mass [4] (Daltons), accession number obtained from NCBInr [5], and Mowse score obtained from Mascot searching [6] are also given. The identified proteins belong to a range of biological processes [7] CO&B: Cytoplasm organisation and biogenesis, NG: neurogenesis, VMT: vesicular mediated transport, PM&T: protein modification and transport

Figure 4.2. Annotated 2D gel image from BVA analysis of control vs. glutamate treated CA1 regions.



Gel image indicating the protein spots identified by BVA analysis as differentially expressed following glutamate receptor stimulation (100μM, 4hrs). Protein samples were run on pH4-7 IPG strips over a molecular weight range of 10 – 250 KDa. Both high and low molecular weight proteins are regulated, and there is also a wide scatter over the entire pH range.

Figure 4.3. Regulation of post translationally modified forms of beta actin.



Beta actin regulation by glutamate treatment of the CA1 mouse hippocampus. A. Gel images of (i) control and (ii) 100μM glutamate treated tissue. B. Magnified gel images of 3 sequential protein spots, identified as beta actin, which were significantly different ($p<0.01$) after glutamate application (c) 3D views generated by DeCyder BVA software highlighting the train of protein spots significantly up regulated as a result of glutamate treatment of hippocampal CA1 sub domains. (d) Graphical representation of the standardized log abundance for the protein spot highlighted in the 3D views. Note that the standard values lie exactly half way between the control and glutamate values and the effect of glutamate is consistent throughout all the gels.

4.4.2 Glutamate treatment of the CA1 region of mouse hippocampus - the contribution of the NMDA receptor

A more detailed analysis of glutamate receptor mediated protein modulation in hippocampal pyramidal cells incorporated the use of the NMDA receptor antagonist D-AP5 to focus on the role of the NMDA receptor in glutamate mediated differential protein expression.

Using a significance value of $p<0.05$, 79 protein spots were differentially expressed as a result of glutamate treatment (figure 4.4, table 4.6). Of these 79, 35 increased in their expression (1.07 to 1.97 fold change) with the remaining 44 decreasing (-1.07 to –1.91 fold change). Treatment with the NMDA receptor blocker D-AP5 prevented the majority (56 out of 79, $p>0.05$) of the changes in spot abundance. Identification of 39 of these differentially expressed protein spots were obtained by either Maldi-tof or LC/MS/MS mass spectrometric analysis (table 4.6). Those spots identified by LC/MS/MS contain, with one exception (spot# 62), multiple protein complements. For example, spot number 60 was subsequently identified by LC/MS/MS mass spectrometry as a mixture of Cab39 and LASP-1. Its regulation by NMDA receptor activation is illustrated in figure 4.5. The change in expression for this protein is clear not only in the gel images, but also in the 3D views and graphical representation. For this particular spot a small but significant decrease in abundance (-1.21 fold change, $p=0.038$) following glutamate application is observed, which is prevented by the co-incubation of glutamate with D-AP5 (-1.11 fold change, $p=0.24$).

Table 4.6 Protein modulation by glutamate (100μM) and D-AP5 (50μM) in the CA1 region of mouse hippocampus

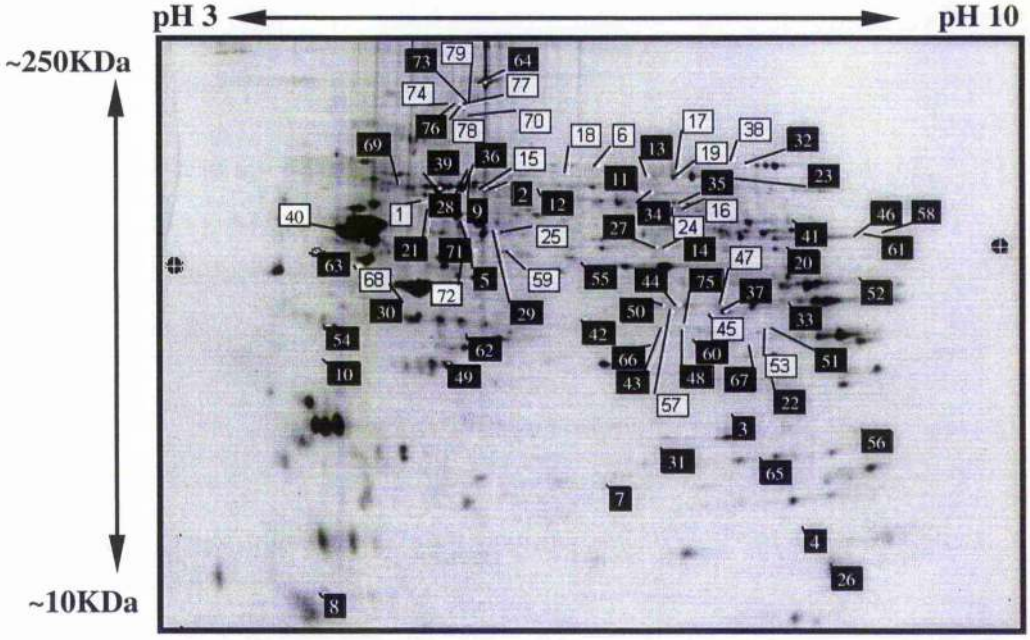
Spot number (1)	Master number (2)	Protein ID (3)	Spot abundance ratios (4)	Glutamate (5)	Glutamate + D-AP5 (6)	Mass (Da) (7)	Accession number (8)	Mowse Score (9)	Biological process (10)
				T-test values					
1	1349		1.97	0.042	0.0034				
2	1172	CRMP2	1.7	0.023	0.073	62531	gi 3122040	104	NG
3	2445		1.59	0.015	0.29				
4	2712		1.5	0.016	0.053				
5	1722		1.4	0.011	0.17				
6	1001		1.35	0.0013	0.031				
7	2633	DJ-1	1.34	0.0015	0.29	18569	gi 10181132	51	CO & B
8	2863		1.32	0.011	0.36				
9	1143		1.28	0.026	0.088				
10	2296	OUT domain 1	1.28	0.046	0.086	31478	gi 19527388	254	PM&T
11	1246		1.26	0.043	0.73				
12	1252	CRMP2	1.26	0.024	0.73	62531	gi 3122040	71	NG
13	1081	Enolase 1	1.24	0.017	0.41	47453	gi 13637776	77	E/M
14	1678		1.24	0.0088	0.25				
15	1171	Plastin 3 precursor	1.23	0.0038	0.038	71210	gi 21704120	**839**	PM&T
		CRMP2				62531	gi 3122040	**564**	NG
		Plastin 1				70763	gi 38090003	**180**	PM&T
		Hsc70				73701	gi 1072476	**70**	PM&T
16	1373	Stress induced phosphoprotein 1	1.23	0.028	0.044	63170	gi 14389431	**614**	RTS
		CRMP3				62484	gi 1915915	**518**	NG
		CRMP1				62410	gi 2342488	**486**	NG
		GAD-65				66037	gi 1352217	**286**	NT
		CRMP1				62493	gi 34328211	**106**	NG
17	1021		1.22	0.038	0.038				
18	1056		1.21	0.0044	0.012				
19	1086		1.21	0.037	0.026				
20	1699		1.21	0.025	0.19				
21	1410	Alpha internexin	1.2	0.03	0.32	55520	gi 17390900	**551**	CO&B
		Calcineurin				59291	gi 8394030	**330**	SIG
		Hsp60				61074	gi 72957	**111**	PM&T
		Neurofilament H				116828	gi 128127	**104**	CO&B
		Alpha tubulin 2				50818	gi 135412	**69**	CO&B
22	2337		1.19	0.014	0.37				
23	1103		1.18	0.002	0.12				
24	1372	CRMP1	1.18	0.013	0.023	62484	gi 1915915	**512**	NG
		VLCAD				69177	gi 31559883	**272**	E/M
		Phosphoglucomutase 2				61665	gi 12847638	**163**	CM
		CRMP4				62493	gi 34328211	**95**	NG
		GAD-65				66037	gi 1352217	**88**	NT
25	1572	v ATP synthase subunit B	1.18	0.013	0.043	56948	gi 1184661	180	NM
26	2783	Cofilin 1	1.18	0.046	0.12	18776	gi 6680924	69	ST
27	1677		1.17	0.0092	0.12				
28	1063		1.15	0.037	0.82				
29	1568		1.15	0.012	0.22				
30	1961	Gamma actin	1.14	0.0076	0.99	41335	gi 809561	91	CO&B

31	2531	Proteosome 26s subunit alpha 6	1.13	0.029	0.41	27811	gi 6955198	62	PM&T
32	989	Aconitase 2 (mitochondrial)	1.11	0.044	0.47	86151	gi 18079339	76	CM
33	2028	Aldolase 1, A isoform	1.11	0.047	0.18	39787	gi 6671539	91	E/M
34	1253	Sdha protein	1.1	0.045	0.19	73363	gi 15030102	**416**	CM
		WD40 repeat protein 1				67049	gi 6755995	**73**	NP
35	1321	CRMP2	1.07	0.0058	0.8	62531	gi 3122040	**302**	NG
		T-complex protein 1, gamma				61021	gi 631730	**247**	PM&T
36	1199	v ATP synthase subunit A	-1.07	0.00037	0.4	68567	gi 1718086	154	NM
37	2037	v ATP synthase subunit C	-1.07	0.0081	0.098	44004	gi 12585525	132	NM
		Aldolase 3, C isoform				39706	gi 13435924	132	E/M
38	947		-1.08	0.045	0.037				
39	1212	Hsc70	-1.1	0.017	0.53	71021	gi 476850	124	PM&T
40	1570	Beta tubulin	-1.1	0.016	0.033	50377	gi 1746161	160	CO&B
41	1447	T-complex protein 1, eta	-1.11	0.013	0.14	60127	gi 549060	**546**	PM&T
		Catalase				60013	gi 115704	**415**	RTS
		Pyruvate kinase 3				58378	gi 31981562	**385**	E/M
		Alpha tubulin 2				50818	gi 135412	**303**	CO&B
		Glucose phosphate isomerase 1				62956	gi 6680067	**206**	E/M
		T complex protein 1, delta				58543	gi 6753322	**185**	CO&B
		Farsla				57723	gi 13905140	**175**	T/T
		Alpha tubulin 4				50634	gi 6678467	**168**	CO&B
		Synapsin II				52818	gi 8567410	**160**	NT
42	2086	Calcineurin	-1.11	0.022	0.57	36972	gi 20455200	286	SIG
43	2118		-1.11	0.032	0.95				
44	2001	Enolase 2	-1.12	0.024	0.053	47437	gi 12963491	116	E/M
		ERK 2				33979	gi 2506073	46	ST
45	2045	v ATP synthase subunit C	-1.12	0.04	0.024	44105	gi 13384916	**455**	NM
		Aldolase 3, C isoform				39706	gi 13435924	**286**	E/M
		N-acetylneuraminic acid synthase				40455	gi 16716467	**217**	E/M
		Isocitrate dehydrogenase 3, beta				42453	gi 18700024	**173**	CM
		v ATP synthase subunit A				59830	gi 6680748	**94**	NM
		Acetyl-Co A acetyltransferase 3				41895	gi 23346599	**84**	E/M
46	1605	Pyruvate kinase3	-1.13	0.041	0.52	58378	gi 31981562	53	E/M
47	1992	Aspartate transaminase, cytosolic	-1.13	0.032	0.0082	46489	gi 90313	95	E/M
48	2123	Glycerol-3-phosphate dehydrog	-1.13	0.018	0.21	43202	gi 22902419	439	E/M
49	2315	NSF	-1.13	0.011	0.48	33878	gi 29789104	129	PM&T
50	2016		-1.15	0.0053	0.14				
51	2116		-1.15	0.043	0.42				
52	1908		-1.16	0.038	0.47				
53	2139		-1.16	0.04	0.0085				
54	2166	Glycerol-3-phosphate dehydrog	-1.16	0.025	0.42	43202	gi 22902419	201	E/M
55	1783		-1.17	0.026	0.14				
56	2484		-1.17	0.025	0.28				
57	2041		-1.18	0.0038	0.00049				
58	1590		-1.2	0.032	0.18				
59	1731		-1.21	0.017	0.00036				
60	2193	Cab39	-1.21	0.038	0.24	39989	gi 18044843	**412**	CIB
		LIM & SH3 protein 1 (LASP 1)				30374	gi 6754508	**269**	CO&B
61	1596		-1.22	0.0088	0.85				
62	2203	Lactate dehydrogenase 2, beta	-1.23	0.0035	0.063	36834	gi 6678674	**420**	E/M
63	1758		-1.24	0.039	0.44				
64	346	Clathrin, heavy polypeptide	-1.25	0.04	0.062	194523	gi 38091541	152	VMT
65	2557		-1.26	0.025	0.79				
66	2225		-1.28	0.018	0.064				

67	2213	GAPDH	-1.29	0.024	0.054	36072	gi 6679937	**566**	E/M
		Putative kinase				35651	gi 12843937	**198**	ST
		Aldose reductase				36052	gi 1351911	**155**	E/M
		Malate dehydrogenase (m)				36030	gi 126897	**81**	E/M
68	1855		-1.41	0.0018	0.043				
69	1152	Hsc 70	-1.42	0.0034	0.075	71056	gi 42542422	153	PM&T
70	553		-1.43	0.0077	0.038				
71	1475		-1.47	0.031	0.36				
72	1644		-1.49	0.042	0.012				
73	468	Vimentin	-1.51	0.02	0.17	53746	gi 55408	171	CO&B
74	502		-1.62	0.023	0.035				
75	2085	Vimentin	-1.63	0.00082	0.081	53698	gi 202368	45	CO&B
76	517	Vimentin	-1.65	0.027	0.092	53746	gi 55408	66	CO&B
77	508		-1.67	0.00034	0.016				
78	518	Spna2	-1.73	0.0073	0.017	156610	gi 20380003	136	CO&B
79	461		-1.91	0.0012	0.01				

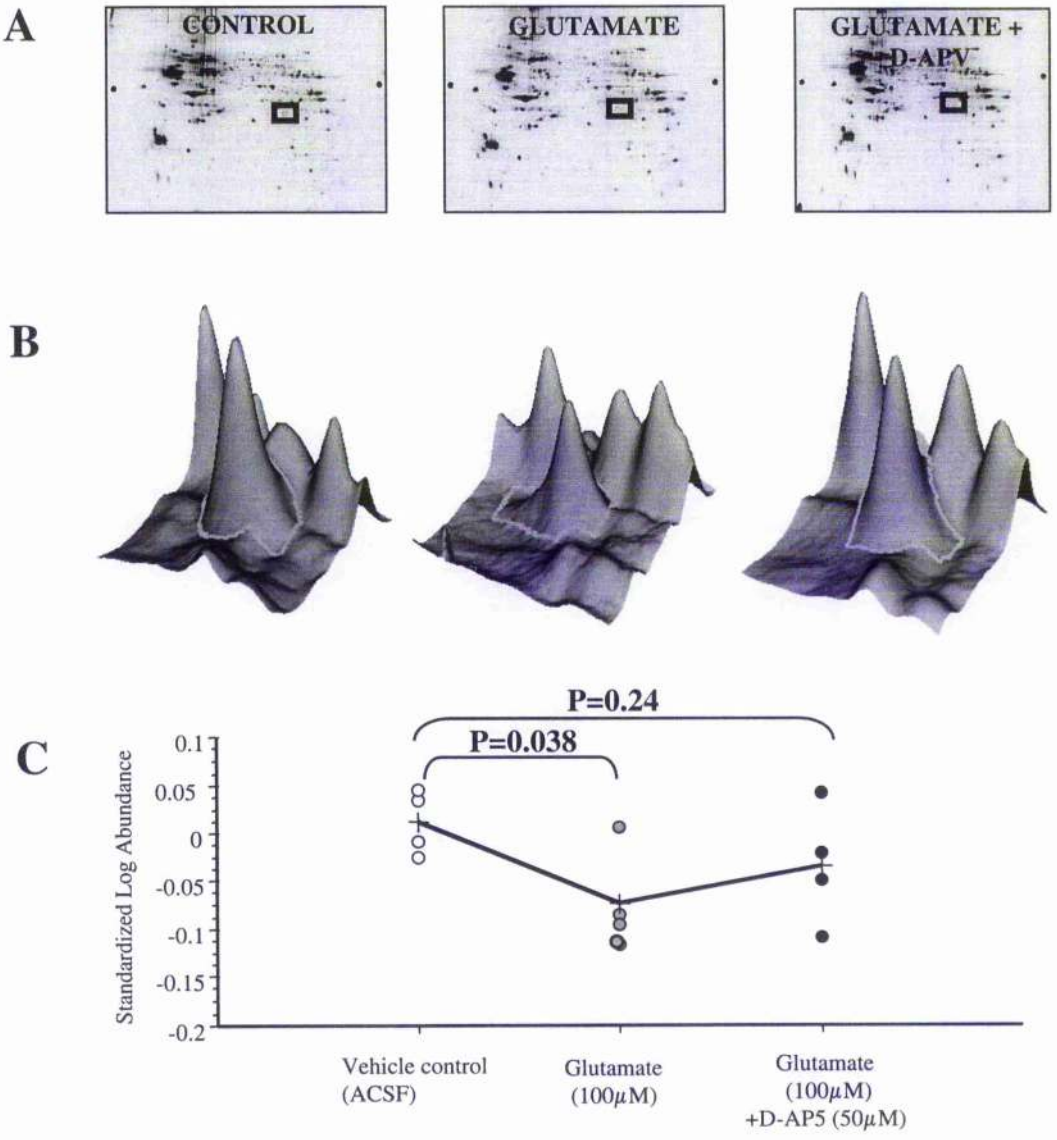
Table listing the proteins spots differentially expressed as a result of glutamate treatment (100μM, 4hrs) of mouse hippocampal CA1 regions following in-depth biological variance analysis. [1] Spot number corresponds to the number annotated on the gel image in figure 4.4 [2] Master number is allocated to each individual protein spot by DeCyder BVA software. Those protein spots significantly modulated by glutamate receptor stimulation (4), $p<0.05$, were subjected to mass spectrometric analysis for protein identification [3] by either Maldi or LC/MS/MS mass spectrometry. Greyed out boxes represent those proteins whose regulation by glutamate is blocked by D-AP5. [d] Mowse scores greater than 61 for PMF analysis and greater than 36 for LC/MS/MS analysis were thought to be significant hits. [e] NG; neurogenesis, T/T; transcription/ translation, E/M; energy/ metabolism, CO&B cytoplasmic organisation and biogenesis, NP; neurotransmitter production, CIB; calcium ion binding, PM&T; protein modification and transport, ST; signal transduction, VMT; vesicular mediated transport, CM; carbohydrate metabolism, NM; nucleotide metabolism, RTS; response to stress.

Figure 4.4. Annotated gel image highlighting differentially labeled hippocampal proteins following glutamate or glutamate + D-AP5 treatment as revealed by biological variance analysis.



Gel image (Cy2) highlighting protein spots identified by BVA as differentially expressed ($p<0.05$) at 4 hours following application of 100μM glutamate to mouse hippocampal slices (CA1 subfields). Numbered boxes represent the 79 proteins displaying differential expression following glutamate application. Parallel samples run in the same DiGE experiment showed that co-incubation of tissues with the NMDA receptor antagonist D-AP5 (50μM) prevented the differential expression a large proportion of the protein spots (black filled boxes). Protein samples were run on pH3-10 non-linear IPG strips over an approximate molecular weight range of 10 – 250 KDa. Note that regulated proteins span both the molecular weight and pI range.

Figure 4.5. Regulation of protein spot number 60 by treatment of hippocampal CA1 regions with glutamate (100μM) and D-AP5 (50μM)

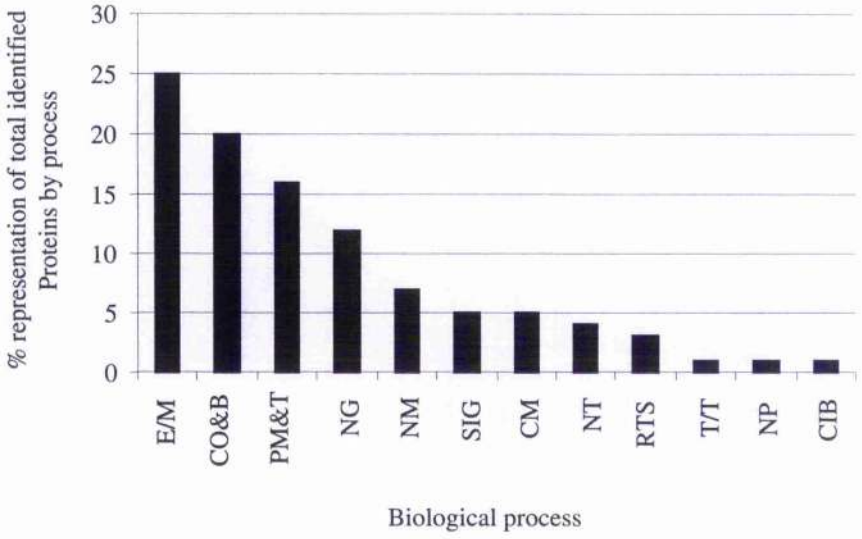


Differential expression of protein spot number 60 as identified by BVA mode of DeCyder software. A) Gel images of the three experimental groups and corresponding magnified views from 2D gels show a decrease in abundance following glutamate treatment, which is not seen when glutamate is given in the presence of the NMDA receptor blocker D-AP5. B) 3D contour plots reiterate this differential expression. C) Scatter plot of the standardized log abundance values for each group in each gel shows a significant decrease in the standardized log abundance following glutamate treatment which is not apparent when D-AP5 is included in the bathing medium.

As illustrated in figure 4.6, the majority of proteins fall into 4 main groupings – energy and metabolism, cytoplasmic organisation and biogenesis, protein metabolism and transport and neurogenesis. The NMDA receptor antagonist D-AP5 was effective in preventing the significant alterations in spot abundance in several of the biological processes, in particular the cytoplasm organisation and biogenesis proteins, molecules involved in the metabolism and transport of proteins, and proteins involved in signal transduction and transcription (figure 4.6b).

Figure 4.6 Illustration of the relative abundance of functional classes modulated following glutamate receptor stimulation.

A



B

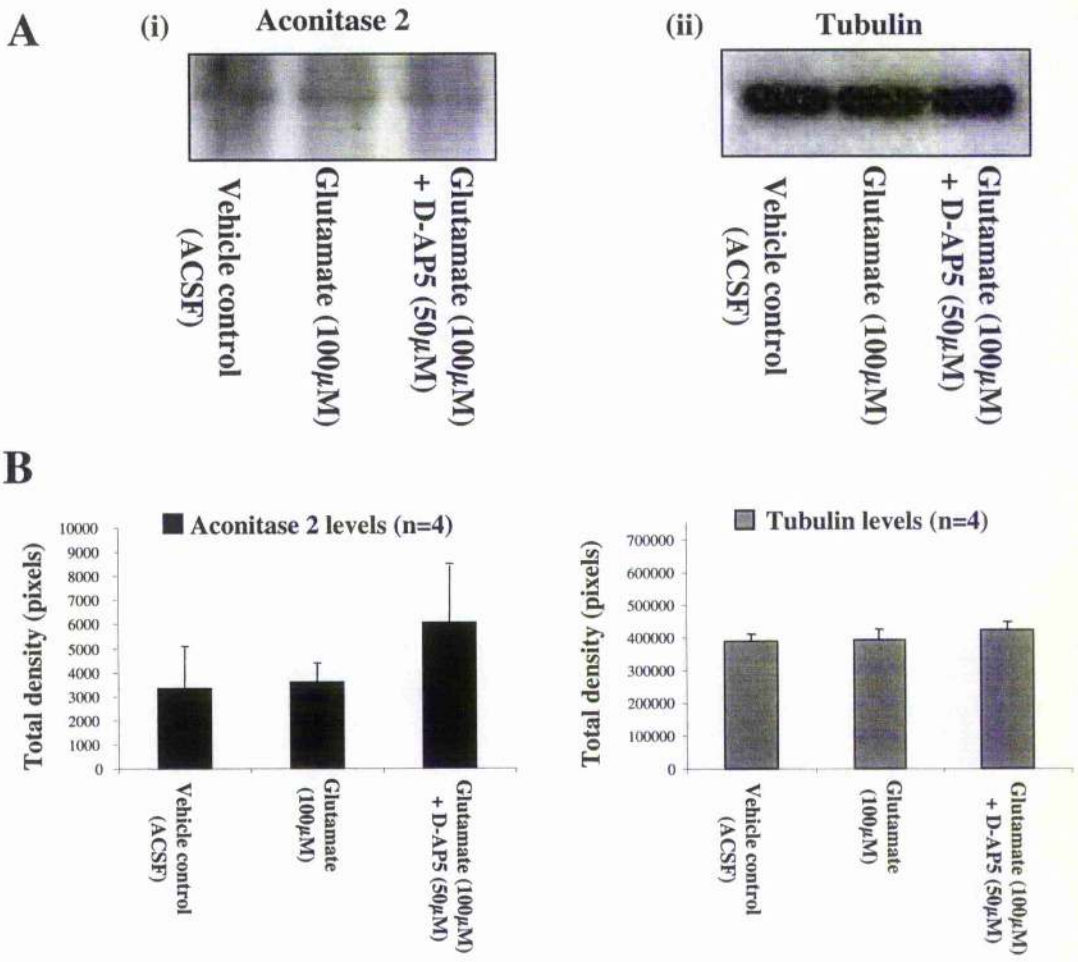
	Number of protein spots changing in abundance											
	NG	CO&B	PM&T	EM	RTS	NT	CM	NM	SIG	T/T	NP	CIB
Glutamate (100μM)	9	14	11	18	2	3	4	5	5	1	1	1
Glutamate (100μM) & D-AP5 (50μM)	6	2	3	5	1	2	2	3	0	0	0	0
% blocked by D -AP5	33	71	75	72	50	33	50	40	100	100	100	100

A) Illustration of the distribution of the functional classes modulated by glutamate receptor stimulation in the CA1 region of the hippocampus. B) Table detailing the effect of the NMDA receptor antagonist D-AP5 (50μM) on the glutamate modulation of the various biological processes. Abbreviations: E/M. energy & metabolism; CO&B, cytoplasm organisation and biogenesis; PM&T, protein metabolism and transport; NG, neurogenesis; NM, nucleotide metabolism; SIG, signal transduction; CM, carbohydrate metabolism; NT, neurotransmitter synthesis/ release; T/T, transcription and translation; RTS, response to stress; NP, neurophysiological process; CIB, calcium ion binding.

4.4.3 Western blot analysis of aconitase 2 and tubulin levels in glutamate treated CA1 subfields of mouse hippocampus

To confirm the differential expression of both aconitase 2 (1.11 fold change, p=0.044) and tubulin (several differentially expressed protein spots 1.2, -1.1, -1.1, -1.1 p=0.03, 0.016, 0.013, 0.013) western blot techniques were employed. CA1 regions from hippocampal slices were harvested 4hrs following treatment with either control bathing medium (ACSF), glutamate bathing medium (100μM) or glutamate + D-AP5 bathing medium (100μM + 50μM). Following tissue lysis and protein extraction samples were separated according to their molecular weight and probed with either primary antibody for aconitase 2 or tubulin followed by incubation with an HRP tagged secondary antibody. Following visualisation using enhanced chemi-luminescence the abundance of protein was calculated for each treatment group. An average of 4 slices in each group was used. An example of a blot for both aconitase and tubulin is shown in figure 4.7. The antibody used for western blot analysis of aconitase 2 did not produce clear defined bands, and resulted in a large amount of background staining. The tubulin blot on the other hand shows clear precise bands. For both proteins no significant changes in expression levels were detected. That said, it appears from the bar chart in figure 4.7b that following co-incubation with glutamate and D-AP5 there is an increase in aconitase 2 protein level although this effect was not significant (p=0.347).

Figure 4.7 Investigation into aconitase and tubulin expression levels following glutamate receptor stimulation using western blots.



Western blot analysis of protein levels of aconitase 2 and tubulin in CA1 hippocampal regions following glutamate receptor stimulation. A. Images of blots showing Aconitase 2 (I) and tubulin (ii) levels in control slices, glutamate (100μM) treated slices and Slices bathed in glutamate (100μM) in the presence of D-AP5 (50μM). B. Bar charts illustrating the actual levels of protein measured in pixel density across all groups for 4 different animals. No significant changes in expression levels were detected.

4.4.4 Differential protein expression in hippocampal CA1 dendritic fractions following pharmacological stimulation of glutamate receptors.

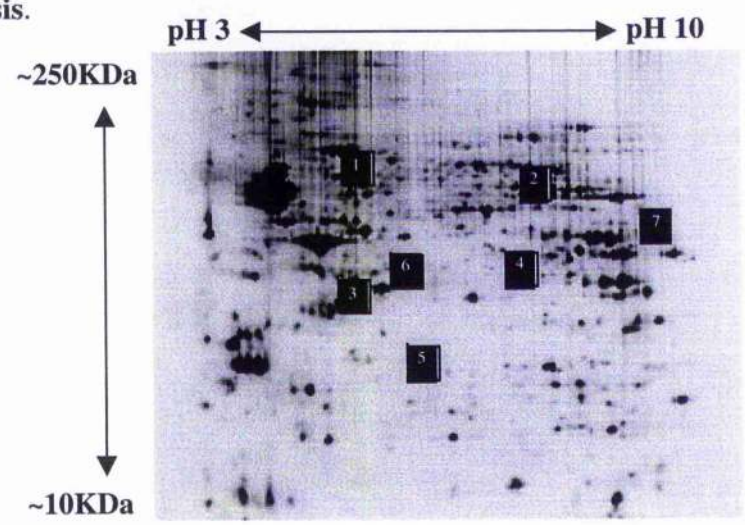
To determine the localisation of the previously identified changes in protein expression, the final experiment in this chapter investigated differential protein expression occurring at local synaptic sites following glutamate receptor activation. To carry out this study in isolated dendritic fractions of CA1 hippocampal neurons were harvested following glutamate (100μM, 4hrs, n=4) treatment alone and in the presence of the NMDA receptor blocker D-AP5 (50μM, 4hrs, n=4). Seven proteins were identified as differentially expressed ($p<0.05$) as a result of glutamate treatment, and all regulation was blocked by D-AP5 (figure 4.8, table 4.7). Very small changes in expression were observed, with only one protein increasing in expression (HSP8, 1.18 fold change) and the remaining 6 decreasing in expression (-1.08 to −1.13 fold change). From the seven proteins highlighted the identities of only 2 have been determined: beta actin, which is involved in cytoplasm organisation and biogenesis and HSP8 also involved in the same biological process.

Table 4.7 Protein modulation by glutamate (100μM) and D-AP5 (50μM) in isolated dendritic fractions of CA1 subfields of mouse hippocampus

Spot number (1)	Master number (2)	Protein ID (3)	Glutamate (4)	Glutamate + D-AP5 (5)	Fold change (6)	Mass (Da) (7)	Accession number (8)	Mowse Score (9)	Biological process (10)
			T-test value						
1	945	HSP 8	0.00083	0.32	1.18				CO&B
2	1944		0.0095	0.24	-1.08				
3	1817		0.019	0.67	-1.11				
4	1103		0.031	0.88	-1.06				
5	1800	Beta actin	0.034	0.073	-1.12				CO&B
6	2162		0.044	0.18	-1.11				
7	1510		0.047	0.96	-1.13				

Table listing the proteins spots differentially expressed as a result of glutamate treatment (100μM, 4hrs) of isolated dendrites from mouse hippocampal CA1 regions following in-depth biological variance analysis. (1) Spot number corresponds to the number annotated on the gel image in figure 4.7 (2) Master number is allocated to each individual protein spot by DeCyder BVA software. Those protein spots significantly modulated by glutamate receptor stimulation (4), $p<0.05$, were subjected to mass spectrometric analysis for protein identification (3) by either Maldi or LC/MS/MS mass spectrometry. Greyed out boxes represent those protein spots whose modulation by glutamate is prevented by co-incubation with D-AP5. (d) Mowse scores greater than 61 for PMF analysis and greater than 36 for LC/MS/MS analysis were thought to be significant hits. (e) CO&B; cytoplasmic organisation and biogenesis.

Figure 4.8 Annotated gel image highlighting differentially labeled hippocampal proteins following glutamate or glutamate + D-AP5 treatment of isolated pyramidal cell dendrites as revealed by biological variance analysis.



Gel image (Cy2) highlighting protein spots identified by BVA as differentially expressed ($p<0.05$) at 4 hours following application of 100μM glutamate to isolated dendritic fractions of the CA1 subfield of mouse hippocampal slices. Numbered boxes represent the 7 proteins displaying differential expression following glutamate application and whose differential expression was prevented by co-incubation with D-AP5. Protein samples were run on pH3-10 non-linear IPG strips over an approximate molecular weight range of 10 – 250 KDa.

4.5 Discussion

The combined approach of brain slice pharmacology and proteomics has been shown to be successful for the identification of proteins whose expression is changed by the pharmacological stimulation of hippocampal glutamate receptors. The activation of these receptors will most likely occur not only on the post synaptic membrane of CA1 hippocampal pyramidal cells but also at pre-synaptic sites, on other neuronal and non-neuronal cell types and in addition, have an effect on glutamate transporters. Whilst pyramidal cells make up the bulk of the neuropil and the identified changes in protein expression will likely represent pyramidal cell proteins, this does not exclude a contribution from other cell types.

Initial proteomics experiments used the DIA mode of DeCyder software to make simple comparisons between two groups – a control group consisting of protein extracts from hippocampal slices bathed in control medium and a treatment group consisting of proteins from slices bathed in medium containing 100μM glutamate. DIA is routinely used for preliminary investigations, in studies where tissue availability is a problem or when time, cost and labour are pertinent issues. DIA is a pair wise comparison between two or three samples all run on a single gel. This has the distinct advantage over normal 2D gel electrophoresis methods of identical running conditions for each sample. Therefore, if the expression of any particular protein is reduced or absent as a result of the running of the 2D electrophoresis process this reduction or absence will be the same for all samples in the gel. The DIA software is able to co-detect spots on each of the sample images and subsequently calculate volume ratios for each. It is thought that threshold settings greater than 2 model SD (standard deviations) for normally distributed data should be utilised as this threshold gives the user a >95% confidence that the differences observed are not due to random chance. The data from this initial experiment appears normally distributed and, at a threshold of 2 S.D. (corresponding to a threshold setting of approximately 1.6), 121 protein spots showed a decrease in log volume ratio whilst 145 showed an increase. Using a different filtration method, for example fold changes in protein abundance, similar results were obtained – 153 decreased and 194 increased at a threshold setting of 1.5. Therefore stimulation of glutamate receptors appears to induce the altered expression of a large number of protein spots. As

previously mentioned however, this degree of confidence is only relevant if data is normally distributed. Natasha Karp and colleagues have carried out in-depth investigations into the use of DIA in terms of statistical significance (Karp *et al.*, 2004a, b). Studies carried out using a bacterial sample, *Erwinia carotovora*, the authors identified a large degree of experimental variance i.e. deviation from the expected log volume ratio of zero, when labelling this sample with either Cy3 or Cy5 (same sample experiment). They found that comparing ratios of various dyes would result in heavy tails and asymmetry in the distribution of the log volume ratios. This deviation was found to largely implicate low volume spots. Differences in background surrounding low volume spots from the fluorescent dyes can result in a large degree of experimental variation. A similar same sample experiment was discussed in Chapter 3 of this thesis. The DIA data from that experiment over four gels appeared to be normally distributed with very little gel to gel variation in the spot volume ratio distributions, therefore problems associated with this kind of experimental variance are not thought to be a major issue with protein samples derived from hippocampal tissue. Hence, the changes identified in this preliminary study are thought to be representative of what is occurring as a result of pharmacological activation of glutamate receptors. However, they are subject to change when a more thorough analysis is carried out in BVA. Not only does BVA take into account biological variance enabling a more robust statistical comparison, but also helps to negate such deviations and false positive results by the inclusion of a dye swap.

The natural progression therefore was to investigate changes in protein expression in hippocampal slices following pharmacological stimulation of glutamate receptors using biological replicates and BVA. The initial BVA study once again compared the effects of glutamate receptor stimulation on protein expression. As this experiment did not include the aforementioned dye swap a more stringent statistical threshold was employed to aid in the exclusion of false positives. As a result, at a significance level of $p<0.01$ only 20 protein spots changed in abundance and at a significance value of $p<0.05$ around 120 spots changed in abundance. A more detailed and controlled study using a dye swap and this time 3 treatment groups (control, glutamate, glutamate & D-AP5) identified a large number of protein spots (79, $p<0.05$) that were differentially expressed following glutamate receptor stimulation.

Interestingly, at a significance threshold of $p<0.01$, 22 protein spots changed in expression -- a very similar figure to that previously obtained providing confidence in the accuracy of the previous experiment even taking into account the absence of a dye flip. Additional information regarding the involvement of the NMDA subtype of glutamate receptor was obtained by the use of the NMDA receptor antagonist D-AP5. As a result of the co-incubation of glutamate with D-AP5 fewer changes in protein expression occurred. In fact the majority (56 out of 79) of the protein spots that changed in abundance following glutamate treatment did not change in expression when D-AP5 was present in the bathing medium, thus implying that the majority of changes resulting from glutamate receptor stimulation in the CA1 region of mouse hippocampus occur as a result of NMDA receptor activation. The differential expression of those spots unaffected by D-AP5 may occur as a result of mGluRs or AMPA receptors. MGluRs show increased sensitivity following LTP in the CA1 region of rat hippocampus, although this effect was shown to be NMDA dependent (Aronica *et al.*, 1991). Subsequent studies by Bortolotto & Collingridge have provided evidence for an NMDA receptor independent form of LTP mediated via mGluRs (Bortolotto & Collingridge, 1992, 1993, 1995).

As is evident from the first two studies, the same protein can be found in multiple positions on a 2D gel. Individual protein types are often found adjacent to each other with slight shifts in the pH dimension due to shifts in charge, representing post-translational modifications such as phosphorylation, glycosylation and acetylation. Of the 10 protein spots identified in the initial BVA investigation into the role of glutamate receptor stimulation in protein modulation, 7 of the 10 can be found in multiple positions on the 2D gel. Clathrin was identified from two spots positioned adjacent to each other as was dynactin-2 and beta actin which was actually found in 3 adjacent protein spots. This regulation of beta actin is further illustrated in the results section (figure 4.3) and highlights the increase in expression of three different protein spots subsequently identified as beta actin and presumably representing three post translationally modified forms of the protein. Methods exist for the identification of such post-translational modifications of which more than 200 have been identified (Krishna & Wold, 1993), although these largely rely on mass spectrometric based determination. Analyses of PTMs are certainly more complex than the relatively

straightforward proteomics approach taken here, as proteins are often modified to a low stoichiometry and the identification of any PTM requires the isolation and analysis of the specific peptide that contains the modified residue. Moreover, the bond between the PTM and the peptide can also be extremely labile. Nevertheless, many groups have focussed their research on the post-translational modification of proteins (Inzitari *et al.*, 2005; Metodiev *et al.*, 2004; Misumi *et al.*, 2002). Other options which avoid the complexity, cost and labour intensive nature of mass spectrometry based techniques include the use of fluorescent stains which specifically label proteins that have been subjected to a particular form of post translational modification. ProQ-Diamond stain is thought to be highly selective for phosphoproteins (Steinberg *et al.*, 2003; Schulenberg *et al.*, 2003). There also exists fluorescent stains for glycoproteins including Pro-Q Emerald stain, which has been successfully used by Kanninen *et al* in 2004 to investigate the glycosylation changes associated with AD.

The final experiment in this chapter examined the modulation of proteins at glutamatergic synapses. Isolating the dendrites of hippocampal pyramidal cells from the CA1 region of rat slices provided an opportunity not only to investigate local changes, but also to aid in the 'pre fractionation' of our protein sample. A study by Oguri *et al* in 2002 revealed, using multiple large 2D gels, over 6500 protein spots in whole rat hippocampal tissue. We routinely detect just short of 3000 protein spots, therefore the resolution of our protein extracts is by no means optimal. One way to overcome this problem and detect less abundant protein entities is to reduce the complexity of the starting material. Therefore the dissection of dendrites from cell bodies reduces the sub-cellular intricacy of our samples. More importantly however, we hoped to determine which proteins identified in the previous studies in this chapter would be similarly modulated in the absence of signal transduction from the cell body. As a result of these experiments only 7 proteins appeared to change in expression. Given that the post-synaptic density comprises of approximately 250 proteins (Li *et al.*, 2004) not including membrane bound receptors (mGlu, AMPA, kainate and NMDA) and membrane associated proteins, this number appears to be relatively small. There are a few possible explanations for this. Firstly, and most likely, is that during the dissection process the dendrites are no longer viable when

they are transferred to the bathing medium. The dissection process is lengthy and whilst care was taken to maintain the slices in an oxygenated ice-cold environment and to minimise the time taken, there is still the possibility that irreparable damage was done to the tissue. In this respect, from the changes observed at least one of the identified proteins, Hsp8, is a known marker for cell stress. However, co-incubation with the NMDA receptor blocker D-AP5 prevented any differential expression previously observed, indicating that the changes seen actually resulted form glutamate receptor stimulation and not purely as a result of tissue damage per se. Moreover, both test and control groups were treated similarly. In addition, others have previously shown that the separation of dendrites results in electrophysiologically healthy tissue (Cracco *et al.*, 2005). An alternative explanation could be that changes in protein expression ultimately require signal transduction from the soma of the cell. This is unlikely, as many groups have shown rapid translation of mRNAs in the dendrites of neurons following glutamatergic afferent stimulation following the induction of LTP (Cole *et al.*, 1989; Moga *et al.*, 2004; Tsokas *et al.*, 2005). As the changes observed were of very small magnitude (in terms of both number of protein spots altering and the fold change of those that were significantly differentially expressed) it is possible that many other changes in expression were occurring, but were too small to detect even using a highly sensitive technique such as DiGE or that changes were transient in nature and therefore out with the time points chosen for this investigation. Indeed, those that did change may show greater expression differences at earlier or later time points. Also, as many of the molecular components of the post-synaptic density will be membrane bound or membrane associated the possibility of identifying these types of protein molecule are greatly reduced when using 2D gel electrophoresis as, due to their hydrophobicity, they do not enter into the first dimension of the 2D process.

From all the studies in this chapter a large number of protein spots have been highlighted as being differentially expressed following pharmacological stimulation of glutamate receptors. Due to the nature of the system (low spot volume, sensitivity of mass spectrometry) the mass spectrometric identification of all of the protein spots was not possible. Following Fatigo gene ontological analysis it was clear that a functional expression pattern was emerging. Energy and metabolism, protein

metabolism and transport, cytoplasm organisation and biogenesis and neurogenesis were amongst those processes with the greatest representation of protein spots demonstrating differential spot abundance following glutamate treatment. The following sections will discuss the possible or established role of these processes in synaptic transmission and plasticity systems resulting from glutamate receptor activation. Discussion will focus purely on the results obtained from the second experiment on this chapter, as adequate controls relating to both experimental design and to the use of a specific NMDA receptor antagonist were in place for this study.

4.5.1 Proteins assigned to the biological process of cytoplasmic organisation and biogenesis

Cytoplasmic organisation and biogenesis was found to represent large percentages of the regulated proteins in all of the DiGE experiments described in this chapter, perhaps highlighting its importance in our pharmacological stimulation of the hippocampus by glutamate. Cytoplasmic organisation and biogenesis encompasses the cytoskeleton and its modulation. The cytoskeleton is responsible for overall neuronal morphology and, in particular, dendritic and spine morphology. The cytoskeleton is also for scaffolding the internal architecture of the neurone and in the positioning and translocation of other protein molecules. Structural remodelling of synaptic connections is essential for enduring forms of synaptic plasticity possibly by anchoring receptors such as AMPA type glutamate receptors and by localising mRNAs, to the post-synaptic membrane. Spines are found on post-synaptic sites of glutamatergic synapses and activation of glutamate receptors on these spines promotes activity dependent structural changes. For example, focal un-caging of glutamate causes an increase in the size of hippocampal pyramidal cell spines (Matsuzaki *et al.*, 2004; Okamoto *et al.*, 2004) and NMDA receptor activation results in segmental beading of dendrites and loss of dendritic spines. These structural changes require NMDA receptor mediated Ca^{2+} influx and actin polymerisation. Synaptic activity in hippocampal neurons can also induce the appearance of new spines (Toni *et al.*, 1999; Engert & Bonhoeffer, 1999) and the retraction of existing spines (Korkotian & Segal, 2001).

The cytoskeleton is a major component of all living cells and comprises microfilaments (actin), microtubules (tubulin) and neurofilaments (intermediate filaments) all of which were identified as differentially regulated following glutamate receptor activation of hippocampal CA1 neurons. Although it is perhaps not surprising that protein molecules of this kind were identified due to their high abundance in neuronal cells, the fact remains that their expression did change as a result of glutamate receptor activation, consistent with concomitant changes in neuronal morphology as a result of this activation. That the differential expression of the majority (71%) of the proteins involved in cytoplasmic organisation and biogenesis was prevented by the co-application of the NMDA receptor antagonist D-AP5 suggests that much of the altered abundance (representing either modification or altered synthesis/ degradation) was downstream of NMDA receptor activation.

The microfilament actin can be found in high concentrations in dendritic spines (Fifkova & Delay, 1982; Matus *et al.*, 1982; Gotow *et al.*, 1991) in both monomeric (G) and polymerised (F) isoforms. Beta actin is also prevalent (Kaech *et al.*, 1997). Indeed beta actin was one of the two identified proteins shown to be differentially expressed when isolated dendritic fractions were treated with glutamate, an effect which was NMDA receptor dependent. One of the major roles of actin in adult synapses is thought to be in the regulation of spine morphology (Krucker *et al.*, 2000; Desmond & Levy, 1983; Geinisman *et al.*, 1991; Hatada *et al.*, 2000) and in providing structures by which proteins can travel to and from the postsynaptic membrane (Kaech *et al.*, 2001). The anchoring of receptors at the postsynaptic membrane may also be a feature of actin regulation in excitatory post synaptic cells as latrunculin A, an actin filament inhibitor as well as reducing spine number, also reduce AMPA receptor clusters (Allison *et al.*, 1998). Application of NMDA induces spine collapse as was shown by Halpain and colleagues in 1998, an effect that was prevented by jasplakinolide, a compound that stabilises polymerised actin. The actin cytoskeleton lies beneath the plasma membrane and in most cases is attached to the plasma membrane by actin binding proteins. We have identified a large number of actin binding proteins including cofilin, spectrin, and Lasp-1 that showed concomitant differential expression following glutamate treatment. TCP-1 is not actually grouped under cytoplasmic organisation and biogenesis in terms of this

discussion but can be classed as an actin-binding protein as it additionally mediates the folding of other proteins (more specifically actin and tubulin (Rommelaere *et al.*, 1993)). It also demonstrates a high sequence homology to other molecular chaperones such as Hsp60 and Hsp70 (Gupta, 1990). Likewise, WDR1 is grouped under a different classification (neurophysiological process) but this protein molecule is also known as actin interacting protein 1 (Aip1). Spectrin levels have been shown to be significantly decreased upon glutamate receptor stimulation in this study. Indeed, it was one of the largest changes observed, with nearly a two-fold change. Seubert *et al* in 1988 reported that stimulation of NMDA receptors induced a cleavage of spectrin in hippocampal slices, an NMDA receptor dependent effect. In our hands, the decrease in spectrin, which could be interpreted as an increase in spectrin proteolysis, was not blocked by D-AP5 (glutamate alone fold change=-1.73 p=0.0073, glutamate + D-AP5 fold change=-1.75 p=0.017). Lasp-1 has also very recently been identified by Phillips *et al* (2004) to be strongly expressed at synaptic sites presumably binding to F-actin in a phosphorylation (activity) dependent manner as has been shown in other cell types (Chew *et al.*, 2002). Lasp-1 belongs to the LIM protein subfamily and has an actin-binding domain in its core (Schreiber *et al.*, 1998). Initially it was identified as an over-expressed gene in human breast cancers (Tomasetto *et al.*, 1995). Other members of this family, the LIM kinases have been shown to phosphorylate members of the ADF/ cofilin family of actin binding proteins, another actin binding protein identified in this study, as part of the Rho kinase pathway (Sumi *et al.*, 2001) Following glutamate receptor stimulation of hippocampal CA1 tissues, Lasp-1 was shown to decrease in expression (-1.21 fold change) whereas cofilin increased by a similar degree (1.18 fold change). Possible reasons for this could be that the particular isoform of Lasp-1 may be the inactive (dephosphorylated) form which is decreasing in expression whereas cofilin-1 could be the active (phosphorylated) form subsequently increasing in expression. The use of the previously discussed phosphor protein stains would represent a useful means by which to investigate this question.

In the dendritic shaft the cytoplasm is dominated by microtubules (Peters *et al.*, 1976; Matus *et al.*, 1983). Tubulin is one of the many 'housekeeping genes' commonly used as a control for western blot analysis. In this study several isoforms

of tubulin were identified, some of which exhibited increased expression and others decreased expression. Western blot analysis of tubulin expression levels was inconclusive presumably due to the variation in protein levels of several different isoforms counteracting one another. Not only does this current study highlight problems associated with the continued use of proteins such as tubulin for western blot analysis, but other researchers have also indicated that the use of tubulin and GAPDH (also identified as differentially expressed in this study) may pose problems with normalisation as frequently they change in expression in a number of conditions (Ferguson *et al.*, 2005). Ferguson and colleagues investigated the expression of actin, tubulin and GAPDH in malignant kidney tissue versus normal kidney tissue and found differential expression of all three proteins. In fact, in one sample there was a complete absence of beta tubulin. This highlights the importance of choosing an appropriate internal standard for western blot techniques. This aside, several protein spots identified as differentially regulated following glutamate treatment of the hippocampal neuropil were found to contain tubulin. This is suggestive not only of several post-translational modifications of this protein but in addition highlights the appearance of different isoforms of the protein (alpha, beta etc.). Beta tubulin has been implicated in glutamate induced neuronal apoptosis in cerebellar granule cells (Ankarcrona *et al.*, 1996). Lopez-Colome and Casas (1984) have also shown that kainate, a heterocyclic analogue of glutamate, induced a dose dependent aggregation of tubulin in rat brains. Furthermore, a specific isoform of tubulin (beta 4) has itself been identified as an actin binding protein. Considerable evidence has accumulated for the involvement of microtubule-associated proteins (MAPs) in synaptic activity in the hippocampus. The phosphorylation state of one of these proteins, MAP2, regulates its interaction with both actin and tubulin, thereby playing a major role in the modulation of neuronal morphology (Quinlan & Halpain, 1996). MAP2 phosphorylation is reported to be increased following metabotropic receptor activation, which was followed by a persistent de-phosphorylation mediated by NMDA receptors and calcineurin (Sanchez *et al.*, 1997). Another microtubule-associated protein, Tau, is a prominent marker in the pathology of Alzheimer's disease (Grundke-Iqbal *et al.*, 1986). Glutamate has been shown to induce a de-phosphorylation of tau in cultured primary rat hippocampal neurons (Hull *et al.*, 1999) and *in vivo* application of high concentrations of glutamate resulted in the differential expression of phosphorylated MAP2 and tau in the rat parietal cortex

(Irving *et al.*, 1996). Microtubule formation and stability is regulated not only by NMDA type glutamate receptors, but also via mGluRs (Huang & Hampson, 2000). This is perhaps the reason that the differential expression of a small proportion of the molecules (tubulin and spectrin) was not prevented when slices were co-treated with D-AP5 as well as glutamate.

A number of neurofilaments were found to change in expression as a result of glutamate treatment of hippocampal slices. Two of these proteins increased in their expression (alpha internexin and neurofilament H). All three identified vimentin isoforms however, decreased in expression. Intermediate filaments, named in relation to their size compared to microfilaments and microtubules, have differential localisation throughout the neuronal architecture of the hippocampus. Alpha internexin is localised to dendritic spines (Suzuki *et al.*, 1997) whereas NF triplet proteins (L, M and H) are predominantly found in the axons of neurons (Benson *et al.*, 1996). The cellular location of NF-H is dependent on its phosphorylation state – active (phosphorylated) NF-H is located in the axons of neuronal cells whereas inactive (nonphosphorylated) NF-H can be found within dendrites, suggesting a translocation of this molecule from dendrites into axons upon stimulation of the cell (Sternberger & Sternberger, 1983; Lee *et al.*, 1987). However, other studies report the phosphorylation of NF-H retards its transport allowing for structural stability of the axon (Carden *et al.*, 1987) an effect which precedes dendritic sprouting (Hall *et al.*, 1991). NF-H levels have been shown to decrease in the brain following ischaemia (Kaku *et al.*, 1993). This was confirmed by a proteomics study by Krapfenbauer *et al* in 2001 who demonstrated that following intra-peritoneal injection of kainic acid, levels of both alpha internexin and neurofilaments were decreased. Alpha internexin was identified in a recent proteomics study by Paulsen and colleagues (2004) as differentially down-regulated following MK801 treatment of the rat thalamus, which ties in with our finding that glutamate receptor activation, more specifically NMDA receptor activation increases alpha internexin levels. It is worth mentioning at this stage that the model adopted for this investigation into protein regulation resulting from glutamate receptor activation could be interpreted as a model for neurotoxicity. However, there does not appear to be an abundance of apoptotic markers identified in this study. NF-H, although highlighted as regulated

(decreased) following kainic acid administration, in our hands actually increased in expression. Another identified protein, stress induced protein-1, may well be an indicator of a degree of cellular damage as this protein has been shown to induce neuroprotective signals that rescue cells from apoptosis (Zanata *et al.*, 2002). However, it is not improbable that this protein has more than one function and may also be involved in the plasticity related process. In addition, a study by Gozal *et al* in 2002 investigated protein changes in the CA1 region of rat hippocampus following intermittent hypoxia (IH) which had previously been shown by this group to impair performance of spatial learning tasks as measured in the Morris water maze (Gozal *et al.*, 2001). There also appeared to be cellular changes and architechtural disorganisation in the cortex and hippocampus compatible with excitotoxicity processes. Following IH 32 proteins in the CA1 region of the rat hippocampus wee identified as changed. Of these 32 only 5 can be found in the studies described in this chapter. Moreover, only one of these 5 proteins, Hsp60, has been classified by Gozal and colleagues as an apoptotic marker. It is unlikely therefore, that this pharmacological model of glutamatergic afferent stimulation is representative of a model for neurotoxicity.

The discovery of changes in the neurofilament protein vimentin in this study indicates a degree of neuritogenesis, as vimentin is initially expressed by most neuronal precursors *in vivo* after which it is replaced by neurofilaments although both vimentin and other neurofilaments can be detected transiently and simultaneously indicating neuritogenesis. Treatment of cultured hippocampal neurons with vimentin sense-oriented deoxyoligonucleotides resulted in neurite outgrowth, which was blocked by anti sense oligonucleotides (Boyne *et al.*, 1996). Vimentin in this study was shown to decrease in its expression following glutamate receptor stimulation by very similar amounts (-1.51, -1.63 and –1.65) and all changes in expression were prevented by D-AP5. Previous studies exploring the exposure of cultured rat cortical astrocytes to glutamate demonstrate an increase in phosphorylated vimentin and GFAP (Yano *et al.*, 1994). As vimentin is not normally expressed in mature neurons its appearance, regardless of expression levels, indicates neurite outgrowth. Vimentin is also expressed in cases of reactive gliosis, such as occurs following seizures in the hippocampus (Stringer, 1996).

Finally, the protein DJ-1 also known as PARK 7 was found to increase in expression following glutamate receptor stimulation; an effect blocked by the NMDA receptor antagonist D-AP5. The biochemical function and sub-cellular localization of DJ-1 protein has not yet been clarified. DJ-1 is found to be mutated in autosomal recessive early onset Parkinson's disease, and is highly expressed in the hippocampus (Shang *et al.*, 2004). Its involvement in excitatory neurotransmission is unclear, and the discovery of its modulation following glutamate receptor activation in the hippocampus may indicate a putative role in glutamate receptor mediated synaptic transmission in the hippocampus.

4.5.2 Proteins involved in energy and metabolism processes

Given that glutamate treatment will result in the significant activation of major excitatory receptors it is unsurprising that such a treatment will result in modulating the expression of molecules associated with energy metabolism. Indeed, given the large degree of structural changes mediated by glutamate receptor activation the fact that energy demands are significantly altered as a result is perhaps to be expected. Energy and metabolism, including nucleotide and carbohydrate metabolism comprises more than a third of the identified proteins. Substantial restructuring and an anticipated increase in cell activity, synaptic transmission and connectivity following stimulation of glutamatergic synapses will undoubtedly result in a large change in metabolism levels and energy requirements within the cell and its support network indicated by the presence of molecules involved in glycolysis, the tricarboxylic acid (TCA) cycle and nucleotide metabolism.

Certain molecular components have individual roles in neuronal function out with their role in energy and metabolism pathways. For example, glucose phosphate isomerase, also known as neuroleukin, the expression of which was shown to significantly decrease following glutamate treatment of hippocampal slices, may promote neurite outgrowth (Gurney *et al.*, 1986). More recent studies have highlighted a central role for neuroleukin and its receptor gp78 in hippocampal learning (Luo *et al.*, 2002). The enolase identified here is of the non-neuronal

subtype, most likely present in the supporting network of the hippocampus to aid in the production of ATP. Enolase was found in more than one position on the 2D gel (spots 13 and 44). As the position of this protein differed along the MW axis of the 2D gel it is possible that the 2 spots represent cleaved/ uncleaved forms of enolase. This is further suggested by the fact that as one spot increases in expression the other decreases.

Aconitase 2 and succinate dehydrogenase (Sdha) are two components of the TCA cycle that may have an essential role in glutamatergic synaptic transmission out with carbohydrate metabolism. Inhibition of Sdha (succinate dehydrogenase) results in an increase in the EPSP slope as a result of increased succinate (Roehrs *et al.*, 2004) an effect dependent on NMDA receptor activation. Excitatory synaptic stimulation by glutamate application actually caused an NMDA dependent increase in Sdha spot abundance, which would be indicative of a decrease in succinate, thereby contradicting the study by Roehrs and colleagues.

Aconitase 2, also known as Iron Regulating Protein 2 (IRP2) is an mRNA binding protein which regulates intracellular iron levels by binding to iron response elements (IREs). Paulson *et al* in 2004 highlighted aconitase as a potential modulator in schizophrenia using a model of hypoglutamatergic brain function in the thalamus. The authors observed a decrease in aconitase expression following treatment with the NMDA receptor antagonist MK801. Stimulation of glutamatergic synapses by glutamate in our hands produces an NMDA receptor dependent increase in aconitase 2 expression. It is possible that aconitase activity is regulated via nitric oxide production which itself results from glutamate receptor activation (Jaffrey *et al.*, 1994). Western blot analysis was unable to confirm the subtle changes in aconitase levels detected by DiGE. Not only did the antibody appear to be of poor quality, but a 10% change seen in our DiGE experiment, is much more difficult to detect using less sensitive western blot techniques due to the nature of the protein visualisation.

Finally in this section the identification of differentially expressed N-acetylneuraminic acid, which is involved in the production of sialic acid, is another

indicator that glutamate receptor activation triggers biochemical events previously associated with synaptogenesis or neuritic outgrowth. Sialic acid is involved in the sialylation of glycoproteins and glycolipids, which in turn is thought to affect neuronal plasticity. For example, synaptogenesis, induced by the lesioning of the CA3-CA1 synaptic connections, is associated with the expression of sialylated cell adhesion molecules on sprouting nerves. Subsequent removal of the sialylated portion delays the observed neurite outgrowth (Muller *et al.*, 1994).

4.5.3 Proteins involved in protein metabolism and transport

This functional group comprises a wide variety of proteins including molecular chaperones (Hsc70, Hsp60, and TCP-1), ubiquitination molecules (OTUBAIN 1, proteasome 26s), actin-binding proteins (Plastin), endocytotic proteins (Clathrin) and signalling molecules (calcineurin). Whilst a number of these molecules could fall into other categories, the FatiGo gene ontology system, which was used throughout this thesis, classified as a protein metabolism and transport molecule.

OTUBAINs can reverse the ubiquitination of proteins. This chapter reports a significant increase in OTUBAIN 1 spot abundance in hippocampal tissues following glutamate receptor stimulation. Recent work by Michael Ehlers (2003) has demonstrated that long term changes in synaptic activity cause postsynaptic proteins to become highly ubiquitinated and for the turnover of certain components of the PSD to increase. Furthermore, pharmacological activation of NMDA receptors has also been shown to produce ubiquitination of PSD-95 and its subsequent degradation by proteasome 26s protein, another molecule that is found to show glutamate induced increased abundance. The fact that we see an increase in both may either represent increased production due to an increased demand for the ubiquitin-proteasome pathway or may represent an increased availability of these molecules due to greater dissociation from their substrates.

The abundance of a number of molecular chaperone protein molecules was altered following glutamate treatment of the CA1 region of mouse hippocampus. The majority of the effects observed were prevented by D-AP5. Heat shock cognate (Hsc) 70 is part of a 70kDa family of heat shock proteins that is expressed under normal conditions (Gething & Sambrook, 1992). As molecular chaperones they play an important role in the synthesis, transport and correct folding of proteins (Sharp *et al.*, 1999; Craig *et al.*, 1993, 1994; Flaherty 1990). HSC70 is also thought to form part of a trimeric complex with cysteine string protein and SGT, which acts as a synaptic chaperone machine possibly involved in synaptic vesicle recycling (Tobaben *et al.*, 2001). HSC70 mRNA levels have been shown to increase following psychophysiological stress in the CA3 region of the hippocampus (Fukudo *et al.*, 1999) and after exposure to NMDA receptor agonists (Kwong *et al.*, 2003). Expression of Hsc70 was found to be highest in the large neurons of the cerebellum and hippocampus, (Morrison-Bogorad *et al.*, 1994), and in the hippocampus both Heat shock protein (Hsp) 70 and Hsc70 are distributed throughout the soma and dendrites. In dendrites Hsc70 appears to co-localise with PSD-95 and has been proposed by Moon *et al* in 2001 as a potential candidate for a synaptic tag (Frey & Morris 1997, 1998). With its association with the PSD, Hsc70 may serve to function as an anchor for certain proteins at synapses and upon stimulation mediate correct folding and subsequent release of the protein. Indeed HSP70 proteins have previously been shown to act in this manner (Zeiner *et al.*, 1997; Bimston *et al.*, 1998; Takayama *et al.*, 1999). Another protein found to display altered abundance following glutamate receptor activation was stress-inducible protein 1, a co-chaperone that is homologous with Hsc70/ Hsp90. As yet no relationship between this molecule and glutamate receptor activation or synaptic plasticity related events has been proposed.

TCP-1 is a molecular chaperone highly homologous to Hsp60 and Hsp70 that is responsible for the assembly and release of tubulin and actin (Chen *et al.*, 1994; Miklos *et al.*, 1994; Ursic *et al.*, 1994; Vinh & Drubin 1994). The difference in expression levels of several isoforms of TCP-1 (delta, eta and gamma) is in keeping with a difference in expression levels of the many isoforms of tubulin.

N-ethylmaleimide sensitive fusion protein (NSF) displays a pre-synaptic location and is reported to be involved in neurotransmitter release and protein transport (Sollner *et al.*, 1993). NSF was shown by Nishimune and colleagues in 1998 to interact directly and selectively with the intracellular C-terminal domain of the GluR2 subunit of AMPA receptors (Nishimune *et al.*, 1998). Blockade of the NSF-GluR2 interaction prevented long-term depression (LTD) (Luthi *et al.*, 1999). As an ATPase it drives the fusion of synaptic vesicles with the presynaptic plasma membrane through its interaction with SNAPs (soluble NSF-attachment proteins) (Rothman 1994; Sudhof 1995; Calakos & Scheller 1996; Goda 1997). Cells exposed to N-ethylmaleimide (NEM) show no change in basal synaptic transmission, but have a reduced level of potentiated synaptic responses at glutamatergic synapses (Lledo *et al.*, 1998) therefore the decrease in NSF abundance observed following glutamate receptor stimulation is in keeping with this finding. Recent evidence indicates that AMPARs are selectively and acutely regulated by NSF, which forms part of a multi-protein complex with AMPARs (Lim and Isaac, 2005).

Clathrin is present as either a heavy chain (CHC), the product of a single gene or as 2 light chains (CLa & CLb). Here, we have identified CHC spot abundance to be significantly decreased following pharmacological activation of glutamate receptors in the CA1 region of the hippocampus. In the dentate gyrus of the hippocampus, CHC has been found to be induced by kainate (Konopka *et al.*, 1995) and the numbers of clathrin-coated pits, (CCPs), are also found to increase following LTP (Toni *et al.*, 2001). It has been suggested that CCPs are responsible for the internalisation of GluRs including AMPARs (Man *et al.*, 2000; Wang & Linden 2000; Lee *et al.*, 2002) and NMDARs (Roche *et al.*, 2001; Vissel *et al.*, 2001; Nong *et al.*, 2003). CCPs are commonly found at glutamatergic synapses early on in development (Petralia *et al.*, 2003) and at the tips of dendritic filopodia in young cultures (Blanpied *et al.*, 2002). A role for glutamate receptor activation in receptor mediated endocytosis, neurogenesis or synaptic vesicle recycling is indicated by a decrease in CHC, an effect mediated by NMDARs. The initial investigation into glutamate-mediated regulation of proteins identified an increase in clathrin expression, whereas the second study identified a significant decrease in clathrin spot expression. As with many of the proteins in this study it is not known whether or not each protein is identical in its PTMs or isoform. As such, it is not possible to draw

conclusions as to the role of clathrin in this model of pharmacological stimulation of glutamate receptors. Suffice to say that clathrin is involved in this process in an NMDA receptor dependent fashion and as such may mediate any number of the effects listed above.

4.5.4 Proteins involved in neurogenesis

The observed differential expression of 33% of the proteins belonging to the biological process of neurogenesis was prevented by D-AP5 co-incubation. As neurogenesis in the dentate gyrus region of the hippocampus is known to be dependent on NMDA receptor activation (Cameron *et al.*, 1995) it is possible that in the CA1 region of the hippocampus neurogenesis is in fact dependent on another glutamate receptor subtype. The neurogenesis group comprises solely of the collapsing response mediator protein (CRMP) family. In 1996 Wang & Strittmatter characterised all 4 members of this family in the rat. They are highly homologous with each other and also to unc-33, a gene known to be required for axonal and synaptic organisation of neurons in the nematode (Li *et al.*, 1992). More recently, a fifth member of this group, the CRMP-associated molecule, (CRAM, CRMP5) has been identified and characterised (Inatome *et al.*, 2000; Hotta *et al.*, 2005). In this proteomics study all CRMP members (with the exception of CRMP5) are present and display increased expression following glutamate treatment. The increase of CRMP-2 is almost completely (3 out of 4) blocked by D-AP5. The differential expression of the remaining family members (1, 3 & 4) is unaffected by the NMDA receptor blocker. All CRMP molecules are exclusive to the nervous system and are thought to be involved in neuronal development (Wang & Strittmatter, 1996). CRMP-2 was shown to be the most widely expressed of the CRMP family in developing neurons, but was also identified in adult hippocampal pyramidal cells. CRMP 1 and 4 are expressed during late embryonic and early postnatal development, whilst CRMP 3 is almost exclusive to postnatal granule cells of the cerebellum.

The identification of CRMP-2 in our model is therefore not surprising given that it has been shown in adult hippocampal neurons. However, the expression of CRMP-1, -3 and -4 indicates an alternative role for these molecules in mature hippocampus. As

these molecules could be considered markers for neurogenesis (Parent *et al.*, 1997; Seki 2002), treatment of hippocampal CA1 regions with glutamate is possibly inducing neurogenesis in this region. It is known that, especially in the dentate gyrus region of the hippocampus, new neurons are formed in adult hippocampus. It is possible from our studies that this effect is also apparent in the CA1 region of adult hippocampus.

The literature on CRMP-2 is indeed more extensive than on the other three family members. It has been reported to induce axons in cultured hippocampal neurons (Inagaki *et al.*, 2001) and along with CRMP-4 is a substrate for GSK-3, the increased activity of which is associated with Alzheimer's disease. CRMP-2 has also been shown to bind to tubulin heterodimers thereby enhancing microtubule assembly and subsequent axon elongation (Fukata *et al.*, 2002).

Our results indicate that a certain degree of neurogenesis is being induced by glutamate treatment of the hippocampus, although not via NMDA receptors. The effects of CRMP-2 however, perhaps on axon elongation and microtubule assembly appear to be in part mediated by NMDA receptors.

4.5.5 Proteins involved in the response to stress

Only 2 of the many proteins identified suggest any kind of neuronal damage occurring as a result of glutamate application to hippocampal slices. In actual fact, the role of one of the molecules STIP1 has already been discussed with respect to its role as a co-chaperone with Hsc70. The other response to stress molecule catalase actually decreases in abundance. It is therefore possible that although classically these molecules are markers in response to cellular stress, that this is not the case here.

4.5.6 Proteins involved in neurotransmitter production and release

The identity of proteins involved in neurotransmitter production and neurotransmitter release such as synapsin and GAD65 illustrate the fact that the adopted proteomics approach whilst in some ways is limited in its ability to detect low abundance molecules and membrane bound protein molecules, nevertheless is inclusive in that both presynaptic, postsynaptic and neuroglia are all sampled for possible changes in their protein expression content. This inclusive approach is in some way a reverse of the traditional reductionist approach in which a single protein is investigated in great molecular and mechanistic detail.

Glutamic acid decarboxylase 65, (GAD65), expression was up regulated following the treatment of hippocampal slices with glutamate. Whilst GAD67 is considered by most authors to be responsible for the production of GABA (Asada *et al.*, 1997) the primary role of GAD65 is suggested to be in the packaging and release of the inhibitory neurotransmitter from pre-synaptic terminals, although the literature on this is somewhat mixed. Previous investigations into GAD65 and its role in synaptic transmission have shown that whereas the genetic deletion of GAD65 has no effect on basal GABAergic transmission, there was an inability to sustain inhibitory neurotransmission following repetitive activation. This was seen in the visual cortex and in the hippocampus where the inhibitory response to prolonged stimulation was reduced in GAD65 knock out mice, including a decrease in post tetanic potentiation of inhibitory responses (Tian *et al.*, 1999). It therefore appears that GAD65 may have a central role in the release of GABA at synapses undergoing increased stimulation. Our results indicate an increase in GAD65, which would possibly suggest an increase in the release of inhibitory GABA following glutamate receptor activation. This perhaps represents a mechanism to prevent the hyper-excitability of pyramidal neurons following glutamate treatment.

Synapsin II was one protein of several contained within a single protein spot that decreased in abundance following pharmacological activation of glutamate receptors. Along with syntaxin and synapsin I, synapsin II is involved in the trafficking, docking and fusion of synaptic vesicles required for neurotransmitter release. Their

roles in presynaptic terminals are largely controlled by phosphorylation (Chi *et al.*, 2001, Hosaka *et al.*, 1999). In a dephosphorylated form, synapsins trigger actin polymerisation (Greengard *et al.*, 1993; Bloom *et al.*, 2003) and phosphorylation results in the dissociation of synapsins from their synaptic vesicles (Hosaka *et al.*, 1999). The phosphorylation state of synapsin I is enhanced with increased Ca^{2+} dependent release of neurotransmitters induced by glutamate or LTP (Nayak *et al.*, 1996). Deletion of synapsin II was also reported to result in the disruption of the actin cytoskeleton (Ferreira *et al.*, 1998). Initial studies by Ferriera *et al.*, in 1995 provided evidence that synapsin II depletion by antisense oligonucleotides resulted in the absence of synapses in hippocampal neurons whereas later studies identified that depletion of synapsin II results in a reduction in axon elongation, whereas deletion of synapsin I only, resulted in a reduction in synapse formation (Ferreira *et al.*, 1998). Other evidence supports a role for synapsin II in synaptogenesis (Han *et al.*, 1991). Unfortunately it is not possible at this stage to determine whether or not the synapsin II identified in this study as regulated following glutamate receptor stimulation is either the phosphorylated or non-phosphorylated form. The decrease observed here could be indicative of a decrease in the non-phosphorylated form and a subsequent decrease in actin polymerisation for example. Although concomitant increases in synapsin II have not been identified, it may well be that unidentified protein spots hold this information.

4.5.7 Proteins involved in signal transduction

Perhaps not surprisingly, all proteins involved in signalling cascades (protein phosphatases and protein kinases) were modulated by NMDA receptor activation. Calcineurin (PP2A) a protein phosphatase was identified from two individual differentially expressed protein spots, one increasing whilst the other decreasing; effects that were prevented by the co-application of the NMDA receptor antagonist D-AP5. Calcineurin is activated by calcium influx mainly through the NMDA receptor where it suppresses protein phosphatase 1 activity and is ultimately linked to a depression of the synaptic response (Mulkey *et al.*, 1994). Calcineurin is also known to activate MAP2, ERK1/ 2, MEK 1/ 2 and p70S6 kinase (Pei *et al.*, 2003; Sanchez-Martin *et al.*, 1998; Norman *et al.*, 2000) all of which are involved in

signalling cascades, which result from NMDA receptor activation. ERK II was also identified as differentially expressed in this proteomics investigation. Several studies have examined the effect of ERK I/ II on plasticity in the hippocampus. Neuronal depolarisation by KCl was seen to enhance ERK activity, an effect completely blocked by the AMPA receptor antagonists CNQX and partially blocked by D-AP5 (Baron *et al.*, 1996). Extracellular regulated kinases (ERKI/II) have an important role in both long-term memory and long-term potentiation (Davis and Squire 1984; Walz *et al.*, 1999; Rosenblum *et al.*, 2002) and are abundant kinases present in the nervous system activated, amongst other conditions, by stimulation of glutamate receptors. ERK II was shown to translocate from the nucleus upon stimulation (either BDNF or HFS) of PC12 cell lines and primary cortical cultures. The inhibition of ERK II we see in this study is possibly a decrease in the inactivated or dephosphorylated isoform of this protein, as we see a decrease in PP2A, which we would expect to accompany an increase in activated ERK II. Although this finding is in keeping with current literature, it would require further confirmation.

4.5.8 Proteins involved in transcription and translation

Farsla protein is also known as phenylalanine-tRNA synthetase-like protein. Whilst very little is known about this specific protein molecule the aminoacyl-tRNA synthetases, a similar group of proteins, are enzymes required for protein synthesis. Their role is in the correct formation of proteins by catalysing the appropriate attachment of specific amino acids to their corresponding tRNAs. In murine erythroleukaemia (MEL) cultured cells, the expression of phenylalanine-tRNA synthetase regulatory alpha subunit like mRNA was decreased during differentiation of these cells (Zhou *et al.*, 1999). In our hands this protein also shows a decrease in expression suggesting a degree of cellular differentiation. However, the two systems used are very different making interpretation of these results problematic.

4.5.9 Proteins involved in calcium ion binding

Cab39, also known as Mo25 is a calcium ion binding protein. There is very little evidence pertaining to the function of this protein molecule in neurones. Cab39 has been shown to complex with LKB1 and STRAD • and in doing so anchors LKB1 to the cytoplasm and enhances its catalytic activity. LKB1 itself has been implicated in regulating cell proliferation and polarity and thereby is involved in oncogenesis (Boudeau *et al.*, 2003). As its name also indicates its role of calcium binding, a decrease in the abundance of this molecule following glutamate receptor activation suggests a reduced requirement possibly as a result of reduced calcium levels. This is likely at late time points following the initial receptor activation. However, its role in glutamate receptor mediated synaptic transmission is far from clear and would therefore make a potential candidate for further mechanistic investigation.

In summary, the use of the hippocampal slice preparation in conjunction with DiGE has provided a useful and informative investigation into differential protein expression following pharmacological activation of glutamate receptors. This chapter not only illustrates the progression of using this technique from early proof of concept studies through to robust controlled investigations with statistical rigour but also reveals a wide variety of protein classes that are differentially expressed as a result of glutamate receptor activation. Additionally, the relationship of the NMDA type glutamate receptor to these changes in protein abundance was also investigated, further elucidating their role in excitatory neurotransmission in the hippocampus. Finally, using a sub-fractionation method to isolate dendrites of hippocampal neurons it was possible to identify local (synaptic) changes in protein expression levels following treatment of isolated tissue with glutamate. Such methodology could provide invaluable for the identification of potential synaptic tags following synaptic stimulation of glutamatergic afferents.

Chapter 5

Differential protein expression in early and late phases of long term potentiation in the CA1 region of mouse hippocampus.

5.1 INTRODUCTION

This chapter investigates changes in protein expression associated with the induction of long-term potentiation (LTP) in the CA1 subfield of mouse hippocampus, where high frequency stimulation of glutamatergic afferents results in an increase in the post-synaptic response of CA1 pyramidal cells (Schwartzkroin & Wester, 1975). LTP is considered an important experimental model for the cellular correlates of learning and memory (Morris *et al.*, 1986; McNaughton *et al.*, 1986; Castro *et al.*, 1989; Moser *et al.*, 1998). It can be described in three temporal phases. The initial phase (phase I), lasting from shortly after the induction stimulus for up to 1-2 hours, is dependent on covalent modification of pre-existing proteins (eg. phosphorylation) (Lovinger *et al.*, 1987; Reymann *et al.*, 1988a, b; Malenka *et al.*, 1989). The intermediate phase (phase II) - apparent around 2 hours following the stimulus - is dependent on the synthesis of new protein via mRNA translation, but is not dependent on gene transcription (Stanton & Sarvey, 1984; Frey *et al.*, 1988). In contrast, the slowest but most sustained phase (phase III, 3+hours) is dependent not only on the synthesis of new protein, but also on gene transcription and the synthesis of new mRNA (Nguyen *et al.*, 1994; Frey *et al.*, 1996). The evidence suggests that these three phases can be observed in all the different models of neuronal plasticity, whether derived from *Aplysia* (Barzilai *et al.*, 1989), (Ghirardi *et al.*, 1995), *helix*, (Schilhab & Christofferson, 1996), *hermissenda* (Crow & Bridge, 1985), lamprey (Parker & Grillner, 1999), *drosophila* (Xia *et al.*, 1998) or hippocampal LTD (Manahan-Vaughan, 2000) as well as LTP in the amygdala (Bailey *et al.*, 1999), and in the dentate gyrus (Otani *et al.*, 1989), CA1 (Frey *et al.*, 1996; Nguyen & Kandel, 1997) and CA3 (Huang *et al.*, 1994) regions of the hippocampal formation. Hence these three sequential and mechanistically distinct phases are likely to represent a general feature of sustained plasticity.

Despite intensive research over a number of years, there is no clear picture as to the identity of the proteins involved in phases II and III of the plasticity response. Currently, elevated expression of around 60 distinct mRNAs has been demonstrated after the induction of LTP in the hippocampus, with roughly half of these induced sufficiently rapidly for the resulting protein to contribute to phase II of LTP (Morris, 2003). However, these genes have generally been identified via changes in mRNA

expression, and there have been recurring concerns about the potential divergence between changes in protein and mRNA content. Hence these mRNAs may not be predictive of the protein changes occurring during neuronal plasticity. Proteomics is defined as the study of proteins expressed by an organism, tissue or cell and the changes in protein expression occurring in different environments and conditions. Surprisingly, to date there have been no proteomic studies aimed at identifying protein changes during synaptic plasticity. This may simply reflect the relative novelty of the techniques, or perhaps it points to the fact that 2D gel electrophoresis is commonly associated with problems such as reproducibility and quantification of findings. These barriers have been overcome by the introduction of a new technique known as difference gel electrophoresis (DiGE) (Unlu *et al.*, 1997). The availability of 3 spectrally resolvable fluorescent dyes, the ability to pre-label protein samples and co-run them within the one 2D gel, along with the incorporation of an internal standard, has revolutionized 2D gel electrophoresis in the last ten years.

Therefore, in order to address the issue of alterations in protein expression following enduring synaptic plasticity, proteomics and more specifically DiGE, in combination with *in vitro* slice electrophysiology has been employed. Global changes in protein expression levels were examined shortly after LTP induction (10minutes) and following a more prolonged potentiation of the synaptic response (240minutes).

5.2 Study Aims

Current theory suggests that temporal components of LTP involve an initial modification of pre-existing protein molecules followed by longer-term modifications. The following experiments were designed to investigate the differential expression of components of hippocampal proteome associated with early and late phases in the plasticity process:

1. To investigate global changes in the hippocampal proteome at 10 minutes following bulk induction of LTP in area CA1.

2. To investigate global changes in the hippocampal proteome at 240 minutes following bulk induction of LTP in area CA1.

3. To map the time-course of expression from early phases through to late phases of potentiation for particular protein spots, samples were run within a single DiGE experiment.

5.3 METHODS

Methods are as described in chapter 2, with further details as follows. Hippocampal slices were obtained from male ICR mice (aged 4-8weeks) and all protein extracts from synaptically stimulated tissue were run on pH3-10 non linear IPG gels.

Table 5.1 Experimental design of short and long-term potentiation DiGE experiment

Gel Number	Cy2	Cy3	Cy5
1	Standard	10minute control	4hr LTP
2	Standard	10minute LTP	4hr control
3	Standard	10minute control	4hr control
4	Standard	10minute LTP	4hr LTP
5	Standard	4hr LTP	10minute control
6	Standard	4hr control	10minute LTP
7	Standard	4hr control	10minute LTP

Table detailing the experimental design adopted for analysis of 10minute control, 10minute LTP, 4hr control and 4hr LTP treated hippocampal CA1 regions using DiGE. Only 7 gels are present due to the loss of one gel in the running of the 1st dimension.

5.4 Results

5.4.1 Development of a robust model of late phase LTP.

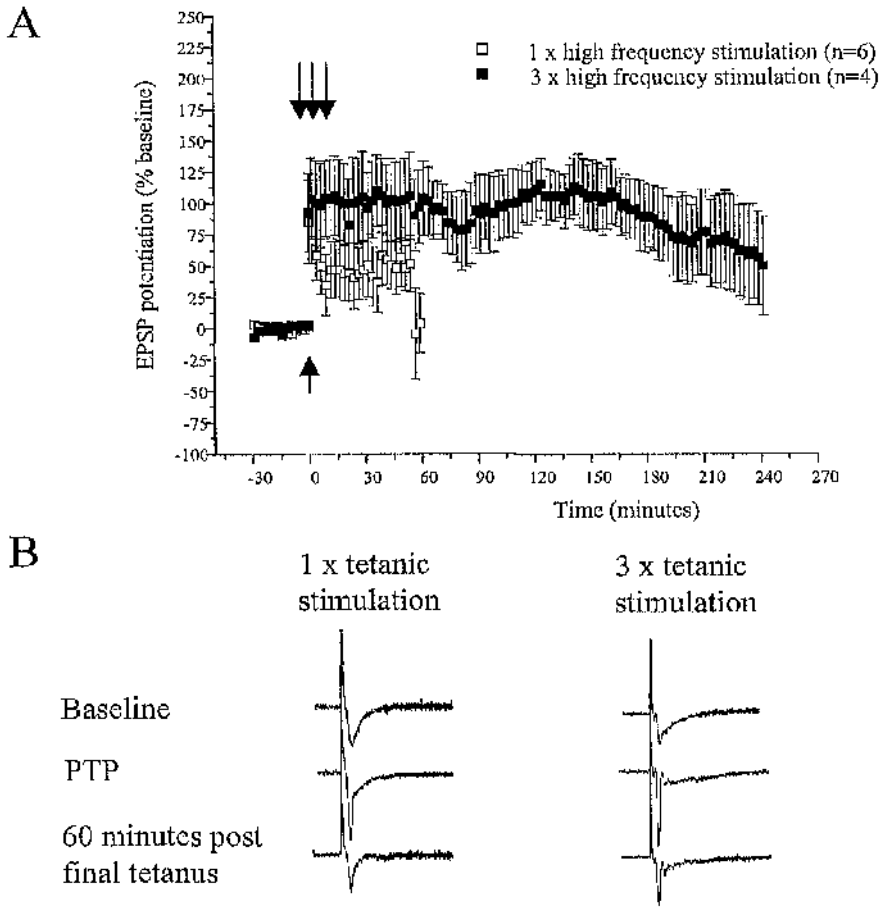
Initial experiments using acute hippocampal slices (n=6) investigated the ability of a single tetanic stimulation (100Hz 1s, 1ms pulse width) to induce a long lasting increase in synaptic strength. This type of stimulus failed to produce a potentiation lasting longer than 1hr (figure 5.1a, open squares). After approximately 5 minutes potentiation had reached 58.87 ±20.4% of baseline. By 60 minutes this had reduced to 4.5 ± 23.5%.

Using a stronger stimulation protocol that is favoured by several groups (3 x 100Hz for 1s, 10 minute inter-train interval, n=4), (Lynch *et al.*, 1983; Frey & Morris 1998), it was possible to induce an enduring potentiation of synaptic strength in excess of 4hrs (49 ±39%) (figure 5.1a, filled boxes). Not only did the single tetanus fail to induce a long lasting potentiation, it also produced a potentiation of smaller amplitude than that of the 3x 100Hz stimulus. (50.9 ± 3.457% compared to 99.9 ±1.61%, $p<0.0001$, students t-test. Readings are an average of the first 30 minutes).

5.4.2 NMDA receptor and protein synthesis dependent LTP in the CA1 region of mouse hippocampus

LTP in the CA1 region of rodent hippocampus is known to be both NMDA receptor and protein synthesis dependent (Harris *et al.*, 1984; Morris *et al.*, 1986; Stanton & Sarvey 1984; Frey *et al.*, 1988). It was necessary therefore to confirm these molecular requirements in our model of LTP. Indeed, the same LTP induction protocol used previously (3x 100Hz for 1s, 10 minute inter-train interval), when delivered in the presence of the NMDA receptor blocker D-AP5 (50μM) did not result in a potentiation of synaptic responses, with the exception of the initial post tetanic potentiation which itself is thought to be NMDA receptor independent in nature (Frey *et al.*, 1996), (Figure 3.2a).

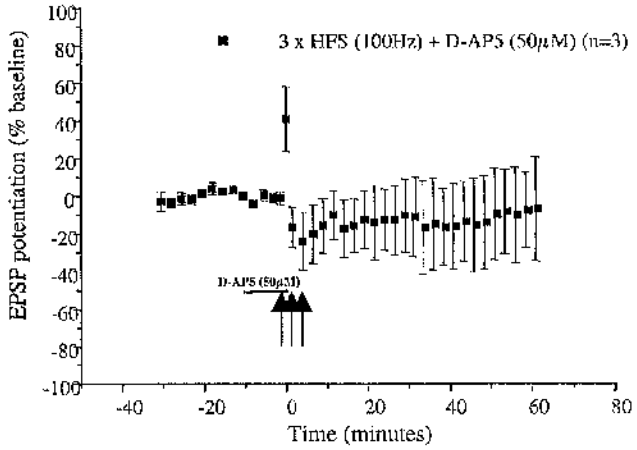
Figure 5.1 LTP of the synaptic response induced by 3 high frequency stimulations of the CA1 region of mouse hippocampus



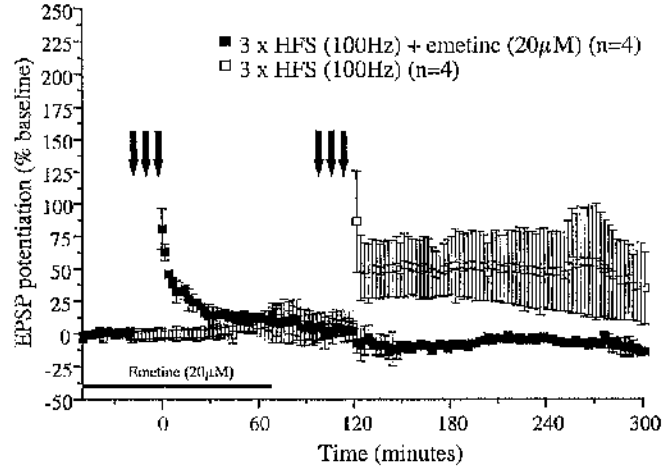
A. Scatter plot of the induction of late phase LTP following the stimulation of the Schaffer collateral afferents in the CA1 region of mouse hippocampal slices with three high frequency bursts (100Hz for 1s, 1mS pulse width, 10 minute intertrain interval, 3 arrows). This robust potentiation lasted 240minutes (n=6). A single tetanic stimulation on the other hand (100Hz for 1s, 1ms pulse width, 1 black arrow) produced a short term potentiation of the synaptic response, returning to it's original strength after approximately 60minutes (n=4). B. Representative EPSP waveforms from a single and triple tetanus experiment. Post tetanic potentiation (PTP) was evident for both experimental paradigms, although the EPSP had returned to its original size 60 minutes following a single burst, but was still potentiated 60 minutes after three HFS. Error bars =± s.e.m.

Figure 5.2 LTP induced by three high frequency stimulations of the CA1 region of mouse hippocampus is both NMDA receptor and protein synthesis dependent

A



B



A. Scatter plot of the effect of the NMDA receptor blocker D-AP5 (50μM) on the potentiation of synaptic responses in the CA1 region of mouse hippocampus 1hr following 3 high frequency stimulations (100Hz for 1s, 1ms pulse width, 10 minutes intertrain interval, 3 arrows). With the exception of post tetanic potentiation there is no increase in EPSP as is normally evident following this LTP induction protocol (n=3). B. The same LTP induction protocol given in the presence of the translational inhibitor emetine (20μM) failed to induce a long lasting potentiation of synaptic response in the CA1 region of rodent hippocampus. Tetanic stimulation of a second pathway in the same slice (open squares) in the absence of emetine results in a long lasting potentiation of the synaptic response (n=4). Error bars = ± s.e.m.

Figure 5.2b illustrates the effect of the translational inhibitor emetine (20μM) on the late phase of LTP in the CA1 region of adult rodent hippocampus. Using 2 separate pathways within a single slice, 3 100Hz bursts delivered in the presence of emetine did not produce late phase LTP, but in fact produced a gradual decline in response, which reached baseline levels within 2hrs (% at 2hrs = 7.5 ±7%, filled boxes). The second pathway given the same stimulation protocol, this time in the absence of emetine, resulted in a long lasting (>2hrs) potentiation of synaptic strength (% at 2hrs = 50 ±30.79%, open boxes).

Hence, LTP induced by 3 x 100Hz high frequency stimulations in the CA1 region of adult rodent hippocampus was found to be both NMDA receptor and protein synthesis dependent.

5.4.3 Differential protein expression in the early stages of LTP

Acute hippocampal slices (n=12) were subjected to 3 bursts of high frequency stimulation (100Hz, 1s) given 10 minutes apart following a stable 30minute baseline recording, which resulted in a potentiation of the EPSP as is shown in figure 5.3a. Initial post titanic potentiation (78.9% ± 12.62%) plateaued to a 52.9 ± 4.9 % increase in synaptic response 10 minutes after the final high frequency burst. Recordings were made for 10 minutes after the final high frequency burst (i.e. 30 minutes following the initial HFS train) and potentiated CA1 regions dissected free from surrounding tissue and snap frozen for further proteomic analysis. Control slices (n=12) did not receive any high frequency stimulation, but field EPSP responses monitored for an equivalent period, before CA1 regions of control slices were dissected free from surrounding hippocampal tissue and snap frozen prior to proteomic analysis (4.2 ± 3.4% at the equivalent 10 minute time point).

Tissue from the electrophysiology experiments shown in figure 5.3a were subjected to DiGE for investigation into the differential protein expression associated with the initial increase in synaptic strength resulting from high frequency stimulation. Representative Cy3 gel images of potentiated (Figure 5.3b (i)) and control (Figure 5.3b (ii)) tissue contained 2997 protein spots in each image. A number of protein spots appear to show differential levels of expression between the two gel images

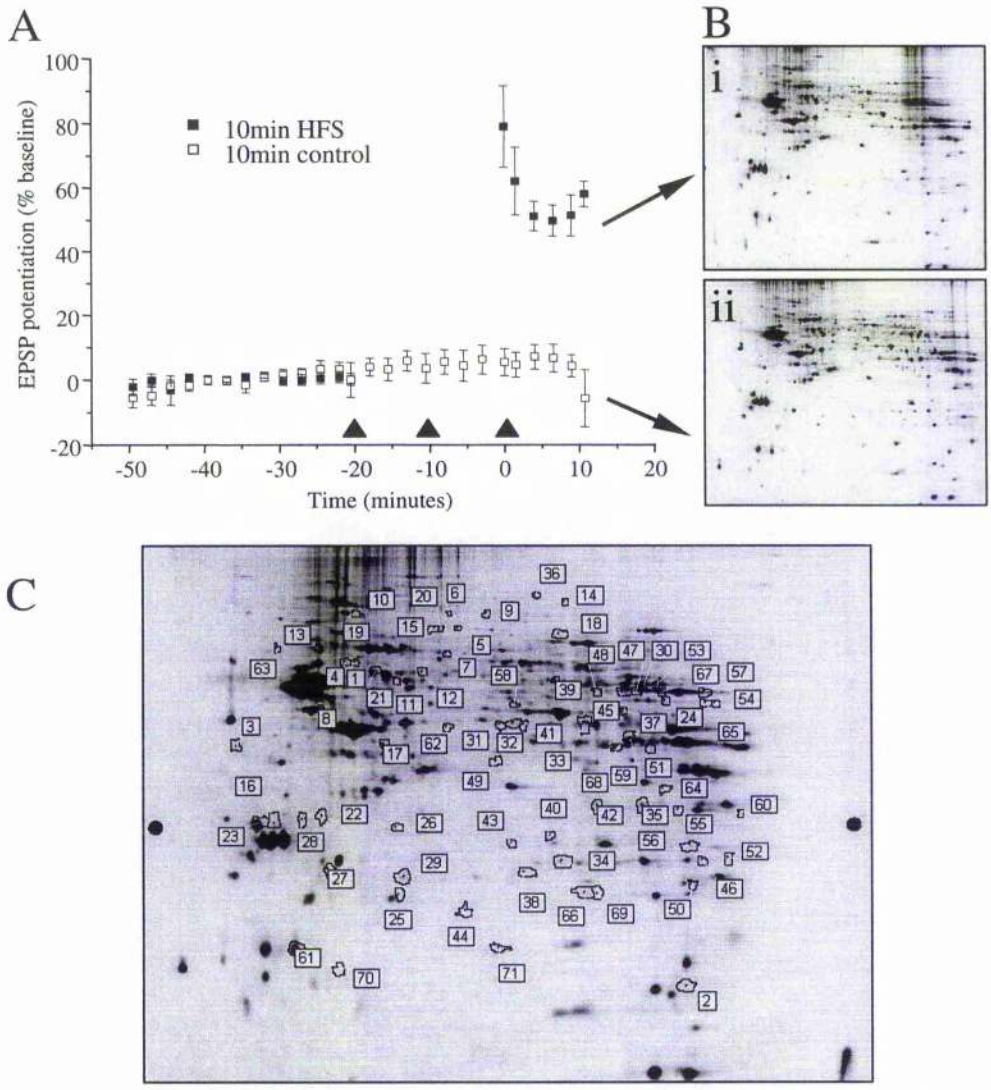
(circles). Further detailed analysis using DeCyder software in BVA mode incorporating biological replicates shows a small fraction of the protein spot population to display significant changes in abundance levels (figure 5.3c).

A total of 71 protein spots demonstrated a significant ($p<0.05$) change in protein spot abundance in tissues harvested at 10minutes following the LTP induction protocol. These protein spots were found to span the entire molecular weight and pI range of the 2D gel as is shown in Figure 5.3c. Some protein spots are clearly visible (spot numbers 13, 23), whilst others were only detected by DeCyder software (spot numbers 2, 66). The majority of the protein spots decreased in expression (50 out of 71). The decreases in fold change ranged from -1.79 to -1.09. The remaining 21 protein spots displayed an increase in their spot density with fold changes ranging from 1.12 to 1.51 (table 5.2).

Out of the 71 protein spots identified as significantly modulated early on in the potentiation of the synaptic response, 29 were identified using LC/ MS/ MS mass spectrometry. Of these 29, 13 protein spots represented multiple protein complements, giving rise to a total of 44 proteins

Protein spots 1 and 4, subsequently identified as a single protein hnRNPK, were both found to significantly ($p<0.05$) increase in their expression in phase I of LTP. These protein spots were also found to lie adjacent to each other on the 2D gel, likely representing post-translationally modified isoforms of this protein (figure 5.4).

Figure 5.3 Differential regulation of hippocampal proteins at 10 minutes following the induction of LTP in area CA1 of mouse hippocampus.



A. Time plot showing the increase in the slope of the field EPSP, recorded for 10 minutes following the high frequency stimulation paradigm (HFS, indicated by ▲) of the Schaffer-collateral fibres in the CA1 region of mouse hippocampus (filled squares) (n=12). Stable baseline recordings were made for 30 minutes prior to HFS. Control tissue (open squares) was not subjected to HFS (n=12). The CA1 region of both control and potentiated tissue was dissected, snap frozen and stored at -70°C prior to DiGE analysis. B. 2D gel images of Cy3 control (i) and Cy3 HFS (ii) treated hippocampal tissue pooled from electrophysiology experiments in A. C. Annotated 2D gel image (Cy2) of 10minute HFS treated tissue with protein spots, identified by DiGE as differentially expressed, highlighted across the gel. A total of 71 protein spots were regulated in this treatment group, spanning both the molecular weight and pI ranges.

Table 5.2 Differential protein expression 10minutes following HFS of glutamatergic afferents.

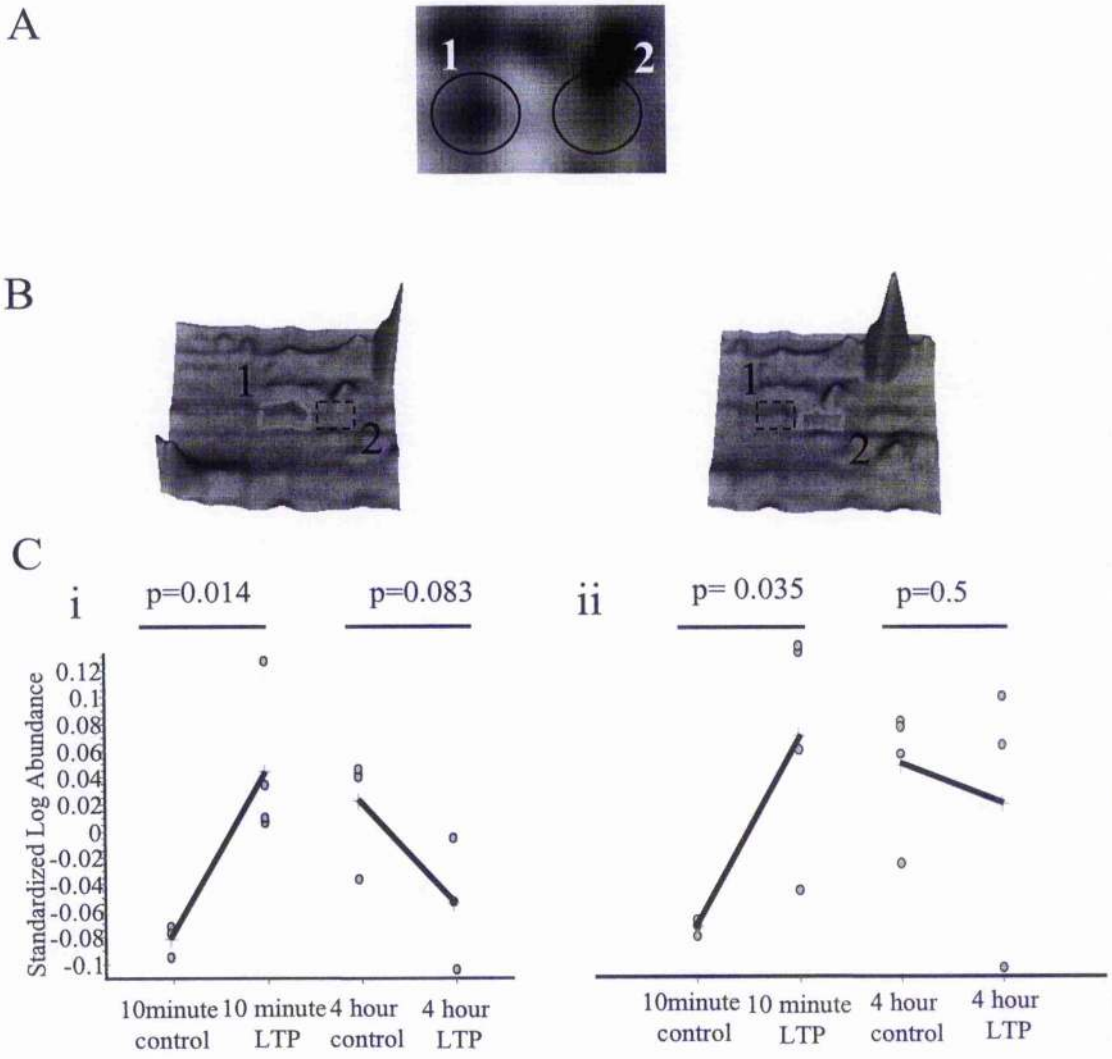
Spot Number (1)	Master Number (2)	Protein ID (3)	Av. Ratio (4)	10 min HFS v's control (T-test value) (5)	Mass (6)	Accession number (7)	Mowse Score (8)	Biologi proces
1	1417	hnRNPK	1.51	0.035	51230	gi 13384620	102	T/T
2	2823		1.5	0.025				
3	2138		1.36	0.028				
4	1412	hnRNPK	1.35	0.014	51230	gi 13384620	131	T/T
		Copine 6			62597	gi 6753510	84	ST
5	1024		1.3	0.028				
6	862		1.29	0.0091				
7	1855	Enolase 2	1.27	0.0092	47609	gi 7305027	303	E/M
8	1325		1.27	0.046				
9	885	Transitional endoplasmic reticulum ATPase	1.21	0.015	89936	gi 400712	302	PM&
10	877		1.21	0.023				
11	1589	Hsp60	1.21	0.026	61074	gi 72957	197	CO&
		CRMP2			62531	gi 3122040	85	NG
12	1492		1.2	0.023				
13	1252	vATPase β	1.19	0.028	56632	gi 23272966	409	NM
		α tubulin 2			50818	gi 35412	97	CO&
14	714		1.17	0.02				
15	1031		1.17	0.041				
16	2503	Tropomyosin 1 α	1.16	0.038	32746	gi 31560030	129	MC
		14-3-3 (epsilon)			28352	gi 1526541	71	PM&
17	2114		1.15	0.031				
18	1095		1.14	0.036				
19	871	Transitional endoplasmic reticulum ATPase	1.13	0.042	89936	gi 400712	302	PM&
20	1027		1.13	0.049				
21	1496		1.12	0.035				
22	2471	Glyoxalase	-1.09	0.0065	31107	gi 12840311	89	CM
23	2500	14-3-3 (epsilon)	-1.1	0.0078	29326	gi 5803225	363	PM&
24	2710		-1.11	0.013				
25	1797		-1.11	0.047				
26	2646		-1.12	0.01				
27	2515	Pyridoxal phosphate phosphatase	-1.12	0.037	31891	gi 44888293	46	E/M
28	2678		-1.14	0.023				
29	2488		-1.14	0.03				
30	1626		-1.15	0.0018				
31	1986	Pyruvate dehydrogenase E1 α	-1.15	0.016	43888	gi 6679261	314	E/M
32	1985		-1.15	0.029				
33	1969		-1.16	0.018				
34	2630		-1.16	0.039				

35	2429	VDAC2	-1.17	0.018	32340	gi 6755965	188	ION
36	638		-1.18	0.019				
37	2656		-1.19	0.01				
38	1875		-1.19	0.012				
39	2540	γ catenin	-1.2	0.0063	68620	gi 423532	165	CA
40	1595	Pyruvate kinase M2	-1.2	0.025	58448	gi 1405933	119	E/M
		CRMP1			62484	gi 1915915	108	NG
41	1987	MEK1	-1.21	0.00011	43788	gi 6678794	48	ST
42	2738		-1.21	0.0014				
43	2568		-1.21	0.017				
44	2434	VDAC2	-1.21	0.029	32340	gi 6755965	188	ION
45	2618		-1.22	0.012				
46	1691	vATPase α	-1.22	0.038	59830	gi 6680748	668	NM
		β tubulin 2			36587	gi 91856	147	CO&
		Aldehyde dehydrogenase 6 (A1)			50018	gi 21410418	103	E/M
47	1692	vATPase α	-1.23	0.0054	59830	gi 6680748	620	NM
		Aldehyde dehydrogenase 6 (A1)			58335	gi 19527258	259	E/M
48	2226		-1.23	0.0099				
49	2689		-1.23	0.033				
50	1712	Pyruvate kinase M2	-1.23	0.038	58394	gi 1363219	134	E/M
51	2141	Aldolase 1, A	-1.24	0.00091	39787	gi 6671539	326	E/M
		Isocitrate dehydrogenase 3 β			42453	gi 18700024	173	CM
		Glutamate oxaloacetate transaminase, mitochondrial			48123	gi 90311	85	PM&
52	2610		-1.24	0.00092				
53	1825		-1.25	0.02				
54	2450	SLC25A22	-1.25	0.026	35104	gi 21311045	96	AT
55	1641	3-oxoacid CoA transferase	-1.25	0.039	51636	gi 12843573	157	E/M
		Synapsin II			52818	gi 8567410	151	ST
56	2583	Dodecenoyl-CoA δ isomerase	-1.27	0.014	32515	gi 319818	46	E/M
57	1725	vATP synthase α	-1.28	0.0099	59830	gi 6680748	644	NM
		GAPDH			36072	gi 6679957	147	E/M
58	2462	VDAC 1	-1.29	0.016	30851	gi 6755963	96	ION
59		SLC25A22			35104	gi 21311845	73	AT
60	2789		-1.29	0.033				
61	1826		-1.29	0.042				
62	2088		-1.29	0.044				
63	2004	Succinate CoA ligase, (ADP) β	-1.32	0.036	50424	gi 20876884	123	CM
		β tubulin 2			36587	gi 91856	96	CO&
64	1272		-1.33	0.021				
65	2375	Lactate dehydrogenase (α)	-1.33	0.037	36816	gi 65923	64	E/M
66	1822		-1.36	0.036				
67	2706		-1.39	0.0049				
68	2144	Poly (rC) binding protein 1	-1.51	0.0031	37987	gi 6754994	161	T/T
		Glutamate oxaloacetate transaminase, cytosolic			46489	gi 90313	129	PM&
69	2711		-1.51	0.049				

70	2808	-1.69	0.014
71	2787	-1.79	0.014

Table listing the protein spots identified by BVA as significantly different 10 minutes after the HFS of Schaffer collateral afferents. 71 protein spots were significantly ($p<0.05$) modulated. 20 of these increased in their expression and the remaining 51 decreased. (1) The spot number represents the number highlighted on Figure 5.1c. (2) The master number represents the number assigned to each spot by DeCyder software and is also illustrative of the large number of protein spots found on this 2D gel. In this instance protein identities (3) of spots with average ratios (4) significantly different between stimulated and un-stimulated (5) were obtained by LC/ MS/MS analysis followed by MS/ MS database searching in Mascot, from where protein mass (6), NCBInr accession numbers (7) and Mowse scores (8) were also obtained. Proteins were assigned to a number of different biological classes using the FatiGo gene ontology tool set at level 5: T/ T, transcription and translation; VMT, vesicular mediated transport; E/M, energy/ metabolism; PM&T; protein metabolism and transport; CO&B, cytoplasmic organization and biogenesis; NG, neurogenesis; NM, nucleotide metabolism; MC, muscle contraction; CM, carbohydrate metabolism; ION, ion channel; CA, cell adhesion; ST, signal transduction; AT, amino acid transport.

Figure 5.4 Post translational modifications of hnRNPK 10 minutes following LTP induction.



Differential expression of a train of protein spots identified as hnRNPK protein 10minutes after LTP induction. (A) Zoomed in 2D gel image showing the two protein spots, identified by BVA in DeCyder software as differentially expressed, lying adjacent to each other. (B) 3D view generated by DeCyder software illustrating the location and proximity of the two protein spots. (C) Graphical representation of the standardised log abundance for each protein spot on each gel at 10 minutes and 240 minutes post HFS. Significant changes in protein expression levels were evident at the 10minutes time point, with no significant change in abundance at the 240 minute time point.

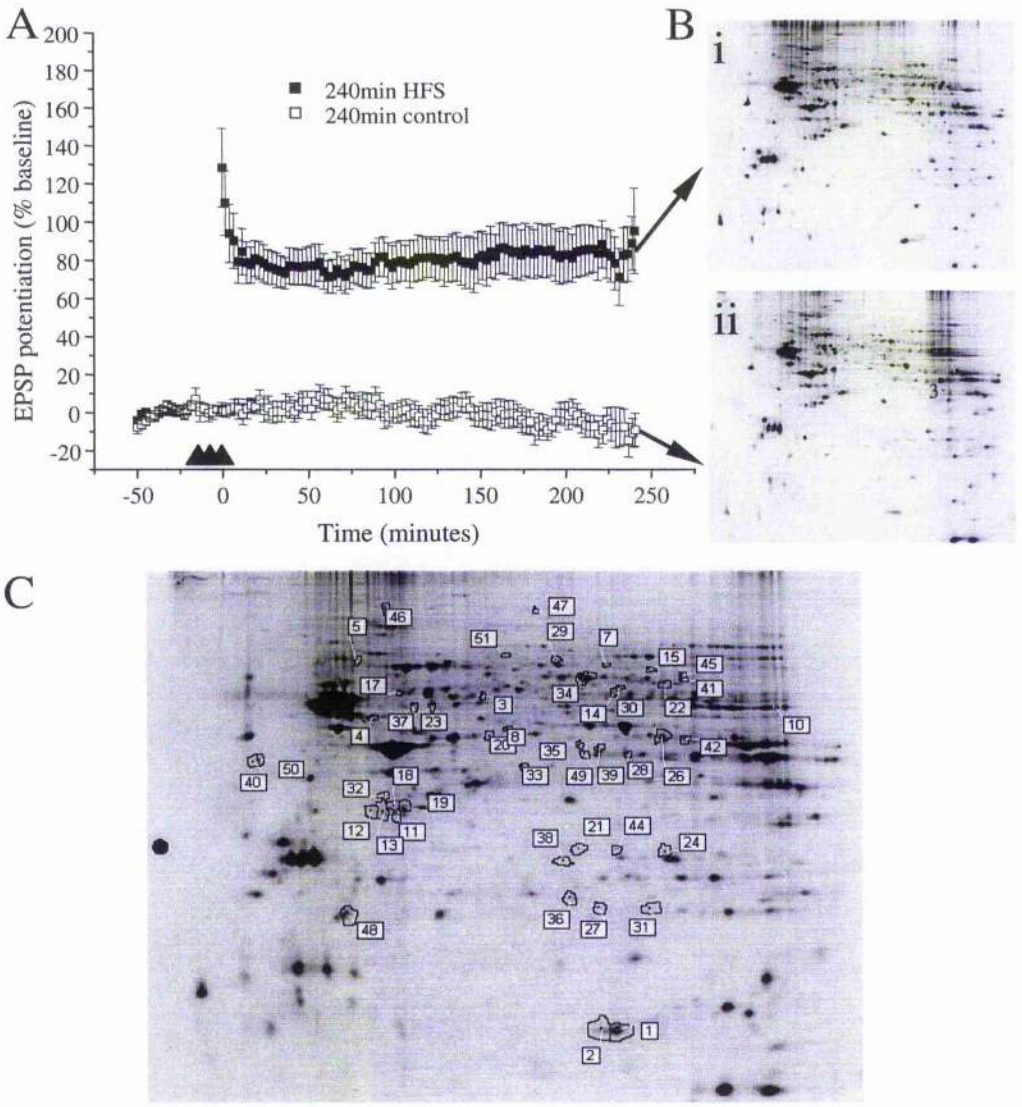
5.4.4 Differential protein expression in later phases of LTP

To assess the differential protein expression associated with a more enduring phase of synaptic potentiation acute mouse hippocampal slices (n=12) were again subjected to three bursts of high frequency stimulation (100Hz, 1s) delivered 10minutes apart in order to induce a significant potentiation of synaptic strength in the CA1 region of the hippocampus lasting 240minutes. Initial post tetanic potentiation reached 128.2 ± 20.8 % of baseline, which plateaued to 83.2 ± 3.4 % of baseline (taken over final 10 minutes of recording). Control slices were not subjected to HFS, instead were repeatedly stimulated once every 30 seconds for the duration of the experiment, which in this case was 290minutes. The average baseline value was -0.6 ± 0.4 % (figure 5.5a). The CA1 regions of all hippocampal slices in which electrophysiology recordings from Figure 5.5a were carried out were removed from surrounding tissue and snap frozen for proteomic analysis.

Representative Cy3 gel images of stimulated and control regions are shown in Figure 5.5b (i & ii). These gel images show a large number of protein spots (2997) ranging from high (~250,000 Da) to low molecular weight (~20,000Da) and low (3) to high isoelectric point (10) resolved in a non-linear fashion.

Following biological variation analysis of the complete set of DiGE gels 51 protein spots were identified as showing significantly ($p<0.05$) altered spot density at the 4 hr time point following the LTP induction protocol (figure 5.5, table 5.3). Of the 51 regulated protein spots, 21 were up-regulated and the remaining 30 down-regulated. Increases in protein expression ranged from 2.00 fold to 1.09 fold. Decreases ranged from –1.43 to –1.13. As with the investigation into proteins modulated in the early phase of LTP, spots highlighted as differentially expressed were picked, digested and subsequently analyzed by LC/ MS/ MS mass spectrometry. Out of the 51 differentially expressed protein spots 23 were identified. Of these 23, 9 had multiple identities due to the insufficient separation of proteins in the 2D gel, giving rise to a total of 34 proteins.

Figure 5.5 The differential regulation of hippocampal proteins at 4hrs following the induction of LTP in area CA1



A. Time plot showing the increase in the slope of the field EPSP, recorded for 240 minutes following the high frequency stimulation paradigm (HFS, indicated by ▲) of the Schaffer-collateral fibres in the CA1 region of mouse hippocampus (filled squares, n=12). Stable baseline recordings were made for 30 minutes prior to HFS. Control tissue (open squares) was not subjected to HFS (n=12). The CA1 region of both control and potentiated tissue was harvested for DiGE analysis. B. 2D gel images of Cy3 control (i) and Cy3 HFS (ii) treated hippocampal tissue pooled from electrophysiology experiments in A. C. Annotated 2D gel image (Cy2) of 10minute HFS treated tissue with protein spots, identified by DiGE as differentially expressed, highlighted across the gel. A total of 51 protein spots were regulated in this treatment group, spanning both the molecular weight and pI ranges.

Table 5.3 Differential protein expression 4hrs following HFS of glutamatergic afferents.

Spot Number [1]	Master Number [2]	Protein ID [3]	Av. Ratio [4]	4hr HFS v's control (T-test) [5]	Mass (Da) [6]	Accession number [7]	Mowse Score [8]	Biological process [9]
1	2861		2	0.049				
2	2859		1.71	0.01				
3	1600	α tubulin 2	1.64	0.021	50818	gi 135412	47	CO&B
4	1760	vATPase β	1.46	0.049	56344	gi 2623222	282	NM
		Dynactin 2			44204	gi 28076935	183	CO&B
5	1196		1.45	0.02				
6	2030		1.42	0.047				
7	1289		1.37	0.032				
8	1899		1.31	0.0092				
9	2480		1.28	0.023				
10	1717	GFAP	1.27	0.033	49935	gi 319914	48	CO&B
11	2407		1.26	0.019				
12	2388		1.24	0.046				
13	2389	Syntaxin 1A	1.23	0.025	33205	gi 15011853	61	NT
		Spermidine synthase			34543	gi 6678131	54	PM&T
14	1582	Munc18	1.21	0.047	69091	gi 4507297	101	VMT
		α tubulin 2			50818	gi 135412	66	CO&B
		Synapsin II			52818	gi 8567410	54	ST
15	1363		1.2	0.019				
16	1265		1.19	0.02				
17	2378	α tubulin 2	1.17	0.029	50818	gi 135412	195	CO&B
18	1521	Alpha internexin	1.17	0.04	55520	gi 17390900	505	CO&B
		Calcineurin			59291	gi 8394030	172	ST
		Hsp60			61074	gi 72957	85	CO&B
19	2377		1.14	0.035				
20	1968		1.13	0.0062				
21	2538		1.09	0.003				
22	1669	vATPase β	-1.13	0.005	56857	gi 17105370	514	NM
		CRMP2			62531	gi 3122040	357	NG
		Calcineurin			58822	gi 1357674	65	PM&T
23	2556		-1.13	0.034				
24	1490	CRMP1	-1.13	0.049	62484	gi 1915915	147	NG
25	1150	β tubulin 2	-1.15	0.017	50255	gi 5174735	536	CO&B
26	1998	Glutamine synthetase	-1.15	0.038	42904	gi 68581	72	E/M
27	2703		-1.16	0.014				
28	2108	Isovaleryl CoA dehydrogenase	-1.17	0.0076	46695	gi 9789985	316	E/M
		Glycoprotein 1B			44244	gi 38080101	125	CO&B
29	1206		-1.18	0.0053				
30	1523	CRMP 4	-1.19	0.035	62484	gi 1915915	147	NG

31	2706		-1.2	0.037				
32	2317	α tubulin 2	-1.23	0.011	50818	gi 35412	195	CO&B
33	2160		-1.24	0.03				
34	1426	CRMP2	-1.25	0.037	62638	gi 40254	774	NG
35	2690		-1.27	0.0056				
36	2050	Glycoprotein 1b	-1.27	0.0087	44244	gi 38080101	117	CO&B
37	2582		-1.28	0.026				
38	1679	β tubulin 2	-1.28	0.031	50255	gi 5174735	253	CO&B
		Endophilin B2			44303	gi 16518438	97	VMT
		vATPase β			56948	gi 1184661	90	NM
		RAP 1			59815	gi 26342380	87	ST
39	2138		-1.3	0.0094				
40	2076		-1.3	0.043				
41	2449	Electron transferring flavoprotein alpha	-1.31	0.0081	35316	gi 26328615	435	E/M
		VDAC 2			32340	gi 6755965	93	ION
		VDAC 1			30851	gi 6755968	58	ST
42	1441		-1.31	0.033				
43	1993	Pyruvate dehydrogenase E1 α	-1.31	0.038	43888	gi 6679261	314	E/M
		Nitrogen fixation gene			50951	gi 14714866	73	PM&T
44	2540	γ catenin	-1.33	0.037	68620	gi 423532	165	CA
45	1404	Hnrpl	-1.35	0.035	22726	gi 2760632	48	T/T
46	600		-1.36	0.0085				
47	512		-1.36	0.03				
48	2726		-1.4	0.047				
49	2095		-1.41	0.03				
50	2122	GAP43	-1.42	0.0073	23732	gi 6679935	68	NG
51	1135	Thimet oligopeptidase 1	-1.43	0.0032	78775	gi 21619359	191	PM&T
		CRMP1			74631	gi 40675740	112	NG

Table listing the protein spots identified by BVA as significantly different 240 minutes after the HFS of Schaffer collateral afferents. 51 protein spots were significantly ($p<0.05$) modulated. 21 of these increased in their expression and the remaining 30 decreased. (1) The spot number represents the number highlighted on Figure 5.1c. (2) The master number represents the number assigned to each spot by DeCyder software and is also illustrative of the large number of protein spots found on this 2D gel. In this instance protein identities (3) of spots with average ratios (4) significantly different between stimulated and un-stimulated (5) were obtained by LC/ MS/MS analysis followed by MS/ MS database searching in Mascot, from where protein mass (6), NCBInr accession numbers (7) and Mowse scores (8) were also obtained. Proteins were assigned to a number of different biological classes using the

FatiGo gene ontology tool set at level 5 [9]: T/ T, transcription and translation; VMT, vesicular mediated transport; E/M, energy/ metabolism; PM&T; protein metabolism and transport; CO&B, cytoplasmic organization and biogenesis; NG, neurogenesis; NM, nucleotide metabolism; CM, carbohydrate metabolism; ION, ion channel; CA, cell adhesion; ST, signal transduction.

5.4.5 Temporal protein expression following high frequency stimulation of hippocampal CA1 glutamatergic afferents

BVA investigated differential protein expression not only between stimulated and un-stimulated tissue, but also allowed the comparison of protein expression over time. The entire list of identified proteins and their relationship (if any) between the early and late phase of LTP is shown in table 5.4.

The expression of individual protein spots was followed through from early to late time points. For example, spot number 2429, (subsequently identified as VDAC2), showed a significant decrease in abundance in early phases of the plasticity response, but was unchanged in late phases (figure 5.6a). Conversely, spot number 1600 (alpha tubulin) showed no change in abundance levels shortly after LTP induction but was significantly ($p=0.021$) increased in more enduring phases (figure 5.6b). Some protein spots, in particular number 2540 (gamma catenin), were modulated at both time points (figure 5.6c).

In addition to the investigation of particular protein spots over time, the expression levels of individual proteins could also be analyzed. As previously mentioned the protein spot containing VDAC2 (number 2429) decreased in expression (-1.17 fold change, $p=0.018$) 10minutes following LTP induction, but remained unchanged (-1.07, $p=0.5$) 240minutes post LTP induction. Another protein spot, 2434, also identified as VDAC2 displayed the same expression profile (10min fold change = -1.21, $p=0.029$, 240min fold change = -1.07, $p=0.53$). However, whilst the expression of these individual spots was confined to the early phase of LTP, the expression of the protein molecule was not. VDAC2, identified as part of protein spot number 2449, decreased in abundance (-1.31 fold change, $p=0.0081$) 240minutes following the LTP induction stimulus but was unaffected at the earlier time point. The appearance of this protein in several regions of the gel is indicative of post-translational modifications of this molecule (figure 5.7).

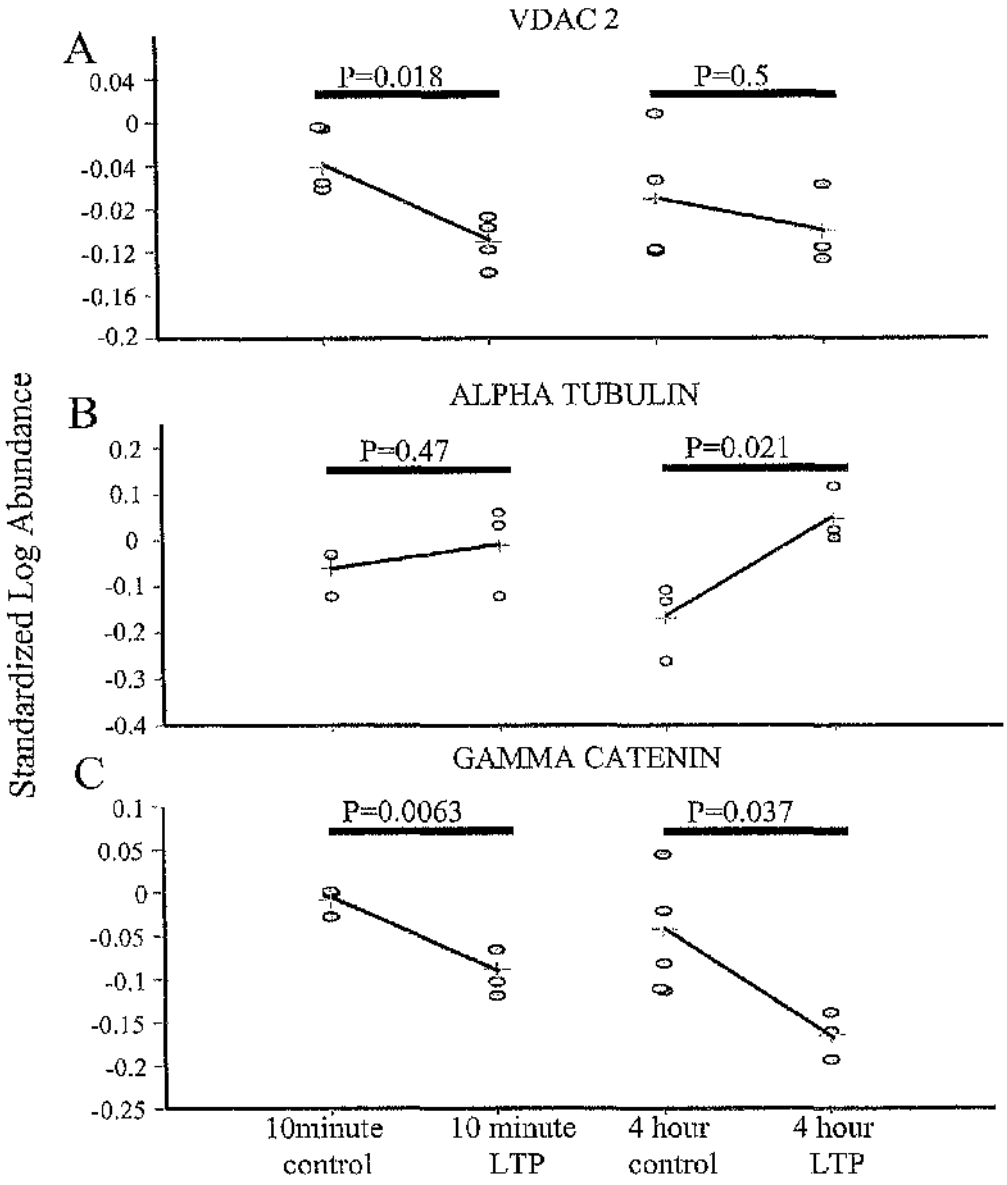
Table 5.4 Relationship of proteins at early and late phases of LTP

Protein	LTP (10min)	LTP (240min)
3-oxoacid CoA transferase	↓	
Aldehyde dehydrogenase family 6, subfamily A1	↓	
Aldolase 1, A isoform	↓	
Alpha tubulin 2	↑	↑↑↑↓
ATP synthase alpha subunit	↓↓↓	
ATP synthase beta subunit	↑	↑↓↓
Beta tubulin 2	↓↓	↓↓
Chaperonin groEL precursor (HSP60)	↑	↑
Copine 6, neuronal copine	↑	
Creatine kinase, mitochondrial 1, ubiquitous		↓
Dihydropyrimidinase related protein - 1 (CRMP 1)	↓	↓
Dihydropyrimidinase related protein - 2 (CRMP 2)	↑	↓↓
Dihydropyrimidinase related protein - 3 (CRMP 3)		↓
Dodecenoyl-Coenzyme A delta isomerase	↓	
Dynactin 2		↑
Electron transferring flavoprotein, alpha polypeptide		↓
Endophilin B2		↓
GFAP		↑
Glutamate ammonia ligase (Glutamine synthetase)		↓
Glutamate oxaloacetate transaminase 1, cttosolic	↓	
Glutamate oxaloacetate transaminase 2, mitochondrial	↓	
Glyceraldehyde - 3 - phosphate dehydrogenase (P)	↓	
Glycoprotein IB (platelet), beta polypeptide		↓↓
Glyoxalase	↓	
Growth associated protein 43 (neuromodulin)		↓
hnRNPK	↑↑	
hnRNPL		↓
Internexin neuronal intermediate filament protein (alpha)		↑
Isocitrate dehydrogenase 3, beta	↓	↓
Isovaleryl coenzyme A dehydrogenase		↓
Gamma catenin	↓	↓
Lactate dehydrogenase (α)	↓	
MEK1	↓	
Nitrogen fixation gene (cysteine disulphurase, mitochondrial precursor)		↓
Poly (rC) binding protein I	↓	
Pyridoxal phosphate phosphatase	↓	
Pyruvate dehydrogenase E1 alpha 1	↓	↓
Pyruvate kinase M2 type	↓↓	
RAP I (guanine nucleotide exchange factor)		↓
SLC25a22	↓↓	
Spermidine synthase		↑
Succinate co-enzyme A ligase, ADP forming, beta subunit	↓	
Synapsin II	↓	↑
Syntaxin 1A		↑
Munc18		↑
TBP-interacting protein b		↓
Thimet oligopeptidase I		↓
Transitional endoplasmic reticulum ATPase	↑↑	
Tropomyosin 1, alpha	↑	
14-3-3 (epsilon)	↑↓	

Voltage dependent anion channel 1	↓	↓
Voltage dependent anion channel 2	↓↓	↓

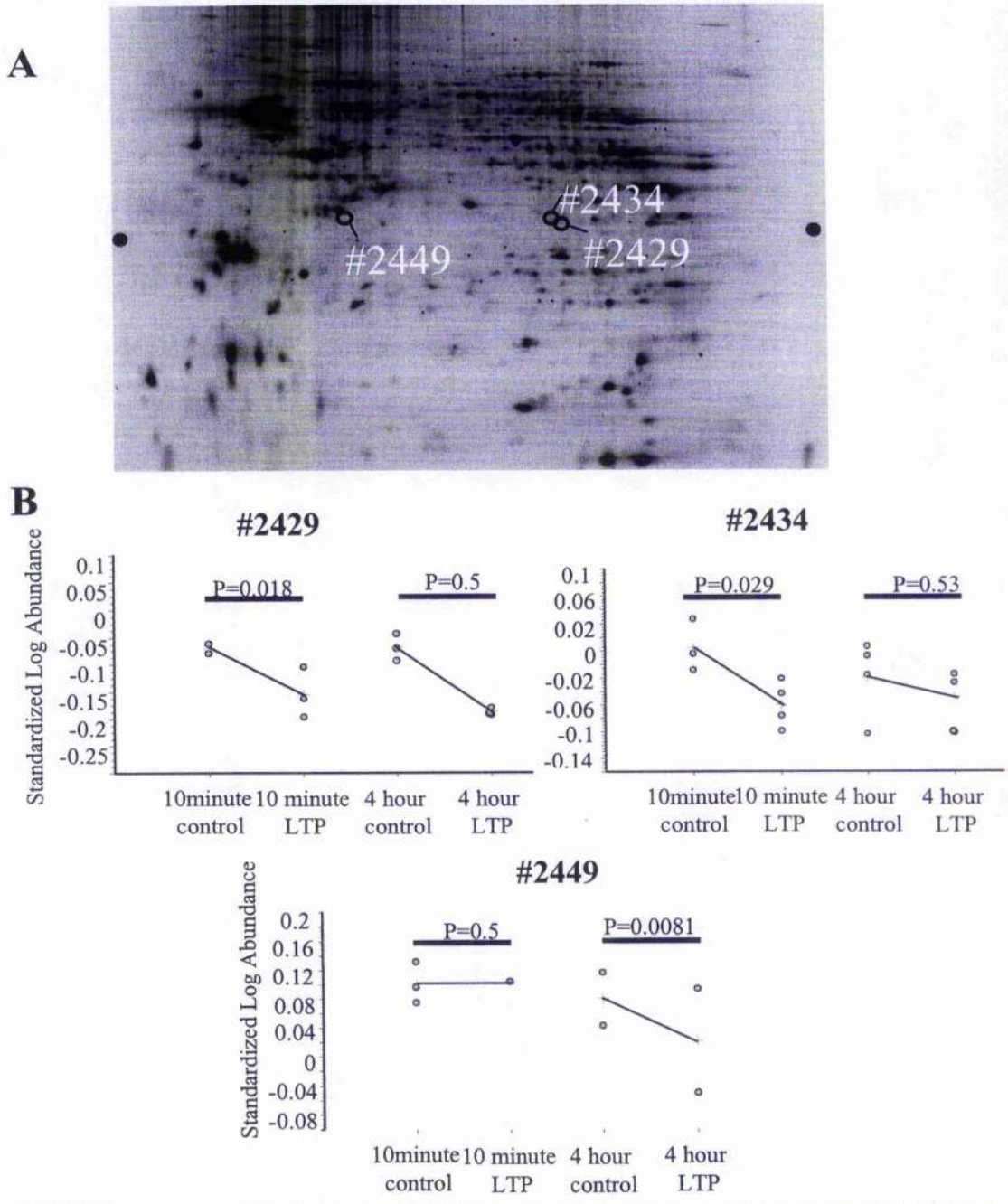
Summary of identified hippocampal CA1 proteins modulated in both early and late phases of LTP at the CA3-to-CA1 glutamatergic synapse. Up and down arrows indicate significant increased or decreased spot density respectively. Multiple arrows in a single box indicate proteins occurring at multiple sites on the master gel, presumably representing multiple modified versions of that protein. All changes indicated reached statistical significance ($p<0.05$).

Figure 5.6 Regulation of VDAC2, alpha tubulin and gamma catenin by high frequency stimulation of the CAI region of mouse hippocampus



Graphical representation of the standardised log abundance values for the proteins VDAC2, alpha tubulin and gamma catenin 10 minutes and 240 minutes after the LTP induction stimulus. A. 10 minutes following the HFS there is a significant (p=0.018) decrease in expression of VDCA2 (spot#2429), not evident at the 240 minute time-point. B. Conversely, standardised log abundance values indicate that the regulation of spot number 1600, identified as alpha tubulin, is significant (p=0.021) at the 240minute time-point compared to its respective 4hr control, an effect not seen early on in the time course. C. Gamma catenin (spot# 2540) is significantly decreased in expression at both early and late phases of LTP, p values of 0.0063 and 0.037 respectively.

Figure 5.7 Differential expression of VDAC2 in phase I and phase III of LTP



VDAC2 expression 10minutes and 240 minutes following LTP induction. (A) 2D gel image illustrating several VDAC2 containing protein spots. Two protein spots (2429 & 2434) lie in close proximity, possibly representing PTMs of VDCA2. One protein spot (2422) lies some distance from the others possibly representing a different isoform that has not undergone any post translational modifications. (B) Scatter plots showing the standardised log abundance for each protein spot in each 2D gel. Significant decreases in expression were evident at both 10 minutes and 240 minutes for VDAC2.

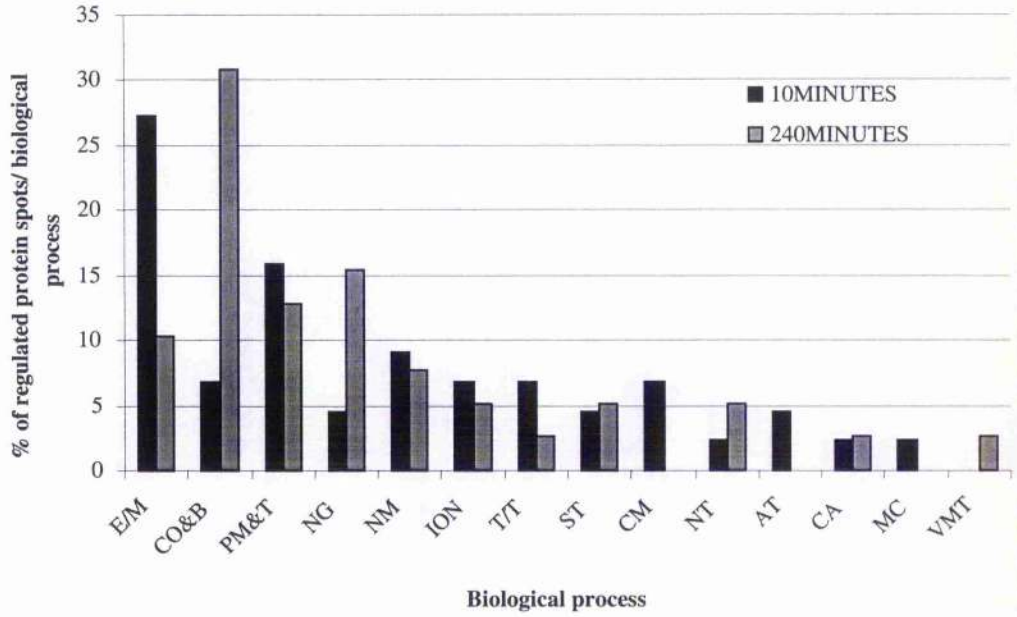
5.4.6 Assignment of biological process to modulated proteins.

To identify the biological processes involved in the plasticity response under investigation here, individual proteins were assigned to particular functional groupings using the FatiGO gene ontology bioinformatics tool as previously described.

Shortly after LTP induction the majority of identified proteins are found to be associated with energy and metabolism processes (12 out of 44), which contrasted with the later phase where cytoplasmic organization and biogenesis dominates (12 out of 39) and energy and metabolism proteins represent a small proportion of the total number (4 from 39). The number of molecules assigned to protein metabolism and transport processes is relatively similar for both phases (6 and 4). Other groupings are dominant in either the early phase (carbohydrate metabolism, amino acid transport, transcription & translation) or in the more enduring phase (neurogenesis, neurotransmitter production/ release) of the synaptic response (figure 5.8).

Figure 5.8 Distribution of biological processes modulated following HFS in the CA1 region of mouse hippocampus.

A



B

	NUMBER OF PROTEIN SPOTS CHANGING IN EXPRESSION													
	BIOLOGICAL PROCESS													
	E/M	CO&B	PM&T	NG	NM	ION	T/T	ST	CM	NT	AT	CA	MC	VMT
10minutes (44)	12	3	7	2	4	3	3	2	3	1	2	1	1	0
240minutes (39)	4	12	5	6	3	2	1	2	0	2	0	1	0	1

(A) Bar chart illustrating the percentage distribution of a wide range of biological processes in terms of differential protein expression in early and late phases of LTP as determined using the gene ontology tool FatiGo. (B) Table further detailing the numbers of differentially regulated protein spots belonging to 14 different biological processes for each phase of LTP. Percentages for (A) are calculated from numbers in (B). Numbers in parentheses indicate total number of identified proteins for each group. E/M, energy & metabolism; CO&B, cytoplasmic organisation and biogenesis; PM&T, protein metabolism and transport; NG, neurogenesis; NM, nucleotide metabolism; ST, synaptic transmission; T/T, transcription and translation; CM, carbohydrate metabolism; ION, ion channel; VMT, vesicular mediated transport; AT, amino acid transport; CA, cell adhesion; MC, muscle contraction; NT, neurotransmitter production and release.

5.5 DISCUSSION

This chapter focuses on differential protein expression resulting from high frequency stimulation of glutamatergic afferents in the hippocampal slice preparation. Long-term potentiation is associated with three temporal phases each one reliant on differing but overlapping molecular mechanisms. Phase I requires the covalent modification of pre- existing protein molecules (Lovinger *et al.*, 1987; Reymann *et al.*, 1988), phase II the translation of mRNAs (Frey *et al.*, 1988) and phase III ultimately requires gene transcription and *de novo* mRNA synthesis (Frey *et al.*, 1996). A study by Thompson and colleagues in 2003 investigated gene expression associated with the early phase of LTP in the CA3 region of the hippocampus *in vivo*, but as yet no study has focused on protein expression resulting from LTP in the hippocampus. To identify the proteins modulated in the early and late stages of this *in vitro* slice model of synaptic plasticity we induced LTP in the CA1 region of the mouse hippocampus using 3 bursts of high frequency stimulation and harvested tissue at 10minutes and 240minutes following the induction stimulus. Such an induction protocol has reliably been used by many researchers to induce a robust and long lasting long term potentiation of the synaptic response (Lynch *et al.*, 1983; Frey & Morris 1998). Protein extracts from these tissue samples were analyzed using DIGE and differentially regulated protein spots identified using mass spectrometry and Mascot database searching.

5.5.1 Long-term potentiation in the hippocampal slice

LTP is established in many laboratories throughout the world and it was necessary as a starting point to establish this model prior to any proteomics studies. Using a protocol described by Frey & Morris (1997) I was able to induce a robust long lasting potentiation of synaptic response in the CA1 region of mouse hippocampus. Initial experiments focusing on the late phase of LTP were variable in their potentiation as can be seen by the large degree of error in the scatter plot in figure 5.1a. However, in subsequent experiments in this chapter the error is much reduced. Repeated experimentation often resulted in increased reliability and reproducibility. Other induction protocols such as single tetanus and theta burst stimulation proved to

be unsuccessful in their ability to induce enduring forms of LTP, although successful application of theta burst has routinely been demonstrated by others (Larson *et al.*, 1986; Kramar & Lynch, 2003). Indeed, theta burst stimulation is considered to be a more physiological induction paradigm for increased synaptic strength and comparing proteins regulated following this type of induction to the one used in this thesis would certainly be of great interest. The next step was to identify the molecular components associated with an increase in synaptic strength such as that seen with LTP. The NMDA receptor is required for LTP induction (Harris *et al.*, 1984; Collingridge *et al.*, 1988), and *de novo* protein synthesis is a requirement for the later stages of this synapse specific plasticity (Stanton & Sarvey, 1984). Consistent with previous findings, application of the NMDA receptor blocker D-AP5 to prevent activation of this receptor was found to completely block LTP. Post-tetanic potentiation, which is pre-synaptic in nature, (Eccles & Krnjevic, 1959; Frey *et al.*, 1989) was not blocked by application of D-AP5. Similarly the translational inhibitor emetine (20μM) given for 1hr prior to, during and 1hr after the LTP induction stimulus, prevented the development of the late phase of LTP. It is worth noting that the general trend of the emetine curve appears to decline gradually back to baseline immediately after LTP induction as is seen in other studies which have used this inhibitor (Bradshaw *et al.*, 2003). Transcriptional inhibitors such as actinomycin D on the other hand show a different profile (Frey *et al.* 1996; Nguyen *et al.*, 1996) where a decline in potentiation does not commence until approximately 1hr after induction, possibly reflecting the translational and transcription dependent temporal phases that are associated with LTP.

High frequency stimulation reliably induced a robust potentiation of the synaptic response in the CA1 region of mouse hippocampus that appears, following the initial decay of post tetanic potentiation, to show no further indication of a decline in the response, even 240 minutes after the final high frequency burst. As previous experiments in this chapter (figure 5.2b) with emetine have shown a definitive requirement for protein synthesis at 240 minutes post tetanus, this time point was chosen for further proteomic investigation into protein regulation in a late phase of LTP. Ideally the use of a transcriptional inhibitor such as actinomycin D would have illustrated the time point where gene transcription and mRNA synthesis takes place and hence the third phase of the plasticity process, although all phases will certainly

overlap to some extent. However, substantial literature points to the fact that 240 minutes post LTP induction is indeed representative of phase III of the plasticity process. Additionally it would have been interesting to investigate the role of protein kinases in the earlier stages of LTP in our hands as classically, protein kinase inhibition blocks the early phase of LTP (Malenka *et al.*, 1989). The most elegant use of the inhibitors of protein kinases, translation and transcription would be to analyze, using DiGE, the protein expression profiles of hippocampal tissues subjected to LTP in their presence and absence.

5.5.2 Analysis of protein expression following LTP induction using DiGE

As has been discussed in previous chapters, the possibility of a dye bias occurring for certain proteins within a particular protein sample is a concern with proteomic experiments of this nature. The DiGE experiment performed in this chapter inadvertently lacked a dye flip for the 10minute control (Cy3 bias) and the 240minute LTP samples (Cy5 bias), due to the loss of a single gel in the 1st dimension running. As previously described, a same sample experiment carried out in chapter 3 investigated whether or not dye bias was a problem for tissue of this kind. It was found that there was only one protein spot from 2604 detected whose abundance significantly changed thereby indicating that CyDye bias is not a significant problem given that there is a 0.00038% chance of this occurring. Therefore the loss of a single gel, whilst not ideal, does not pose a problem in this instance. A possible solution to overcome this potential hazard in future experiments is to run more gels. Amersham Biosciences, the supplier of the DiGE equipment, CyDyes and software, recommend running a minimum of 6 gels, representing 6 biological replicates. Loss of one or two of these gels would still allow for a dye bias in all samples. However, the running of extra gels results in greater cost and is very labour intensive, factors that would have to be weighed up against the potential loss of sample and its consequences. In addition, the time required to harvest late LTP tissues is substantial and gel loss would not only be costly in terms of time.

Differential in gel analysis (DIA) was not carried out on these protein samples, as an in-depth, statistically robust analysis was instead carried out across all replicates

using BVA. Nevertheless, by visually comparing pairs of gel images relating to stimulated and unstimulated tissue, several differences in spot abundance were apparent. For some of these changes, confirmation was made following analysis in BVA mode of DeCyder software. However, for the majority of these changes biological variation analysis demonstrated that there was actually no significant change in expression. This further illustrates the problems commonly associated with 2D gel electrophoresis, even between protein samples prepared and run in almost identical conditions. However it should be noted that these comparisons were made between two separate gels, and DIA, which also uses a single pair of gel images, compares these images from a single gel, which further increases the reproducibility of the samples.

A larger number of protein spots were found to change in expression at earlier time points compared to late (71 compared to 51). This is indicative of the large degree of protein modulation no doubt occurring in this phase of LTP. Pre-existing protein molecules are modified in the induction phase of LTP. For example, phosphorylation by protein kinase C (Malenka *et al.*, 1986), protein kinase A (Roberson and Sweatt, 1996), MAPK (Wu *et al.*, 1999) and CaMKII (Malinow *et al.*, 1988.) results from high frequency stimulation of Schaffer collateral afferents. In addition, rapid induction of immediate early genes such as zif268 (Cole *et al.*, 1989), Arc (Moga *et al.*, 2004) and tissue plasminogen activator (Qian *et al.*, 1993) is also taking place in phase I of the plasticity response. Therefore, a plethora of molecules are modified within a very short space of time following LTP induction. It appears from our proteomics investigation that this degree of modification is somewhat less in the latter stages of synaptic potentiation, where protein synthesis and gene transcription play a central role and modifications to pre-existing protein molecules is minimal as protein kinase inhibitors are relatively ineffective at preventing the later stages of LTP when given after the LTP induction stimulus (Malinow *et al.*, 1989).

Spot resolution is a pertinent issue in terms of protein identification by mass spectrometry. DiGE analysis highlights individual protein spots to be differentially regulated. The identification of this spot would theoretically lead to the identification of the regulated protein. In reality, protein spots often contain multiple protein complements, and the actual protein or proteins responsible for the differential

expression are listed amongst several possible candidates. In this study all protein spots that were picked and digested were subsequently analyzed solely by LC/MS/MS mass spectrometry and not Maldi-tof mass spectrometry. LC/MS/MS requires only a few, or sometimes, only a single peptide to obtain significant protein identification. Yet, 29 out of the 52 protein spots that were identified by mass spectrometric analysis were found to contain a single protein indicating that protein spot resolution does not pose an overwhelming problem in this instance. For those protein spots associated with a number of protein identities, the use of zoom gels, as was previously discussed in chapter 3 of this thesis would aid in the identification of the proteins actually being modified as a result of synaptic activation. This is also a possible solution to the identification of low abundance proteins. It is also clear from tables 5.2 and 5.3 in the results section that not all protein spots were identified as for some protein spots detected by DeCyder software insufficient protein content was available for successful mass spectrometric analysis.

A major advantage of DiGE and its use of an internal standard is the ability to design and run experiments that allow comparison between any number of groups. For example, time, drug treatment and drug concentration all within the one DiGE experiment. In this study not only was it possible to compare between stimulated and un-stimulated protein extracts at a single time-point, but it was also possible to compare over time for individual protein spots. The examples shown in Figure 5.7 further illustrate this point. The expression of three individual protein spots, numbers 2429, 1600 and 2540 from early through to late phases of LTP is shown. The first spot 2429 changes in expression in early phase LTP, but is unchanged in late phase LTP, conversely spot number 1600 is unaffected at 10 minutes but significantly increases after 240 minutes, and spot number 2540 changed in expression levels over the entire time course studied. Whilst the addition of other groups in a single experiment results in a costly and more labour intensive experiment, it does allow for a more in depth analysis of a particular system.

From the 122 protein spots highlighted as differentially expressed in both early and late phases of LTP three were regulated in both phases with only one being identified following mass spectrometric analysis. This protein spot (#2540) was subsequently identified as gamma catenin or plakoglobin. To date I am unaware of any evidence

for the involvement of this isoform of catenin in synaptic plasticity events in the CNS. The localization of catenins at the synapse however does suggest a role for these molecules in synaptic plasticity and LTP. Indeed synaptic activity modulates the distribution of beta catenin (Murase *et al.*, 2002) in that depolarization brought about by high frequency stimulation causes an increased affinity between beta catenin and cadherin via cyclin-dependent kinase 5 activity. A study by Bamji *et al* in 2003 investigated the *in vivo* and *in vitro* effects of beta catenin disruption on synapse formation. It was discovered that this isoform of catenin plays a role in the localization of synaptic vesicles at presynaptic sites by acting as a scaffolding protein to link cadherin clusters to PDZ domain containing proteins via its PDZ binding motif. Removal of beta catenin also caused an increase in the number of synapses, confirmed by an increase in synaptic response as illustrated by input/ output curves. These changes are thought to be compensatory mechanisms for loss of function. Beta catenin is found in high levels in neuronal processes early in development, in fact even before the detection of synaptic proteins (Benson & Tanaka, 1998). Yu and Malenka (2003) demonstrated that over-expression of beta catenin (and other members of this family) enhanced dendritic arborisation, whereas sequestering endogenous beta catenin caused a decrease in dendritic branch tip number and prevented the enhancement of dendritic morphogenesis caused by neural activity. These effects on dendritic arborisation are most likely mediated through beta catenins relationship with the actin cytoskeleton. Indeed, mice depleted of delta-catenin, a neuron-specific catenin, demonstrate impairments in hippocampal short- and long-term synaptic plasticity as well as severe impairments in cognitive function (Israely *et al.*, 2004). The novel finding reported here that gamma catenin is differentially expressed at both early and late time points following the induction of LTP further suggests an important role for this family of protein molecules in the plasticity process.

It is perhaps surprising that there is not a greater degree of overlap between the two phases in terms of differentially expressed protein spots however, there is a greater degree of overlap with regards to particular proteins which were encountered at multiple positions on the gel and thus presumably reflect different modified versions of the same protein. One such example is the differential expression of VDAC2. This

protein can be found in several positions on the 2D gel. All protein spots identified as containing VDAC2 significantly ($p<0.05$) decrease in abundance all by similar amounts (-1.17, -1.21, -1.31 fold changes). VDAC2 is one of three mitochondrial porin proteins that are considered to be the most abundant proteins in the mitochondrial outer membrane. At least half of all presynaptic terminals in the rat hippocampal CA3-CA1 region contain at least one mitochondrion (Shepherd & Harris 1998) and it is known that these structures have an important role in basal synaptic transmission (Doolette 1997; Nguyen *et al.*, 1997; David *et al.*, 1998; Sandoval 1980) and possibly synaptic potentiation (Tang & Zucker 1997). Mammals express three VDAC isoforms (VDAC1, 2 & 3) and have significant roles in diverse cellular processes including regulation of mitochondrial ATP and calcium flux. VDAC1 was additionally identified in our proteomics study as differentially expressed following both phases of the plasticity response. Weeber *et al* in 2002 showed that mice deficient in porins had impaired fear conditioning and spatial learning. These deficits were isoform dependent. For example VDAC3 but not VDAC 1 was found to be necessary for hippocampus dependent contextual fear conditioning. Following electrophysiological studies it was also discovered that these knock out mice (VDAC1 & 3 $^{-/-}$) had deficits in short and long term potentiation. Furthermore, inhibition of the mitochondrial permeability transition pore (MPT) by cyclosporin A in wild type hippocampal slices reproduced the electrophysiological effect seen in the porin deficient mice. This MPT appears to be functional in physiological conditions such as calcium induced calcium release and has been shown to control synaptic calcium buffering (Ichas *et al.*, 1997; Brustovetsky & Dubinsky, 2000; Levy *et al.*, 2003).

The relationship between individual proteins in early and late phases of LTP is summarized in Table 5.4. From the 122 protein spots analyzed, 52 proteins were identified, several with multiple isoforms across the 2D gel. Only 12 of these proteins were found to be common to both phases of LTP. The others remain exclusive to either the early phase (21) or more enduring phases (19). Some of the protein molecules found in common to both time points show similar trends in expression levels (beta tubulin, Hsp60 and CRMP1) whereas others show very different expression profiles (CRMP2 and synapsin II). This could indicate that, in

general, some classes are either up or down regulated in a relatively straightforward manner. However, protein classes which show both up and down regulation could indicate the transformation from one form to a modified form i.e. non-phosphorylated to phosphorylated.

5.5.3 Functional grouping of proteins regulated in the early and late phase of LTP

To obtain a clearer picture of the protein expression profiles arising from early and late phases of LTP protein molecules were once again assigned to a particular biological process using a Gene Ontology bioinformatics tool, FatiGO. A specific level of ontology was chosen in order to obtain the optimum information on a particular protein. For example, using level 3 for alpha tubulin would assign it to the category cellular physiological process. On the other hand, using level 5 would assign this protein to cell organization and biogenesis – a more detailed and descriptive definition of its biological process. Hence, all protein molecules were searched at the gene ontology level 5.

Interestingly, certain functional groupings demonstrate quite different distribution profiles in relation to early and late phase LTP.

5.5.3.1 Proteins involved in energy and metabolism

Proteins belonging to the biological process encompassing energy and metabolism (including carbohydrate metabolism, but excluding nucleotide metabolism) change in abundance levels very early on in the plasticity process compared to later stages with 27% of the total proteins identified showing altered expression at 10 minutes compared to just over 10% at 4hrs. As the initial induction period of LTP involves a multitude of processes including neurotransmission across synaptic clefts, receptor activation, calcium influx, phosphorylation of proteins, AMPA and NMDA receptor recycling, retrograde signalling, the induction of several signal transduction pathways, mRNA sequestration and translation and possibly protein degradation pathways it is not surprising that this phase is heavily dependent on energy flux and

the metabolism of various cellular components. Additionally a large number of the proteins belonging to the energy and metabolism grouping are involved in cellular glycolysis – for example pyruvate kinase and enolase. Suppression of glycolysis is itself a model of long-term potentiation in the hippocampus (Tekkok & Krnjevic, 1995; Zhao *et al.*, 1997) perhaps indicating a role out with energy supply for these molecules in the induction of LTP. Clearly, nucleotide metabolism is an ongoing energy providing process required for the duration of the synaptic enhancement, as expression levels of the proteins involved in nucleotide metabolism (i.e. ATPases) are very similar in both phases of LTP.

5.5.3.2 Proteins involved in cytoplasmic organization and biogenesis

In contrast to proteins involved in energy metabolism, proteins associated with cellular organization and biogenesis were shown predominantly to demonstrate altered expression at the later time point following the induction of LTP. This functional grouping comprises cytoskeletal proteins such as tubulin, dynactin and internexin as well as other molecules including the cell adhesion molecule gamma catenin. Synaptic morphological changes are likely to be a key feature for the long-term enhancement of synaptic transmission. LTP induction has been associated with the formation of new dendritic spines (Engert & Bonhoeffer, 1999; Maletic-Savatic *et al.*, 1999; Toni *et al.*, 1999) and changes in spine morphology (Popov *et al.*, 2004). In contrast, long-term depression of the synaptic response by low frequency stimulation is accompanied by shrinkage in spine density (Zhou *et al.*, 2004). It has been postulated that rearrangements of the postsynaptic cytoskeleton are involved in these morphological adaptations (Cumming & Burgoyne, 1983; Burns, 1985). LTP induction by theta patterned stimulation was shown by Lin *et al* (2005) to rapidly (within minutes) increase actin polymerization in hippocampal dendritic spines and stabilization of actin filaments. Kim & Lisman were the first group to demonstrate that LTP is depleted in slices exposed to actin filament destabilisers such as latrunculin B (1999). Similar experiments by Krucker and colleagues in 2000 confirmed these findings. Interestingly, actin was not amongst the cytoskeletal proteins identified in either early or late phases of LTP in this proteomics study. Of course, it still remains a possibility that actin is one of the proteins not identified due

to low abundance of protein, but given the highly abundant nature of these molecules, it would be expected that mass spectrometric identification would be possible. It is also possible that the actin dynamics are so rapid that they are not being detected (Matus *et al.*, 2000).

Whereas in this study we see a large degree of differential protein expression associated with the microtubule protein tubulin (9 out of 15 protein spots identified as tubulin show differential spot density at 4 hrs post LTP induction), gene expression studies have previously demonstrated no change in tubulin expression levels following LTP induction (Roberts *et al.*, 1996), although microtubule associated proteins such as MAP2 have a definitive role in synaptic plasticity. Significant phosphorylation of MAP2 occurs following high frequency stimulation of CA1 afferents (Fukunaga *et al.*, 1996). This phosphorylation influences the state of neuronal microtubule polymerization and subsequent morphological changes – an increase in phosphorylation inhibits microtubule binding whereas dephosphorylation facilitates tubulin binding (Jameson & Caplow, 1981; Brugg & Matus, 1991). Therefore, it is not unrealistic to expect such changes in tubulin levels in the enduring phases of LTP. MAPs are substrates for several protein kinases including PKC (Ainsztein & Purich, 1992), PKA (Goldenring *et al.*, 1985), CaMKII (Schulman, 2004) and MAPK (Hoshi *et al.*, 1992; Berling *et al.*, 1994) all of which are induced following high frequency stimulation. Such a large degree of kinase activity surrounding microtubules certainly points towards a substantial alteration in microtubule formation.

The global proteomics approach adopted here has the advantage that it can investigate protein changes occurring in a number of different cell types, including the supporting neuronal network comprised of glial cells. This is demonstrated by the discovery that glial fibrillary acidic protein (GFAP) is significantly increased in expression 240minutes post LTP induction. Several molecules of GFAP, an intermediate filament protein, bind together to form the main intermediate filament found in glial cells. Loss of GFAP results in an increased susceptibility to ischemic insults (Tanaka *et al.*, 2002), enhanced hippocampal LTP (McCall *et al.*, 1996) and decreased cerebellar long-term depression (Shibuki *et al.*, 1996). In relation to this, ischemic insult resulted in the ablation of LTP in the hippocampus of GFAP deficient

mice (Tanaka *et al.*, 2002). Recent studies have also shown a requirement for glia in the regulation of synapse formation (Ullian *et al.*, 2004) and synapse efficiency (Pfrieger & Barres, 1997).

Tropomyosin was assigned by FatiGO to the biological process of muscle development and contraction, but it is also a recognised actin binding protein, which has two brain specific isoforms TMBr-1 (TM-4) and TMBr-3 (Stamm *et al.*, 1993). Subsequent studies by Had *et al* in 1994 demonstrated that TM-4 was concentrated in the growth cones of cultured neurons and *in vivo* in areas where neurites were growing. On completion of development, TM-4 is found to be restricted to postsynaptic sites and TMBr-3 to presynaptic terminals. No information is available concerning the relationship of this actin-binding protein with synaptic plasticity events in the CNS, but it is yet another possible candidate for mediating structural changes required for the enhancement of synaptic strength as observed in LTP.

5.5.3.3 Proteins involved in neurogenesis

At the 10 minute time point 4% of the total number of identified proteins showed altered levels of expression compared to 15% of the total number of identified proteins at the 4 hr time point. The structural changes that are suggested by the presence of several cytoskeleton-related proteins late on in the plasticity process are in keeping with the higher percentage of neurogenesis related proteins also identified at this stage. This process comprises mainly the collapsing response mediator protein (CRMP) family and GAP43. Neurogenesis in the dentate gyrus region of adult hippocampus is well documented particularly following neuronal injury (Sharp *et al.*, 2002) and also as a result of learning and physical activity (Bruel-Jungerman *et al.*, 2005; Lu *et al.*, 2003; Shors *et al.*, 2002). In the CA1 region of the adult hippocampus, less information is available. However in 2002, Schmidt & Reymann demonstrated proliferating cells in the CA1 region of gerbil hippocampus following global ischemia.

Growth associated protein (GAP) 43 also known as neuromodulin or B-50 is involved in the development of synaptic networks and axonal growth. GAP43 is a

presynaptic protein and is used as a marker of newly formed synapses (Benowitz & Routtenberg, 1997). A well documented PKC substrate, it has been implicated in the modulation of different forms of plasticity in the brain. For example, over-expression of GAP43 results in spontaneous nerve sprouting in several brain regions (Aigner *et al.*, 1995; Harding *et al.*, 1999; Klein *et al.*, 1999) and depletion of GAP43 disrupted axonal path-finding and resulted in deficient spreading, branching or adhesion of growth cones (Strittmatter *et al.*, 1995; Aigner & Caroni, 1993, 1995). It has also been implicated in synaptic plasticity and related events such as LTP. During LTP PKC is activated by HFS and calcium and there is also evidence that prolonged phosphorylation of GAP43 is associated with LTP in the CA1 region of the hippocampal slice (Gianotti *et al.*, 1992; Ramakers *et al.*, 1995; Oestreicher *et al.*, 1997) an effect prevented by the blockade of the NMDA receptor (Linden *et al.*, 1988). Hulo *et al* in 2002 utilized transgenic mice that either over-expressed different mutated forms of GAP43 or were deficient in GAP43 and investigated these genetic manipulations in terms of both short and long term synaptic plasticity. Over-expression of a constitutively phosphorylated form of GAP43 resulted in an enhancement of LTP in the CA1 region of hippocampal slices an effect that was NMDA receptor dependent. Routtenberg and colleagues had previously demonstrated enhanced learning in these mice (Routtenberg *et al.*, 2000). However, over expression or depletion of wild type GAP43 had no effect on LTP in the CA1 region of adult mouse hippocampal slices suggesting that GAP43 is not required for LTP induction (Hulo *et al.*, 2002).

Additionally the presence of other molecules, unrelated to the cytoskeleton, also indicates a degree of neurogenesis as a result of high frequency stimulation. The identification of neuronal specific enolase (NSE) is not only indicative of an increase in glycolysis but is also an indication of extensive re-modelling and neuronal morphogenesis as described by Sensenbrenner *et al* in 1997 and Huang & Lim in 1990, who used NSE as a marker for mitotic cells following neuronal injury.

5.5.3.4 Proteins involved in protein metabolism & transport

Protein metabolism and transport represents one of the biological process associated with a large number of modulated proteins. In the early phase of LTP 18% of the identified molecules belonged to this process and slightly less (13%) were represented the latter phase of LTP. Given that there are fairly similar levels of regulation in both phases this implies a constant turnover of protein molecules commencing at the induction stimulus and continuing on through the maintenance phase of the synaptic enhancement. Within this grouping are the proteins transitional endoplasmic reticulum ATPase, 14-3-3 (epsilon), glutamate oxaloacetate transaminase, spermidine synthase, calcineurin, nitrogen fixation gene and thimet oligopeptidase and Hsp60 and MEK1, some of which are discussed in greater detail below.

Heat shock proteins are, contrary to their name, constitutively expressed in most cells under normal physiological conditions. D'Souza & Brown in 1998 demonstrated a developmentally related increase in expression of Hsp60 mRNA in rat brain. Hsp60 was also found in locations associated with mitochondria in the differentiated neuron (Martin *et al.*, 1993). The identification in this study of the groEL heat shock protein of bacteria is likely to be a human mitochondrial protein epitope that is homologous with the bacterial form (Martin *et al.*, 1993). Heat shock protein 60 is also known as a stress-inducible mitochondrial matrix protein that forms a chaperonin complex required for mitochondrial protein folding and function.

Discovered more than thirty years ago by Moore *et al* (1968) in the bovine brain, 14-3-3 protein associates with phosphor-serine or phosphor-threonine containing peptides (Aitken *et al.*, 2002). In terms of synaptic plasticity and more specifically LTP, a requirement for 14-3-3 has been shown for a presynaptic form of LTP in the mouse cerebellum representing forms of motor learning such as adaptation of the vestibule-ocular reflex and associative eyelid conditioning (Simsek-Duran *et al.*, 2004)

Transitional endoplasmic reticulum ATPase (TER ATPase) also known as p97, valosin containing protein (VCP) or CDC48 provides a link between the endoplasmic reticulum and the proteasome as disruption of CDC48 in yeast and VCP in mammals results in the accumulation of polyubiquitinated proteins (Dai and Li 2001; Ye *et al.*, 2001; Rabinovich *et al.*, 2002; Jarosch *et al.*, 2002; Braun *et al.*, 2002). TER ATPase is a member of a family of ATPases with multiple cellular activities (AAA). The VCP complex binds to polyubiquitin chains and facilitates the untethering of ubiquitinated proteins from their binding partners. VCP has been identified as a polyglutamine (polyQ)-interacting protein and hence may play a vital role in polyQ diseases such as Prion disease and AD and a general role in neurodegeneration. Its role in synaptic plasticity however is unclear. VCP is ubiquitous, essential and highly abundant in cells, accounting for more than 1% of the total cellular protein and is involved in numerous cellular activities including membrane fusion (Latterich *et al.*, 1995; Zhang *et al.*, 1994), ER associated degradation (Bays *et al.*, 2003; Hitchcock *et al.*, 2001; Jarosch *et al.*, 2002; Rabinovich *et al.*, 2002), transcriptional activation (Asai *et al.*, 2002; Dai *et al.*, 1998), cell cycle control (Moir *et al.*, 1982; Egerton *et al.*, 1992) and apoptosis (Shirogane *et al.*, 1999) Therefore its role in LTP could be one of several, but it is thought that VCP mediates all these biological functions listed above via its actions as a molecular chaperone in the ubiquitin-proteasome system (Dai and Li 2001; Ye *et al.*, 2001; Rabinovich *et al.*, 2002; Jarosch *et al.*, 2002 & Braun *et al.*, 2002).

Neurons of adult brain are able to remodel their synaptic connections in response to various stimuli. Modifications of the peridendritic environment, including the extracellular matrix (ECM), are likely to be important in this morphogenesis. Proteolytic disassembly of ECM is a complex process using the regulated actions of specific extracellular proteinases – matrix metalloproteinases (MMPs). Thimet oligopeptidase 1 is an enzyme linked to the proteolytic cleavage of amyloid precursor protein in AD (Papastoitsis *et al.*, 1994). This aside, a role for Thimet oligopeptidase in synaptic plasticity has yet to be determined. MMPs on the other hand, in particular MMP-9 and TIMP-1 are elevated in neurons in response to enhanced neuronal activity for example, following seizures (Nedivi *et al.*, 1993; Rivera *et al.*, 1997; Jaworski *et al.*, 1999). Seizures were shown to elevate protein and enzymatic levels of MMP-9 after 6h. Levels of mRNA however peaked at 24-

72h in both neuronal cell bodies and in the dendritic layer suggesting activity dependent translocation of this mRNA (Szklarczyk *et al.*, 2002) to the dendrites of activated synapses as is thought to be required for LTP in the hippocampus (Frey & Morris 1998; Martin *et al.*, 2000). Finally, Wright *et al.* (2002) showed that MMP-9 enzymatic activity in the hippocampus was enhanced following spatial learning in the rat.

The ERK/ MAPK signalling pathway is a highly conserved kinase cascade linking transmembrane receptors to downstream effector mechanisms (Chang & Karin 2001). In neurons, the ERK pathway is activated by stimuli associated with synaptic activity and plasticity (Ca^{2+} influx, neurotrophins) (McAllister *et al.*, 1999, Tyler *et al.*, 2002). Previous studies have demonstrated general involvement of the ERK pathway in synaptic plasticity and learning and memory (Impey *et al.*, 1999; Mazzucchelli & Brambilla 2000; Sweatt 2001).

High frequency synaptic stimulation of pyramidal cells of the CA1 region of the hippocampus resulted in the decreased expression of MEK1. The cellular distribution of MEK1, (mitogen-activated protein kinase kinase 1), an upstream activator of MAPK, was elucidated by Schipper and colleagues in 1999. They showed wide distribution of MEK1, with prominence in the cortex, hippocampus, brainstem, hypothalamus and cerebellum. Sub-cellular distribution in neurons is confined to perikarya and dendrites, with a portion co-localizing with microtubules. MAPK kinase itself is activated by glutamate receptor activation and calcium influx into the post-synaptic cell, also a requirement for LTP (Fiore *et al.*, 1993, Farnsworth *et al.*, 1995; Rosen *et al.*, 1994) suggesting effects on upstream molecules such as MEK. Glutamate receptor activation results in tyrosine kinase activation, with phosphorylated kinases leading to the formation of a Ras-GTP complex which in turn binds and activates Raf and subsequently phosphorylates MEK. Raf is also only detectable in neuronal dendrites (Mihaly *et al.*, 1991) suggesting that neurons utilize the Raf/ MEK/ MAPK pathway for signalling in dendrites. MEK then phosphorylates MAPK on both its Thr and Tyr residues. MAPK or ERK activation has been linked to several learning behaviours and activity dependent neuronal function. Dominant negative MEK1 mice exhibit a significant impairment in

contextual fear conditioning resulting from the decreased activation of ERK 1 & 2 in the hippocampus (Shalin *et al.*, 2004). These mice also had selective deficits in the translation dependent, transcription independent phase of L-LTP (phase II). In hippocampal neurons, ERK inhibition blocked neuronal activity induced translation as well as phosphorylation of several translation factors (eIF4E, 4EBP1 and ribosomal protein S6). Protein synthesis and translation factor phosphorylation induced in control slices by the L-LTP stimulus were significantly reduced in mutant slices.

RAP1 was identified from a single protein spot shown to be significantly decreased 240 minutes following LTP in the mouse hippocampus. Although classed as a signalling molecule, its connection to the MAPK signalling pathway merits its discussion at this stage. RAP1 (guanine nucleotide exchange factor) has been shown to oppose the actions of the Ras superfamily of small G proteins in terms of synaptic plasticity, and has been shown to activate the MAP kinase cascade in several cell types. In PC12 cells depolarization led to the PKA dependent stimulation of RAP1 and the formation of a Rap1/ B-Raf signalling complex. The major action of this interaction in hippocampal neurons is the activation of ERKs (Grewal *et al.*, 2000). Rap1 is highly expressed in the CNS. Multiple second messengers such as cAMP, PKA, Ca^{2+} and DAG can control ERK signalling via the small G proteins Ras and Rap1. Learning induced synaptic plasticity commonly involves the interaction between cAMP and ERK signalling. Morozov *et al* in 2003 developed a mouse with interfering Rap1 mutant in the mouse forebrain. The consequence of this was a decreased basal phosphorylation of a membrane associated pool of ERK, impaired cAMP-dependent LTP in CA1 region induced by either forskolin or theta frequency stimulation. These changes were associated with impaired spatial memory and context discrimination. Rap1 couples cAMP signalling to a selective membrane associated pool of ERK to control excitability of pyramidal cells, the early and late phases of LTP and the storage of spatial memory.

5.5.3.5 Proteins involved in transcription and translation

There is a greater representation of protein molecules belonging to the functional grouping of transcription and translation in the earlier stages of LTP (3) in comparison to the more enduring stages (1), although the numbers of identified proteins in this grouping are very small indeed. The molecules identified, hnRNPK and PCBP-1 are both involved in mRNA binding.

Proteins involved in the translation of pre-existing mRNAs are crucial to phase II of LTP, thought to be around 3hrs following induction. Indeed we have identified a number of molecules that are involved in the translational control of a number of mRNAs. These include heterogeneous ribonucleoprotein K (hnRNPK) (which increases in expression) and poly (rC) binding protein (PCBP-1). HnRNPK is thought to recruit various molecular partners and therefore play a role in a number of different processes including transcription and RNA processing as well as translation. PCBP-1 and -2 along with hnRNPK correspond to the major cellular poly (rC)-binding proteins. These proteins have been shown to stimulate the activity of the internal ribosome entry segment (IRES) required for translation of mRNAs (Evans *et al.*, 2003). Pinkstaff *et al* in 2001 suggest IRESes (internal ribosome entry sites) increase translation efficiency at postsynaptic sites after synaptic activation. Although translation mediated by this IRES occurred throughout the cell, translation was relatively more efficient in dendrites possibly indicating a change in dendritic translation shortly after the induction of LTP in the CA1 region of the hippocampus. As previously described in chapter 4, a train of protein spots is indicative of post translational modifications of particular proteins (Chapter 4, Figure 4.3). In the example shown in Figure 5.2, hnRNPK is present within two adjacent protein spots. It is known that increases in phosphorylation are known to decrease the resultant isoelectric point of a particular protein on a 2D gel. HnRNPK is phosphorylated by protein kinase C *in vivo* and *in vitro* at Ser^{302}. In *vitro*, PKCδ binds hnRNPK via the highly interactive KI domain, an interaction that is blocked by poly(C) binding protein RNA (Schullery *et al.*, 1999). Indeed PCBP-1 was found to decrease significantly in expression in the early stage of LTP in this proteomics investigation. HnRNPK has also been shown by Ostrowski *et al.* in 2000 to be tyrosine-

phosphorylated *in vitro* by Src and Lck. Translation-based assays show that K protein is constitutively bound to many mRNAs *in vivo*. Native immuno-precipitated K protein-mRNA complexes were disrupted by tyrosine phosphorylation, suggesting that the *in vivo* binding of K protein to mRNA may be responsive to the extracellular signals that activate tyrosine kinases. This study shows that tyrosine phosphorylation of K protein regulates K protein-protein and K protein-RNA interactions. These data are consistent with a model in which functional interaction of K protein is responsive to changes in the extracellular environment. Acting as a docking platform, K protein may bridge signal transduction pathways to sites of nucleic acid-dependent process such as transcription, RNA processing, and translation. Another mRNA binding protein from the same family, hnRNPQ1 is found to be distributed throughout the cytosol, and in a proteomic study investigating the protein complexes associated with hnRNPQ1, Bannai *et al* (2004a) found it to be preferentially associated with ribosomal proteins and RNA binding proteins. It was also found contained within mRNA granules also containing IP3R1 mRNA to be transported within dendrites in a microtubule dependent manner.

5.5.3.6 Proteins involved in amino acid transport

Modulation of amino acid transport was only identified in the early stages of LTP. The two protein spots that significantly decrease in expression were subsequently identified at SLC25A22 – a mitochondrial carrier. Mitochondrial carriers shuttle a variety of metabolites across the inner mitochondrial membrane. These carriers are encoded by the SLC25 genes of which there are several. They are thought to function as homodimers, with each monomer being folded in the membrane into six transmembrane segments. Therefore, the sample preparation described in chapter 2 has therefore optimized the release of some membrane bound proteins, commonly thought to be absent from 2D gels. The common function of these carriers is to provide a link between mitochondria and cytosol by facilitating the flux of a large variety of solutes through the permeability barrier of the inner mitochondrial membrane. The SLC25 gene identified in these LTP studies, SLC25A22, belongs to a group of electrogenic carriers that transport glutamate across the mitochondrial membrane. There are 2 glutamate carriers GC1 and GC2 with 63% homology to each other (Fiermonte *et al.*, 2002). The main physiological role is to import glutamate

from the cytosol, to the mitochondrial matrix where glutamate dehydrogenase is exclusively located. The specific isoform of this protein (either GC1 or GC2) was not identified but it is thought that GC2 matches the basic requirements of all tissues with respect to amino acid degradation and GC1 becomes operative to accommodate higher demands associated with specific metabolic functions. As with other protein molecules involved in energy and metabolism processes these proteins were exclusively found in the early stages of LTP, providing further evidence of an increased metabolic demand shortly after exposure of the hippocampal cells to the induction stimulus.

5.5.3.7 Proteins involved in vesicle mediated transport, neurotransmitter transport and synaptic transmission

Vesicle mediated transport, neurotransmitter transport and synaptic transmission are three very similar biological processes which are nevertheless listed here as individual classes. Within each class are very small numbers of regulated proteins, one protein assigned to vesicle mediated transport regulated at the 4 hr time point (endophilin 2), 2 proteins assigned to neurotransmitter transport also at the 4 hr time point (Munc18 & syntaxin 1A) and 2 proteins assigned to synaptic transmission regulated at 10 minutes (MEK1 & synapsin II) and 1 regulated at 240 minutes after LTP induction (RAP1 & synapsin II).

Expression of endophilin 2 in the CNS was determined by Ringstad *et al* back in 2001. It is highly expressed in the brain and found concentrated at nerve terminals in complexes with synaptojanin and dynamin. Earlier studies by this group in 2000 showed that disruption of this complex resulted in the impairment of synaptic vesicle endocytosis in a living synapse. In particular, the SH3 domain of endophilin is thought to participate in both fission and un-coating of clathrin coated vesicles (Gad *et al.*, 2000). Synaptic vesicles are quickly and efficiently recycled at nerve terminals. Part of this recycling involves clathrin-mediated endocytosis. Once again the dynamin-endophilin-synaptojanin complex plays an important role. Endophilin/ SH3p4 is a protein highly enriched in nerve terminals that binds the GTPase dynamin

and the polyphosphoinositide phosphatase synaptojanin, two proteins implicated in synaptic vesicle endocytosis.

As is the case for vesicle-mediated transport, the modulation of proteins involved in neurotransmitter transport is exclusively found in the later stages of LTP. Following one trial aversive learning several genes were identified as regulated in the hippocampus (Igaz *et al.*, 2004). One of the genes up-regulated 24h after training was syntaxin 1A. Syntaxin 1A is predominantly localized to the plasma membrane, with a small proportion present within an intracellular compartment (Mitchell & Ryan, 2004). Syntaxin-1 is a key component of the synaptic vesicle docking/ fusion machinery that binds with VAMP/ synaptobrevin and SNAP-25 to form the SNARE complex. It also inhibits GABA uptake of an endogenous GABA transporter in neuronal cultures from rat hippocampus (Beckman *et al.*, 1998) therefore not only is it involved in neurotransmitter release but also in neurotransmitter uptake. This was confirmed in a study by Yamaguchi *et al* in 1997, which showed that an antibody to syntaxin 1A enhanced the EPSC measured from cultured rat hippocampal neurons, an effect attributable to an increase in neurotransmitter release from the presynaptic terminal. A second isoform of syntaxin 1, syntaxin 1B is up regulated following LTP in the dentate gyrus (Hicks *et al.*, 1997; Davis *et al.*, 2000). Accompanying this increase in syntaxin 1B and synapsin II protein levels (which was also identified in our study) was an increase in glutamate release, all of which were prevented by blocking the NMDA receptor. Syntaxin binding protein or Munc18 was also shown to be differentially expressed.

A presynaptic location for LTP induction and maintenance has been documented previously by several groups (Baxter *et al.*, 1985; Nelson *et al.*, 1989; Malinow & Tsein 1990). Enhancement of presynaptic neurotransmitter release is thought to be a possible mechanism for the enhancement of synaptic strength. Copine 6 and synapsin II are two protein molecules identified in this study known to be involved in synaptic transmission. As very small numbers of these proteins were identified, it is not possible to determine whether or not this functional grouping dominates in either the early or late phase of LTP.

The regulation of neuronal copine as a result of HFS is very interesting. Copines are ubiquitous, Ca^{2+} dependent phospholipid binding proteins, thought to be involved in membrane trafficking. N-copine or Copine 6 is a relatively novel protein whose expression is brain specific and is up-regulated following hippocampal CA1 LTP. N-copine mRNA is expressed exclusively in neurons of the hippocampus (cell bodies and dendrites) and also in the main and accessory olfactory bulb where various forms of synaptic plasticity and memory formation are known to occur. Its association with cell membranes occurred in the presence of Ca^{2+} not in its absence. N-copine contains 2 C2 domains. The C2 domain was originally identified as a Ca^{2+} binding domain of PKC gamma (Ono *et al.*, 1989). It is now known that its function includes Ca^{2+} dependent and Ca^{2+} independent phospholipids binding, inositol polyphosphate binding, Ca^{2+} binding and interaction with other proteins (Fukuda *et al.*, 1994; Sheng *et al.*, 1994; Ullrich *et al.*, 1994; Chapman *et al.*, 1995; Sugita *et al.*, 1996; Schiavo *et al.*, 1997). Other proteins are known to also carry two C2 domains aptly named the "double C2 protein family" (Sudhof & Rizo, 1996). In neurons these proteins (synaptotagmins, rabphilin-3A) are found in presynaptic terminals and play roles in the transport of synaptic vesicles during neurotransmitter release (Li *et al.*, 1994; Ullrich *et al.*, 1994). The difference between N-copine and the other double C2 proteins is that Copines C2 domains are on the N terminus as opposed to the C terminus therefore it is thought that the roles are also different. In 1998 Nakayama's group showed an up regulation of N-copine mRNA after kainate administration and following LTP, an NMDA receptor dependent effect (Nakayama *et al.*, 1999a). Further studies by Nakayama and colleagues detected N-copine in both the cytosol and membrane of mouse brain cells. Membrane association was seen to be Ca^{2+} dependent. N-copine positive staining was found in cell bodies and dendrites of pyramidal cells and not in stratum oriens in contrast to presynaptic proteins such as synaptotagmin and rab3A suggesting a postsynaptic role and localization of N-copine. N-copine up-regulation following kainate administration was NMDA receptor dependent (Nakayama *et al.*, 1999b). Although Nakayama and colleagues have previously described a role for N-copine in LTP, its discovery in this proteomics study is still of great interest. Firstly, Nakayama looked at late phase LTP (6 hours) and we see a change here at 10 minutes. The up-regulation of this protein spot is not an enduring change. This is not to say however that N-copine is not amongst some of the unidentified protein spots differentially expressed in the latter

stages of synaptic plasticity. Secondly, this can be seen as an important validation of these proteomic experiments. Not only are we able to identify potential novel targets but we are also able to confirm the presence of proteins previously associated with LTP in the CA1 hippocampal sub field.

In summary, LTP of the synaptic responses of hippocampal pyramidal cells resulting from high frequency stimulation of glutamatergic afferents results in the significant differential expression of a number of protein molecules. These proteins belong to several functional groups including protein metabolism and transport, neurogenesis, cell organization and biogenesis and ion channels. The early phase of LTP appears to require increased energy and metabolism demands whilst long-term enhancement of synaptic transmission appears to involve molecules associated with cellular organization and biogenesis and neurogenesis, supporting the theory that enduring forms of synaptic plasticity requires morphological adaptation. The modulation of a portion of the identified proteins has previously been reported, for example GAP43 and copine 6. Additionally, modulation of potentially novel modulators (hnRNPK, gamma catenin) was also highlighted.

Chapter 6

In vivo protein regulation in models associated with learning and memory

6.1 INTRODUCTION

The adult mammalian brain has the capacity to form new cells out with developmental stages. Such neurogenesis has been identified in the striatum (Collins *et al.*, 2005), substantia nigra (Yoshimi *et al.*, 2005) cortex (Jiang *et al.*, 2003) and the hippocampus (Arvidsson *et al.*, 2001; Seki, 2002). Neurogenesis occurs following exposure of rodents to an enriched environment (Auvergne *et al.*, 2002), increased social interaction (Lu *et al.*, 2003) and exercise (van Pragg *et al.*, 1999) but is also apparent following neuronal damage such as ischaemia (Liu *et al.*, 1998) and ethanol-induced neurotoxicity (Pawlak *et al.*, 2002). There is also evidence of increased hippocampal neurogenesis in AD (Jin *et al.*, 2004) although some animal models exhibit impaired neurogenesis (Haughey *et al.*, 2002). In the dentate gyrus region of the hippocampus newly formed cells are thought to form part of a functional network by sending and receiving connections from other cells in the CA3 region (van Praag *et al.*, 2002). This increase in connectivity is thought to be involved in the formation of new memories. Indeed, learning and memory is shown to be enhanced with and increase in the number of neuronal cells (Bruel-Jungermann *et al.*, 2005; Shors *et al.*, 2002). Contradictory studies do exist however, and Ambrogini *et al* (2004) have very recently shown that learning actually reduces the degree of neurogenesis, although this occurs at very specific time points following new cell formation.

One way to induce neurogenesis is to expose rodents for example, to an enriched environment. This exposure promotes structural changes (i.e. higher dendritic spine densities, increases in dendritic arborization (Turner *et al.*, 2003; Faherty *et al.*, 2003)) and in doing so, or possibly as a result of, enhances learning and memory in these rodents (Duffy *et al.*, 2001). Several other groups have also demonstrated an improved performance in spatial learning tasks and avoidance tasks following enrichment (Iuvone *et al.*, 1996; Pham *et al.*, 1999; Young *et al.*, 1999). EE has been shown to improve performance in spatial memory tasks in aged animals (Frick & Fernandez, 2003; Kobayashi *et al.*, 2002). Kobayashi and colleagues also identified increased efficacy of environmental enrichment during the developmental stages of young animals on maze learning. Similarly, Venable *et al.*, 1988 showed that exposure of pre-weaned animals to an enriched environment improved the

performance in the Hebb-Williams maze compared to the performance of slightly older post-weaned rats. Jankowsky and colleagues have also shown that exposure of environmental enrichment can result in the amelioration of cognitive deficits associated with AD (Jankowsky *et al.*, 2005). It is possible that this improvement in cognitive performances could be as a result of decreased Abeta levels and amyloid deposition as has been shown by Lazarov *et al.*, (2005).

The structural and molecular changes that occur following enrichment will undoubtedly accompany, or indeed result from gene transcription and mRNA translation and may account for improved performance in learning and memory tasks seen in older animals (Esconhuela *et al.*, 1995). Short and long-term gene expression in the cortex of mice following exposure to an enriched environment has been investigated by Rampon *et al* (2000). They found several genes both up and down regulated in the period shortly after exposure (2hrs) and in the days (14) following. Short term genes included large increases genes known to be involved in DNA replication and neuronal cell differentiation and genes involved in new synapse formation. Longer exposure to an enriched environment resulted in the differential expression of genes involved in NMDA receptor function as well as structural proteins.

In contrast to EE-induced enhancement of hippocampal-dependent tasks, many disease models exist which replicate deficits in learning and memory. One of the most widely studied models is that for the neurodegenerative condition AD. To date over 120 different genetically modified models mouse strains mimicking various aspects of AD have been developed. Most such models have mutations in a number of key proteins including amyloid precursor protein (Moechars *et al.*, 1999), apolipoprotein (Mann *et al.*, 2004), Cox-2 (Andreasson *et al.*, 2001), presenilin (Wang *et al.*, 2004; Oyama *et al.*, 1998), tau (Lewis *et al.*, 2000) or a combination of some of these (Siman *et al.*, 2000). As previously mentioned, AD has been associated with both increased and decreased neurones (Jin *et al.*, 2004; Haughey *et al.*, 2002). Additionally, some models of familial Alzheimer's disease (FAD) such as mouse models harbouring mutations in the presenilin-1 gene also accompany decreases in adult neurogenesis in the dentate gyrus (Wang *et al.*, 2004). The transgenic mouse line TAS10 over expresses the 695-amino acid isoform of the

human amyloid precursor protein which itself contains the Swedish double familial Alzheimer's disease formation (Vidal *et al.*, 1990). These mice show an age dependent increase in the deposition of beta amyloid in the brain. Spatial learning and working memory deficits have also been reported (Richardson *et al.*, 2003). Amyloid plaque deposition and associated pathologies were increasingly evident by around 18 months, especially in the somatosensory cortex of these animals when compared to the hippocampus (Richardson *et al.*, 2003).

The following chapter describes a series of investigations initially to identify changes in the protein expression in both the somatosensory cortex and the hippocampus of aged TAS10 transgenic mice and subsequently to examine protein expression in a rodent model of environmental enrichment in the CA1 region of the rat hippocampus. In doing so it investigates the global proteome changes associated with a treatment representative of increased neurogenesis, learning and plasticity and a treatment representative of neurodegeneration and subsequent associated decreases in learning and plasticity.

6.2 Study Aims

Environmental enrichment is widely documented as a stimulus likely to induce a number of adaptive processes including increased neurogenesis, an increased capacity for learning and increased neuronal plasticity. Conversely, AD is a neurodegenerative disease associated with cognitive decline and decreased neuronal plasticity. The specific aims of the current chapter were thus:

- To investigate global alterations in the hippocampal proteome following exposure of rats to a novel and complex environment (environmental enrichment)

- To investigate global differences in the hippocampal and neocortical proteome of the TAS10 mouse model of AD from WT age-matched littermates.

6.3 METHODS

As described in full in chapter 2 of this thesis with the following additional information. Enrichment studies used rat male hooded Listers (aged 4 weeks at commencement of study, 10 weeks old at end of study). Protein extracts from dendritic and somatic regions of hippocampal area CA1 were run on pH 3-10NL gradient IPG strips. The experimental design adopted for this set of experiments was as follows:

Table 6.1 Experimental design of environmental enrichment DiGE study

Gel	Cy2	Cy3	Cy5
1	Standard	Enriched (dendritic)	Control (somatic)
2	Standard	Enriched (somatic)	Control (dendritic)
3	Standard	Enriched (somatic)	Enriched (dendritic)
4	Standard	Control (somatic)	Enriched (dendritic)
5	Standard	Control (somatic)	Enriched (somatic)
6	Standard	Control (dendritic)	Control (somatic)
7	Standard	Control (dendritic)	Enriched (somatic)

Table illustrating the experimental design used to examine the effect of 4 treatment groups (control (somatic region) (n=4), control (dendritic region) (n=4), enriched environment (somatic region) (n=4) and enriched environment (dendritic region) (n=4)) using incorporation of an internal standard all labelled with Cy2 and the randomised labelling of the 4 treatment groups with either Cy3 or Cy5.

TAS10 transgenic mice (12-13 months old) (male (n=3) and female (n=1)) and their WT littermates (male (n=3) and female (n=1)) were used for the TAS10 AD model studies. Protein extracts from both the somatosensory cortex and hippocampus of these mice were run on pH 3-10NL gradient IPG strips. The experimental design adopted for this set of experiments was as follows:

Table 6.2 Experimental design for TAS10 transgenic mice (entire hippocampus (H)) DiGE study.

Gel	Cy2	Cy3	Cy5
1	Standard	TAS10 TG - H	WT - H
2	Standard	TAS10 TG - H	WT - H
3	Standard	WT - H	TAS10 TG - H
4	Standard	WT - H	TAS10 TG - H

Table illustrating the experimental design used to examine the effect of 2 treatment groups (TAS10 transgenic hippocampus (TG - H) (n=4) and wild type hippocampus (WT - H) control (n=4)) using incorporation of an internal standard all labelled with Cy2 and the randomised labelling of the 2 treatment groups with either Cy3 or Cy5.

Table 6.3 Experimental design for TAS10 transgenic mice (cortex (C)) DiGE study.

Gel	Cy2	Cy3	Cy5
1	Standard	TAS10 TG - C	WT - C
2	Standard	TAS10 TG - C	WT - C
3	Standard	WT - C	TAS10 TG - C
4	Standard	WT - C	TAS10 TG - C

Table illustrating the experimental design used to examine the effect of 2 treatment groups (TAS10 transgenic cortex (TG - C) (n=4) and wild type cortex (WT - C) control (n=4)) using incorporation of an internal standard all labelled with Cy2 and the randomised labelling of the 2 treatment groups with either Cy3 or Cy5.

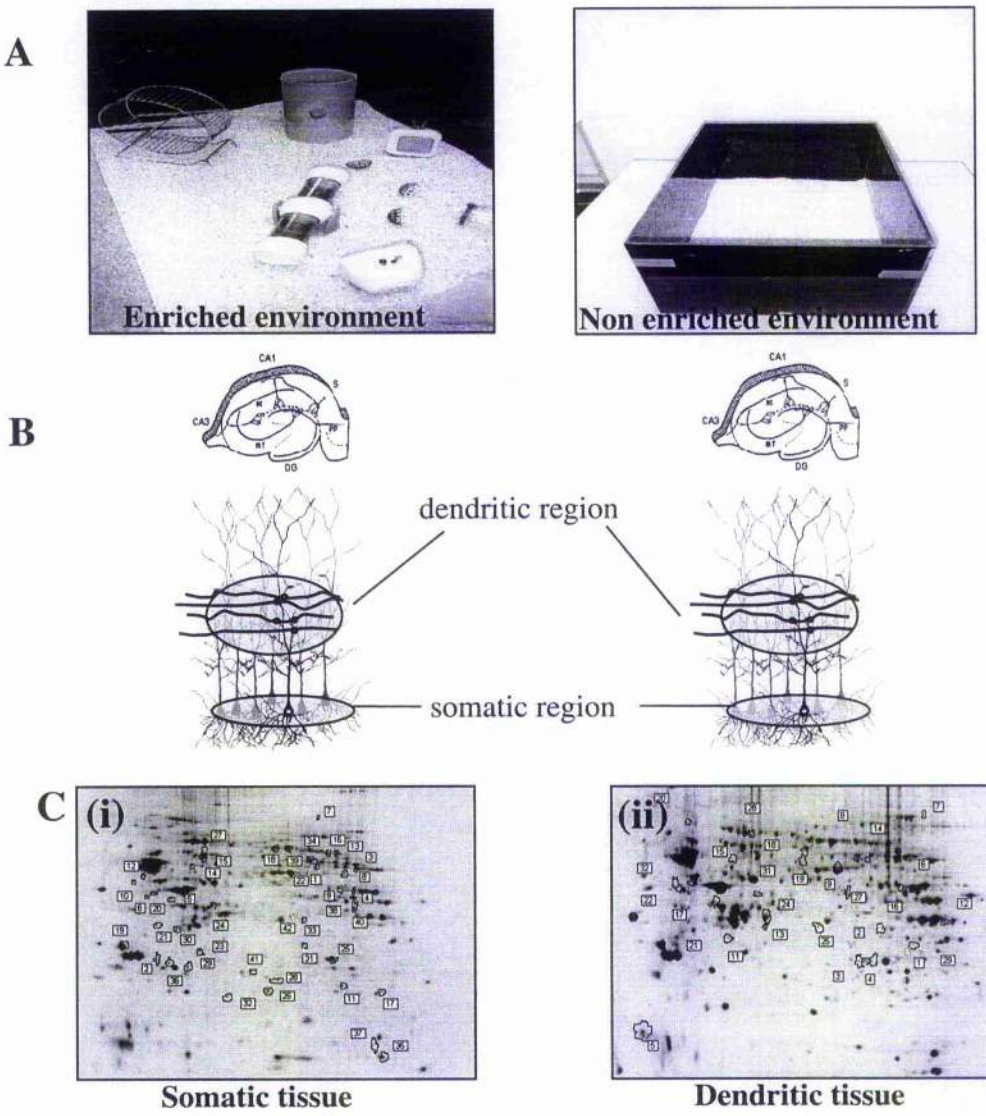
6.4 RESULTS

6.4.1 Protein regulation in CA1 pyramidal neurons following animal exposure to a stimulating and complex environment

Environmental enrichment is thought to involve an increase in adult neurogenesis and as a consequence improved cognitive performance in tasks that investigate spatial learning (Leggio *et al.*, 2005). In an attempt to identify the range and form of molecules that may play a role in this enhanced cognition and neurogenesis we exposed male hooded Lister rats to a complex and enriched environment for a total experimental period of 6 weeks to identify any possible changes in protein expression that may be associated with these previous findings. This complex environment comprised of several toys, hiding places and activity wheels which not only promoted exploration but also potentially increased the amount of exercise taken by the animals. In contrast, control animals were transferred to identical open arenas but which lacked enrichment tools for the same period of time (Figure 6.1a).

Following the enrichment period the hippocampi of all animals (enriched and non-enriched, n=6/group) were removed and the somatic and dendritic regions from area CA1 micro-dissected and snap frozen for subsequent DiGE analyses (Figure 6.1b). Total CyDye labelled protein extracts from dendritic and cell bodies layers of area CA1 of enriched and non-enriched hippocampi were resolved over two dimensions (pI and MW), and protein abundance levels quantified and compared using the BVA component of DeCyder software. Such biological variance analysis identified 42 protein spots showing significantly ($p<0.05$) altered abundance in the cell body layer fraction from enriched animals compared to non-enriched controls (Table 6.4) and 32 protein spots showing significant ($p<0.05$) differential expression in the dendritic layer fraction of hippocampal CA1 area from enriched animals compared to non-enriched controls (Table 6.5). The scatter of regulated protein spots spans the majority of the pI and MW ranges for both sets of tissues (Figure 6.1c).

Figure 6.1 DIGE analysis of dendritic and somatic hippocampal subfields from rats exposed to an enriched environment and a control non-enriched environment.



A. Images of the two different environment in which rats were housed. The enriched environment comprised a selection of toys and activity wheels and the non-enriched environment consisted of an empty arena. Rats were exposed to these environments for 15 hours per day, 5 days per week for a total experimental period of 6 weeks. B. Schematic diagram illustrating the procedure for tissue collection. Following the experimental period, hippocampi were removed, somatic and dendritic regions harvested and corresponding protein extracts resolved and compared by 2D gel electrophoresis and DiGE analysis (C). Representative annotated 2D gel images containing the 32 proteins isolated from dendritic regions (i) and 42 proteins isolated from somatic regions (ii) differentially expressed as a result of exposure of rats to a complex environment spanning a wide pI (ph3-10 NL) and MW (10 – 200kDa) range.

Table 6.4. List of extracted somatic proteins differentially expressed following long term exposure of rodents to an enriched environment.

Spot # (1)	Master # (2)	Protein ID (3)	Av. Ratio (4)	Control v enriched (somatic) (T-test) (5)	Mass (6)	NCBI Acc# (7)	Mowse Score (8)	Clas (9)
1	1297		-1.4	0.034				
2	2081		-1.28	0.0017				
3	1312	ATP synthase α (M)	-1.28	0.021	58904	gi 114523	95	NM
4	1584	Citrate synthase	-1.26	0.0063	52176	gi 18543177	174	E/M
		Phosphoglycerate kinase 1			44909	gi 40254752	111	CM
5	1672	Actin, ß	-1.2	0.036	42066	gi 71620	261	CO&
6	1642		-1.19	0.0055				
7	491	Dynamin 1	-1.18	0.00048	96209	gi 18093102	215	CO&
8	1395	4-aminobutyrate transaminase	-1.16	0.0055	57178	gi 2143559	66	NT
9	1566		-1.16	0.045				
10	1465		-1.13	0.043				
11	2232	Proteasome subunit, ß type 2	-1.12	0.033	23069	gi 8394079	272	PM&
		Auh protein			33605	gi 34873875	132	E/M
		ATP synthase α (M)			58904	gi 114523	82	NM
		RAB7			23075	gi 92022	39	ST
12	1400		-1.1	0.011				
13	1098		-1.1	0.046				
14	1253		-1.07	0.02				
15	1141	Protein phosphatase 3 (α)	1.1	0.046	59291	gi 8394030	105	ST
16	1101	Isoform of PSD-95/SAP90	1.11	0.028	92685	gi 1517938	49	NT
17	2270	Dynactin 3	1.11	0.029	24867	gi 34867161	144	CP
		ATP synthase, subunit D (M)			18827	gi 220904	104	NM
18	1034	CRMP-2 (Mus musculus)	1.12	0.023	62638	gi 40254595	125	NG
19	1998	14-3-3	1.13	0.0095	29274	gi 13928824	63	PM&
		Tropomyosin isoform 6			29245	gi 29336093	71	MC
		GFAP δ			48809	gi 5030428	50	CO&
20	1608		1.13	0.02				
21	1898	Tubulin, ß chain 15	1.13	0.027	50361	gi 92930	148	CO&
22	1210		1.14	0.032				
23	2034		1.14	0.035				
24	1723	Eno1 protein	1.14	0.045	51736	gi 38649320	355	E/M
		Tubulin ß chain 15			50361	gi 92930	319	CO&
		Tubulin, ß2-like			50225	gi 40018568	291	CO&
		Tubulin, ß5			50095	gi 27465535	287	CO&
		Tubulin, α2			50804	gi 34740335	256	CO&
		Tubulin, α1			50788	gi 11560133	237	CO&
		GTP-binding regulatory protein Go			40568	gi 71911	135	ST
		Isocitrate dehydrogenase 3 (NAD+)			40044	gi 16758446	102	E/M
		Tubulin, ß3			50711	gi 21245098	90	CO&
		Enolase (neuronal)			47495	gi 1363309	87	E/M
		Actin ß			42066	gi 71620	75	CO&
25	2086	Phosphoglycerate mutase (B)	1.16	0.012	28942	gi 8248819	121	CM
26	2253		1.16	0.032				
27	1054	hnRNPK protein	1.17	0.02	51230	gi 38197650	63	T/T
28	2209	RhoA	1.18	0.015	22110	gi 31542143	142	ST
29	2133	Ubiquitin thiolesterase	1.18	0.021	25096	gi 92934	168	PM&
30	1919	Tubulin, ß	1.18	0.04	50377	gi 34876329	72	CO&
31	1995	Glutathione transferase ωa 1	1.18	0.049	27936	gi 12585231	56	E/M
32	2287		1.19	0.017				
33	1865	Dynamin 1	1.21	0.037	96209	gi 18093102	235	CO&
		Malate dehydrogenase 1 NAD			36631	gi 15100179	226	E/M
34	1150	Synapsin IIb	1.22	0.0057	52822	gi 112350	329	ST
		Tyrosyl-tRNA synthetase			63385	gi 34871588	241	PM&
		Mammalian fusca gene			53964	gi 871528	214	NG
35	2431	Peptidylprolyl isomerase A	1.28	0.028	18091	gi 8394009	74	PM&
36	2105		1.29	0.022				
37	2405	Destrin	1.31	0.013	18661	gi 7441446	205	CO&
38	1724		1.39	0.0029				
39	1032	CRMP-2 (Mus musculus)	1.4	0.006	62638	gi 40254595	157	NG
40	1768		1.41	0.011				
41	2156		1.5	0.0023				
42	1824	Malate dehydrogenase 1 NAD	1.5	0.0066	36631	gi 15100179	214	E/M
		Glycerol 3-phosphate dehydrog			38001	gi 2317252	57	E/M

Table 6.4 List of proteins found to be significantly ($p<0.05$) differentially expressed in the somatic region of hippocampal CA1 neurons following long term exposure of Lister hooded rats to an enriched environment compared to somatic CA1 hippocampal regions in non-enriched rats. [(1)] Spot number corresponds to the annotation found in figure 6.1(i). [(2)] Master number is the number assigned by DeCyder BVA software to each individual protein spot. [(3)] Protein i.d. assigned to each individual protein spot following mascot database searching using the Rattus taxonomy [(4)] Average abundance ratio of individual protein spots (+ = increase, - = decrease in expression). Fold changes ranged from 40% decrease in expression (-1.4) to a 50% increase in expression (1.5) [(5)] Students T-test value assigned by DeCyder BVA software, $p<0.05$ determines significance. [(6), (7) & (8)] Mass, NCBI accession number and Mowse score obtained following Mascot PMF or MS/MS ion searching following either Maldi-tof (bold) or Qstar mass spectrometry. [(9)] Proteins biological processes were determined using the Fatigo gene ontology database. Proteins belong to a wide variety of functional classes. Abbreviations: CO&B, cytoplasmic organisation and biogenesis; E/M, energy & metabolism; PM&T, protein modification and transport; ST, signal transduction; NM, nucleotide metabolism; NG, neurogenesis; CM, carbohydrate metabolism; NT, neurotransmitter release and production; CP, cell proliferation ; MC, muscle contraction; T/T, transcription and translation.

Table 6.5 List of extracted dendritic proteins significantly differentially expressed following long term exposure of rodents to an enriched environment.

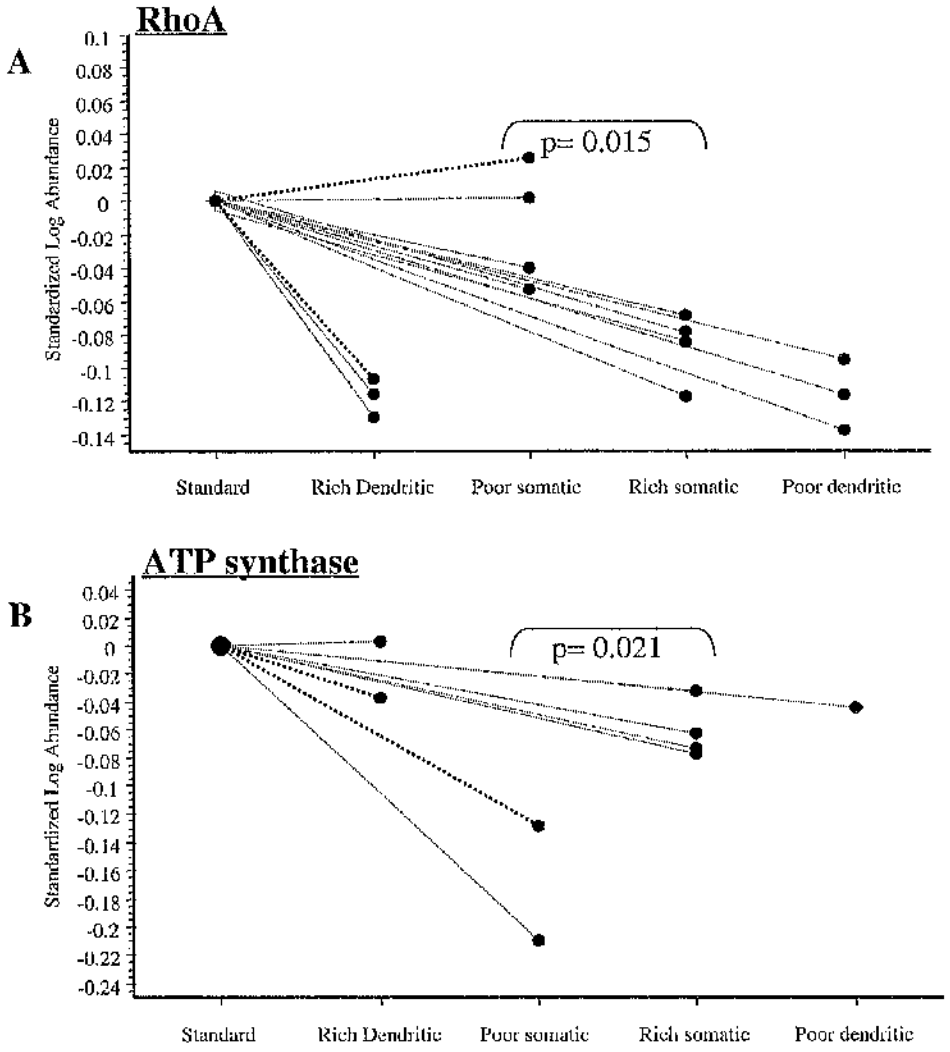
Spot # [1]	Master # [2]	Protein ID [3]	Av. Ratio [4]	Control v's enriched (dendritic) (T-test) [5]	Mass [6]	NCBI Acc# [7]	Mowse Score [8]	Class
1	2030	ATP synthase α (M)	-2.98	0.0058	58904	gi 114523	293	NM
		VAMP-associated protein B						ST
		VAMP-associated protein A			27476	gi 61889097	59	ST
		Glyceraldehyde 3-phosphate-dehydrogenase			36098	gi 56188	320	E/M
2	2080	Phosphoglycerate mutase B	-1.48	0.00083	28942	gi 8248819	98	E/M
		Hsc70-ps1			71112	gi 56385	402	CO&B
		Rho-associated coiled-coil forming kinase 2			160475	gi 6981478	31	ST
3	2075	Pgam1 protein	-1.43	0.025	28928	gi 12805529	97	CM
		Chain F, Enoyl-Coa hydratase complexed with octanoyl-Coa			28555	gi 3212683	145	E/M
4	2078	Pgam1 protein	-1.41	0.0096	28928	gi 12805529	225	CM
		Proteasome 26S non-ATPase subunit 9			24985	gi 18426862	121	PM&T
5	2365	ATP synthase, subunit D (M)	-1.4	0.043	18809	gi 9506415	424	NM
		Enolase (neuronal)			47495	gi 1363309	58	E/M
		Transgelin 3			24981	gi 13928938	48	CO&B
		Ubiquitin-conjugating enzyme			42505	gi 34933268	36	PM&T
6	1579	Pyruvate dehydrogenase E1 α	-1.27	0.014	43853	gi 57657	781	E/M
		Phosphoglycerate kinase 1			44909	gi 40254752	281	E/M
		SEC14-like protein 2			46593	gi 21542226	158	T/T
		Citrate synthase			52176	gi 18543177	104	E/M
		RNA binding protein p42 AUF1			36164	gi 9588098	72	T/T
7	757	Aconitase 2, mitochondrial	-1.26	0.035	86121	gi 40538860	682	CM
		GFAPδ			48809	gi 5030428	63	CO&B
8	1288	Heat shock protein 60 precursor	-1.25	0.024	58061	gi 1334284	87	CO&B
9	1365	Enolase 1	-1.23	0.036	47428	gi 56107	98	E/M
10	1289	Aldehyde dehydrogenase 7, A1	-1.21	0.0097	25313	gi 25090044	49	E/M
11	1967	Actin, ß	-1.21	0.019	42066	gi 71620	260	CO&B
		Tubulin, ß			50095	gi 27465535	39	CO&B
12	1844	Malate dehydrogenase (M)	-1.18	0.027	36117	gi 42476181	112	E/M
13	1858	GFAPδ	-1.15	0.043	48809	gi 5030428	122	CO&B
14	1301		-1.15	0.047				
15	1266		-1.1	0.041				
16	1931	Fumarylacetoacetate hydrolase	-1.08	0.038	40884	gi 34858672	66	E/M
		Creatine kinase (M)			47398	gi 57539	44	E/M
		Glyceraldehyde 3-phosphate-dehydrogenase			36090	gi 37590767	43	E/M
17	1574	GFAPδ	1.08	0.047	48809	gi 5030428	86	CO&B
18	1144	GRP58	1.09	0.021	57044	gi 38382858	174	PM&T
19	1296		1.13	0.019				
20	859	Heat shock 70kD protein 5	1.15	0.016	72473	gi 38303969	78	CO&B
21	1719	GNBP α inhibiting 2	1.17	0.019	41043	gi 13591955	511	ST
		GTP-binding regulatory protein Go α chain, splice form α-2			40568	gi 71911	222	ST
		A326s Mutant Of An Inhibitory α Subunit			36325	gi 3891516	166	ST
		Tubulin, α			50804	gi 34740335	162	CO&B
		Actin, ß			42066	gi 71620	88	CO&B
		GNBP3			40781	gi 27465609	81	ST
		GNBP α 13			44326	gi 61557003	63	ST
22	1588	Actin, ß	1.2	0.03	42066	gi 71620	276	CO&B
		Tubulin, ß5			50095	gi 27465535	153	CO&B
		Tubulin ß chain 15			50361	gi 95030	146	CO&B
		GFAPδ			48809	gi 5030428	127	CO&B
		Hsc70-ps1			71112	gi 56385	68	CO&B
23	1663		1.21	0.044				
24	1793	Lactate dehydrogenase B	1.21	0.047	36874	gi 6981146	79	E/M

25	1908	Septin 5	1.23	0.035	44349	gi 16758814	66	ST
		Crystallin, λ 1			35717	gi 28461157	78	E/M
26	2133	Ubiquitin thiolesterase	1.24	0.032	25096	gi 92934	168	PM&T
27	1637		1.25	0.023				
28	934	ATPase, H+ transporting, V1 subunit A, isoform 1	1.28	0.036	68584	gi 34869154	56	NM
29	2005	Cyclin-dependent kinase 5	1.29	0.0073	33689	gi 18266682	232	NG
		VDAC 3			31178	gi 3786204	69	ST
		Inducible carbonyl reductase			30920	gi 1906812	80	E/M
		B-36 VDAC			32327	gi 299036	68	ST
		Lymphocyte protein tyrosine kinase			58366	gi 34871627	55	ST
		ATP synthase γ			29972	gi 310190	55	NM
		Protein tyrosine kinase			57290	gi 57582	52	ST
30	1526		1.3	0.028				
31	1617		1.31	0.017				
32	1586	Diacylglycerol kinase ζ	1.58	0.006	105426	gi 13592131	56	E/M

Table 6.5 List of proteins found to be significantly ($p<0.05$) differentially expressed in the dendritic region of hippocampal CA1 neurons following long term exposure of Lister hooded rats to an enriched environment compared to dendritic CA1 hippocampal proteins in non-enriched rats. [(1)] Spot number corresponds to the annotation found in figure 6.1(ii). [(2)] Master number is the number assigned by DeCyder BVA software to each individual protein spot. [(3)] Protein i.d. assigned to each individual protein spot following mascot database searching using the Rattus taxonomy [(4)] Average abundance ratio of individual protein spots (+ = increase, - = decrease in expression). Fold changes range from a 198% decrease (-2.98) in expression to a 58% increase in expression (1.58). [(5)] Students T-test value assigned by DeCyder BVA software [(6), (7) & (8)] Mass, NCBI accession number and Mowse score obtained following Mascot PMF or MS/MS ion searching following either Maldi-tof (bold) or Qstar mass spectrometry. [(9)] Proteins biological processes were determined using the Fatigo gene ontology database. Proteins belong to a wide variety of functional classes. Abbreviations: CO&B, cytoplasmic organisation and biogenesis; E/M, energy & metabolism; PM&T, protein modification and transport; ST, signal transduction; NM, nucleotide metabolism; NG, neurogenesis; CM, carbohydrate metabolism; NT, neurotransmitter release and production; CP, cell proliferation; T/T, transcription and translation.

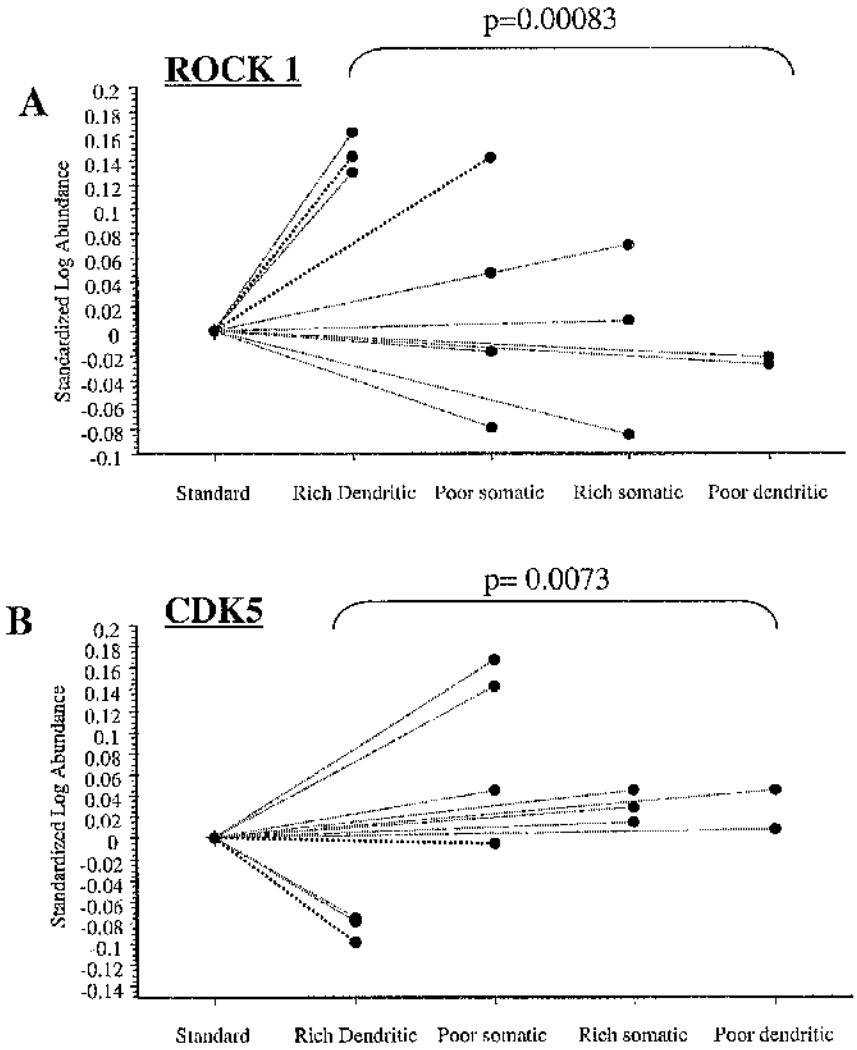
Although the effect of environmental enrichment on behavioural and neuro-anatomical changes associated with the hippocampus is well documented the molecular changes which presumably underlie such events are less well known. In this current investigation several proteins have been identified as differentially expressed following long term exposure of rats to a complex, stimulating environment (Figures 6.2 and 6.3) one of which was the small GTPase RhoA. This molecule was found to significantly ($p=0.015$) increase in expression by 1.18 fold in the hippocampal CA1 dendritic region of rats previously exposed to an enriched environment. In contrast, ATP synthase (mitochondrial type) significantly ($p=0.021$) decreased in expression (-1.28 fold change) (Figure 6.2a, b). A. ROCK1, a Rho-associated serine/ threonine kinase was found to significantly ($p=0.00083$) decrease in expression by -1.48 fold in the somatic region of area CA1 of rats previously exposed to an enriched environment. Cyclin-dependent kinase 5 significantly ($p=0.0073$) increased in expression (1.29 fold change) (Figure 6.3a, b).

Figure 6.2 Regulation of plasticity related proteins in the cell body layer fraction of area CA1 of rat hippocampus after long term exposure to an enriched environment.



A. Graphical representation of the regulation of the small GTPase RhoA following BVA of hippocampal protein extracts following a period of environmental enrichment using DeCyder software. RhoA is found to significantly (p=0.015) increase in expression by 1.18 fold in the somatic region of area CA1 in the hippocampus of rats previously exposed to an enriched environment. B. A similar graphical representation of the regulation of ATP synthase (mitochondrial type) following BVA of hippocampal protein extracts following a period of environmental enrichment using DeCyder software. This molecule significantly (p=0.021) decreases in expression (-1.28 fold change) in the somatic region of area CA1 in the hippocampus of rats previously exposed to an enriched environment. Both have previously been implicated in the plasticity process (Wang *et al.*, 2005; Lim & Isaac, 2005).

Figure 6.3 Regulation of plasticity related proteins in the dendritic layer fraction of area CA1 of rat hippocampus after long term exposure to an enriched environment.



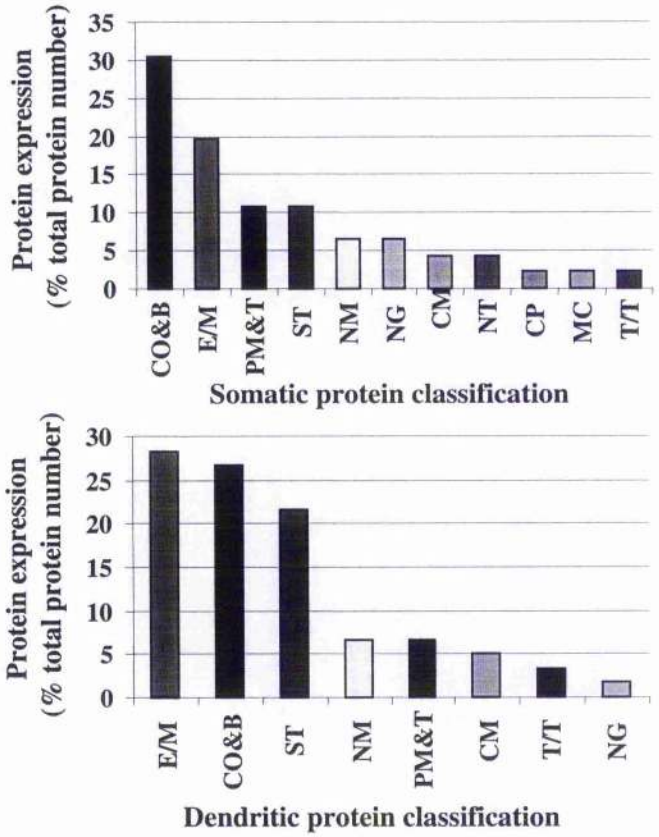
A. Graphical representation of the regulation of ROCK1, a Rho-associated serine/ threonine kinase following BVA of hippocampal protein extracts following a period of environmental enrichment using DeCyder software. ROCK1 is found to significantly (p=0.00083) decrease in expression by -1.48 fold in the dendritic region of area CA1 of rats previously exposed to an enriched environment. B. A similar graphical representation of the regulation of Cyclin-dependent kinase 5 (CDK5) following BVA of hippocampal protein extracts following a period of environmental enrichment using DeCyder software. CDK5 significantly (p=0.0073) increases in expression (1.29 fold change) in the dendritic region of area CA1 of rats previously exposed to an enriched environment. Both have been previously implicated in the plasticity process (O'Kane *et al.*, 2004; Schuman & Murase, 2003).

6.4.2 Functional cluster analysis of proteins identified as differentially expressed following environmental enrichment and DiGE analysis.

As with the previous chapters in this thesis, protein identities from spots previously identified by BVA as displaying altered protein spot abundance were subjected to cluster analysis using the Fatigo data-mining software. As a result of this functional cluster analysis for both somatic and dendritic regions it is apparent that once again, a large proportion of protein molecules identified belong to the biological process of energy / metabolism (19.6% - somatic, 28.3% - dendritic) and cytoplasmic organisation and biogenesis (30.4% - somatic, 26.7% - dendritic). Signal transduction molecules also account for a large percentage of the total protein number (10.9% - somatic, 21.7% - dendritic). Other functional groupings including protein metabolism and transport, nucleotide metabolism, neurogenesis and transcription and translational processes made up the residual 39.1% of somatically located proteins and 23.3% of dendritically located proteins (Figure 6.4a, b).

Figure 6.4 Functional cluster analysis of proteins identified as potentially regulated following exposure of rats to an enriched environment

A



B

Biological process	CO&B	E/M	PM&T	ST	NM	NG	CM	NT	CP	MC	T/T
Somatic											
No. of proteins	14	9	5	5	3	3	2	2	1	1	1
% of total protein no.	30.4	19.6	10.9	10.9	6.5	6.5	4.3	4.3	2.2	2.2	2.2
Dendritic											
No. of proteins	16	17	4	13	4	1	3	0	0	0	2
% of total protein no.	26.7	28.3	6.7	21.7	6.7	1.7	5.0	0.0	0.0	0.0	3.3

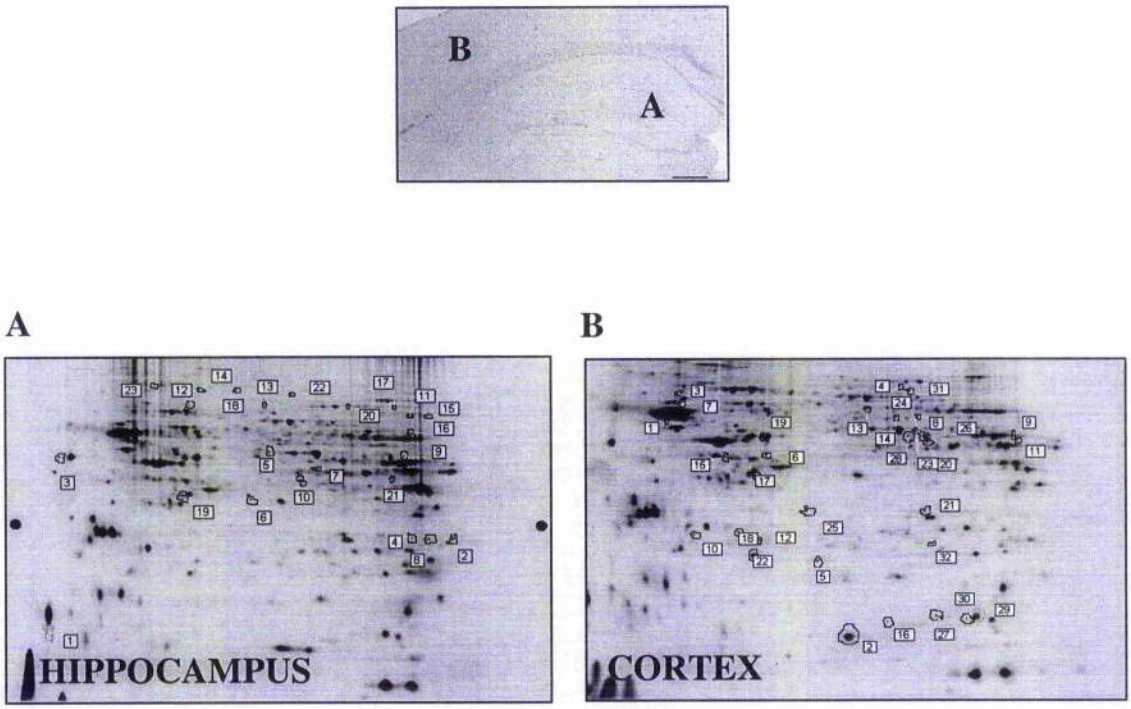
A. Bar charts illustrating the functional groupings of proteins identified as significantly differentially expressed in the soma and dendrites of rats exposed to an enriched environment. B. Table summarising the actual numbers and relative percentages of proteins which are contained within each biological process. Abbreviations: CO&B, cytoplasmic organisation and biogenesis; E/M, energy & metabolism; PM&T, protein modification and transport; ST, signal transduction; NM, nucleotide metabolism; NG, neurogenesis; CM, carbohydrate metabolism; NT, neurotransmitter release and production; CP, cell proliferation ; MC, muscle contraction; T/T, transcription and translation.

6.4.3 Differential protein expression associated with APP over-expression in a transgenic mouse model of Alzheimer's disease, the TAS10 model.

The previous section detailed the investigation of differential protein expression in an animal model representative of increased cognitive function. In contrast, the next step was to investigate differential protein expression in an animal model representative of cognitive decline. One of the most common conditions demonstrating such a deficit is Alzheimer's disease. Therefore, using a model illustrative of a rapid onset of AD we used brain tissue from the TAS10 transgenic mouse model of AD to investigate the possible molecular events involved in the associated pathology. Previous studies by Jill Richardson and colleagues at GlaxoSmithKline pharmaceuticals, from where these tissue samples were obtained, provide a detailed description of the neuro-anatomical, electrophysiological and behavioural characteristics of these mice (Richardson *et al.*, 2003; Brown *et al.*, 2003).

Using the hippocampus and cortex from the animals used in the electrophysiological studies carried out by Jon Brown and colleagues (Brown *et al.*, 2003), the molecular components possibly involved in the above neuroanatomical, behavioural and physiological observations were investigated. Protein extracts from the cortex and hippocampus of WT and Tg (TAS10) mice (12-13 months old) were labelled with CyDyes and compared using DiGE in two separate experiments. In the first DiGE experiment a total of 23 protein spots were identified as significantly ($p<0.05$) differentially regulated in the hippocampus of TG mice compared to WT controls (Figure 6.5a, Table 6.6). The second DiGE study comparing the cortex of TG mice to WT controls identified 31 significantly ($p<0.05$) different protein spots (Figure 6.5b, Table 6.7).

Figure 6.5 Annotated 2D gel maps of protein samples from the hippocampus and cortex of TAS10 transgenic mice



DiGE analysis of protein extracts comparing the hippocampus (A) and cortex (B) proteomes from Tg TAS10 and WT littermate control mice. Of the 2347 protein spots detected in the hippocampal proteome 23 demonstrated differential expression, whereas 32 out of 2423 were identified as differentially expressed in the cortex of TAS10 Tg mice compared to WT littermate controls.

Table 6.6 List of differentially expressed proteins in the hippocampus of TAS10 transgenic mice compared to littermate WT controls.

Spot #	Master #	Av. Ratio	T-test value (WT vs. Tg)
1	2237	-1.87	0.027
2	2098	-1.46	0.023
3	1705	-1.43	0.019
4	2083	-1.29	0.012
5	1981	-1.22	0.049
6	1655	-1.22	0.0089
7	1782	-1.19	0.00059
8	2093	-1.15	0.0058
9	1673	-1.13	0.035
10	1847	-1.1	0.034
11	1216	1.06	0.0041
12	1026	1.07	0.035
13	1019	1.08	0.0079
14	1380	1.09	0.047
15	1227	1.09	0.032
16	796	1.09	0.03
17	1088	1.1	0.025
18	806	1.12	0.041
19	1963	1.12	0.037
20	1065	1.14	0.049
21	1848	1.16	0.01
22	894	1.17	0.022
23	682	1.18	0.038

Table listing the 23 protein spots identified by DIGE analysis as differentially expressed in the hippocampus of TAS10 transgenic mice compared to WT littermate controls. (1) Spot number corresponds to the annotation on the gel image in figure 6.7a. (2) Master number is the number assigned to each spot by DeCyder BVA software. (3) Average ratios ranged from an 87% decrease in expression (-1.87) to an 18% increase in expression (1.18) and were identified as significant differences if p values were less than 0.05.

Table 6.7 List of differentially expressed proteins in the somatosensory cortex of TAS10 transgenic mice compared to littermate WT controls.

Spot # (1)	Master # (2)	Fold change (3)	T-test value (WT vs. Tg)
1	869	-1.23	0.0091
2	2255	-1.23	0.021
3	553	-1.21	0.042
4	454	-1.18	0.038
5	2039	-1.18	0.016
6	1303	-1.17	0.047
7	667	-1.14	0.036
8	881	-1.13	0.026
9	1077	-1.09	0.035
10	1879	-1.07	0.026
11	1139	1.09	0.04
12	1929	1.1	0.025
13	762	1.11	0.012
14	894	1.11	0.034
15	1325	1.13	0.03
16	2220	1.14	0.035
17	1481	1.17	0.034
18	1869	1.17	0.041
19	1120	1.18	0.049
20	1176	1.18	0.035
21	1727	1.2	0.016
22	2015	1.21	0.045
23	1056	1.22	0.037
24	1112	1.22	0.0064
25	1731	1.24	0.046
26	1123	1.26	0.017
27	2202	1.3	0.046
28	1111	1.32	0.0022
29	2205	1.35	0.03
30	2211	1.36	0.039
31	543	1.6	0.048
32	1948	2.15	0.01

Table listing the 32 protein spots identified by DIGE analysis as differentially expressed in the hippocampus of TAS10 transgenic mice compared to WT littermate controls. (1) Spot number corresponds to the annotation on the gel image in figure 6.7b. (2) Master number is the number assigned to each spot by DeCyder BVA software. (3) Fold changes ranged from a 23% decrease in expression (-1.23) to a 115% increase in expression (2.15) and were identified as significant differences if p values were less than 0.05.

6.5 DISCUSSION

In previous chapters this thesis has described protein regulation following pharmacological activation of glutamate receptors (Chapter 4) and in relation to the induction of synaptic plasticity at glutamatergic synapses (Chapter 5). The focus of the current chapter concentrates on protein regulation *in vivo* in two animal models representative of two extremes of cognitive function. Differential protein spot abundance was apparent in both plasticity-related models, the first a model of enhanced cognition – environmental enrichment, the second a model representative of cognitive decline – TAS10 transgenic mouse model of AD.

In our enrichment studies rats had made available to them toys, various hiding places and exercise wheels, all of which were not only conducive to increased exploration but also to increased voluntary exercise. Following 6 weeks of exposure to novel and complex environments hippocampal protein extracts (from somatic and dendritic regions) were compared to that of non-enriched animals. A total of 74 protein spots (42 somatic, 32 dendritic) were highlighted by DiGE analysis as differentially expressed.

Interestingly, whilst there appears to be a larger number of protein spots regulated in the somatic region of CA1 pyramidal cells (42 protein spots compared to 32 in the dendritic region of CA1 pyramidal cells) there is also an apparent increase in the resolution of these spots. Out of the 42 somatic spots highlighted as significantly different, 46 individual proteins were identified. In contrast, from the 32 protein spots demonstrating significant regulation in the dendritic region of area CA1, nearly double the number of individual protein identities were obtained (60), indicating that dendritically isolated tissue appears to be less well resolved following 2D gel electrophoresis. Moreover and perhaps not surprisingly, a more diverse range of functional classes exists in the somatic region of CA1 pyramidal cells (11 classes) compared to the dendritic region (8 classes). This was touched upon in chapter 3 of this thesis. Analysis of differential protein expression between somatic and dendritic regions of naïve hippocampal CA1 regions highlighted a number of differences between the two regions (42 at $p<0.01$) possibly due to the localisation of different organelles in each sub-region.

Once again two biological processes, energy & metabolism and cytoplasmic organisation & biogenesis represent the majority of the identified protein molecules (>50% representation for each region) which were regulated following environmental enrichment. This is consistent with previous findings investigating pharmacological and synaptic stimulation of glutamate receptor induced protein regulation (chapters 4 & 5) of an increased energy requirement and substantial remodelling of neural circuitry. It is also consistent with the documented findings of other research groups that have detailed enhanced dendritic growth (Leggio *et al.*, 2005), increased spine density (Turner *et al.*, 2003) and increased cell volume and dendritic length (Faherty *et al.*, 2003). However, the overall distribution of other functional classes appears somewhat different. For example, signal transduction molecules account for over 10% of the identified somatic proteins modulated by exposure to an enriched environment and remarkably, over 20% of the identified dendritic modulated proteins belong to signal transduction pathways. This is the third highest representation in this subfield suggesting a large increase in signalling taking place in the dendritic fields of CA1 hippocampal neurons in animals exposed to a complex and stimulating environment. The identified signal transduction molecules mainly represent previously identified plasticity-related molecules such as calcineurin (Lu *et al.*, 1996; Xia & Storm, 2005), GTP binding regulatory protein (Baker *et al.*, 1999), several isoforms of guanine nucleotide binding protein (Katsuki *et al.*, 1992; Satoh & Kaneko, 1994), VAMP associated protein (Pozzo-Miller *et al.*, 1999), septin 5 (Xue *et al.*, 2004), ROCK1 (O'Kane *et al.*, 2004; Niisato *et al.*, 2005) and protein tyrosine kinases (O'Dell *et al.*, 1991; Ho *et al.*, 2004). One particularly interesting signalling molecule identified in this study is the small GTPase, RhoA which was found to increase in expression in the cell body layer fraction of area CA1 in enriched rodent hippocampi. RhoA belongs to the Rho subfamily of Rho, Rac and Cdc42. These proteins are molecular switches in various signalling pathways. The expression of Rho GTPases in adult rat brain was carried out by Olenik and colleagues in 1997. They discovered large amounts of RhoA, RhoB, Rac1 and Cdc42 mRNA in neurons of the hippocampus - pyramidal cells of the CA1-CA4 regions as well as in granule cells of the dentate gyrus and in hilar cells. It is also thought that RhoA is involved in the maintenance of dendritic structures in rat hippocampal slice cultures (Nakayama *et al.*, 2000). RhoA was seen to induce a drastic simplification of dendritic branch patterns which was found to be dependent on the activity of a

downstream kinase ROCK – another molecule identified in our study this time to significantly decrease in expression in the dendrites of CA1 pyramidal cells. Several studies carried out in the laboratory of Professor Brian Morris have investigated the role of Rho GTPases in synaptic transmission and long term potentiation. The first such study reported the strong localisation of RhoA, RhoB, RhoG, Cdc42, and Rac1 in hippocampal tissue of adult rat brain (O'Kane *et al.*, 2003a). This was followed by the report that both RhoA and interestingly, another GTPase RhoB were activated in the CA1 region of the hippocampus by low frequency stimulation. Moreover, LTP induction was accompanied by an increase in RhoB activation only and not RhoA (O'Kane *et al.*, 2003b). As previously mentioned they also identified a role for Rho kinases in increasing the magnitude of LTP. A more recent study by Wang and colleagues suggests that Rho GTPases, including RhoA play a central role in potentiation of the synaptic response by increasing the concentration of synaptic proteins (Wang *et al.*, 2005).

Given the substantial amount of evidence regarding the relationship between environmental enrichment and neurogenesis (van Praag *et al.*, 1999; Kempermann *et al.*, 2002; Bruel-Jungerman *et al.*, 2005) it is perhaps surprising that molecules which are markers for the formation of new neurones such as the CRMP family of proteins commonly found in the previous chapters are somewhat limited in this study. It may be that such morphological enhancement may well have taken place early on in the study and hence markers for new neurones are no longer present. This may also explain the increase in other classes of molecules – an increase in neuronal number would almost certainly result in increased signalling, metabolism and cytoskeletal and associated products.

Several plasticity-related protein molecules were identified in this study. Namely isoforms of PSD-95 and several of the previously discussed signalling molecules such as tyrosine kinases and the protein phosphatase calcineurin. In fact, an increase in hippocampal, cortical and subcortical PSD-95 levels has already been demonstrated by Nithianantharajah and colleagues in 2004 (Nithianantharajah *et al.*, 2004). Hence, it is suggestive that our current enrichment studies as carried out here are indeed representative of a plasticity related process. Given that environmental enrichment has been shown to lead to an increase in learning capabilities and long

term memory function (Gardner *et al.*, 1975; Duffy *et al.*, 2001; Leggio *et al.*, 2005) it is not surprising that many of these protein molecules have also been identified as regulated by or even a requirement for the acquirement and retention of memories (PSD-95, Migaud *et al.*, 1998; Tyrosine kinases, Grant *et al.*, 1992; calcineurin, Mansuy *et al.*, 1998).

The global approach to tissue sampling again adopted here allowed the incorporation of numerous cell types and was not biased towards either pre- or post- synaptic regions as is evident from the discovery of glial fibrillary acidic protein and synapsin thought to be a marker for presynaptic terminals.

Another interesting finding was the discovery of ATP synthase protein molecules in several spots on the gel given that this molecule has been identified as being differentially expressed in all of the proteomic investigations of this thesis. A recent publication by John Isaac's group identifies a requirement for ATP hydrolysis in synaptic transmission and plasticity. By applying the non-hydrolysable ATP analogue adenosine 5'-[beta, gamma-methylene] triphosphate (AMP-PCP) into hippocampal CA1 pyramidal cells in hippocampal slices they showed an increase in basal AMPA receptor (AMPAR)-mediated transmission. In addition, infusion of AMP-PCP blocked the induction of both LTD and LTP. Therefore, the regulation of ATP synthase molecules following environmental enrichment may not purely be a requirement for increased energy metabolism, but is also a definitive requirement for synaptic plasticity related events (Lim & Isaac, 2005).

Another interesting finding is that of the mammalian fusca gene protein. The only information on this molecule was published in 1998 by Nitz and colleagues. Interestingly this protein was found to be highly expressed in kidney, pineal and retina, and particularly strong expression was found in the testes of rats, but no CNS localisation was described. The discovery that this protein undergoes regulation following a period of environmental enrichment not only shows, for the first time, a brain localisation for this protein but, in addition, demonstrates a novel role for this protein in the plasticity process.

Given the increased opportunity of the rats to increase their exercise levels over the six week trial period, it is possible that the observed changes in protein expression did not occur purely as a result of exposure to a complex environment but also as a result of increased exercise. Indeed, exercise has been shown to promote BDNF and NGF mRNA expression in the hippocampus of rats previously given *ad libitum* access to running wheels (Neeper *et al.*, 1996; Oliff *et al.*, 1998). In addition, such voluntary running elicits sustained activation of CREB and MAPK, two plasticity-related molecules, in the rat hippocampus (Shen *et al.*, 2001). The same group carried out an in depth investigation into gene expression levels following exercise (Tong *et al.*, 2001). Some of the identified regulated genes are consistent with our findings in this proteomics study, confirming the role of exercise in this enrichment study (for example tropomyosin, calcineurin and the CRMP family of proteins).

Environmental enrichment not only promotes increases in cognitive function but in addition alleviates cognitive deficits in mouse models of Alzheimer's disease (Arendash *et al.*, 2004) possibly by reducing Abeta levels and amyloid deposition (Lazarov *et al.*, 2005) or reducing amyloid plaque formation (Jankowsky *et al.*, 2003). As a disease most commonly associated with severe cognitive decline and an important therapeutic target for pharmaceutical companies throughout the world, the search for molecules involved in such a devastating dementia related disorder is an important and necessary task. In this chapter we investigated protein expression in a transgenic mouse model of Alzheimer's disease known as the TAS10 model which harbours genetic mutations to the APP protein (K670N and M671L) located close to the beta-secretase cleavage site which are thought to cause increased cleavage by BACE1 resulting in an over-expression of Abeta (Citron *et al.*, 1992). This so-called 'Swedish' mutation occurs in a particular family in Sweden that suffers from early onset AD (Mullan *et al.*, 1992). Several studies have adopted this proteomics style approach to identify the effects of amyloid deposition and the related protein molecules associated with the observed severe cognitive dysfunction (Tsuji *et al.*, 2002; Castegna *et al.*, 2002a, b, 2003). The study reported in this chapter is somewhat limited in its information. Although DiGE analysis was successful in pinpointing the number of regulated protein spots in the hippocampus and somatosensory cortex of TG mice, subsequent mass spectrometric analysis was unable to identify these protein spots due to problems associated with the spot

handling workstation. Nevertheless, the information obtained illustrates an increased number of protein spots differentially expressed in the cortex of these mice compared to the hippocampus. This is in keeping with the findings by Richardson and colleagues who documented that the neuropathology was increased in the cortex when compared to the hippocampus of these mice (Richardson *et al.*, 2003). It is also clear that although such mouse models are associated with specific changes in protein levels such as amyloid precursor protein and beta secretase, there are a number of other changes in protein expression occurring at the same time.

In summary, two animal models, one representative of cognitive enhancement, the other of cognitive decline were used to investigate associated protein regulation. Environmental enrichment led to the regulation of several protein molecules in both the dendritic and somatic regions of area CA1 in the rat hippocampus some of which appear to be modulators of or indeed modulated by such plasticity related events associated with exposure of animals to a complex environment. The proteomics based approach used here was limited in terms of discovering the identity of specific proteins associated with the pathology of Abeta over-expression, but it indicates that several proteins over and above the targeted mutation are in fact regulated in AD throughout the hippocampus and associated structures.

Chapter 7

General discussion

The main aim of this thesis was to investigate protein regulation involved in or responsible for synaptic plasticity-related processes. It was hoped that such an investigation would provide an abundance of information as to the nature and scale of protein modification involved in synapse specific plasticity events which are ultimately thought to underlie adaptive cognitive processing and memory formation in the mammalian brain. It was originally envisaged that the concomitant detection of several thousand protein spots on a gel would enable the unbiased demonstration of differential changes in expressional patterns in relation to the activation of excitatory neurotransmission and activity-dependent induced changes in excitatory neurotransmission. This chapter will initially highlight the major findings of such an investigation including novel and significant discoveries. It will also emphasise the importance of these findings in relation to neuroscience research and in particular the area of learning and memory. Furthermore, I will also draw attention to some of the more noteworthy discoveries not covered in previous chapters. I will discuss various technical considerations of the present work and in particular the advantages and limitations of the methodology used and possible solutions to overcome these limitations. Finally, I will discuss the direction that future studies may take as a result of this investigation.

7.1 Major findings

The major findings reported this thesis were as follows:

- Pharmacological stimulation of glutamatergic afferents in the CA1 region of mouse hippocampus results in the differential expression of around 3% of the total hippocampal protein spots that can be resolved on a 2D gel.

- The NMDA receptor is central to the regulation of around 75% of spots showing differential abundance following glutamate receptor activation.

- Energy metabolism and cytoplasmic organisation and biogenesis-related proteins are amongst the highest represented biological processes occurring following glutamate receptor stimulation indicative of substantial energy

demands and morphological alterations in hippocampal pyramidal cells following activation of glutamate receptors.

- Pharmacological activation of glutamate receptors in isolated dendritic fractions of area CA1 results in the modulation of 0.2% of the rat dendritic layer proteome.

- High frequency stimulation of glutamatergic afferents in area CA1 of mouse hippocampus results in a similar percentage of protein regulation at both early (2.8%) and late (2%) phases of long-term potentiation.

- In the early phase LTP proportionately more protein spots appear to be differentially regulated that at more enduring time points following LTP induction.

- Potentiation of Schaffer collateral • CA1 synapses associated with particularly pronounced differential expression of proteins associated with energy metabolism followed by a delayed altered expression of cytoskeletal associated/ structural proteins as the plasticity process matures.

- Exposure of rats to an enriched environment results in the differential expression of approximately 3% of the total resolved hippocampal proteome. Around half of these proteins are found within the somatic region of area CA1 and half within the dendritic region.

- Whilst energy metabolism and cytoplasmic organisation and biogenesis groupings represent those largest groups of identified regulated proteins, signal transduction molecules also represented a large proportion (21.7%) of the differentially expressed proteins identified in the dendritic layer of stratum radiatum.

- In the TAS10 transgenic mouse model of AD both the hippocampus and cortex of these animals, when compared to their littermate WT controls, contain a number of regulated proteins as a result of the genetic mutation.

- A diverse range of protein classes was identified in all studies. Energy metabolism and cytoplasmic organisation processes were prominent in all investigations. Also identified were proteins involved in neurogenesis, protein modification and transport, signal transduction and neurotransmitter release.

7.2 Novel findings

The development of this technique and its implementation as described in previous chapters has, to my knowledge, not as yet been adopted by any other researchers as a global and unbiased experimental approach with which to identify protein regulation associated with synapse specific forms of plasticity.

From the many hundreds of protein molecules identified in all of the individual studies described in this thesis, a number of novel and important findings have been identified. These include the identification of individual proteins that have, to date, no previous link to plasticity-related processes such as LTP (e.g. CRMP family of proteins, gamma catenin). Indeed, some proteins showing altered abundance have not even, as yet, been found within the hippocampus

For example, the role of actin in plasticity related processes such as LTP is well documented (Fukazawa *et al.*, 2003; Kim & Lisman, 1999). The identity of several forms of actin as well as numerous actin-binding proteins (cofilin, spectrin, LASP-1) throughout this investigation provides an insight into actin regulation following glutamate receptor stimulation. For example, the role of actin in plasticity related processes such as glutamate receptor activation and LTP could be one of many, hence the large number of actin-associated proteins identified. It is possible that actin serves to aid in the development and maintenance of new neurones, as was suggested by Zhang and Benson in 2001. There is also a definitive role for actin in the anchoring of molecules and receptors in or near the postsynaptic membrane. Allison

et al reported that CaMKII was completely dependent on an intact cytoskeleton to remain localised to the PSD (Allison *et al.*, 2000). Indeed, the PSD has been shown to contain both actin and spectrin (Walsh and Kuruc, 1992). A previous study by Allison and colleagues in 1998 demonstrated that the depolymerization of F-actin led to a 40% decrease in both the number of synaptic NMDA receptor clusters and the number of AMPA receptor (GluR1)-labelled spines. Moreover they showed that the actin binding protein alpha-Actinin-2 dissociated from the receptor clusters following actin depolymerization. This echoes a study by Wyszynski *et al* in 1998 who showed that the actin binding protein alpha-actinin-2 binds directly to the NMDA receptor subunits NR1 and NR2B, suggesting that alpha-actinin-2 may function to attach NMDA receptors to the actin cytoskeleton. Another possible function of actin and its associated binding proteins could be to define and change the shape and/ or number of postsynaptic spines and dendrites. This was suggested by Fukazawa and colleagues in 2003 who demonstrated a large increase in synapse diameter in spines which were shown to contain F-actin. Although actin depolymerization was not shown to affect the shape of dendrites and spines in a similar study by Kim and Lisman in 1999.

Another possible role for actin and its associated binding proteins could be to modify the number of synapses on existing spines and the shape of existing spines. For example, Popov and colleagues in 2004 found an increase in the percentage of synapses on thin dendritic spines, a decrease in synapses on both stubby spines and dendritic shafts, but no change in the proportion of synapses on mushroom spines. Additional analysis on thin and mushroom shaped spines following LTP induction revealed a significant increase in their volume and area with shaft and stubby spines transforming to thin dendritic spines, and mushroom spines changing only in shape and volume (Popov *et al.*, 2004). This follows an earlier developmental study on spine size and shape by Harris and colleagues (1992). They show a near doubling in the total density of synapses between postnatal day 15 (PND 15) and adult ages. Thin and mushroom shaped spines increase by four-fold in density between PND 15 and adult ages. In contrast, stubby spines decrease by more than half. It has been suggested that changes in spine shape and density are in fact transient and serve to act as a marker for synaptic activation (Lang *et al.*, 2004).

These changes in spine shape could in fact result from or, indeed, result in spine neck constriction. In 1990, Holmes postulated that spine neck constriction was in fact a model by which activated synapses could concentrate Ca^{2+} locally. In doing so it is possible this serves as a synaptic tag, and also prevents the activation of adjacent, unstimulated synapses. Sabatini and colleagues later demonstrated this in 2002. Indeed, LIM kinase 1, an actin binding protein which regulates actin binding through inhibition of ADF/ cofilins has been reported to be responsible for a reduction in the size of spine necks (Meng *et al.*, 2002). Indeed, in this current study cofilin and LASP-1, a LIM and SH3 actin binding protein, have both been identified as differentially expressed following glutamate receptor activation. Interestingly these molecules may in fact be acting to regulate spine neck thickness and localise the postsynaptic signal.

One other possibility is that the structural alterations suggested by the large number of cytoplasmic organisation and biogenesis proteins is in fact occurring in the dendritic shafts of the neurones as mRNAs or other molecules are transported from the cell body to the stimulated synapses. However, it is thought that it is in fact microtubules function to regulate axonal and dendritic structure (Kaech *et al.*, 2001). Interestingly, several microtubules, including several isoforms of tubulin and some microtubule associated protein kinases were identified following both pharmacological and synaptic activation of glutamate receptors. This suggests that as well as structural alterations at the synaptic level, there are also alterations along the somatodendritic axis.

Although the discovery of actin and several microfilament associated proteins is in some ways a validation of the technique being used here, it is also extremely suggestive of a large degree of actin dynamics occurring downstream of glutamate receptor activation. These actin dynamics associated with plasticity at glutamatergic synapses may act to anchor various molecules and receptors at the postsynaptic membrane, to modify the shape and number of dendritic spines or to transport or localise Ca^{2+} or mRNA to activated synapses.

One of the most exciting proteins to come out of the first study in this thesis, which focussed on glutamate receptor activation, is the protein DJ-1 also known as PARK7.

A relatively novel protein (discovered less than 10 years ago by Nagakubo *et al.*, 1997), mutations in the DJ-1 gene have been associated with autosomal recessive early-onset Parkinsonism (Bonifati *et al.*, 2003). It is also associated with male fertilization (Wagenfeld *et al.*, 1998). However, its localisation within the hippocampus was only discovered last year (Shang *et al.*, 2004), and no connection of this protein to plasticity in the mammalian brain has yet been ascertained. It may function as a molecular chaperone (Shendelman *et al.*, 2004) or to activate transcription of RNA (Xu *et al.*, 2005). Investigations of this protein are in their infancy and its identification here in relation to synaptic plasticity is an exciting novel discovery that merits further mechanistic and biochemical investigation.

Another interesting protein shown by this work to be regulated by pharmacological stimulation of glutamate receptors is Farsla protein. The literature on this protein is scant but suggests this molecule to be a phenylalanine-tRNA synthetase-like protein. There is no previous link for this protein to the hippocampus or even the CNS making its identification an intriguing finding. As previously mentioned in chapter 4 it is possible that this protein is acting to produce a degree of cellular differentiation (Zhou *et al.*, 1999) although further studies would certainly be required to confirm such a possibility as it is unknown if such a process could in fact occur in mature CNS tissue.

Other novel findings include the discovery of the increased expression of protein spots representing hnRNPK that is thought to be involved in the translation of mRNAs (Evans *et al.*, 2003). Given the known requirement for local mRNA translation possibly in the early stages of LTP (Tsokas *et al.*, 2005) and given that increases in hnRNPK were identified 10 minutes after LTP induction, it is entirely possible that this protein molecule serves to locally transcribe mRNA at synapses, as has been suggested in several studies. For example, Tsokas and colleagues that the induction of protein synthesis-dependent hippocampal LTP increases the expression of elongation factor 1A (eEF1A). They also show that the mRNA for eEF1A is present in pyramidal cell dendrites and that the LTP-associated increase in eEF1A expression was intact in dendrites that had been severed from their cell bodies before stimulation. eEF1A levels increased within 5 min after stimulation in a translation-dependent manner, and this effect remained stable for 3 h. Local mRNA translation

has also been demonstrated by Miller and colleagues in 2002. Using a mutant mouse in which the protein-coding region of CaMKIIalpha is intact, but mRNA is restricted to the soma a reduction in late-phase long-term potentiation (LTP), and impairments in spatial memory, associative fear conditioning, and object recognition memory. These results demonstrate that local translation is important for synaptic delivery of the kinase and that local translation contributes to synaptic and behavioural plasticity. Local protein synthesis has also been demonstrated by Cracco and colleagues (2005) and by Bradshaw *et al* in 2003. Cracco *et al* showed that isolated apical dendrites in area CA1 of pyramidal cells were unable to convert early phase LTP into a more enduring form. Bradshaw however, utilised local application of a protein synthesis inhibitor to demonstrate the same finding. Not only does this suggest that late phases of LTP require somatically driven protein synthesis, it also highlights the fact that the early phases of LTP are dependent on local mRNA translation. Hence, the novel discovery of the regulation of hnRNPK in the early time points following LTP induction is extremely significant.

Finally, the large representation of the CRMP family of proteins following both pharmacological and synaptic activation of glutamate receptors is noteworthy. Not only because of their novelty in terms of synaptic plasticity, which in itself is an important discovery, but also in terms of the relationship to the enrichment studies. Following environmental enrichment there was a relatively small percentage (6.5% in somatic regions, 1.7% in dendritic regions) of differentially expressed proteins which belonged to the process of neurogenesis. Environmental enrichment is widely associated with an increase in neurogenesis (van Praag *et al.*, 1999; Bruel-Jungermann *et al.*, 2005). Certain members of the CRMP family (more specifically CRMP-1, -3 and -4) may be considered markers for new cells when identified in adult neurones (Parent *et al.*, 1997). Therefore in this current investigation there appears to be a large degree of neurogenesis following both pharmacological and synaptic activation of glutamate receptors but interestingly, where such markers would be expected, i.e. in the EE study, none were found. Therefore, it would be of great interest to identify the exact role of this family of proteins. Are they indeed markers for the generation of new neuronal cells or do they have another function?

An additional surprising and extremely informative finding from this investigation was the results from the cluster analysis of the identified proteins. This analysis was carried out to investigate protein-class dependent alterations in expression following the pharmacological activation of glutamate receptors, the induction of LTP and also following the environmental enrichment studies. The trend that emerges from the analysis of each study is very interesting. If you take the processes that have the highest representation throughout all the studies (for example energy metabolism and cytoplasmic organisation and biogenesis) and compare across all investigations a clear pattern emerges. Glutamate receptor stimulation occurs constantly throughout the experimental period. LTP induction stimulus occurs only at the very beginning of the experimental period whilst environmental enrichment occurs constantly throughout the experimental period. Energy and metabolism processes are at their highest representation following glutamate receptor stimulation, shortly after the LTP induction protocol and following environmental enrichment. Cytoplasmic organisation and biogenesis however is at its highest representation following pharmacological glutamate receptor stimulation, at later stages of the plasticity process following LTP and also following environmental enrichment. This indicates that energy metabolism occurs immediately or shortly after the stimulus and changes in the cellular organisation occurs after a more prolonged period of time.

Cluster analysis also highlighted that a large amount of signalling molecules were identified as differentially expressed following environmental enrichment. Given that these molecules are relatively low in abundance and that in the other studies the representation of this protein class was considerably lower suggests a very prominent role for signal transduction in the CA1 pyramidal cells of the enriched hippocampi. Further mechanistic and biochemical studies possibly focussing on phosphorylation of proteins are required to confirm this finding and find out why such increases in signalling occur and the functional outcome of this increased signal transduction.

7.3 Significance of investigation

The importance of this investigation takes two forms. Firstly, the development of a combined proteomic and brain slice pharmacology/ electrophysiology approach paves the way for future investigation focussing on more detailed pharmacology/

temporal considerations and specific biochemical pathways. A substantial amount of time was spent developing this experimental approach and optimising it to obtain reliable and meaningful data. Hopefully, this development will be of great benefit to others who wish to adopt this technique for their own research. Indeed, the fast emerging new technology in the proteomics field will only serve to further compliment what is reported here.

This investigation also highlights the advantages of adopting new disciplines and approaches so as to provide new angles and avenues of research within a relatively saturated field of investigation. These new angles almost certainly complement what has already been discovered as well as adding to previous studies. Indeed, the current investigations described in this thesis hopefully provide researchers with a substantial platform with which further investigations may build upon.

Not only does this study have the advantage of reporting a large number of potential modulators of synaptic plasticity related events, but it also illustrates the simultaneous regulation of a large number of individual proteins and individual biological processes as was illustrated by the cluster analysis. Perhaps the latter information is the most important as it provides a greater indication of the global molecular events occurring in for example, excitatory neurotransmission and LTP. The importance of such findings cannot be stressed enough as in any biological event one protein molecule will not act alone but in concert with others. It is this coincident protein regulation which is the next step in plasticity-related research.

7.4 Technical considerations

7.4.1 Implementation of 2D gel electrophoresis

In order to carry out such a study it was first necessary to develop and subsequently employ a global approach to identify those molecules central to activity-dependent changes in neural circuitry occurring in the mammalian brain. A global approach would allow the study of numerous protein molecules simultaneously. Large-scale protein analysis of this kind is termed proteomics. The method of choice for initial separation is based upon recent technological advances in automated 2-dimension

(2D) gel electrophoresis a process by which proteins are separated initially according to their charge and secondly by their molecular weight. It has the distinct advantage over 1 dimensional (1D) analysis in that there is increased resolution as gel bands visualised on a 1D gel are often found to contain several proteins of similar molecular weights. 2D gels provide one further degree of separation and therefore increased resolution. Other possible approaches include western blot analysis, *in situ* hybridisation and microarray studies. The first two are largely dependent on a single target protein molecule and are limiting in the number of molecules that can be investigated at the one time and the availability of suitable antibodies. Moreover, you can only see what you are looking for. Microarray studies are similar and in fact excel in their globalised approach but are limited by the lack of functional information they provide – mRNA expression does not always correspond with the expression of a functional, unmodified protein molecule. Hence, 2D-gel electrophoresis was adopted as an experimental approach for the investigation of proteome wide changes in protein expression resulting from synaptic plasticity in the rodent brain.

Experiments initially required the modification of standard 2D gel electrophoresis conditions for optimised use with hippocampal brain slices. The main problem faced here was the lack of tissue available for protein extraction. Many of the planned experiments entailed the use of CA1 subfields of 400 μm thick hippocampal slices, which provided very small amounts of tissue for analysis. Not only is the amount of total protein important for visualisation of as many individual protein in the extract as was possible, it was also crucial for subsequent mass spectrometric identification. To obtain identification of even the more abundant proteins in the hippocampal proteome around 300-500μg of total protein was required to be loaded onto a single gel. For mass spectrometric identification using preparative gels practically it was not possible to use slices which had undergone for example, enduring forms of long term potentiation for this purpose due to the time consuming nature of such experiments. It was therefore necessary to use either whole naïve hippocampus or many (up to 10) naïve hippocampal slices. Using naïve, untreated tissue did not pose a problem as the changes observed in this investigation occurred in pre-existing protein molecules. There were no new protein spots appearing or existing protein spots disappearing following any of the experimental paradigms investigated.

However, if this were not the case then it would be necessary to obtain very large amounts of protein extracts from experimental tissue. One possible solution to address this problem is to progress to the use of whole animal models. This would not only provide more experimental tissue but would circumvent any possible differential protein modifications as a result of slice preparation i.e. a more physiological preparation.

7.4.2 Development of DiGE

Standard 2D gel electrophoresis is notably fraught with limitations (poor replication, quantification and gel to gel comparisons), many of which are discussed in detail in a review by Angelika Görg (Görg *et al.*, 2004). It was believed that highly accurate analysis would be a definitive requirement in this study of protein regulation in synapse specific plasticity processes as it was not anticipated that any changes would be colossal due not only to sample size but also as a result of the physiological nature of the experimental approaches being investigated. Indeed even given the electrode array in place for the LTP experiments and the high stimulation paradigm used, it is still anticipated that a small subset of synapses would in fact be stimulated. Therefore, an alternative 2D gel electrophoresis approach was pursued which was not only able to increase reproducibility between gel replicates but also provided accurate, quantifiable and statistically robust data on protein expression profiles. Difference gel electrophoresis (DiGE) is a relatively novel system designed to overcome some of the problems commonly associated with 2D gel electrophoresis (Unlu *et al.*, 1997).

To use DiGE it was necessary to further optimise this relatively new proteomics technique for use with brain tissue as, at the time of development, very few researchers had utilised DiGE for use with CNS tissue. The only investigations of this kind had been carried out by Swatton and colleagues at the Cambridge centre for proteomics (CCP) using human post mortem tissue (Swatton *et al.*, 2004) and Van den Bergh *et al* in 2003 investigating protein expression differences in the striate cortex of kittens and cats. Following discussions with Dr Kathryn Lilley at the CCP it was apparent that interfering substances found in abundance in brain tissue i.e. lipids and salts, results in a much reduced expression of labelled protein samples and

hence fewer protein spots available for the final analysis. Therefore, in removing these contaminants labelling would be optimised and a greater number of proteins would be visualised on the final 2D gel. Indeed this was found to be the case and represented a major breakthrough. When protein extracts from hippocampal slices were labelled with CyDyes following ammonium acetate precipitation of these extracts a larger proportion of protein spots were represented on a single 2D gel. Other precipitation methods were used including GE Healthcare's 2D clean up kit and more traditional methods such as TCA and acetone precipitations. Such methods were found to be unsuitable for the hippocampal tissue samples I was using mainly due to the reduced recovery of protein following precipitation. Such consequences were not ideal given the protein amount limitations already faced.

7.4.3 CyDyes

Using 3 spectrally resolvable CyDyes, DiGE allows up to three protein samples to be resolved on a single 2D gel simultaneously. Using a third marker has an additional benefit of including an internal standard which can be run on all gels providing a point of reference across these gels and allowing the standardisation and normalisation of each individual protein spot. In doing this there is an accurate value assigned to the respective change in abundance level of each individual protein spot.

The CyDyes used in DiGE have many advantages over other protein stains. Detection limits of CyDyes compared to other commonly used protein stains are one such advantage. CyDyes have close to five orders of linear detection levels whereas Sypro stains (Ruby and Orange) have only 2-3 orders of linearity in addition to being less sensitive. Silver staining has a similar sensitivity level but can only be visualised over one order of magnitude. However, the sensitivity of these dyes is not complimented by the sensitivity of the mass spectrometric analyses being carried out following DiGE. Given that these dyes are sensitive down to the sub pico-gram range, and that mass spectrometry has a much higher protein concentration requirement (nanogram) it is not entirely surprising that several of the protein spots labelled and detected by the CyDyes and DeCyder software respectively, are not identified following either Maldi-tof or Q star mass spectrometric analysis. It is

therefore to be expected that not all of the protein spots identified as changing in abundance will be identified.

Two different kinds of CyDyes are available – one suitable for minimal labelling of proteins using 50μg of total protein extract, the other suitable for saturation labelling of scarce samples which requires only 5μg of protein. Indeed, this would have been a welcome alternative for this study given the problems associated with tissue availability. However, only 2 saturation dyes are currently available, Cy3 and Cy5, thus, not allowing the incorporation of an internal standard into the DiGE experiments.

7.4.4 Limitations of DiGE

Despite the finding that DiGE vastly improves the quality and quantity of protein analysis and data output from 2D gel experiments, it is not without limitations. One of the main limitations was possibly the cost of running these experiments although probable that the expense may ultimately overshadow the substantial advantages. Whilst the CyDyes themselves may seem relatively expensive, the reduced number of replicates required to obtain a large amount of robust, quantifiable data compared to standard 2D methods makes the initial outlay worthwhile and may actually, in the long run, be more economical. In addition, much of the equipment required to run standard 2D gel electrophoresis experiments is the same as is required for DiGE experimentation with the exception of the DeCyder analysis software. It is possible to design DiGE experiments, which will result in the generation of a vast amount of data from a single experiment. Another consideration is the time consuming nature of the DiGE approach. Nevertheless, whilst a higher throughput methodology would almost certainly be welcomed, careful experimental design using DiGE can provide the experimenter with an abundance of information relating to as many different conditions (time, concentration, age) as is desired. Moreover, the incorporation of brain slice pharmacology, electrophysiology and DiGE provides a unique and novel approach and with it some unique and novel discoveries.

Undoubtedly, DiGE provides a number of benefits over standard 2D gel methods, but also many of the same problems are encountered. The major problem is the

inability to resolve hydrophobic proteins on a 2D gel as their hydrophobicity prevents them from entering into the 1st dimension. There is so far no way to analyse the vast majority of these proteins in gel format. Given that many of the proteins known to be central to synapse specific forms of plasticity are hydrophobic membrane bound receptors (i.e. NMDA and AMPA receptors) there remains a specific and important area not covered by DiGE analysis. This is a recognised limitation for this technique and as a result there has been much effort to try and identify alternative methods to study the regulation of this group of proteins (Alexandersson *et al.*, 2004; Gauthier *et al.*, 2004; Sazuka *et al.*, 2004; Fan *et al.*, 2004). Indeed, the choice of detergent used to extract proteins from hippocampus throughout this study was chosen as due to the evidence provided that it was thought to increase the representation of membrane associated proteins in 2D gels (Taylor & Pfeiffer, 2003). However, the use of surfactants such as ASB14 to solubilise these proteins can suppress peptide ionization and interfere with chromatographic separation of proteins. It is thought that to be able to fully investigate the regulation of membrane bound proteins such as receptors and ion channels a shift to less gel based technology is required, for example ICAT which provides the user with the ability to identify and quantitate differences in integral membrane proteins between samples. This technique was first described by Goshe and colleagues in 2003 and has recently been utilised to identify the effect of chronic morphine exposure on the synaptic plasma-membrane sub-proteome of rats (Prokai *et al.*, 2005).

By its nature, 2D-gel electrophoresis is known to preferentially involve the investigation of the most abundant proteins in a proteome, masking many of the proteins that exist in a low copy number throughout a cell. The low abundance of important physiologically relevant proteins has rendered their analyses almost impossible without some means of prior purification and enrichment from tissue lysates or biological fluids. Cellular fractionation techniques followed by specific affinity probes for tracking target proteins have been developed to deplete the proteome of high abundance proteins in order to increase the sample loading for achieving greater sensitivity for proteins present in low abundance. Those applications can entail the removal of one protein or a class of proteins that interferes with the resolution of proteins in a 2-DE map. Moreover, the use of superior detergents in combination with narrow immobilized pH gradients, results in higher

resolution by stretching the protein pattern in the first dimension. Removal of the most abundance proteins prior to 2D gel electrophoresis has been adopted by several groups including Fountoulakis and colleagues who removed albumin and immunoglobulin from plasma samples prior to 2D gel electrophoresis (Fountoulakis *et al.*, 2004). In doing so several new proteins were visualised on the 2D gel. Moreover, capillary HPLC in conjunction with nanoESI/MS also allows for the detection of low-abundance tryptic peptides, a method implemented by Stevens *et al* in 2003 to carry out an in depth analysis of the synaptic plasma membrane fraction isolated from rat forebrain. In the current study however, it is important to remember that a global approach requires the investigation of all of the proteins in a proteome – the nature of this study was unbiased and removing some of the proteins from an extract does not fit with this thinking. Activity-dependent changes in neuronal connectivity/ communication do not occur purely through low abundance signalling type protein molecules. Indeed, the identification of several abundant cytoskeletal proteins including tubulin and actin, which are consistently regulated following synaptic plasticity, related events in the hippocampus highlight to the importance of implementing such an unbiased approach.

7.4.5 Interpretation of mass spectrometry data

In addition to the limitations posed by DiGE technology, there should also be some caution associated with the results obtained from the mass spectrometry analysis of statistically significant protein spots. As has previously been mentioned, the Mowse score generated following either a PMF search or MS/MS ion search in Mascot is a probability-based score where P is the probability that the observed match is a random event. Protein scores greater than 61 are thought to be significant ($p<0.05$) following PMF analysis and individual ion scores greater than 35 indicate identity or extensive homology ($p<0.05$) following MS/MS ion searching. Hence for both types of analysis the chances of generating a false positive result are as high as 1 in 20. Therefore, for those proteins close to the threshold level (i.e. close to $p=0.05$) the chance of them being a random event increases. On the other hand, those proteins with very high Mowse scores are associated with an increased confidence in their identity. To further increase the confidence in a particular protein identity being 'real' it is also worth determining the number of peptides that have been matched and

the sequence coverage of these peptides. Greater numbers of peptides matched and a greater percentage of sequence coverage are further indicative of a true identity.

Another issue, more commonly associated with MS/ MS analysis is the identification of multiple proteins within a single spot. This results from poor resolution of particular protein spots on a 2D gel hence several proteins co-migrate to a single spot on a gel. The nature of MS/MS analysis means that it is more capable of identifying these multiple components compared to Maldi-Tof mass spectrometric analysis. As a result, for a particular protein spot which has significantly changed in expression, but which contains three, four perhaps more proteins, it is not possible to state which of the identified components are in fact responsible for the change in expression. Indeed, it could be one or several but this is not discernable with the information available.

Whilst both these caveats apply to several of the proteins identified in this study it is also worth reiterating that the global nature of this approach doesn't permit the exclusion of such proteins, but that careful interpretation of the results should be used.

7.5 Future studies

The studies detailed in this thesis have not only imparted a substantial amount of valuable information regarding the diversity and type of proteins involved in or responsible for synaptic plasticity in the hippocampus but have also paved the way for further, more detailed analyses of a similar vein. Some of these are discussed further below.

7.5.1 Protein-protein interactions

As previously mentioned, proteins do not act in isolation in a cell, but usually form transitory or stable complexes in order to participate in pathways and act in networks. Protein-protein interactions thus constitute an essential aspect of the normal workings of the living cell and unravelling the various interactions in which individual proteins are involved constitutes an invaluable way of understanding

protein function. Much work has been done to develop Protein Interaction Maps (PIMs) for whole cells and proteomes and this approach – now called interactomics – has recently been adopted by several groups (Nabieva *et al.*, 2005; Ramani *et al.*, 2005). Although no such work has as yet been carried out in relation to the CNS.

In a similar vein is the use of protein-protein interactions in terms of isolating certain protein complexes and resolving them on a 2D gel. For example, the family of kinesins are proteins responsible for the shuttling of cargo proteins throughout cells including neuronal cells by using adaptor or scaffolding proteins to recognise and bind cargoes. Such cargoes have been shown to include NMDA receptor subunits including the NR2B subunit via interaction with Kif17 (Guillaud *et al.*, 2003) and over expression of Kif17 in transgenic mice was shown by Wong and colleagues to enhance spatial and working memory (Wong *et al.*, 2002). Bannai *et al* in 2004b demonstrated that sub-compartments of ER moved rapidly along the dendrites in both anterograde and retrograde directions and that depletion of microtubules. Over-expression of dominant-negative kinesin and kinesin depletion by antisense DNA reduced the number and velocity of the moving vesicles, suggesting that kinesin may drive the transport of the vesicular sub-compartment of ER along microtubules in the dendrite. Importantly, it has been shown that kinesins, more specifically KIF5, are responsible for the transport of RNA-transporting granules (Kanai *et al.*, 2004). It is therefore possible that the sub-cellular translocation of molecules associated with synapse specific forms of plasticity can be identified by isolating the protein complex that is Kif5 and associated cargo. To determine whether or not this is in fact the case, immunoprecipitation studies that utilised both generic forms of kinesin (i.e. kinesin light or heavy chains, or both) and more specific forms of kinesin (i.e. Kif17, Kif5) would be a valid approach. The protein complexes obtained from such immunoprecipitation assays could then be resolved on a 2D gel and the resultant protein spots identified by mass spectrometric analysis. Ideally, such an approach would be carried out on stimulated and unstimulated hippocampal tissue. If kinesins are indeed involved in the trafficking of molecules to activated synapses from somatic regions of the neurone, as is suggested by the studies of Kanai *et al.* in 2004 and Gillaud and colleagues in 2003, then such an approach would identify these proteins. Such a global investigation could prove invaluable as it could identify a

proportion of the molecules involved in late phases of LTP.

7.5.2 *De novo* protein synthesis

An investigation into the role and variety of proteins displaying altered rates of *de novo* protein synthesis following the induction of hippocampal LTP was carried by Fazeli and colleagues in 1993. Although advanced in its thinking at the time, methodological restraints prevented the study from providing all the desired information. Using ^{35}S methionine labelling of *de novo* proteins synthesised following the induction of LTP they resolved these newly formed proteins on a 2D gel and visualised them using autoradiography. Although several new proteins were synthesised 3 hrs post LTP induction they were not subsequently identified by mass spectrometry. This is an experiment, which to all extents and purposes could be reproduced now, but would have the distinct advantage of using mass spectrometric technology to identify the proteins.

7.6 Summary

This investigation into protein regulation associated with synapse specific forms of hippocampal plasticity has been extremely fruitful in many aspects. Not only has it resulted in the development of a methodology that allows an unbiased investigation into protein regulation following synaptic plasticity related events, but it also has permitted the investigation of almost the entire hippocampal proteome. In doing so it has provided information on the regulation of a large component of the hippocampal proteome. As a result several novel, plasticity-related protein molecules have been identified which will merit further investigation. Not only this, but activity dependent changes in functional expression patterns have also been highlighted for the first time. Clearly, this investigation is not exhaustive, but it certainly lays the foundation for further studies and in doing so will hopefully go some way to identify the variety of molecules involved in or responsible for synapse specific forms of plasticity and hence provide an insight into the diversity of proteins underlying the complex molecular workings of cognitive processes in the mammalian brain.

A

Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER, Bourtchouladze R (1997). Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. *Cell.* **88(5)**: 615-26.

Abele AE, Scholz KP, Scholz WK, Miller RJ (1990). Excitotoxicity induced by enhanced excitatory neurotransmission in cultured hippocampal pyramidal neurons. *Neuron.* **4(3)**: 413-9.

Abeliovich A, Chen C, Goda Y, Silva AJ, Stevens CF, Tonegawa S (1993a). Modified hippocampal long-term potentiation in PKC gamma-mutant mice. *Cell.* **75(7)**: 1253-62.

Abeliovich A, Paylor R, Chen C, Kim JJ, Wehner JM, Tonegawa S (1993b). PKC gamma mutant mice exhibit mild deficits in spatial and contextual learning. *Cell.* **75(7)**: 1263-71.

Acsady L, Gores TJ, Freund TF (1996). Different populations of vasoactive intestinal polypeptide-immunoreactive interneurons are specialized to control pyramidal cells or interneurons in the hippocampus. *Neuroscience.* **73(2)**: 299-315.

Adams MD, Dubnick M, Kerlavage AR, Moreno R, Kelley JM, Utterback TR, Nagle JW, Fields C, Venter JC (1992). Sequence identification of 2,375 human brain genes. *Nature.* **355(6361)**: 632-4.

Aggleton JP & Brown MW (1999). Episodic memory, amnesia, and the hippocampal-anterior thalamic axis. *Behav Brain Sci.* **22(3)**: 425-44.

Agoston DV, Kuhnt U (1986). Increased Ca-uptake of presynaptic terminals during long-term potentiation in hippocampal slices. *Exp Brain Res.* **62(3)**: 663-8.

Aiba A, Chen C, Herrup K, Rosenmund C, Stevens CF, Tonegawa S (1994). Reduced hippocampal long-term potentiation and context-specific deficit in associative learning in mGluR1 mutant mice. *Cell.* **79(2)**: 365-75.

Aigner L, Caroni P (1993). Depletion of 43-kD growth-associated protein in primary sensory neurons leads to diminished formation and spreading of growth cones. *J Cell Biol.* **123(2)**: 417-29.

Aigner L, Arber S, Kapfhammer JP, Laux T, Schneider C, Botteri F, Brenner HR, Caroni P (1995). Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. *Cell.* **83(2)**: 269-78.

Aigner L, Caroni P (1995). Absence of persistent spreading, branching, and adhesion in GAP-43-depleted growth cones. *J Cell Biol.* **128(4)**: 647-60.

Ainsztein AM, Purich DL (1992). Cleavage of bovine brain microtubule-associated protein-2 by human immunodeficiency virus proteinase. *J Neurochem.* **59(3)**: 874-80.

Aitken A, Baxter H, Dubois T, Clokie S, Mackie S, Mitchell K, Peden A, Zemlickova E (2002). Specificity of 14-3-3 isoform dimer interactions and phosphorylation. *Biochem Soc Trans.* **30(4)**: 351-60.

Akers RF, Lovinger DM, Colley PA, Linden DJ, Routtenberg A (1986). Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science.* **231(4738)**: 587-9.

Akers RF & Routtenberg A (1987). Calcium-promoted translocation of protein kinase C to synaptic membranes: relation to the phosphorylation of an endogenous substrate (protein F1) involved in synaptic plasticity. *J Neurosci.* **7(12)**: 3976-83.

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(1)**: 36-44.

Alexandersson E, Saalbach G, Larsson C, Kjellbom P (2004). Arabidopsis plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. *Plant Cell Physiol.* **45(11)**: 1543-56.

Allison DW, Gelfand VI, Spector I, Craig AM (1998). Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors. *J Neurosci.* **18(7)**: 2423-36.

Allison DW, Chervin AS, Gelfand VI, Craig AM (2000). Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. *J Neurosci* **20(12)**: 4545-54.

Amaral DG (1999). Anatomical Organization of the Central Nervous System. Chapter 17 in Principles of Neural Science (Fourth Edition) E.R. Kandel, J.H. Schwartz, T.M. Jessel (Eds.). pp. 317-336

Ambrogini P, Orsini L, Mancini C, Ferri P, Ciaroni S, Cuppini R (2004). Learning may reduce neurogenesis in adult rat dentate gyrus. *Neurosci Lett.* **359(1-2)**: 13-6.

Anderson KJ, Scheff SW, DeKosky ST (1986). Reactive synaptogenesis in hippocampal area CA1 of aged and young adult rats. *J Comp Neurol.* **252(3)**: 374-84.

Anderson WW & Collingridge GL (2001). The LTP Program: a data acquisition program for on-line analysis of long-term potentiation and other synaptic events. *J Neurosci Methods.* **108(1)**: 71-83.

Andreasson KI, Savonenko A, Vidensky S, Goellner JJ, Zhang Y, Shaffer A, Kaufmann WE, Worley PF, Isakson P, Markowska AL (2001). Age-dependent cognitive deficits and neuronal apoptosis in cyclooxygenase-2 transgenic mice. *J Neurosci.* **21(20)**: 8198-209.

Ankarcrona M, Zhivotovsky B, Holmstrom T, Diana A, Eriksson JE, Orrenius S, Nicotera P (1996). Lamin and beta-tubulin fragmentation precede chromatin degradation in glutamate-induced neuronal apoptosis. *Neuroreport.* **7(15-17)**: 2659-64.

Anwyl R, Mulkeen D, Rowan MJ (1989). The role of N-methyl-D-aspartate receptors in the generation of short-term potentiation in the rat hippocampus. *Brain Res.* **503(1)**: 148-51.

Arendash GW, Garcia MF, Costa DA, Cracchiolo JR, Wefes IM, Potter H (2004). Environmental enrichment improves cognition in aged Alzheimer's transgenic mice despite stable beta-amyloid deposition. *Neuroreport.* **15(11)**: 1751-4.

Aronica E, Frey U, Wagner M, Schroeder H, Krug M, Ruthrich H, Catania MV, Nicoletti F, Reymann KG (1991). Enhanced sensitivity of "metabotropic" glutamate receptors after induction of long-term potentiation in rat hippocampus. *J Neurochem.* **57(2)**: 376-83.

Arvidsson A, Kokaia Z, Lindvall O (2001). N-methyl-D-aspartate receptor-mediated increase of neurogenesis in adult rat dentate gyrus following stroke. Eur J Neurosci. 2001 Jul;14(1):10-8.

Asada H, Kawamura Y, Maruyama K, Kume H, Ding RG, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K (1997). Cleft palate and decreased brain gamma-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. *Proc Natl Acad Sci U S A.* **94(12)**: 6496-9.

Asai T, Tomita Y, Nakatsuka S, Hoshida Y, Myoui A, Yoshikawa H, Aozasa K (2002). VCP (p97) regulates NFkappaB signaling pathway, which is important for metastasis of osteosarcoma cell line. *Jpn J Cancer Res.* **93(3)**: 296-304.

Asztely F, Xiao MY, Gustafsson B (1996). Long-term potentiation and paired-pulse facilitation in the hippocampal CA1 region. *Neuroreport.* **7(10)**: 1609-12.

Auvergne R, Lerc C, El Bahh B, Arthaud S, Lespinet V, Rougier A, Le Gal La Salle G (2002). Delayed kindling epileptogenesis and increased neurogenesis in adult rats housed in an enriched environment. *Brain Res.* **954(2)**: 277-85.

B

Bailey CH & Kandel ER (1993). Structural changes accompanying memory storage. *Annu Rev Physiol.* **55**: 397-426

Bailey CH, Bartsch D, Kandel ER (1996). Toward a molecular definition of long-term memory storage. *Proc Natl Acad Sci U S A.* **93(24)**: 13445-52

Bailey DJ, Kim JJ, Sun W, Thompson RF, Helmstetter FJ (1999). Acquisition of fear conditioning in rats requires the synthesis of mRNA in the amygdala. *Behav Neurosci.* **113(2)**: 276-82.

Baimbridge KG, Miller JJ (1981). Calcium uptake and retention during long-term potentiation of neuronal activity in the rat hippocampal slice preparation. *Brain Res.* **221(2)**: 299-305.

Bajo M, Fruehauf J, Kim SH, Fountoulakis M, Lubec G (2002). Proteomic evaluation of intermediary metabolism enzyme proteins in fetal Down's syndrome cerebral cortex. *Proteomics.* **2(11)**: 1539-46.

Baker LP, Nielsen MD, Impey S, Hacker BM, Poser SW, Chan MY, Storm DR (1999). Regulation and immunohistochemical localization of betagamma-stimulated adenylyl cyclases in mouse hippocampus. *J Neurosci.* **19(1)**: 180-92.

Bamji SX, Shimazu K, Kimes N, Huelsken J, Birchmeier W, Lu B, Reichardt LF (2003). Role of beta-catenin in synaptic vesicle localization and presynaptic assembly. *Neuron.* **40(4)**: 719-31.

Banks RE, Dunn MJ, Forbes MA, Stanley A, Pappin D, Naven T, Gough M, Harnden P, Selby PJ (1999). The potential use of laser capture microdissection to selectively obtain distinct populations of cells for proteomic analysis--preliminary findings. *Electrophoresis.* **20(4-5)**: 689-700.

Bannai H, Fukatsu K, Mizutani A, Natsume T, Iemura S, Ikegami T, Inoue T, Mikoshiba K (2004a). An RNA-interacting protein, SYNCRIP (heterogeneous nuclear ribonuclear protein Q1/NSAP1) is a component of mRNA granule transported with inositol 1,4,5-trisphosphate receptor type 1 mRNA in neuronal dendrites. *J Biol Chem.* **279(51)**: 53427-34.

Bannai H, Inoue T, Nakayama T, Hattori M, Mikoshiba K (2004b). Kinesin dependent, rapid, bi-directional transport of ER sub-compartment in dendrites of hippocampal neurons. *J Cell Sci.* **117(Pt 2)**: 163-75.

Barber DS & LoPachin RM (2004). Proteomic analysis of acrylamide-protein adduct formation in rat brain synaptosomes. *Toxicol Appl Pharmacol.* **201(2)**: 120-36.

Barco A, Alarcon JM, Kandel ER (2002). Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. *Cell.* **108(5)**: 689-703.

Baron C, Benes C, Van Tan H, Fagard R, Roisin MP (1996). Potassium chloride pulse enhances mitogen-activated protein kinase activity in rat hippocampal slices. *J Neurochem.* **66(3)**: 1005-10.

Barzilai A, Kennedy TE, Sweatt JD, Kandel ER (1989). 5-HT modulates protein synthesis and the expression of specific proteins during long-term facilitation in Aplysia sensory neurons. *Neuron.* **2(6)**: 1577-86.

Bashir ZI, Bortolotto ZA, Davies CH, Berretta N, Irving AJ, Seal AJ, Henley JM, Jane DE, Watkins JC, Collingridge GL (1993). Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. *Nature.* **363(6427)**: 347-50.

Baumert M, Maycox PR, Navone F, De Camilli P, Jahn R (1989). Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. *EMBO J.* **8(2)**: 379-84.

Baxter DA, Bittner GD, Brown TH (1985). Quantal mechanism of long-term synaptic potentiation. *Proc Natl Acad Sci U S A.* **82(17)**: 5978-82.

Bays NW, Wilhovsky SK, Goradia A, Hodgkiss-Harlow K, Hampton RY (2001). HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. *Mol Biol Cell.* **12(12)**: 4114-28.

Beckman ML, Bernstein EM, Quick MW (1998). Protein kinase C regulates the interaction between a GABA transporter and syntaxin 1A. *J Neurosci.* **18(16)**: 6103-12.

Behnisch T, Matsushita S, Knopfel T (2004). Imaging of gene expression during long-term potentiation. Neuroreport. **15(13)**: 2039-43.

Ben-Ari Y, Tseeb V, Raggozzino D, Khazipov R, Gaiarsa JL (1994). gamma-Aminobutyric acid (GABA): a fast excitatory transmitter which may regulate the development of hippocampal neurones in early postnatal life. *Prog Brain Res.* **102:** 261-73

Benowitz LI, Routtenberg A (1997). GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends Neurosci.* **20(2)**: 84-91.

Benson DL, Mandell JW, Shaw G, Banker G (1996). Compartmentation of alpha-internexin and neurofilament triplet proteins in cultured hippocampal neurons. *J Neurocytol.* **25(3)**: 181-96.

Benson DL, Tanaka H (1998). N-cadherin redistribution during synaptogenesis in hippocampal neurons. *J Neurosci.* **18(17)**: 6892-904.

Beranova-Giorgianni S, Pabst MJ, Russell TM, Giorgianni F, Goldowitz D, Desiderio DM (2002). Preliminary analysis of the mouse cerebellum proteome. *Brain Res Mol Brain Res.* **98(1-2)**: 135-40.

Berling B, Wille H, Roll B, Mandelkow EM, Garner C, Mandelkow E (1994). Phosphorylation of microtubule-associated proteins MAP2a,b and MAP2c at Ser136 by proline-directed kinases in vivo and in vitro. *Eur J Cell Biol.* **64(1)**: 120-30.

Bimston D, Song J, Winchester D, Takayama S, Reed JC, Morimoto RI (1998). BAG-1, a negative regulator of Hsp70 chaperone activity, uncouples nucleotide hydrolysis from substrate release. *EMBO J.* **17(23)**: 6871-8.

Blackstone CD, Moss SJ, Martin LJ, Levey AI, Price DL, Huganir RL (1992). Biochemical characterization and localization of a non-N-methyl-D-aspartate glutamate receptor in rat brain. *J Neurochem.* **58(3)**: 1118-26.

Blalock EM, Chen KC, Sharrow K, Herman JP, Porter NM, Foster TC, Landfield PW (2003). Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. *J Neurosci.* **23(9)**: 3807-19.

Blanpied TA, Scott DB, Ehlers MD (2002). Dynamics and regulation of clathrin coats at specialized endocytic zones of dendrites and spines. *Neuron.* **36(3)**: 435-49.

Bliss, TVP & Lomo, T (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol (London)* **232**: 331-356.

Bliss TV & Collingridge GL (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature. 1993 Jan 7;361(6407):31-9.

Blitzer RD, Wong T, Nouranifar R, Iyengar R, Landau EM (1995). Postsynaptic cAMP pathway gates early LTP in hippocampal CA1 region. *Neuron.* 1995 **15(6)**: 1403-14.

Bloom O, Evergren E, Tomilin N, Kjaerulff O, Low P, Brodin L, Pieribone VA, Greengard P, Shupliakov O (2003). Colocalization of synapsin and actin during synaptic vesicle recycling. *J Cell Biol.* **161(4)**: 737-47.

Boal RW & Gillette RG (2004). Central neuronal plasticity, low back pain and spinal manipulative therapy. *J Manipulative Physiol Ther.* **27(5)**: 314-26

Boguski MS & Jones AR (2004). Neurogenomics: at the intersection of neurobiology and genome sciences. *Nat Neurosci.* **7(5)**: 429-33.

Bonifati V, Rizzu P, Squitieri F, Krieger E, Vanacore N, van Swieten JC, Brice A, van Duijn CM, Oostra B, Meco G, Heutink P (2003). DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. *Neurol Sci.* **24(3)**: 159-60.

Bortolotto ZA & Collingridge GL (1992). Activation of glutamate metabotropic receptors induces long-term potentiation. *Eur J Pharmacol.* **214(2-3)**: 297-8.

Bortolotto ZA & Collingridge GL (1993). Characterisation of LTP induced by the activation of glutamate metabotropic receptors in area CA1 of the hippocampus. *Neuropharmacology.* **32(1)**: 1-9.

Bortolotto ZA, Bashir ZI, Davies CH, Collingridge GL (1994). A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation. *Nature.* **368(6473)**: 740-3.

Bortolotto ZA & Collingridge GL (1995). On the mechanism of long-term potentiation induced by (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) in rat hippocampal slices. *Neuropharmacology.* **34(8)**: 1003-14.

Bortolotto ZA, Bashir ZI, Davies CH, Taira T, Kaila K, Collingridge GL (1995). Studies on the role of metabotropic glutamate receptors in long-term potentiation: some methodological considerations. *J Neurosci Methods.* **59(1)**: 19-24.

Boss BD, Peterson GM & Cowan WM (1985). On the number of neurons in the dentate gyrus of the rat. *Brain Res.* **338(1)**: 144-50.

Boss BD, Turlejski K, Stanfield BB & Cowan WM (1987). On the numbers of neurons in fields CA1 and CA3 of the hippocampus of Sprague-Dawley and Wistar rats. *Brain Res.* **406(1-2)**: 280-7.

Boudeau J, Sapkota G, Alessi DR (2003). LKB1, a protein kinase regulating cell proliferation and polarity. *FEBS Lett.* **546(1)**: 159-65.

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell.* 79(1):59-68.

Boyne LJ, Fischer I, Shea TB (1996). Role of vimentin in early stages of neuritogenesis in cultured hippocampal neurons. *Int J Dev Neurosci.* **14(6)**: 739-48.

Brackmann M, Zhao C, Schmieden V, Braunewell KH (2004). Cellular and subcellular localization of the inhibitory glycine receptor in hippocampal neurons. *Biochem Biophys Res Commun.* **324(3)**: 1137-42.

Bradshaw KD, Emptage NJ, Bliss TV (2003). A role for dendritic protein synthesis in hippocampal late LTP. *Eur J Neurosci.* **18(11)**: 3150-2.

Braun S, Matuschewski K, Rape M, Thoms S, Jentsch S (2002). Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. *EMBO J.* **21(4)**: 615-21.

Breakwell NA, Rowan MJ, Anwyl R (1998). (+)-MCPG blocks induction of LTP in CA1 of rat hippocampus via agonist action at an mGluR group II receptor. *J Neurophysiol.* **79(3)**: 1270-6.

Bruel-Jungerman E, Laroche S, Rampon C (2005). New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment. *Eur J Neurosci.* **21(2)**: 513-21.

Brugg B, Matus A (1991). Phosphorylation determines the binding of microtubule-associated protein 2 (MAP2) to microtubules in living cells. *J Cell Biol.* **114(4)**: 735-43.

Brustovetsky N, Dubinsky JM (2000). Limitations of cyclosporin A inhibition of the permeability transition in CNS mitochondria. *J Neurosci.* **20(22)**: 8229-37.

Brown TH & Johnston D (1983). Voltage-clamp analysis of mossy fiber synaptic input to hippocampal neurons. *J Neurophysiol.* **50(2)**: 487-507.

Brown J, Cooper-Kuhn CM, Kempermann G, Van Praag H, Winkler J, Gage FH, Kuhn HG (2003). Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. *Eur J Neurosci.* **17(10)**: 2042-6.

Brown JT, Richardson JC, Collingridge GL, Randall AD, Davies CH (2005). Synaptic transmission and synchronous activity is disrupted in hippocampal slices taken from aged TAS10 mice. *Hippocampus.***15(1)**: 110-7.

Bundman MC, Pico RM, Gall CM (1994a). Ultrastructural plasticity of the dentate gyrus granule cells following recurrent limbic seizures: I. Increase in somatic spines. *Hippocampus.* **4(5)**: 601-10.

Bundman MC, Gall CM (1994b). Ultrastructural plasticity of the dentate gyrus granule cells following recurrent limbic seizures: II. Alterations in somatic synapses. *Hippocampus.* **4(5)**: 611-22.

Burns NR (1985). Skeleton key to memory? *Nature.* **313(5999)**: 178-9.

C

Calakos N & Scheller RH (1996). Synaptic vesicle biogenesis, docking, and fusion: a molecular description. *Physiol Rev.* **76(1)**: 1-29.

Callahan PM, Paris JM, Cunningham KA, Shinnick-Gallagher P (1991). Decrease of GABA-immunoreactive neurons in the amygdala after electrical kindling in the rat. *Brain Res.* **555(2)**: 335-9.

Cameron HA, McEwen BS, Gould E (1995). Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. *J Neurosci.* **15(6)**: 4687-92.

Carden MJ, Trojanowski JQ, Schlaepfer WW, Lee VM (1987). 'Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. *J Neurosci.* **7(11)**: 3489-504.

Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002). Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev.* **26(3)**: 321-52

Castegna A, Aksenov M, Aksenova M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA (2002a). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. *Free Radic Biol Med.* **33(4)**: 562-71.

Castegna A, Aksenov M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA (2002b). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71. *J Neurochem.* **82(6)**: 1524-32.

Castegna A, Thongboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA (2003). Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem.* **85(6)**: 1394-401.

Castejon OJ, Valero C, Diaz M (1995). Synaptic degenerative changes in human traumatic brain edema. An electron microscopic study of cerebral cortical biopsies. *J Neurosurg Sci.* **39(1)**: 47-65.

Castejon OJ, Castellano A, Arismendi G (2004). Transmission electron microscopy of cortical dendritic spines in the human oedematous cerebral cortex. *J Submicrosc Cytol Pathol.* **36(2)**: 181-91.

Castro CA, Silbert LH, McNaughton BL, Barnes CA (1989). Recovery of spatial learning deficits after decay of electrically induced synaptic enhancement in the hippocampus. *Nature.* **342(6249)**: 545-8.

Cavallaro S, Schreurs BG, Zhao W, D'Agata V, Alkon DL (2001). Gene expression profiles during long-term memory consolidation. *Eur J Neurosci.* **13(9)**: 1809-15.

Cavallaro S, D'Agata V, Manickam P, Dufour F, Alkon DL (2002). Memory-specific temporal profiles of gene expression in the hippocampus. *Proc Natl Acad Sci U S A.* 99(25): 16279-84.

Chang L & Karin M (2001). Mammalian MAP kinase signalling cascades. *Nature.* **410(6824)**: 37-40.

Chapman ER, Hanson PI, An S, Jahn R (1995). Ca2+ regulates the interaction between synaptotagmin and syntaxin 1. *J Biol Chem.* **270(40)**: 23667-71.

Chapman PF, White GL, Jones MW, Cooper-Blacketer D, Marshall VJ, Irizarry M, Younkin L, Good MA, Bliss TV, Hyman BT, Younkin SG, Hsiao KK (1999). Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. *Nat Neurosci.* **2(3)**: 271-6.

Chen X, Sullivan DS, Huffaker TC (1994). Two yeast genes with similarity to TCP-1 are required for microtubule and actin function in vivo. *Proc Natl Acad Sci U S A.* **91(19)**: 9111-5.

Chen W, Ji J, Xu X, He S, Ru B (2003). Proteomic comparison between human young and old brains by two-dimensional gel electrophoresis and identification of proteins. *Int J Dev Neurosci.* **21(4)**: 209-16.

Cheon MS, Fountoulakis M, Dierssen M, Ferreres JC, Lubec G (2001). Expression profiles of proteins in fetal brain with Down syndrome. *J Neural Transm Suppl.* **61**: 311-9.

Chetkovich DM, Gray R, Johnston D, Sweatt JD (1991). N-methyl-D-aspartate receptor activation increases cAMP levels and voltage-gated Ca2+ channel activity in area CA1 of hippocampus. *Proc Natl Acad Sci U S A.* **88(15)**: 6467-71.

Chetkovich DM, Sweatt JD (1993). nMDA receptor activation increases cyclic AMP in area CA1 of the hippocampus via calcium/calmodulin stimulation of adenylyl cyclase. *J Neurochem.* **61(5)**: 1933-42.

Chew CS, Chen X, Parente JA Jr, Tarrer S, Okamoto C, Qin HY (2002). Lasp-1 binds to non-muscle F-actin in vitro and is localized within multiple sites of dynamic actin assembly in vivo. *J Cell Sci.* **115(Pt 24)**: 4787-99.

Chi P, Greengard P, Ryan TA (2001). Synapsin dispersion and reclustering during synaptic activity. *Nat Neurosci.* **4(12)**: 1187-93.

Choe LH, Dutt MJ, Relkin N, Lee KH (2002). Studies of potential cerebrospinal fluid molecular markers for Alzheimer's disease. *Electrophoresis.* **23(14)**: 2247-51.

Choi DW (1985). Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neurosci Lett.* **58(3)**: 293-7.

Choi J, Forster MJ, McDonald SR, Weintraub ST, Carroll CA, Gracy RW (2004). Proteomic identification of specific oxidized proteins in ApoE-knockout mice: relevance to Alzheimer's disease. *Free Radic Biol Med.* **36(9)**: 1155-62.

Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ (1992). Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature.* **360(6405)**: 672-4.

Cohen AS, Abraham WC (1996). Facilitation of long-term potentiation by prior activation of metabotropic glutamate receptors. *J Neurophysiol.* **76(2)**: 953-62.

Colangelo V, Schurr J, Ball MJ, Pelaez RP, Bazan NG, Lukiw WJ (2002). Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling. *J Neurosci Res.* **70(3)**: 462-73.

Cole AJ, Saffen DW, Baraban JM, Worley PF (1989). Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature.* **340(6233)**: 474-6.

Collingridge GL, Kehl SJ, McLennan H (1983a). The antagonism of amino acid-induced excitations of rat hippocampal CA1 neurones in vitro. *J Physiol.* **334**: 19-31.

Collingridge GL, Kehl SJ, Loo R, McLennan H. (1983b). Effects of kainic and other amino acids on synaptic excitation in rat hippocampal slices: 1. Extracellular analysis. *Exp Brain Res.* **52(2)**: 170-8.

Collingridge GL, Herron CE, Lester RA (1988). Frequency-dependent N-methyl-D-aspartate receptor-mediated synaptic transmission in rat hippocampus. *J Physiol.* **399**: 301-12.

Collin T, Arvidsson A, Kokaia Z, Lindvall O (2005). Quantitative analysis of the generation of different striatal neuronal subtypes in the adult brain following excitotoxic injury. *Exp Neurol.* **Jun 1**

Collins DR, Scollon JM, Russell DC, Davies SN (1995). Indirect potentiation of synaptic transmission by metabotropic glutamate receptors in the rat hippocampal slice. *Brain Res.* **684(2)**: 165-71.

Comery TA, Shah R, Greenough WT (1995). Differential rearing alters spine density on medium-sized spiny neurons in the rat corpus striatum: evidence for association of morphological plasticity with early response gene expression. *Neurobiol Learn Mem.* **63(3)**: 217-9.

Cordwell SJ, Nouwens AS, Verrills NM, Basseal DJ, Walsh BJ (2000). Subproteomics based upon protein cellular location and relative solubilities in conjunction with composite two-dimensional electrophoresis gels. *Electrophoresis.* **21(6)**: 1094-103.

Costa E, Panula P, Thompson HK, Cheney DL (1983). The transsynaptic regulation of the septal-hippocampal cholinergic neurons. *Life Sci.* **32(3)**: 165-79.

Cotman CW & Hoff SF (1983). Synapse repair in the hippocampus: the effects of aging. *Birth Defects Orig Artic Ser.* **19(4)**: 119-34.

Cracco JB, Serrano P, Moskowitz SI, Bergold PJ, Sacktor TC (2005). Protein synthesis-dependent LTP in isolated dendrites of CA1 pyramidal cells. *Hippocampus.* Mar 24.

Craig EA, Gambill BD, Nelson RJ (1993). Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol Rev.* **57(2)**: 402-14.

Craig EA, Weissman JS, Horwich AL (1994). Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. *Cell.* **78(3)**: 365-72.

Crochemore C, Pena-Altamira E, Virgili M, Monti B, Contestabile A (2005). Disease-related regressive alterations of forebrain cholinergic system in SOD1 mutant transgenic mice. *Neurochem Int.* **46(5)**: 357-68.

Crow T & Bridge MS (1985). Serotonin modulates photoresponses in Hermissenda type-B photoreceptors. *Neurosci Lett.* **60(1)**: 83-8.

Cumming R, Burgoyne RD (1983). Compartmentalization of neuronal cytoskeletal proteins. Review. *Biosci Rep.* **3(11)**: 997-1006.

D

Dai RM, Chen E, Longo DL, Gorbea CM, Li CC (1998). Involvement of valosin-containing protein, an ATPase Co-purified with IkappaBalpha and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of IkappaBalpha. *J Biol Chem.* **273(6)**: 3562-73.

Dai RM & Li CC (2001). Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nat Cell Biol.* **3(8)**: 740-4.

Datson NA, Meijer L, Steenbergen PJ, Morsink MC, van der Laan S, Meijer OC, de Kloet ER (2004). Expression profiling in laser-microdissected hippocampal subregions in rat brain reveals large subregion-specific differences in expression. Eur *J Neurosci.* **20(10)**: 2541-54.

David G, Barrett JN, Barrett EF (1998). Evidence that mitochondria buffer physiological Ca2+ loads in lizard motor nerve terminals. *J Physiol* **509 (Pt 1)**: 59-65.

Davies SN, Lester RA, Reymann KG, Collingridge GL (1989). Temporally distinct pre- and post-synaptic mechanisms maintain long-term potentiation. *Nature.* **338(6215)**: 500-3.

Davis HP & Squire LR (1984). Protein synthesis and memory: a review. *Psychol Bull.* **96(3)**: 518-59.

Davis S, Salin H, Helme-Guizon A, Dumas S, Stephan A, Corbex M, Mallet J, Laroche S (2000). Dysfunctional regulation of alphaCaMKII and syntaxin 1B transcription after induction of LTP in the aged rat. *Eur J Neurosci.* **12(9)**: 3276-82.

Deadwyler SA, Foster TC & Hampson RE (1987a). Processing of sensory information in the hippocampus. *CRC Crit Rev Clin Neurobiol.* **2(4)**: 335-55

Deadwyler SA, Dunwiddie T, Lynch G (1987). A critical level of protein synthesis is required for long-term potentiation. *Synapse.* **1(1)**: 90-5.

de Lanerolle NC, Kim JH, Robbins RJ, Spencer DD (1989). Hippocampal interneuron loss and plasticity in human temporal lobe epilepsy. *Brain Res.* **495(2)**: 387-95.

Derkach V, Barria A, Soderling TR (1999). Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. *Proc Natl Acad Sci U S A.* **96(6)**: 3269-74.

Desmond NL & Levy WB (1983). Synaptic correlates of associative potentiation/depression: an ultrastructural study in the hippocampus. *Brain Res.* **265(1)**: 21-30.

De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F (1998). Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature.* **391(6665)**: 387-90.

Dickey CA, Loring JF, Montgomery J, Gordon MN, Eastman PS, Morgan D (2003). Selectively reduced expression of synaptic plasticity-related genes in amyloid precursor protein + presenilin-1 transgenic mice. *J Neurosci.* **23(12)**: 5219-26.

Di Giovanni S, Faden AI, Yakovlev A, Duke-Cohan JS, Finn T, Thouin M, Knoblach S, De Biase A, Bregman BS and Hoffman EP (2005). Neuronal plasticity after spinal cord injury: identification of a gene cluster driving neurite outgrowth. *FASEB J.* **19(1)**: 153-4

Dingledine R (1983). Excitatory amino acids: modes of action on hippocampal pyramidal cells. *Fed Proc.* **42(12)**: 2881-5.

Doherty AJ, Palmer MJ, Bortolotto ZA, Hargreaves A, Kingston AE, Ornstein PL, Schoepp DD, Lodge D, Collingridge GL (2000). A novel, competitive mGlu(5) receptor antagonist (LY344545) blocks DHPG-induced potentiation of NMDA responses but not the induction of LTP in rat hippocampal slices. *Br J Pharmacol.* **131(2)**: 239-44.

Doolette DJ (1997). Mechanism of adenosine accumulation in the hippocampal slice during energy deprivation. *Neurochem Int.* **30(2)**: 211-23.

D'Souza SM & Brown IR (1998). Constitutive expression of heat shock proteins Hsp90, Hsc70, Hsp70 and Hsp60 in neural and non-neural tissues of the rat during postnatal development. *Cell Stress Chaperones.* **3(3)**: 188-99.

Duffy SN, Craddock KJ, Abel T, Nguyen PV (2001). Environmental enrichment modifies the PKA-dependence of hippocampal LTP and improves hippocampus-dependent memory. *Learn Mem.* **8(1)**: 26-34.

Duffy SN & Nguyen PV (2003). Postsynaptic application of a peptide inhibitor of cAMP-dependent protein kinase blocks expression of long-lasting synaptic potentiation in hippocampal neurons. *J Neurosci.* **23(4)**: 1142-50.

Dunwiddie TV, Lynch G (1979). The relationship between extracellular calcium concentrations and the induction of hippocampal long-term potentiation. *Brain Res.* **169(1)**: 103-10.

Dvorak K & Feit J (1978). Testing of the course of neurogenesis and gliogenesis in the germinative zones of the CNS of embryonal and early postnatal rats by means of the gel reaction for the histochemical demonstration of the thiamine-pyrophosphatase. Histochemical and autoradiographical study. *Acta Histochem.* **63(1)**: 89-104.

E

Eccles JC & Krnjevic K (1959). Presynaptic changes associated with post-tetanic potentiation in the spinal cord. *J Physiol.* **149**: 274-87.

Edgar PF, Schonberger SJ, Dean B, Faull RL, Kydd R, Cooper GJ (1999a). A comparative proteome analysis of hippocampal tissue from schizophrenic and Alzheimer's disease individuals. *Mol Psychiatry.* **4(2)**: 173-8.

Edgar PF, Douglas JE, Knight C, Cooper GJ, Faull RL, Kydd R (1999b). Proteome map of the human hippocampus. *Hippocampus.* **9(6)**: 644-50.

Edgar PF, Douglas JE, Cooper GJ, Dean B, Kydd R, Faull RL (2000). Comparative proteome analysis of the hippocampus implicates chromosome 6q in schizophrenia. *Mol Psychiatry.* **5(1)**: 85-90.

Edgerton VR, Leon RD, Harkema SJ, Hodgson JA, London N, Reinkensmeyer DJ, Roy RR, Talmadge RJ, Tillakaratne NJ, Timoszyk W, Tobin A (2001). Retraining the injured spinal cord. *J Physiol.* **533(Pt 1)**: 15-22

Egerton M, Ashe OR, Chen D, Druker BJ, Burgess WH, Samelson LE (1992). VCP, the mammalian homolog of cdc48, is tyrosine phosphorylated in response to T cell antigen receptor activation. *EMBO J.* **11(10)**: 3533-40.

Ehlers MD (2003). Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nat Neurosci.* **6(3)**: 231-42.

Elliott RC, Miles MF, Lowenstein DH (2003). Overlapping microarray profiles of dentate gyrus gene expression during development- and epilepsy-associated neurogenesis and axon outgrowth. *J Neurosci.* **23(6)**: 2218-27.

Engert F & Bonhoeffer T (1999). Dendritic spine changes associated with hippocampal long-term synaptic plasticity. *Nature*. 399(6731): 66-70.

Escorihuela RM, Tobena A, Fernandez-Teruel A (1995). Environmental enrichment and postnatal handling prevent spatial learning deficits in aged hypoemotional (Roman high-avoidance) and hyperemotional (Roman low-avoidance) rats. *Learn Mem.* **2(1)**: 40-8.

Eun JP, Choi HY, Kwak YG (2004). Proteomic analysis of human cerebral cortex in epileptic patients. *Exp Mol Med.* **36(2)**: 185-91.

Evans JR, Mitchell SA, Spriggs KA, Ostrowski J, Bomsztyk K, Ostarek D, Willis AE (2003). Members of the poly (rC) binding protein family stimulate the activity of the c-myc internal ribosome entry segment in vitro and in vivo. *Oncogene*. **22(39)**: 8012-20.

F

Fagan AM & Gage FH (1994). Mechanisms of sprouting in the adult central nervous system: cellular responses in areas of terminal degeneration and reinnervation in the rat hippocampus. *Neuroscience*. **58(4)**: 705-25.

Faherty CJ, Kerley D, Smeyne RJ (2003). A Golgi-Cox morphological analysis of neuronal changes induced by environmental enrichment. *Brain Res Dev Brain Res.* **141(1-2)**: 55-61.

Falkenberg T, Mohammed AK, Henriksson B, Persson H, Winblad B, Lindefors N (1992). Increased expression of brain-derived neurotrophic factor mRNA in rat

hippocampus is associated with improved spatial memory and enriched environment. *Neurosci Lett.* **138(1)**: 153-6.

Fan X, She YM, Bagshaw RD, Callahan JW, Schachter H, Mahuran DJ (2004). A method for proteomic identification of membrane-bound proteins containing Asn-linked oligosaccharides. *Anal Biochem.* **332(1)**: 178-86.

Farnsworth CL, Freshney NW, Rosen LB, Ghosh A, Greenberg ME, Feig LA (1995). Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. *Nature.* **376(6540)**: 524-7.

Fazeli MS, Corbet J, Dunn MJ, Dolphin AC, Bliss TV (1993). Changes in protein synthesis accompanying long-term potentiation in the dentate gyrus in vivo. *J Neurosci.* **13(4)**: 1346-53.

Fedorov NB, Brown RE, Reymann KG (1997). Fast increases of AMPA receptor sensitivity following tetanus-induced potentiation in the CA1 region of the rat hippocampus. *Neuroreport.* **8(2)**: 411-4.

Feldblum S, Ackermann RF, Tobin AJ (1990). Long-term increase of glutamate decarboxylase mRNA in a rat model of temporal lobe epilepsy. *Neuron.* **5(3)**: 361-71.

Ferguson RE, Carroll HP, Harris A, Maher ER, Selby PJ, Banks RE (2005). Housekeeping proteins: a preliminary study illustrating some limitations as useful references in protein expression studies. *Proteomics.* **5(2)**: 566-71.

Ferreira A, Han HQ, Greengard P, Kosik KS (1995). Suppression of synapsin II inhibits the formation and maintenance of synapses in hippocampal culture. *Proc Natl Acad Sci U S A.* **92(20)**: 9225-9.

Ferreira A, Chin LS, Li L, Lanier LM, Kosik KS, Greengard P (1998). Distinct roles of synapsin I and synapsin II during neuronal development. *Mol Med.* **4(1)**: 22-8.

Fiermonte G, Palmieri L, Todisco S, Agrimi G, Palmieri F, Walker JE (2002). Identification of the mitochondrial glutamate transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution of two human isoforms. *J Biol Chem.* **277(22)**: 19289-94.

Fifkova E & Delay RJ (1982). Cytoplasmic actin in neuronal processes as a possible mediator of synaptic plasticity. *J Cell Biol.* **95(1)**: 345-50.

Fiore RS, Murphy TH, Sanghera JS, Pelech SL, Baraban JM (1993). Activation of p42 mitogen-activated protein kinase by glutamate receptor stimulation in rat primary cortical cultures. *J Neurochem.* **61(5)**: 1626-33.

Fischer von Mollard G, Mignery GA, Baumert M, Perin MS, Hanson TJ, Burger PM, Jahn R, Sudhof TC (1990). rab3 is a small GTP-binding protein exclusively localized to synaptic vesicles. *Proc Natl Acad Sci U S* A. **87(5)**: 1988-92.

Fitzjohn SM, Morton RA, Kuenzi F, Rosahl TW, Shearman M, Lewis H, Smith D, Reynolds DS, Davies CH, Collingridge GL, Seabrook GR (2001). Age-related impairment of synaptic transmission but normal long-term potentiation in transgenic mice that overexpress the human APP695SWE mutant form of amyloid precursor protein. *J Neurosci.* **21(13)**: 4691-8.

Flaherty KM, DeLuca-Flaherty C, McKay DB (1990). Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature.* **346(6285)**: 623-8.

Floeter MK & Greenough WT (1979). Cerebellar plasticity: modification of Purkinje cell structure by differential rearing in monkeys. *Science.* **206(4415)**: 227-9.

Foster TC, Gagne J, Massicotte G (1996). Mechanism of altered synaptic strength due to experience: relation to long-term potentiation. *Brain Res.* **736(1-2)**: 243-50.

Fountoulakis M, Juranville JF, Dierssen M, Lubec G (2002). Proteomic analysis of the fetal brain. *Proteomics.* **2(11)**: 1547-76.

Fountoulakis M, Juranville JF, Jiang L, Avila D, Roder D, Jakob P, Berndt P, Evers S, Langen H (2004). Depletion of the high-abundance plasma proteins. *Amino Acids*. **27(3-4)**: 249-59

Franck JE, Schwartzkroin PA (1985). Do kainate-lesioned hippocampi become epileptogenic? *Brain Res.* **329(1-2)**: 309-13.

Freir DB, Holscher C, Herron CE (2001). Blockade of long-term potentiation by beta-amyloid peptides in the CA1 region of the rat hippocampus in vivo. *J Neurophysiol.* **85(2)**: 708-13.

Freir DB, Herron CE (2003). Inhibition of L-type voltage dependent calcium channels causes impairment of long-term potentiation in the hippocampal CA1 region in vivo. *Brain Res.* **967(1-2)**: 27-36.

French PJ, O'Connor V, Voss K, Stean T, Hunt SP, Bliss TV (2001). Seizure-induced gene expression in area CA1 of the mouse hippocampus. *Eur J Neurosci.* **14(12)**: 2037-41.

Freund TF & Buzsaki G (1996). Interneurons of the hippocampus. *Hippocampus.* **6(4)**: 347-470.

Frey U, Krug M, Reymann KG, Matthies H (1988). Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. *Brain Res.* **452(1-2)**: 57-65.

Frey U, Krug M, Brodemann R, Reymann K, Matthies H (1989). Long-term potentiation induced in dendrites separated from rat's CA1 pyramidal somata does not establish a late phase. *Neurosci Lett.* **97(1-2)**: 135-9.

Frey U, Huang YY, Kandel ER (1993). Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science.* **260(5114)**: 1661-4.

Frey U, Frey S, Schollmeier F, Krug M (1996). Influence of actinomycin D, a RNA synthesis inhibitor, on long-term potentiation in rat hippocampal neurons in vivo and in vitro. *J Physiol.* **490 (Pt 3)**: 703-11.

Frey U & Morris RG (1997). Synaptic tagging and long-term potentiation. *Nature.* **385(6616)**: 533-6.

Frey U and Morris RG (1998). Weak before strong: dissociating synaptic tagging and plasticity-factor accounts of late-LTP. *Neuropharmacology.* **37(4-5)**: 545-52.

Frick KM, Fernandez SM (2003). Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. *Neurobiol Aging.* **24(4)**: 615-26.

Friedman DB, Hill S, Keller JW, Merchant NB, Levy SE, Coffey RJ, Caprioli RM (2004). Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. *Proteomics.* **4(3)**: 793-811.

Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K (2002). CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nat Cell Biol.* **4(8)**: 583-91.

Fukazawa Y, Saitoh Y, Ozawa F, Ohta Y, Mizuno K, Inokuchi K (2003). Hippocampal LTP is accompanied by enhanced F-actin content within the dendritic spine that is essential for late LTP maintenance in vivo. *Neuron.* **38(3)**: 447-60.

Fukuda M, Aruga J, Niinobe M, Aimoto S, Mikoshiba K (1994). Inositol-1,3,4,5-tetrakisphosphate binding to C2B domain of IP4BP/synaptotagmin II. *J Biol Chem.* **269(46)**: 29206-11.

Fukudo S, Abe K, Itoyama Y, Mochizuki S, Sawai T, Hongo M (1999). Psychophysiological stress induces heat shock cognate protein 70 messenger RNA in the hippocampus of rats. *Neuroscience.* **91(4)**: 1205-8.

Fukunaga K, Muller D, Miyamoto E (1996). CaM kinase II in long-term potentiation. *Neurochem Int.* **28(4)**: 343-58.

G

Gad H, Ringstad N, Low P, Kjaerulff O, Gustafsson J, Wenk M, Di Paolo G, Nemoto Y, Crun J, Ellisman MH, De Camilli P, Shupliakov O, Brodin L (2000). Fission and uncoating of synaptic clathrin-coated vesicles are perturbed by disruption of interactions with the SH3 domain of endophilin. *Neuron.* **27(2)**: 301-12.

Gade D, Thiermann J, Markowsky D, Rabus R (2003). Evaluation of two-dimensional difference gel electrophoresis for protein profiling. Soluble proteins of the marine bacterium Pirellula sp. strain 1. *J Mol Microbiol Biotechnol.* **5(4)**: 240-51.

Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F, *et al* (1995). Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature.* **373(6514)**: 523-7.

Gardner EB, Boitano JJ, Mancino NS, D'Amico DP (1975). Environmental enrichment and deprivation: effects on learning, memory and exploration. *Physiol Behav.* **14(3)**: 321-7.

Gauthier DJ, Gibbs BF, Rabah N, Lazure C (2004). Utilization of a new biotinylation reagent in the development of a nondiscriminatory investigative approach for the study of cell surface proteins. *Proteomics.* **4(12)**: 3783-90.

Geinisman Y, deToledo-Morrell L, Morrell F (1991). Induction of long-term potentiation is associated with an increase in the number of axospinous synapses with segmented postsynaptic densities. *Brain Res.* **566(1-2)**: 77-88.

Geinisman Y, Disterhoft JF, Gundersen HJ, McEchron MD, Persina IS, Power JM, van der Zee EA, West MJ (2000) Remodeling of hippocampal synapses after hippocampus-dependent associative learning. *Comp Neurol.* **417(1)**: 49-59.

Geinisman Y, Berry RW, Disterhoft JF, Power JM, Van der Zee EA (2001). Associative learning elicits the formation of multiple-synapse boutons. J Neurosci. 2001 **21(15)**: 5568-73

Gelfi C & Righetti PG (1983). Preparative isoelectric focusing in immobilized pH gradients. II. A case report. *J Biochem Biophys Methods.* **8(2)**: 157-72.

Gething MJ & Sambrook J (1992). Protein folding in the cell. *Nature.* **355(6355)**: 33-45.

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. *Mol Cell Proteomics.* **1(2)**: 91-8.

Ghirardi M, Montarolo PG, Kandel ER (1995). A novel intermediate stage in the transition between short- and long-term facilitation in the sensory to motor neuron synapse of aplysia. *Neuron.* **14(2)**: 413-20.

Ghosh A, Ginty DD, Bading H, Greenberg ME (1994). Calcium regulation of gene expression in neuronal cells. *J Neurobiol.* **25(3)**: 294-303.

Gianotti C, Nunzi MG, Gispen WH, Corradetti R (1992). Phosphorylation of the presynaptic protein B-50 (GAP-43) is increased during electrically induced long-term potentiation. *Neuron.* **8(5)**: 843-8.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998). Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science.* 1998 **279(5352)**: 870-3.

Gilbert RW, Costain WJ, Blanchard ME, Mullen KL, Currie RW, Robertson HA (2003). DNA microarray analysis of hippocampal gene expression measured twelve hours after hypoxia-ischemia in the mouse. *J Cereb Blood Flow Metab.* **23(10)**: 1195-211.

Globus A, Rosenzweig MR, Bennett EL, Diamond MC (1973). Effects of differential experience on dendritic spine counts in rat cerebral cortex. J Comp Physiol Psychol. 1973 Feb;82(2):175-81.

Goda Y (1997). SNAREs and regulated vesicle exocytosis. Proc *Natl Acad Sci U S A.* **94(3)**: 769-72.

Goldenring JR, Vallano ML, DeLorenzo RJ (1985). Phosphorylation of microtubule-associated protein 2 at distinct sites by calmodulin-dependent and cyclic-AMP-dependent kinases. *J Neurochem.* **45(3)**: 900-5.

Gorg A, Postel W, Gunther S (1988). The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis.* **9(9)**: 531-46.

Goshe MB, Blonder J, Smith RD (2003). Affinity labeling of highly hydrophobic integral membrane proteins for proteome-wide analysis. *J Proteome Res* **2(2)**: 153-61.

Gotow T, Miyaguchi K, Hashimoto PH (1991). Cytoplasmic architecture of the axon terminal: filamentous strands specifically associated with synaptic vesicles. *Neuroscience.* **40(2)**: 587-98.

Gozal E, Gozal D, Pierce WM, Thongboonkerd V, Scherzer JA, Sachleben LR Jr, Brittian KR, Guo SZ, Cai J, Klein JB (2002). Proteomic analysis of CA1 and CA3 regions of rat hippocampus and differential susceptibility to intermittent hypoxia. *J Neurochem* **83(2)**: 331-45.

Grant SG, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER (1992). Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science*. **258(5090)**: 1903-10.

Gray EG. Electron microscopy of synaptic contacts on dendrite spines of the cerebral cortex (1959). *Nature* **183(4675)**: 1592-3

Gray EG, Westrum LE, Burgoyne RD, Barron J (1982). Synaptic organisation and neuron microtubule distribution. *Cell Tissue Res.* **226(3)**: 579-88.

Greengard P, Jen J, Nairn AC, Stevens CF (1991). Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. *Science*. **253(5024)**: 1135-8.

Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science*. **259(5096)**: 780-5.

Greengauz-Roberts O, Stoppler H, Nomura S, Yamaguchi H, Goldenring JR, Podolsky RH, Lee JR, Dynan WS (2005). Saturation labeling with cysteine-reactive cyanine fluorescent dyes provides increased sensitivity for protein expression profiling of laser-microdissected clinical specimens. *Proteomics*. [Epub ahead of print]

Greenough WT & Volkmar FR (1973). Pattern of dendritic branching in occipital cortex of rats reared in complex environments. *Exp Neurol.* **40(2)**: 491-504.

Greenough WT, Volkmar FR, Juraska JM (1973). Effects of rearing complexity on dendritic branching in frontolateral and temporal cortex of the rat. *Exp Neurol*. **41(2)**: 371-8.

Greenough WT, McDonald JW, Parnisari RM, Camel JE (1986). Environmental conditions modulate degeneration and new dendrite growth in cerebellum of senescent rats. *Brain Res.* **380(1)**: 136-43.

Grewal SS, Fass DM, Yao H, Ellig CL, Goodman RH, Stork PJ (2000). Calcium and cAMP signals differentially regulate cAMP-responsive element-binding protein function via a Rap1-extracellular signal-regulated kinase pathway. *J Biol Chem.* **275(44)**: 34433-41.

Griffin TJ, Gygi SP, Rist B, Aebersold R, Loboda A, Jilkine A, Ens W, Standing KG (2001). Quantitative proteomic analysis using a MALDI quadrupole time-of-flight mass spectrometer. *Anal Chem.* **73(5)**: 978-86.

Griffith WH, Brown TH & Johnston D (1986). Voltage-clamp analysis of synaptic inhibition during long-term potentiation in hippocampus. *J Neurophysiol.* 55(4):767-75.

Grosshans DR, Clayton DA, Coultrap SJ, Browning MD (2002). LTP leads to rapid surface expression of NMDA but not AMPA receptors in adult rat CA1. *Nat Neurosci.* **5(1)**: 27-33.

Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM (1986). Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *J Biol Chem.* **261(13)**: 6084-9.

Guillaud L, Setou M, Hirokawa N (2003). KIF17 dynamics and regulation of NR2B trafficking in hippocampal neurons. *J Neurosci.* **23(1)**: 131-40.

Gulyas AI, Hajos N, Freund TF (1996). Interneurons containing calretinin are specialized to control other interneurons in the rat hippocampus. *J. Neurosci.* **16(10)**: 3397-411.

Gupta RS (1990). Sequence and structural homology between a mouse T-complex protein TCP-1 and the 'chaperonin' family of bacterial (GroEL, 60-65 kDa heat shock antigen) and eukaryotic proteins. *Biochem Int.* **20(4)**: 833-41.

Gurney ME, Heinrich SP, Lee MR, Yin HS (1986). Molecular cloning and expression of neuroleukin, a neurotrophic factor for spinal and sensory neurons. *Science*. **234(4776)**: 566-74.

H

Had L, Faivre-Sarrailh C, Legrand C, Mery J, Brugidou J, Rabie A (1994). Tropomyosin isoforms in rat neurons: the different developmental profiles and distributions of TM-4 and TMBr-3 are consistent with different functions. *J Cell Sci*. **107 (Pt 10)**: 2961-73.

Hall GF, Lee VM, Kosik KS (1991). Microtubule destabilization and neurofilament phosphorylation precede dendritic sprouting after close axotomy of lamprey central neurons. *Proc Natl Acad Sci U S A*. **88(11)**: 5016-20.

Halpain S, Hipolito A, Saffer L (1998). Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. *J Neurosci*. **18(23)**: 9835-44.

Han HQ, Nichols RA, Rubin MR, Bahler M, Greengard P (1991). Induction of formation of presynaptic terminals in neuroblastoma cells by synapsin IIb. *Nature*. **349(6311)**: 697-700.

Harding DI, Greensmith L, Mason M, Anderson PN, Vrbova G (1999). Overexpression of GAP-43 induces prolonged sprouting and causes death of adult motoneurons. *Eur J Neurosci*. **11(7)**: 2237-42.

Harris EW, Ganong AH, Cotman CW (1984). Long-term potentiation in the hippocampus involves activation of N-methyl-D-aspartate receptors. *Brain Res*. **323(1)**: 132-7.

Harris KM, Jensen FE, Tsao B (1992). Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages:

implications for the maturation of synaptic physiology and long-term potentiation. *J Neurosci.* **12(7)**: 2685-705.

Harvey J & Collingridge GL (1992). Thapsigargin blocks the induction of long-term potentiation in rat hippocampal slices. *Neurosci Lett.* **139(2)**: 197-200.

Haseloff RF, Krause E, Blasig IE (2003). Proteomics of brain endothelium. Separation of proteins by two-dimensional gel electrophoresis and identification by mass spectrometry. *Methods Mol Med.* **89**: 465-77.

Hata R, Masumura M, Akatsu H, Li F, Fujita H, Nagai Y, Yamamoto T, Okada H, Kosaka K, Sakanaka M, Sawada T (2001). Up-regulation of calcineurin Abeta mRNA in the Alzheimer's disease brain: assessment by cDNA microarray. *Biochem Biophys Res Commun.* **284(2)**: 310-6.

Hatada Y, Wu F, Sun ZY, Schacher S, Goldberg DJ (2000). Presynaptic morphological changes associated with long-term synaptic facilitation are triggered by actin polymerization at preexisting varicositis. *J Neurosci.* **20(13)**: RC82.

Haughey NJ, Nath A, Chan SL, Borchard AC, Rao MS, Mattson MP (2002). Disruption of neurogenesis by amyloid beta-peptide, and perturbed neural progenitor cell homeostasis, in models of Alzheimer's disease. *J Neurochem.* **83(6)**: 1509-24.

Hebb, D. O. (1949). The organization of behavior: A neuropsychological theory. New York: Wiley.

Henderson JT, Georgiou J, Jia Z, Robertson J, Elowe S, Roder JC, Pawson T. The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function. *Neuron.* **32(6)**: 1041-56.

Henningsen R, Gale BL, Straub KM, DeNagel DC (2002). Application of zwitterionic detergents to the solubilization of integral membrane proteins for two-dimensional gel electrophoresis and mass spectrometry. *Proteomics.* **2(11)**: 1479-88.

Herron CE, Lester RA, Coan EJ, Collingridge GL (1986). Frequency-dependent involvement of NMDA receptors in the hippocampus: a novel synaptic mechanism. *Nature*. **322(6076)**: 265-8.

Hestrin S (1992). Developmental regulation of NMDA receptor-mediated synaptic currents at a central synapse. *Nature*. **357(6380)**: 686-9.

Heydorn WE, Creed GJ, Goldman D, Kanter D, Merril CR, Jacobowitz DM (1983). Mapping and quantitation of proteins from discrete nuclei and other areas of the rat brain by two-dimensional gel electrophoresis. *J Neurosci*. **3(12)**: 2597-606.

Hicks RR, Numan S, Dhillon HS, Prasad MR, Seroogy KB (1997a). Alterations in BDNF and NT-3 mRNAs in rat hippocampus after experimental brain trauma. *Brain Res Mol Brain Res*. **48(2)**: 401-6.

Hicks A, Davis S, Rodger J, Helme-Guizon A, Laroche S, Mallet J (1997b). Synapsin I and syntaxin 1B: key elements in the control of neurotransmitter release are regulated by neuronal activation and long-term potentiation in vivo. *Neuroscience*. **79(2)**: 329-40.

Hilfiker S, Benfenati F, Doussau F, Nairn AC, Czernik AJ, Augustine GJ, Greengard P (2005). Structural domains involved in the regulation of transmitter release by synapsins. *J Neurosci*. **25(10)**: 2658-69.

Hitchcock AL, Krebber H, Frietze S, Lin A, Latterich M, Silver PA (2001). The conserved npl4 protein complex mediates proteasome-dependent membrane-bound transcription factor activation. *Mol Biol Cell*. **12(10)**: 3226-41.

Ho OH, Delgado JY, O'Dell TJ (2004). Phosphorylation of proteins involved in activity-dependent forms of synaptic plasticity is altered in hippocampal slices maintained in vitro. *J Neurochem*. **91(6)**: 1344-57.

Hoff SF (1986). Lesion-induced transneuronal plasticity in the adult rat hippocampus. *Neuroscience*. **19(4)**: 1227-33.

Holmes WR (1990). Is the function of dendritic spines to concentrate calcium? *Brain Res.* **519(1-2)**: 338-42.

Hong SJ, Li H, Becker KG, Dawson VL and Dawson TM (2004). Identification and analysis of plasticity-induced late-response genes. *Proc Natl Acad Sci U S A.* **101(7)**: 2145-50.

Hosaka M, Hammer RE, Sudhof TC (1999). A phospho-switch controls the dynamic association of synapsins with synaptic vesicles. *Neuron.* **24(2)**: 377-87.

Hoshi M, Ohta K, Gotoh Y, Mori A, Murofushi H, Sakai H, Nishida E (1992). Mitogen-activated-protein-kinase-catalyzed phosphorylation of microtubule-associated proteins, microtubule-associated protein 2 and microtubule-associated protein 4, induces an alteration in their function. *Eur J Biochem.* **203(1-2)**: 43-52.

Hotta A, Inatome R, Yuasa-Kawada J, Qin Q, Yamamura H, Yanagi S (2005). Critical role of collapsin response mediator protein-associated molecule CRAM for filopodia and growth cone development in neurons. *Mol Biol Cell.* **16(1)**: 32-9.

Hsia AY, Masliah E, McConlogue L, Yu GQ, Tatsuno G, Hu K, Kholodenko D, Malenka RC, Nicoll RA, Mucke L (1999). Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc Natl Acad Sci U S A.* **96(6)**: 3228-33.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996). Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science.* **274(5284)**: 99-102.

Hu GY, Hvalby O, Walaas SI, Albert KA, Skjeflo P, Andersen P, Greengard P (1987). Protein kinase C injection into hippocampal pyramidal cells elicits features of long term potentiation. *Nature.* **328(6129)**: 426-9.

Huang CC & Hsu KS (1999). Protein tyrosine kinase is required for the induction of long-term potentiation in the rat hippocampus. *J Physiol.* **520 Pt 3**: 783-96.

Huang YY, Li XC, Kandel ER (1994). cAMP contributes to mossy fiber LTP by initiating both a covalently mediated early phase and macromolecular synthesis-dependent late phase. *Cell.* **79(1)**: 69-79.

Huang LB, Lim R (1990). Identification of injury-induced mitotic cells in adult rat cerebral cortex by neuron-specific markers. *Brain Res Dev Brain Res.* **51(1)**: 123-7.

Huang XP, Hampson DR (2000). Inhibition of microtubule formation by metabotropic glutamate receptors. *J Neurochem.* **74(1)**: 104-13.

Hull M, Eistetter J, Fiebich BL, Bauer J (1999). Glutamate but not interleukin-6 influences the phosphorylation of tau in primary rat hippocampal neurons. *Neurosci Lett.* **261(1-2)**: 33-6.

Hulo S, Alberi S, Laux T, Muller D, Caroni P (2002). A point mutant of GAP-43 induces enhanced short-term and long-term hippocampal plasticity. *Eur J Neurosci.* **15(12)**: 1976-82.

Husi H, Ward MA, Choudhary JS, Blackstock WP, Grant SG (2000). Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat Neurosci.* **3(7)**:661-9.

Husi H & Grant SG (2001). Isolation of 2000-kDa complexes of N-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. J Neurochem. 2001 Apr;77(1):281-91.

Hussain I, Powell D, Howlett DR, Tew DG, Meek TD, Chapman C, Gloger IS, Murphy KE, Southan CD, Ryan DM, Smith TS, Simmons DL, Walsh FS, Dingwall C, Christie G (1999). Identification of a novel aspartic protease (Asp 2) as beta-secretase. *Mol Cell Neurosci.* **14(6)**: 419-27.

I

Ichas F, Jouaville LS, Mazat JP (1997). Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. *Cell.* **89(7)**: 1145-53.

Ickes BR, Pham TM, Sanders LA, Albeck DS, Mohammed AH, Granholm AC (2000). Long-term environmental enrichment leads to regional increases in neurotrophin levels in rat brain. *Exp Neurol.* **164(1)**: 45-52.

Igaz LM, Bekinschtein P, Izquierdo I, Medina JH (2004). One-trial aversive learning induces late changes in hippocampal CaMKIIalpha, Homer 1a, Syntaxin 1a and ERK2 protein levels. *Brain Res Mol Brain Res.* **132(1)**: 1-12.

Impey S, Mark M, Villacres EC, Poser S, Chavkin C, Storm DR (1996). Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. *Neuron.* **16(5)**: 973-82.

Impey S, Obrietan K, Storm DR (1999). Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. *Neuron.* **23(1)**: 11-4.

Inagaki N, Chihara K, Arimura N, Menager C, Kawano Y, Matsuo N, Nishimura T, Amano M, Kaibuchi K (2001). CRMP-2 induces axons in cultured hippocampal neurons. *Nat Neurosci.* **4(8)**: 781-2.

Inatome, R., Tsujimura, T., Hitomi, T., Mitsui, N., Hermann, P., Kuroda, S., Yamamura, H., and Yanagi, S. (2000). Identification of CRAM, a novel unc-33 gene family protein that associates with CRMP3 and protein-tyrosine kinase(s) in the developing rat brain. *J. Biol. Chem.* **275:** 27291-27302.

Inzitari R, Cabras T, Onnis G, Olmi C, Mastinu A, Sanna MT, Pellegrini MG, Castagnola M, Messana I (2005). Different isoforms and post-translational

modifications of human salivary acidic proline-rich proteins. *Proteomics*. **5(3)**: 805-15.

Irving EA, McCulloch J, Dewar D (1996). Intracortical perfusion of glutamate in vivo induces alterations of tau and microtubule-associated protein 2 immunoreactivity in the rat. *Acta Neuropathol (Berl)*. **92(2)**: 186-96.

Isaac JT, Nicoll RA, Malenka RC (1995). Evidence for silent synapses: implications for the expression of LTP. *Neuron*. **15(2)**: 427-34.

Israely I, Costa RM, Xie CW, Silva AJ, Kosik KS, Liu X (2004). Deletion of the neuron-specific protein delta-catenin leads to severe cognitive and synaptic dysfunction. *Curr Biol*. **14(18)**: 1657-63.

Itoh A, Akaike T, Sokabe M, Nitta A, Iida R, Olariu A, Yamada K, Nabeshima T (1999). Impairments of long-term potentiation in hippocampal slices of beta-amyloid-infused rats. *Eur J Pharmacol*. **382(3)**: 167-75.

Iuvone L, Geloso MC, Dell'Anna E (1996). Changes in open field behavior, spatial memory, and hippocampal parvalbumin immunoreactivity following enrichment in rats exposed to neonatal anoxia. *Exp Neurol*. **139(1)**: 25-33.

J

Jaffrey SR, Cohen NA, Rouault TA, Klausner RD, Snyder SH (1994). The iron-responsive element binding protein: a target for synaptic actions of nitric oxide. *Proc Natl Acad Sci U S A*. **91(26)**: 12994-8.

Jameson L, Caplow M (1981). Modification of microtubule steady-state dynamics by phosphorylation of the microtubule-associated proteins. *Proc Natl Acad Sci U S A*. **78(6)**: 3413-7.

Janigro D & Schwartzkroin PA (1988). Effects of GABA on CA3 pyramidal cell dendrites in rabbit hippocampal slices. *Brain Res*. **453(1-2)**: 265-74.

Jankowsky JL, Melnikova T, Fadale DJ, Xu GM, Slunt HH, Gonzales V, Younkin LH, Younkin SG, Borchelt DR, Savonenko AV (2005). Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. *J Neurosci.* **25(21)**: 5217-24.

Jarosch E, Taxis C, Volkwein C, Bordallo J, Finley D, Wolf DH, Sommer T (2002). Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat Cell Biol.* **4(2)**: 134-9.

Jaworski J, Biedermann IW, Lapinska J, Szklarczyk A, Figiel I, Konopka D, Nowicka D, Filipkowski RK, Hetman M, Kowalczyk A, Kaczmarek L (1999). Neuronal excitation-driven and AP-1-dependent activation of tissue inhibitor of metalloproteinases-1 gene expression in rodent hippocampus. *J Biol Chem.* 1999 Oct 1;274(40):28106-12.

Jenkins LW, Peters GW, Dixon CE, Zhang X, Clark RS, Skinner JC, Marion DW, Adelson PD, Kochanek PM (2002). Conventional and functional proteomics using large format two-dimensional gel electrophoresis 24 hours after controlled cortical impact in postnatal day 17 rats. *J Neurotrauma.* **19(6)**: 715-40.

Jia Z, Lu Y, Henderson J, Taverna F, Romano C, Abramow-Newerly W, Wojtowicz JM, Roder J (1998). Selective abolition of the NMDA component of long-term potentiation in mice lacking mGluR5. *Learn Mem.* **5(4-5)**: 331-43.

Jiang L, Lindpaintner K, Li HF, Gu NF, Langen H, He L, Fountoulakis M (2003a). Proteomic analysis of the cerebrospinal fluid of patients with schizophrenia. *Amino Acids.* **25**(1): 49-57.

Jiang W, Gu W, Brannstrom T, Rosqvist R, Wester P (2003b). Cortical neurogenesis in adult rats after transient middle cerebral artery occlusion. *Stroke.* **32(5)**: 1201-7.

Jimenez CR, Eyman M, Lavina ZS, Gioio A, Li KW, van der Schors RC, Geraerts WP, Giuditta A, Kaplan BB, van Minnen J (2002). Protein synthesis in synaptosomes: a proteomics analysis. *J Neurochem.* **81(4)**: 735-44.

Jin K, Mao XO, Eshoo MW, Nagayama T, Minami M, Simon RP, Greenberg DA (2001). Microarray analysis of hippocampal gene expression in global cerebral ischemia. *Ann Neurol.* **50(1)**: 93-103.

Jin K, Peel AL, Mao XO, Xie L, Cottrell BA, Henshall DC, Greenberg DA (2004). Increased hippocampal neurogenesis in Alzheimer's disease. *Proc Natl Acad Sci U S A.* **101(1)**: 343-7

Johansson BB & Ohlsson AL (1996). Environment, social interaction, and physical activity as determinants of functional outcome after cerebral infarction in the rat. *Exp Neurol.* **139(2)**: 322-7.

Johnston D & Brown TH (1984). The synaptic nature of the paroxysmal depolarizing shift in hippocampal neurons. *Ann Neurol.* **16** Suppl: S65-71.

Johnston D & Brown TH (1986). Control theory applied to neural networks illuminates synaptic basis of interictal epileptiform activity. *Adv Neurol.* **44**: 263-74

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(2)**: 142-9.

Jolas T, Zhang XS, Zhang Q, Wong G, Del Vecchio R, Gold L, Priestley T (2002). Long-term potentiation is increased in the CA1 area of the hippocampus of APP(swe/ind) CRND8 mice. *Neurobiol Dis.* **11(3)**: 394-409.

Jordan BA, Fernholz BD, Boussac M, Xu C, Grigorean G, Ziff EB, Neubert TA (2004). Identification and verification of novel rodent postsynaptic density proteins. *Mol Cell Proteomics.* **3(9)**: 857-71.

Junker H, Spate K, Suofu Y, Walther R, Schwarz G, Kammer W, Nordheim A, Walker LC, Runge U, Kessler C, Popa-Wagner A (2005). Proteomic identification of

the involvement of the mitochondrial rieske protein in epilepsy. *Epilepsia.* **46(3)**: 339-43.

K

Kaech S, Fischer M, Doll T, Matus A (1997). Isoform specificity in the relationship of actin to dendritic spines. *Neurosci.* **17(24)**: 9565-72.

Kaech S, Parmar H, Roelandse M, Bornmann C, Matus A (2001). Cytoskeletal microdifferentiation: a mechanism for organizing morphological plasticity in dendrites. *Proc Natl Acad Sci U S A.* **98(13)**: 7086-92.

Kaiser S, Foltz LA, George CA, Kirkwood SC, Bemis KG, Lin X, Gelbert LM, Nisenbaum LK (2004). Phencyclidine-induced changes in rat cortical gene expression identified by microarray analysis: implications for schizophrenia. *Neurobiol Dis.* **16(1)**: 220-35

Kaku Y, Yonekawa Y, Tsukahara T, Ogata N, Kimura T, Taniguchi T (1993). Alterations of a 200 kDa neurofilament in the rat hippocampus after forebrain ischemia. *J Cereb Blood Flow Metab.* **13(3)**: 402-8.

Kamme F, Salunga R, Yu J, Tran DT, Zhu J, Luo L, Bittner A, Guo HQ, Miller N, Wan J, Erlander M (2003). Single-cell microarray analysis in hippocampus CA1: demonstration and validation of cellular heterogeneity. *J Neurosci.* **23(9)**: 3607-15.

Kanai Y, Dohmae N, Hirokawa N (2004). Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. *Neuron.* **43(4)**: 513-25.

Kandel ER, Tauc L (1965). Heterosynaptic facilitation in neurones of the abdominal ganglion of Aplysia depilans. *J Physiol.* **181(1)**: 1-27.

Kanninen K, Goldsteins G, Auriola S, Alafuzoff I, Koistinaho J (2004). Glycosylation changes in Alzheimer's disease as revealed by a proteomic approach. *Neurosci Lett.* **367(2)**: 235-40.

Kaplan MS & Hinds JW (1977). Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science.* **197(4308)**: 1092-4.

Kapus G, Szekely JI, Durand J, Ruiz A, Tarnawa I (2000). AMPA receptor antagonists, GYKI 52466 and NBQX, do not block the induction of long-term potentiation at therapeutically relevant concentrations. *Brain Res Bull.* **52(6)**: 511-7.

Karp NA, Kreil DP, Lilley KS (2004a). Determining a significant change in protein expression with DeCyder during a pair-wise comparison using two-dimensional difference gel electrophoresis. *Proteomics.* **4(5)**: 1421-32.

Karp NA, Griffin JL, Lilley KS (2004b). Application of partial least squares discriminant analysis to two-dimensional difference gel studies in expression proteomics. *Proteomics.* **5(1)**: 81-90

Kasahara J, Fukunaga K, Miyamoto E (2001). Activation of calcium/calmodulin-dependent protein kinase IV in long term potentiation in the rat hippocampal CA1 region. *J Biol Chem.* **276(26)**: 24044-50

Katsel PL, Davis KL, Haroutunian V (2005). Large-scale microarray studies of gene expression in multiple regions of the brain in schizophrenia and Alzheimer's disease. *Int Rev Neurobiol.* **63** :41-82.

Katsuki H, Kaneko S, Satoh M (1992). Involvement of postsynaptic G proteins in hippocampal long-term potentiation. *Brain Res.* **581(1)**: 108-14.

Kauer JA, Malenka RC, Nicoll RA (1988). A persistent postsynaptic modification mediates long-term potentiation in the hippocampus. *Neuron.* **1(10)**: 911-7.

Kempermann G, Kuhn HG, Gage FH (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature.* **386(6624)**:493-5.

Kempermann G, Gage FH (1999). Experience-dependent regulation of adult hippocampal neurogenesis: effects of long-term stimulation and stimulus withdrawal. *Hippocampus*. **9(3)**: 321-32.

Kempermann G, Gast D, Gage FH (2002). Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Ann Neurol*. **52(2)**: 135-43.

Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH (2003). Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development*. **130(2)**: 391-9.

Kessler M, Arai A, Vanderklish P, Lynch G (11991). Failure to detect changes in AMPA receptor binding after long-term potentiation. *Brain Res*. **560(1-2)**: 337-41.

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(4)**: 451-60.

Kiessling M, Dienel GA, Jacewicz M, Pulsinelli WA (1986). Protein synthesis in postischemic rat brain: a two-dimensional electrophoretic analysis. *J Cereb Blood Flow Metab*. **6(6)**: 642-9.

Kim CH, Lisman JE (1999). A role of actin filament in synaptic transmission and long-term potentiation. *J Neurosci*. **19(11)**: 4314-24.

Kirchner L, Weitzdoerfer R, Hoeger H, Url A, Schmidt P, Engelmann M, Villar SR, Fountoulakis M, Lubec G, Lubec B (2004). Impaired cognitive performance in neuronal nitric oxide synthase knockout mice is associated with hippocampal protein derangements. *Nitric Oxide*. **11(4)**: 316-30.

Klein RL, McNamara RK, King MA, Lenox RH, Muzyczka N, Meyer EM (1999). Generation of aberrant sprouting in the adult rat brain by GAP-43 somatic gene transfer. *Brain Res*. **832(1-2)**: 136-44.

Klein JB, Gozal D, Pierce WM, Thongboonkerd V, Scherzer JA, Sachleben LR, Guo SZ, Cai J, Gozal E (2003). Proteomic identification of a novel protein regulated in CA1 and CA3 hippocampal regions during intermittent hypoxia. *Respir Physiol Neurobiol.* 2003 **136(2-3)**: 91-103.

Kleppisch T, Voigt V, Allmann R, Offermanns S (2001). G(alpha)q-deficient mice lack metabotropic glutamate receptor-dependent long-term depression but show normal long-term potentiation in the hippocampal CA1 region. *J Neurosci.* **21(14)**: 4943-8.

Knowles WD (1992). Normal anatomy and neurophysiology of the hippocampal formation. *J Clin Neurophysiol.* **9(2)**: 252-63.

Kobayashi S, Ohashi Y, Ando S (2002). Effects of enriched environments with different durations and starting times on learning capacity during aging in rats assessed by a refined procedure of the Hebb-Williams maze task. *J Neurosci Res.* **70(3)**: 340-6.

Konopka D, Nowicka D, Filipkowski RK, Kaczmarek L (1995). Kainate-evoked secondary gene expression in the rat hippocampus. *Neurosci Lett.* **185(3)**: 167-70.

Korkotian E & Segal M (2001). Regulation of dendritic spine motility in cultured hippocampal neurons. *J Neurosci.* **21(16)**: 6115-24.

Kornau HC, Schenker LT, Kennedy MB, Seeburg PH (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science.* **269(5231)**: 1737-40.

Koski L, Mernar TJ, Dobkin BH (2004). Immediate and long-term changes in corticomotor output in response to rehabilitation: correlation with functional improvements in chronic stroke. *Neurorehabil Neural Repair* **18(4)**: 230-49.

Kramar EA & Lynch G (2003). Developmental and regional differences in the consolidation of long-term potentiation. *Neuroscience.* **118(2)**: 387-98.

Krapfenbauer K, Berger M, Friedlein A, Lubec G, Fountoulakis M (2001a). Changes in the levels of low-abundance brain proteins induced by kainic acid. *Eur J Biochem.* **268(12)**: 3532-7.

Krapfenbauer K, Berger M, Lubec G, Fountoulakis M (2001b). Changes in the brain protein levels following administration of kainic acid. *Electrophoresis.* **22(10)**: 2086-91.

Krapfenbauer K, Fountoulakis M, Lubec G (2003). A rat brain protein expression map including cytosolic and enriched mitochondrial and microsomal fractions. *Electrophoresis.* **24(11)**: 1847-70.

Krishna RG & Wold F (1993). Post-translational modification of proteins. *Adv Enzymol Relat Areas Mol Biol.* **67**: 265-98.

Krucker T, Siggins GR, Halpain S (2000). Dynamic actin filaments are required for stable long-term potentiation (LTP) in area CA1 of the hippocampus. *Proc Natl Acad Sci U S A.* **97(12)**: 6856-61.

Kuenzi FM, Fitzjohn SM, Morton RA, Collingridge GL, Seabrook GR (2000). Reduced long-term potentiation in hippocampal slices prepared using sucrose-based artificial cerebrospinal fluid. *J Neurosci Methods.* **100(1-2)**: 117-22.

Kuhnt U, Mihaly A, Joo F (1985). Increased binding of calcium in the hippocampal slice during long-term potentiation. *Neurosci Lett.* **53(2)**: 149-54.

Kullmann DM & Nicoll RA (1992). Long-term potentiation is associated with increases in quantal content and quantal amplitude. *Nature.* **357(6375)**: 240-4.

Kuo CC, Kuo CW, Liang CM, Liang SM (2005). A transcriptomic and proteomic analysis of the effect of CpG-ODN on human THP-1 monocytic leukemia cells. *Proteomics.* **5(4)**: 894-906.

Kwak S & Matus A (1988). Denervation induces long-lasting changes in the distribution of microtubule proteins in hippocampal neurons. *J Neurocytol.* **17(2)**: 189-95.

Kwong JM, Lam TT, Caprioli J (2003). Hyperthermic pre-conditioning protects retinal neurons from N-methyl-D-aspartate (NMDA)-induced apoptosis in rat. *Brain Res.* **970(1-2)**: 119-30.

L

Lachance PE and Chaudhuri A (2004). Microarray analysis of developmental plasticity in monkey primary visual cortex. *J Neurochem.* **88(6)**: 1455-69

Lang C, Barco A, Zablow L, Kandel ER, Siegelbaum SA, Zakharenko SS (2004). Transient expansion of synaptically connected dendritic spines upon induction of hippocampal long-term potentiation. *Proc Natl Acad Sci U S A* **101(47):**1 6665-70

Larson J, Wong D, Lynch G (1986). Patterned stimulation at the theta frequency is optimal for the induction of hippocampal long-term potentiation. *Brain Res.* 1986 **368(2)**: 347-50.

Larson J, Lynch G, Games D, Seubert P (1999). Alterations in synaptic transmission and long-term potentiation in hippocampal slices from young and aged PDAPP mice. *Brain Res.* **840(1-2)**: 23-35.

Latterich M, Frohlich KU, Schekman R (1995). Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. *Cell.* **82(6)**: 885-93.

Lazarov O, Robinson J, Tang YP, Hairston IS, Korade-Mirnics Z, Lee VM, Hersh LB, Sapolsky RM, Mirnics K, Sisodia SS (2005). Environmental enrichment reduces Abeta levels and amyloid deposition in transgenic mice. *Cell.* **120(5)**: 701-13.

Leahy JC, Luo Y, Kent CS, Meiri KF, Vallano ML (1993). Demonstration of presynaptic protein kinase C activation following long-term potentiation in rat hippocampal slices. *Neuroscience*. **52(3)**: 563-74.

Lee VM, Carden MJ, Schlaepfer WW, Trojanowski JQ (1987). Monoclonal antibodies distinguish several differentially phosphorylated states of the two largest rat neurofilament subunits (NF-H and NF-M) and demonstrate their existence in the normal nervous system of adult rats. *J Neurosci*. **7(11)**: 3474-88.

Lee SH, Liu L, Wang YT, Sheng M (2002). Clathrin adaptor AP2 and NSF interact with overlapping sites of GluR2 and play distinct roles in AMPA receptor trafficking and hippocampal LTD. *Neuron*. **36(4)**: 661-74.

Leggio MG, Mandolesi L, Federico F, Spirito F, Ricci B, Gelfo F, Petrosini L (2005). Environmental enrichment promotes improved spatial abilities and enhanced dendritic growth in the rat. *Behav Brain Res*. **May 20**

Lehrmann E, Hyde TM, Vawter MP, Becker KG, Kleinman JE, Freed WJ (2003). The use of microarrays to characterize neuropsychiatric disorders: postmortem studies of substance abuse and schizophrenia. *Curr Mol Med*. **3(5)**: 437-46.

Leil TA, Ossadtchi A, Nichols TE, Leahy RM, Smith DJ (2003). Genes regulated by learning in the hippocampus. *J Neurosci Res*. **71(6)**: 763-8.

Leinekugel X, Khalilov I, McLean H, Caillard O, Gaiarsa JL, Ben-Ari Y, Khazipov R (1999). GABA is the principal fast-acting excitatory transmitter in the neonatal brain. *Adv Neurol*. **79**: 189-201

Le Naour F, Hohenkirk L, Grolleau A, Misek DE, Lescure P, Geiger JD, Hanash S, Beretta L (2001). Profiling changes in gene expression during differentiation and maturation of monocyte-derived dendritic cells using both oligonucleotide microarrays and proteomics. *J Biol Chem*. **276(21)**:17920-31

Leuner B, Falduto J, Shors TJ (2003). Associative memory formation increases the observation of dendritic spines in the hippocampus. *J Neurosci.* **23(2)**: 659-65.

Levy M, Faas GC, Saggau P, Craigen WJ, Sweatt JD (2003). Mitochondrial regulation of synaptic plasticity in the hippocampus. J Biol Chem. 2003 May 16;278(20):17727-34.

Lewis D, Teyler TJ (1986). Anti-S-100 serum blocks long-term potentiation in the hippocampal slice. *Brain Res.* **383(1-2)**: 159-64.

Lewis J, McGowan E, Rockwood J, Melrose H, Nacharaju P, Van Slegtenhorst M, Gwinn-Hardy K, Paul Murphy M, Baker M, Yu X, Duff K, Hardy J, Corral A, Lin WL, Yen SH, Dickson DW, Davies P, Hutton M (2000). Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nat Genet.* **25(4)**: 402-5.

Li W, Herman RK, Shaw JE (1992). Analysis of the Caenorhabditis elegans axonal guidance and outgrowth gene unc-33. *Genetics.* **132(3)**: 675-89.

Li C, Takei K, Geppert M, Daniell L, Stenius K, Chapman ER, Jahn R, De Camilli P, Sudhof TC (1994). Synaptic targeting of rabphilin-3A, a synaptic vesicle Ca2+/phospholipid-binding protein, depends on rab3A/3C. *Neuron.* **13(4)**: 885-98.

Li KW, Hornshaw MP, Van Der Schors RC, Watson R, Tate S, Casetta B, Jimenez CR, Gouwenberg Y, Gundelfinger ED, Smalla KH, Smit AB (2004a). Proteomics analysis of rat brain postsynaptic density. Implications of the diverse protein functional groups for the integration of synaptic physiology. *J Biol Chem.* **279(2)**: 987-1002.

Li HH, Lee SM, Cai Y, Sutton RL, Hovda DA (2004). Differential gene expression in hippocampus following experimental brain trauma reveals distinct features of moderate and severe injuries. *J Neurotrauma.* **21(9)**: 1141-53.

Liao D, Jones A, Malinow R (1992). Direct measurement of quantal changes underlying long-term potentiation in CA1 hippocampus. *Neuron*. **9(6)**: 1089-97.

Liao D, Hessler NA, Malinow R (1995). Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature*. **375(6530)**: 400-4.

Liberatori S, Canas B, Tani C, Bini L, Buonocore G, Godovac-Zimmermann J, Mishra OP, Delivoria-Papadopoulos M, Bracci R, Pallini V (2004). Proteomic approach to the identification of voltage-dependent anion channel protein isoforms in guinea pig brain synaptosomes. *Proteomics*. **4(5)**: 1335-40.

Lim W, Isaac JT (2005). ATP hydrolysis is required for the rapid regulation of AMPA receptors during basal synaptic transmission and long-term synaptic plasticity. *Neuropharmacology*. **48(7)**: 949-55.

Lin B, Kramar EA, Bi X, Brucher FA, Gall CM, Lynch G (2005). Theta stimulation polymerizes actin in dendritic spines of hippocampus. *J Neurosci*. **25(8)**: 2062-9.

Linden DJ, Wong KL, Sheu FS, Routtenberg A (1988). NMDA receptor blockade prevents the increase in protein kinase C substrate (protein F1) phosphorylation produced by long-term potentiation. *Brain Res*. **458(1)**: 142-6.

Liu J, Solway K, Messing RO, Sharp FR (1998). Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J Neurosci* **18(19)**: 7768-78.

Lledo PM, Zhang X, Sudhof TC, Malenka RC, Nicoll RA (1998). Postsynaptic membrane fusion and long-term potentiation. *Science*. **279(5349)**: 399-403.

Lomax MI, Huang L, Cho Y, Gong TL, Altschuler RA (2000). Differential display and gene arrays to examine auditory plasticity. *Hear Res*. **147(1-2)**: 293-302.

Long Y, Zou L, Liu H, Lu H, Yuan X, Robertson CS, Yang K (2003). Altered expression of randomly selected genes in mouse hippocampus after traumatic brain injury. *J Neurosci Res.* **71(5)**: 710-20.

Lopez-Colome AM & Casas A (1984). Kainate reversibly aggregates brain tubulin in vitro. *Biochem Biophys Res Commun.* **122(3)**: 925-31.

Lopez-Molina L, Boddeke H, Muller D (1993). Blockade of long-term potentiation and of NMDA receptors by the protein kinase C antagonist calphostin C. *Naunyn Schmiedebergs Arch Pharmacol.* **348(1)**: 1-6.

Lorente de No R (1934). Studies on the structure of the cerebral cortex. II. Continuation of the study of the Ammonic system. *J. Psychologie Neurologie.* **46**: 113-177.

Loscher W & Schwark WS (1987). Further evidence for abnormal GABAergic circuits in amygdala-kindled rats. *Brain Res.* **420(2)**: 385-90.

Lovinger DM, Colley PA, Akers RF, Nelson RB, Routtenberg A (1986). Direct relation of long-term synaptic potentiation to phosphorylation of membrane protein F1, a substrate for membrane protein kinase C. *Brain Res.* **399(2)**: 205-11.

Lovinger DM, Wong KL, Murakami K, Routtenberg A (1987). Protein kinase C inhibitors eliminate hippocampal long-term potentiation. *Brain Res.* **436(1)**: 177-83.

Lu YF, Tomizawa K, Moriwaki A, Hayashi Y, Tokuda M, Itano T, Hatase O, Matsui H (1996). Calcineurin inhibitors, FK506 and cyclosporin A, suppress the NMDA receptor-mediated potentials and LTP, but not depotentiation in the rat hippocampus. *Brain Res.* **729(1)**: 142-6.

Lu YM, Jia Z, Janus C, Henderson JT, Gerlai R, Wojtowicz JM, Roder JC (1997). Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J Neurosci.* **17(13)**: 5196-205.

Lu YM, Roder JC, Davidow J, Salter MW (1998). Src activation in the induction of long-term potentiation in CA1 hippocampal neurons. *Science*. **279(5355)**: 1363-7.

Lu YF, Kojima N, Tomizawa K, Moriwaki A, Matsushita M, Obata K, Matsui H (1999). Enhanced synaptic transmission and reduced threshold for LTP induction in fyn-transgenic mice. *Eur J Neurosci*. **11(1)**: 75-82.

Lu L, Bao G, Chen H, Xia P, Fan X, Zhang J, Pei G, Ma L (2003). Modification of hippocampal neurogenesis and neuroplasticity by social environments. *Exp Neurol*. **183(2)**: 600-9.

Lubec G, Nonaka M, Krapfenbauer K, Gratzer M, Cairns N, Fountoulakis M (1999). Expression of the dihydropyrimidinase related protein 2 (DRP-2) in Down syndrome and Alzheimer's disease brain is downregulated at the mRNA and dysregulated at the protein level. *J Neural Transm Suppl*. **57**: 161-77.

Lu L, Bao G, Chen H, Xia P, Fan X, Zhang J, Pei G, Ma L (2003). Modification of hippocampal neurogenesis and neuroplasticity by social environments. *Exp Neurol*. **183(2)**: 600-9.

Luo Y, Long JM, Lu C, Chan SL, Spangler EL, Mascarucci P, Raz A, Longo DL, Mattson MP, Ingram DK, Weng NP (2002). A link between maze learning and hippocampal expression of neuroleukin and its receptor gp78. *J Neurochem*. **80(2)**: 354-61.

Luthi A, Chittajallu R, Duprat F, Palmer MJ, Benke TA, Kidd FL, Henley JM, Isaac JT, Collingridge GL (1999). Hippocampal LTD expression involves a pool of AMPARs regulated by the NSF-GluR2 interaction. *Neuron*. **24(2)**: 389-99.

Lynch G, Kessler M, Halpain S, Baudry M (1983). Biochemical effects of high-frequency synaptic activity studied with in vitro slices. *Fed Proc*. **42(12)**: 2886-90.

M

Mabuchi T, Kitagawa K, Kuwabara K, Takasawa K, Ohtsuki T, Xia Z, Storm D, Yanagihara T, Hori M, Matsumoto M (2001). Phosphorylation of cAMP response element-binding protein in hippocampal neurons as a protective response after exposure to glutamate in vitro and ischemia in vivo. *J Neurosci.* **21(23)**: 9204-13.

Malenka RC, Madison DV, Nicoll RA (1986). Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature.* **321(6066)**: 175-7.

Malenka RC, Kauer JA, Zucker RS, Nicoll RA (1988). Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. *Science.* **242(4875)**: 81-4.

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature.* **340(6234)**: 554-7.

Maletic-Savatic M, Malinow R, Svoboda K (1999). Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science* **283(5409)**: 1923-7.

Malinow R, Madison DV, Tsien RW (1988). Persistent protein kinase activity underlying long-term potentiation. *Nature.* **335(6193)**: 820-4.

Malinow R, Schulman H, Tsien RW (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science.* **245(4920)**: 862-6.

Malinow R & Tsien RW (1990). Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature.* **346(6280)**: 177-80.

Malinow R, Malenka RC (2002). AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci.* **25**: 103-26

Malinow R (2003). AMPA receptor trafficking and long-term potentiation. *Philos Trans R Soc Lond B Biol Sci.* **358(1432)**: 707-14.

Man HY, Ju W, Ahmadian G, Wang YT (2000). Intracellular trafficking of AMPA receptors in synaptic plasticity. *Cell Mol Life Sci.* **57(11)**: 1526-34.

Man HY, Wang Q, Lu WY, Ju W, Ahmadian G, Liu L, D'Souza S, Wong TP, Taghibiglou C, Lu J, Becker LE, Pei L, Liu F, Wymann MP, MacDonald JF, Wang YT (2003). Activation of PI3-kinase is required for AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. *Neuron.* **38(4)**: 611-24.

Manahan-Vaughan D (2000). Long-term depression in freely moving rats is dependent upon strain variation, induction protocol and behavioral state. *Cereb Cortex.* **10(5)**: 482-7.

Mann KM, Thorngate FE, Katoh-Fukui Y, Hamanaka H, Williams DL, Fujita S, Lamb BT (2004). Independent effects of APOE on cholesterol metabolism and brain Abeta levels in an Alzheimer disease mouse model. *Hum Mol Genet* **13(17)**: 1959-68.

Mansuy IM, Mayford M, Jacob B, Kandel ER, Bach ME (1998). Restricted and regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory. *Cell.* **92(1)**: 39-49.

Manzoni OJ, Weisskopf MG, Nicoll RA (1994). MCPG antagonizes metabotropic glutamate receptors but not long-term potentiation in the hippocampus. *Eur J Neurosci.* **6(6)**: 1050-4.

Marco S, Carrascosa JL, Valpuesta JM (1994). Reversible interaction of beta-actin along the channel of the TCP-1 cytoplasmic chaperonin. *Biophys J.* **67(1)**: 364-8.

Martin LJ, Blackstone CD, Huganir RL, Price DL (1992). Cellular localization of a metabotropic glutamate receptor in rat brain. *Neuron.* **9(2)**: 259-70.

Martin JE, Swash M, Mather K, Leigh PN (1993). Expression of the human groEL stress-protein homologue in the brain and spinal cord. *J Neurol Sci.* **118(2)**: 202-6.

Martin KC, Barad M, Kandel ER.Local protein synthesis and its role in synapse-specific plasticity (2000). *Curr Opin Neurobiol.* **10(5)**: 587-92.

Matsui H, Doi A, Itano T, Shimada M, Wang JH, Hatase O (1987). Immunohistochemical localization of calcineurin, calmodulin-stimulated phosphatase, in the rat hippocampus using a monoclonal antibody. *Brain Res.* **402(1)**: 193-6.

Matsushita M, Tomizawa K, Moriwaki A, Li ST, Terada H, Matsui H (2001). A high-efficiency protein transduction system demonstrating the role of PKA in long-lasting long-term potentiation. *J Neurosci.* **21(16)**: 6000-7.

Matsuzaki M, Honkura N, Ellis-Davies GC, Kasai H (2004). Structural basis of long-term potentiation in single dendritic spines. *Nature.* **429(6993)**: 761-6.

Matthies H & Reymann KG (1993). Protein kinase A inhibitors prevent the maintenance of hippocampal long-term potentiation. *Neuroreport.* **4(6)**: 712-4.

Matus A, Ackermann M, Pehling G, Byers HR, Fujiwara K (1982). High actin concentrations in brain dendritic spines and postsynaptic densities. *Proc Natl Acad Sci U S A.* **79(23)**: 7590-4.

Matus A, Huber G, Bernhardt R (1983). Neuronal microdifferentiation. *Cold Spring Harb Symp Quant Biol.* **48 Pt 2**: 775-82.

Matus A, Brinkhaus H, Wagner U (2000). Actin dynamics in dendritic spines: a form of regulated plasticity at excitatory synapses. *Hippocampus.* **10(5)**: 555-60.

Matzilevich DA, Rall JM, Moore AN, Grill RJ, Dash PK (2002). High-density microarray analysis of hippocampal gene expression following experimental brain injury. *J Neurosci Res.* **67(5)**: 646-63.

Mayer ML & Westbrook GL (1987). Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. *J Physiol.* **394**: 501-27.

Mazzucchelli C & Brambilla R (2000). Ras-related and MAPK signalling in neuronal plasticity and memory formation. *Cell Mol Life Sci.* **57(4)**: 604-11.

McAllister AK, Katz LC, Lo DC (1999). Neurotrophins and synaptic plasticity. *Annu Rev Neurosci.* **22**: 295-318.

McCall MA, Gregg RG, Behringer RR, Brenner M, Delaney CL, Galbreath EJ, Zhang CL, Pearce RA, Chiu SY, Messing A (1996). Targeted deletion in astrocyte intermediate filament (Gfap) alters neuronal physiology. *Proc Natl Acad Sci U S A.* **93(13)**: 6361-6.

McGuinness N, Anwyl R, Rowan M (1991). The effects of trans-ACPD on long-term potentiation in the rat hippocampal slice. *Neuroreport.* **2(11)**: 688-90.

McLennan H (1983). Receptors for the excitatory amino acids in the mammalian central nervous system. *Prog Neurobiol.* **20(3-4)**: 251-71.

McMillan TM, Powell GE, Janota I, Polkey CE (1987). Relationships between neuropathology and cognitive functioning in temporal lobectomy patients. *J Neurol Neurosurg Psychiatry.* **50(2)**: 167-76

McNaughton BL, Barnes CA, Rao G, Baldwin J, Rasmussen M (1986). Long-term enhancement of hippocampal synaptic transmission and the acquisition of spatial information. *J Neurosci.* **6(2)**: 563-71.

McWilliams JR & Lynch G (1983). Rate of synaptic replacement in denervated rat hippocampus declines precipitously from the juvenile period to adulthood. *Science.* **221(4610)**: 572-4.

Melchers BP, Pennartz CM, Lopes da Silva FH (1987). Differential effects of elevated extracellular calcium concentrations on field potentials in dentate gyrus and CA1 of the rat hippocampal slice preparation. *Neurosci Lett.* **77(1)**: 37-42.

Meldrum BS & Chapman AG (1986). Benzodiazepine receptors and their relationship to the treatment of epilepsy. *Epilepsia.***27 Suppl 1**: S3-13

Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron.* **35(1)**: 121-33.

Meshul CK & Hopkins WF (1990). Presynaptic ultrastructural correlates of long-term potentiation in the CA1 subfield of the hippocampus. *Brain Res.* **514(2)**: 310-9.

Metodiev MV, Timanova A, Stone DE (2004). Differential phosphoproteome profiling by affinity capture and tandem matrix-assisted laser desorption/ionization mass spectrometry. *Proteomics.* **4(5)**: 1433-8.

Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RG, Morrison JH, O'Dell TJ, Grant SG (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature.* **396(6710)**: 433-9.

Mihaly A, Oravecz T, Olah Z, Rapp UR (1991). Immunohistochemical localization of raf protein kinase in dendritic spines and spine apparatuses of the rat cerebral cortex. *Brain Res.* **547(2)**: 309-14.

Miklos D, Caplan S, Mertens D, Hynes G, Pitluk Z, Kashi Y, Harrison-Lavoie K, Stevenson S, Brown C, Barrell B, *et al* (1994). Primary structure and function of a

second essential member of the heterooligomeric TCP1 chaperonin complex of yeast, TCP1 beta. *Proc Natl Acad Sci U S A.* **91(7)**: 2743-7.

Miles R & Wong RK (1984). Unitary inhibitory synaptic potentials in the guinea-pig hippocampus in vitro. *J Physiol.* **356**: 97-113.

Miles R & Wong RK (1987). Inhibitory control of local excitatory circuits in the guinea-pig hippocampus. *J Physiol.* **388**: 611-29.

Miller EK, Raese JD, Morrison-Bogorad M (1991). Expression of heat shock protein 70 and heat shock cognate 70 messenger RNAs in rat cortex and cerebellum after heat shock or amphetamine treatment. *J Neurochem.* **56(6)**: 2060-71

Miller S, Yasuda M, Coats JK, Jones Y, Martone ME, Mayford M (2002). Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. *Neuron.* **36(3)**: 507-19..

Milner B & Penfield W (1955). The effect of hippocampal lesions on recent memory. Trans Am Neurol Assoc. (80th meeting, 42-48).

Mirnics K, Korade Z, Arion D, Lazarov O, Unger T, Macioce M, Sabatini M, Terrano D, Douglass KC, Schor NF, Sisodia SS (2005). Presenilin-1-dependent transcriptome changes. *J Neurosci.* **25(6)**: 1571-8.

Misumi S, Fuchigami T, Takamune N, Takahashi I, Takama M, Shoji S (2002). Three isoforms of cyclophilin A associated with human immunodeficiency virus type 1 were found by proteomics by using two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Virol.* **76(19)**: 10000-8.

Mitchell SJ & Ryan TA (2004). Syntaxin-1A is excluded from recycling synaptic vesicles at nerve terminals. *J Neurosci.* **24(20)**: 4884-8.

Miura M, Watanabe M, Offermanns S, Simon MI, Kano M (2002). Group I metabotropic glutamate receptor signaling via Galpha q/Galpha 11 secures the induction of long-term potentiation in the hippocampal area CA1. *J Neurosci.* **22(19)**: 8379-90.

Miyamoto E, Fukunaga K (1996). A role of Ca2+/calmodulin-dependent protein kinase II in the induction of long-term potentiation in hippocampal CA1 area. *Neurosci Res.* **24(2)**: 117-22.

Mochida H, Sato K, Sasaki S, Yazawa I, Kamino K, Momose-Sato Y (2001). Effects of anisomycin on LTP in the hippocampal CA1: long-term analysis using optical recording. *Neuroreport.* **12(5)**: 987-91.

Mody M, Cao Y, Cui Z, Tay KY, Shyong A, Shimizu E, Pham K, Schultz P, Welsh D, Tsien JZ (2001). Genome-wide gene expression profiles of the developing mouse hippocampus. *Proc Natl Acad Sci U S A.* **98(15)**: 8862-7.

Moechars D, Dewachter I, Lorent K, Reverse D, Baekelandt V, Naidu A, Tesseur I, Spittaels K, Haute CV, Checler F, Godaux E, Cordell B, Van Leuven F (1999). Early phenotypic changes in transgenic mice that overexpress different mutants of amyloid precursor protein in brain. *J Biol Chem.* **274(10)**: 6483-92.

Mohammed AH, Henriksson BG, Soderstrom S, Ebendal T, Olsson T, Seckl JR (1993). Environmental influences on the central nervous system and their implications for the aging rat. *Behav Brain Res.* **57(2)**: 183-91.

Moir D, Stewart SE, Osmond BC, Botstein D (1982). Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics.* **100(4)**: 547-63.

Moga DE, Calhoun ME, Chowdhury A, Worley P, Morrison JH, Shapiro ML (2004). Activity-regulated cytoskeletal-associated protein is localized to recently activated excitatory synapses. *Neuroscience.* **125(1)**: 7-11.

Molteni R, Ying Z, Gomez-Pinilla F (2002). Differential effects of acute and chronic exercise on plasticity-related genes in the rat hippocampus revealed by microarray. *Eur J Neurosci.* **16(6)**: 1107-16.

Moon IS, Park IS, Schenker LT, Kennedy MB, Moon JI, Jin I (2001). Presence of both constitutive and inducible forms of heat shock protein 70 in the cerebral cortex and hippocampal synapses. *Cereb Cortex.* **11(3)**: 238-48.

Moore BW, Perez VJ, Gehring M (1968). Assay and regional distribution of a soluble protein characteristic of the nervous system. *J Neurochem* **15(4)**: 265-72.

Morgan SL, Teyler TJ (1999). VDCCs and NMDARs underlie two forms of LTP in CA1 hippocampus in vivo. *J Neurophysiol.* **82(2)**: 736-40.

Morozov A, Muzzio IA, Bourtchouladze R, Van-Strien N, Lapidus K, Yin D, Winder DG, Adams JP, Sweatt JD, Kandel ER (2003). Rap1 couples cAMP signaling to a distinct pool of p42/44MAPK regulating excitability, synaptic plasticity, learning, and memory. *Neuron.* **39(2)**: 309-25.

Morris RG, Anderson E, Lynch GS, Baudry M (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature.* **319(6056)**: 774-6.

Morris RG (1989). Synaptic plasticity and learning: selective impairment of learning rats and blockade of long-term potentiation in vivo by the N-methyl-D-aspartate receptor antagonist AP5. *J Neurosci.* **9(9)**: 3040-57.

Morrison-Bogorad M, Pardue S, McIntire DD, Miller EK (1994). Cell size and the heat-shock response in rat brain. *J Neurochem.* **63(3)**: 857-67.

Moser EI, Krobert KA, Moser MB, Morris RG (1998). Impaired spatial learning after saturation of long-term potentiation. *Science.* **281(5385)**: 2038-42.

Mouledous L, Hunt S, Harcourt R, Harry J, Williams KL, Gutstein HB (2003). Navigated laser capture microdissection as an alternative to direct histological staining for proteomic analysis of brain samples. *Proteomics*. **3(5)**: 610-5.

Mulkeen D, Anwyl R, Rowan MJ (1987). Enhancement of long-term potentiation by the calcium channel agonist Bayer K8644 in CA1 of the rat hippocampus in vitro. *Neurosci Lett*. **80(3)**: 351-5.

Mulkeen D, Anwyl R, Rowan M (1988). The effects of external calcium on long-term potentiation in the rat hippocampal slice. *Brain Res*. **447(2)**: 234-8.

Mulkey RM, Endo S, Shenolikar S, Malenka RC (1994). Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature*. **369(6480)**: 486-8.

Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L (1992). A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. *Nat Genet*. **1(5)**: 345-7.

Muller D, Joly M, Lynch G (1988). Contributions of quisqualate and NMDA receptors to the induction and expression of LTP. *Science*. **242(4886)**: 1694-7.

Muller D, Lynch G (1989). Evidence that changes in presynaptic calcium currents are not responsible for long-term potentiation in hippocampus. *Brain Res*. **479(2)**: 290-9.

Muller D, Buchs PA, Dunant Y, Lynch G (1990). Protein kinase C activity is not responsible for the expression of long-term potentiation in hippocampus. *Proc Natl Acad Sci U S A*. **87(11)**: 4073-7.

Muller D, Stoppini L, Wang C, Kiss JZ (1994). A role for polysialylated neural cell adhesion molecule in lesion-induced sprouting in hippocampal organotypic cultures. *Neuroscience*. **61(3)**: 441-5.

Mumby DG, Astur RS, Weisend MP, Sutherland RJ (1999). Retrograde amnesia and selective damage to the hippocampal formation: memory for places and object discriminations. *Behav Brain Res.* **106(1-2)**: 97-107

Murase S, Mosser E, Schuman EM (2002). Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function. *Neuron.* **35(1)**: 91-105.

Murata Y, Doi T, Taniguchi H, Fujiyoshi Y (2005). Proteomic analysis revealed a novel synaptic proline-rich membrane protein (PRR7) associated with PSD-95 and NMDA receptor. *Biochem Biophys Res Commun.* **327(1)**: 183-91.

Musgrave MA, Ballyk BA, Goh JW (1993). Coactivation of metabotropic and NMDA receptors is required for LTP induction. *Neuroreport.* **4(2)**: 171-4.

N

Nabieva E, Jim K, Agarwal A, Chazelle B, Singh M (2005). Whole-proteome prediction of protein function via graph-theoretic analysis of interaction maps. *Bioinformatics.* **21 Suppl 1**: i302-i310.

Nadler JV, Cotman CW, Lynch GS (1974). Biochemical plasticity of short-axon interneurons: increased glutamate decarboxylase activity in the denervated area of rat dentate gyrus following entorhinal lesion. *Exp Neurol.* **45(2)**: 403-13.

Nadler JV, White WF, Vaca KW, Cotman CW (1977). Calcium-dependent gamma-aminobutyrate release by interneurons of rat hippocampal regions: lesion-induced plasticity. *Brain Res.* **131(2)**: 241-58.

Nagakubo D, Taira T, Kitaura H, Ikeda M, Tamai K, Iguchi-Ariga SM, Ariga H (1997). DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. *Biochem Biophys Res Commun.* **231(2)**: 509-13.

Nakayama T, Yaoi T, Yasui M, Kuwajima G (1998). N-copine: a novel two C2-domain-containing protein with neuronal activity-regulated expression. *FEBS Lett.* **428(1-2)**: 80-4.

Nakayama T, Yaoi T, Kuwajima G (1999a). Localization and subcellular distribution of N-copine in mouse brain. *J Neurochem.* **72(1)**: 373-9.

Nakayama T, Yaoi T, Kuwajima G, Yoshie O, Sakata T (1999b). Ca2(+)-dependent interaction of N-copine, a member of the two C2 domain protein family, with OS-9, the product of a gene frequently amplified in osteosarcoma. *FEBS Lett.* **453(1-2)**: 77-80.

Nakayama AY, Harms MB, Luo L (2000). Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *J Neurosci.* **20(14)**: 5329-38.

Nayak AS, Moore CI, Browning MD (1996). Ca2+/calmodulin-dependent protein kinase II phosphorylation of the presynaptic protein synapsin I is persistently increased during long-term potentiation. *Proc Natl Acad Sci U S A.* **93(26)**: 15451-6.

Nayak A, Zastrow DJ, Lickteig R, Zahniser NR, Browning MD (1998). Maintenance of late-phase LTP is accompanied by PKA-dependent increase in AMPA receptor synthesis. *Nature.* **394(6694)**: 680-3.

Nedivi E, Hevroni D, Naot D, Israeli D, Citri Y (1993). Numerous candidate plasticity-related genes revealed by differential cDNA cloning. *Nature.* **363(6431)**: 718-22.

Neeper SA, Gomez-Pinilla F, Choi J, Cotman CW (1996). Physical activity increases mRNA for brain-derived neurotrophic factor and nerve growth factor in rat brain. *Brain Res.* **726(1-2)**: 49-56.

Nelson RB, Linden DJ, Routtenberg A (1989). Phosphoproteins localized to presynaptic terminal linked to persistence of long-term potentiation (LTP): quantitative analysis of two-dimensional gels. *Brain Res.* **497(1)**: 30-42.

Neville H & Bavelier D (2002). Human brain plasticity: evidence from sensory deprivation and altered language experience. *Nat Rev Neurosci.* **3(6)**: 443-52

Nguyen PV, Abel T, Kandel ER (1994). Requirement of a critical period of transcription for induction of a late phase of LTP. *Science.* **265(5175)**: 1104-7.

Nguyen PV & Kandel ER (1996). A macromolecular synthesis-dependent late phase of long-term potentiation requiring cAMP in the medial perforant pathway of rat hippocampal slices. *J Neurosci.* **16(10)**: 3189-98.

Nguyen PV & Kandel ER (1997). Brief theta-burst stimulation induces a transcription-dependent late phase of LTP requiring cAMP in area CA1 of the mouse hippocampus. *Learn Mem.* **4(2)**: 230-43.

Nicoletti F, Meek JL, Iadarola MJ, Chuang DM, Roth BL, Costa E (1986). Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. *J Neurochem.* **46(1)**: 40-6.

Nielsen PA, Olsen JV, Podtelejnikov AV, Andersen JR, Mann M, Wisniewski JR (2005). Proteomic Mapping of Brain Plasma Membrane Proteins. Mol Cell *Proteomics.* **4(4)**: 402-408.

Niisato K, Fujikawa A, Komai S, Shintani T, Watanabe E, Sakaguchi G, Katsuura G, Manabe T, Noda M (2005). Age-dependent enhancement of hippocampal long-term potentiation and impairment of spatial learning through the Rho-associated kinase pathway in protein tyrosine phosphatase receptor type Z-deficient mice. *J Neurosci.* **25(5)**: 1081-8.

Nilsson M, Perfilieva E, Johansson U, Orwar O, Eriksson PS (1999). Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *J Neurobiol.* **39(4)**: 569-78.

Nishimune A, Isaac JT, Molnar E, Noel J, Nash SR, Tagaya M, Collingridge GL, Nakanishi S, Henley JM (1998). NSF binding to GluR2 regulates synaptic transmission. *Neuron.* **21(1)**: 87-97.

Nithianantharajah J, Levis H, Murphy M (2004). Environmental enrichment results in cortical and subcortical changes in levels of synaptophysin and PSD-95 proteins. *Neurobiol Learn Mem.* **81(3)**: 200-10.

Nitz B, Ivell R, Hartung S, Middendorff R, Olcese J (1998). A fusca gene homologue in mammalian tissues: Developmental regulation in the rat testes. *Mol Cell Endocrinol.* **144(1-2)**: 131-8.

Nong Y, Huang YQ, Ju W, Kalia LV, Ahmadian G, Wang YT, Salter MW (2003). Glycine binding primes NMDA receptor internalization. *Nature.* **422(6929)**: 302-7.

Norman ED, Thiels E, Barrionuevo G, Klann E (2000). Long-term depression in the hippocampus in vivo is associated with protein phosphatase-dependent alterations in extracellular signal-regulated kinase. *J Neurochem.* **74(1)**: 192-8.

Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature.* **307(5950)**: 462-5.

O

O'Dell TJ, Kandel ER, Grant SG (1991). Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature.* **353(6344)**: 558-60.

Oestreicher AB, De Graan PN, Gispen WH, Verhaagen J, Schrama LH (1997). B-50, the growth associated protein-43: modulation of cell morphology and communication in the nervous system. *Prog Neurobiol.* **53(6)**: 627-86.

Oguri T, Takahata I, Katsuta K, Nomura E, Hidaka M, Inagaki N (2002). Proteome analysis of rat hippocampal neurons by multiple large gel two-dimensional electrophoresis. *Proteomics.* **2(6)**: 666-72.

Ohlsson AL & Johansson BB (1995). Environment influences functional outcome of cerebral infarction in rats. *Stroke.* **26(4)**: 644-9.

Okamoto K, Nagai T, Miyawaki A, Hayashi Y (2004). Rapid and persistent modulation of actin dynamics regulates postsynaptic reorganization underlying bidirectional plasticity. *Nat Neurosci.* **7(10)**: 1104-12.

O'Kane EM, Stone TW, Morris BJ (2003a). Distribution of Rho family GTPases in the adult rat hippocampus and cerebellum. *Brain Res Mol Brain Res.* **114(1)**: 1-8.

O'Kane EM, Stone TW, Morris BJ (2003b). Activation of Rho GTPases by synaptic transmission in the hippocampus. *J Neurochem.* **87(5)**: 1309-12.

O'Kane EM, Stone TW, Morris BJ (2004). Increased long-term potentiation in the CA1 region of rat hippocampus via modulation of GTPase signalling or inhibition of Rho kinase. *Neuropharmacology.* **46(6)**: 879-87.

O'Keefe J (1979). A review of the hippocampal place cells. *Prog Neurobiol.* **13(4)**: 419-39.

Olenik C, Barth H, Just I, Aktories K, Meyer DK (1997). Gene expression of the small GTP-binding proteins RhoA, RhoB, Rac1, and Cdc42 in adult rat brain. *Brain Res Mol Brain Res.* **52(2)**: 263-9.

Oliff HS, Berchtold NC, Isackson P, Cotman CW (1998). Exercise-induced regulation of brain-derived neurotrophic factor (BDNF) transcripts in the rat hippocampus. *Brain Res Mol Brain Res.* **61(1-2)**: 147-53.

Olney JW, Ho OL, Rhee V (1971). Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system. *Exp Brain Res.* **14(1)**: 61-76.

Olsson T, Mohammed AH, Donaldson LF, Henriksson BG, Seckl JR (1994). Glucocorticoid receptor and NGFI-A gene expression are induced in the hippocampus after environmental enrichment in adult rats. *Brain Res Mol Brain Res.* **23(4)**: 349-53.

Olsson T, Mohammed AK, Donaldson LF, Seckl JR (1995). Transcription factor AP-2 gene expression in adult rat hippocampal regions: effects of environmental manipulations. *Neurosci Lett.* **189(2)**: 113-6.

Ono Y, Fujii T, Igarashi K, Kuno T, Tanaka C, Kikkawa U, Nishizuka Y (1989). Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc Natl Acad Sci U S A.* **86(13)**: 4868-71.

Osten P, Srivastava S, Inman GJ, Vilim FS, Khatri L, Lee LM, States BA, Einheber S, Milner TA, Hanson PI, Ziff EB (1998). The AMPA receptor GluR2 C terminus can mediate a reversible, ATP-dependent interaction with NSF and alpha- and beta-SNAPs. *Neuron.* **21(1)**: 99-110.

Ostrowski J, Schullery DS, Denisenko ON, Higaki Y, Watts J, Aebersold R, Stempka L, Gschwendt M, Bomsztyk K (2000). Role of tyrosine phosphorylation in the regulation of the interaction of heterogenous nuclear ribonucleoprotein K protein with its protein and RNA partners. *J Biol Chem.* **275(5)**: 3619-28.

Otani S, Marshall CJ, Tate WP, Goddard GV, Abraham WC (1989). Maintenance of long-term potentiation in rat dentate gyrus requires protein synthesis but not

messenger RNA synthesis immediately post-tetanization. *Neuroscience*. **28(3)**: 519-26.

Otani S, Ben-Ari Y, Roisin-Lallemand MP (1993). Metabotropic receptor stimulation coupled to weak tetanus leads to long-term potentiation and a rapid elevation of cytosolic protein kinase C activity. *Brain Res*. **613(1)**: 1-9.

Otmakhova NA, Otmakhov N, Mortenson LH, Lisman JE (2000). Inhibition of the cAMP pathway decreases early long-term potentiation at CA1 hippocampal synapses. *J Neurosci*. **20(12)**: 4446-51.

Otmakhov N & Lisman JE (2002). Postsynaptic application of a cAMP analogue reverses long-term potentiation in hippocampal CA1 pyramidal neurons. *J Neurophysiol*. **87(6)**: 3018-32.

Otto T, Eichenbaum H, Wiener SI, Wible CG (1991). Learning-related patterns of CA1 spike trains parallel stimulation parameters optimal for inducing hippocampal long-term potentiation. *Hippocampus*. **1(2)**: 181-92.

Oyama F, Sawamura N, Kobayashi K, Morishima-Kawashima M, Kuramochi T, Ito M, Tomita T, Maruyama K, Saido TC, Iwatsubo T, Capell A, Walter J, Grunberg J, Ueyama Y, Haass C, Ihara Y (1998). Mutant presenilin 2 transgenic mouse: effect on an age-dependent increase of amyloid beta-protein 42 in the brain. *J Neurochem*. **71(1)**: 313-22.

P

Papastoitsis G, Siman R, Scott R, Abraham CR (1994). Identification of a metalloprotease from Alzheimer's disease brain able to degrade the beta-amyloid precursor protein and generate amyloidogenic fragments. *Biochemistry*. **33(1)**: 192-9.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997). Dentate granule cell neurogenesis is increased by seizures and contributes to

aberrant network reorganization in the adult rat hippocampus. *J Neurosci.* **17(10)**: 3727-38.

Parker D & Grillner S (1999). Activity-dependent metaplasticity of inhibitory and excitatory synaptic transmission in the lamprey spinal cord locomotor network. *J Neurosci.* **19(5)**: 1647-56.

Paulson L, Martin P, Persson A, Nilsson CL, Ljung E, Westman-Brinkmalm A, Eriksson PS, Blennow K, Davidsson P (2003). Comparative genome- and proteome analysis of cerebral cortex from MK-801-treated rats. *J Neurosci Res.* **71(4)**: 526-33.

Paulson L, Martin P, Nilsson CL, Ljung E, Westman-Brinkmalm A, Blennow K, Davidsson P (2004). Comparative proteome analysis of thalamus in MK-801-treated rats. *Proteomics.* **4(3)**: 819-25.

Pawlak R, Skrzypiec A, Sulkowski S, Buczko W (2002). Ethanol-induced neurotoxicity is counterbalanced by increased cell proliferation in mouse dentate gyrus. Neurosci Lett. 2002 Jul 19;327(2):83-6.

Pazman C, Bengzon J, McKay RD, Somogyi R (1997). Novel differentially expressed genes induced by kainic acid in hippocampus: putative molecular effectors of plasticity and injury. *Exp Neurol.* **146(2)**: 502-12.

Pei JJ, Gong CX, An WL, Winblad B, Cowburn RF, Grundke-Iqbal I, Iqbal K (2003). Okadaic-acid-induced inhibition of protein phosphatase 2A produces activation of mitogen-activated protein kinases ERK1/2, MEK1/2, and p70 S6, similar to that in Alzheimer's disease. *Am J Pathol.* **163(3)**: 845-58.

Penfield W & Milner B (1958). Memory deficit produced by bilateral lesions in the hippocampal zone. *AMA Arch Neurol Psychiatry.* 79(5): 475-97

Perkins DN, Pappin DJ, Creasy DM, Cottrell JS (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* **20(18)**: 3551-67.

Peters A, Feldman M, Saldanha J (1976). The projection of the lateral geniculate nucleus to area 17 of the rat cerebral cortex. II. Terminations upon neuronal perikarya and dendritic shafts. *J Neurocytol.* **5(1)**: 85-107.

Petit TL, LeBoutillier JC, Markus EJ, Milgram NW (1989). Synaptic structural plasticity following repetitive activation in the rat hippocampus. *Exp Neurol.* **105(1)**: 72-9.

Petralia RS, Yokotani N, Wenthold RJ (1994a). Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. *J Neurosci.* **14(2)**: 667-96.

Petralia RS, Wang YX, Wenthold RJ (1994b). The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1. *J Neurosci.* **14(10)**: 6102-20.

Petralia RS, Wang YX, Wenthold RJ (2003). Internalization at glutamatergic synapses during development. *Eur J Neurosci.* **18(12)**: 3207-17.

Pettit DL, Perlman S, Malinow R (1994). Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons. *Science.* **266(5192)**: 1881-5.

Pfrieger FW, Barres BA (1997). Synaptic efficacy enhanced by glial cells in vitro. *Science.* **277(5332)**: 1684-7.

Pham TM, Soderstrom S, Winblad B, Mohammed AH (1999). Effects of environmental enrichment on cognitive function and hippocampal NGF in the non-handled rats. *Behav Brain Res.* **103(1)**: 63-70.

Phillips GR, Anderson TR, Florens L, Gudas C, Magda G, Yates JR 3rd, Colman DR (2004). Actin-binding proteins in a postsynaptic preparation: Lasp-1 is a component of central nervous system synapses and dendritic spines. *J Neurosci Res.* **78(1)**: 38-48.

Philpot BD, Weisberg MP, Ramos MS, Sawtell NB, Tang YP, Tsien JZ, Bear MF (2001). Effect of transgenic overexpression of NR2B on NMDA receptor function and synaptic plasticity in visual cortex. *Neuropharmacology*. **41(6)**: 762-70.

Pico RM & Gall C (1989). Continuities between outer nuclear membrane and the rough endoplasmic reticulum increase in hippocampal neurons during seizure-induced protein synthesis. *Brain Res.* **497(2)**: 387-92.

Pinkstaff JK, Chappell SA, Mauro VP, Edelman GM, Krushel LA (2001). Internal initiation of translation of five dendritically localized neuronal mRNAs. *Proc Natl Acad Sci U S A.* **98(5)**: 2770-5.

Poduri A, Beason-Held LL, Moss MB, Rosene DL, Hyman BT (1995). CA3 neuronal degeneration follows chronic entorhinal cortex lesions. *Neurosci Lett.* **197(1)**: 1-4.

Poon HF, Castegna A, Farr SA, Thongboonkerd V, Lynn BC, Banks WA, Morley JE, Klein JB, Butterfield DA (2004). Quantitative proteomics analysis of specific protein expression and oxidative modification in aged senescence-accelerated-prone 8 mice brain. *Neuroscience.* **126(4)**: 915-26.

Popov VI, Davies HA, Rogachevsky VV, Patrushev IV, Errington ML, Gabbott PL, Bliss TV, Stewart MG (2004). Remodelling of synaptic morphology but unchanged synaptic density during late phase long-term potentiation (LTP): a serial section electron micrograph study in the dentate gyrus in the anaesthetised rat. *Neuroscience.* **128(2)**: 251-62.

Pozzo-Miller LD, Gottschalk W, Zhang L, McDermott K, Du J, Gopalakrishnan R, Oho C, Sheng ZH, Lu B (1999). Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *J Neurosci.* **19(12)**: 4972-83.

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(7)**: 684-97, 643.

Prokai L, Zharikova AD, Stevens SM Jr (2003). Effect of chronic morphine exposure on the synaptic plasma-membrane subproteome of rats: a quantitative protein profiling study based on isotope-coded affinity tags and liquid chromatography/mass spectrometry. *J Mass Spectrom*. **40(2)**: 169-75.

Prolla TA (2002). DNA microarray analysis of the aging brain. *Chem Senses*. **27(3)**: 299-306.

Pumain R, Kurcewicz I, Louvel J (1987). Ionic changes induced by excitatory amino acids in the rat cerebral cortex. *Can J Physiol Pharmacol*. **65(5)**: 1067-77.

Q

Quinlan EM & Halpain S (1996). Emergence of activity-dependent, bidirectional control of microtubule-associated protein MAP2 phosphorylation during postnatal development. *J Neurosci*. **16(23)**: 7627-37.

Qian Z, Gilbert ME, Colicos MA, Kandel ER, Kuhl D (1993). Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. *Nature*. 361(6411):453-7.

R

Rabilloud T, Valette C, Lawrence JJ (1994). Sample application by in-gel rehydration improves the resolution of two-dimensional electrophoresis with immobilized pH gradients in the first dimension. *Electrophoresis*. **15(12)**: 1552-8.

Rabinovich E, Kerem A, Frohlich KU, Diamant N, Bar-Nun S (2002). AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. *Mol Cell Biol.* **22(2)**: 626-34.

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(23)**: 13892-8.

Ramani AK, Bunescu RC, Mooney RJ, Marcotte EM (2005). Consolidating the set of known human protein-protein interactions in preparation for large-scale mapping of the human interactome. *Genome Biol.* **6(5)**: R40

Rammes G, Zeilhofer HU, Collingridge GL, Parsons CG, Swandulla D (1999). Expression of early hippocampal CA1 LTP does not lead to changes in AMPA-EPSC kinetics or sensitivity to cyclothiazide. *Pflugers Arch.* **437(2)**: 191-6.

Ramón y Cajal S (1894). *Les nouvelles idées sur la fine anatomie de système nerveux.* Paris, 1894

Rampon C, Tang YP, Goodhouse J, Shimizu E, Kyin M, Tsien JZ (2000). Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. *Nat Neurosci.* **3(3)**: 238-44.

Rasmuson S, Olsson T, Henriksson BG, Kelly PA, Holmes MC, Seckl JR, Mohammed AH (1998). Environmental enrichment selectively increases 5-HT1A receptor mRNA expression and binding in the rat hippocampus. *Brain Res Mol Brain Res.* **53(1-2)**: 285-90.

Reddy PH, McWeeney S, Park BS, Manczak M, Gutala RV, Partovi D, Jung Y, Yau V, Searles R, Mori M, Quinn J (2004). Gene expression profiles of transcripts in amyloid precursor protein transgenic mice: up-regulation of mitochondrial metabolism and apoptotic genes is an early cellular change in Alzheimer's disease. *Hum Mol Genet.* **13(12)**: 1225-40.

Regehr WG, Tank DW (1990). Postsynaptic NMDA receptor-mediated calcium accumulation in hippocampal CA1 pyramidal cell dendrites. *Nature*. **345(6278)**: 807-10.

Reilly CE (2001). Hippocampus selectively supports episodic memory retrieval. *J Neurol*. **248(11)**: 1014-5

Represa A, Le Gall La Salle G, Ben-Ari Y (1989). Hippocampal plasticity in the kindling model of epilepsy in rats. *Neurosci Lett*. **99(3)**: 345-50.

Reymann KG, Matthies HK, Frey U, Vorobyev VS, Matthies H (1986). Calcium-induced long-term potentiation in the hippocampal slice: characterization of the time course and conditions. *Brain Res Bull*. **17(3)**: 291-6.

Reymann KG, Frey U, Jork R, Matthies H (1988). Polymyxin B, an inhibitor of protein kinase C, prevents the maintenance of synaptic long-term potentiation in hippocampal CA1 neurons. *Brain Res*. **440(2)**: 305-14.

Reymann KG, Brodemann R, Kase H, Matthies H (1988). Inhibitors of calmodulin and protein kinase C block different phases of hippocampal long-term potentiation. *Brain Res*. **461(2)**: 388-92.

Reymann KG, Matthies HK, Schulzeck K, Matthies H (1989). N-methyl-D-aspartate receptor activation is required for the induction of both early and late phases of long-term potentiation in rat hippocampal slices. *Neurosci Lett*. **96(1)**: 96-101.

Ribak CE (1983). Morphological, biochemical, and immunocytochemical changes of the cortical, GABAergic system in epileptic foci. *Res Publ Assoc Res Nerv Ment Dis*. **61:** 109-30.

Ribak CE (1985). Axon terminals of GABAergic chandelier cells are lost at epileptic foci. *Brain Res*. **326(2)**: 251-60

Richardson JC, Kendal CE, Anderson R, Priest F, Gower E, Soden P, Gray R, Topps S, Howlett DR, Lavender D, Clarke NJ, Barnes JC, Haworth R, Stewart MG, Rupniak HT (2003). Ultrastructural and behavioural changes precede amyloid deposition in a transgenic model of Alzheimer's disease. *Neuroscience.* **122(1)**: 213-28.

Ringstad N, Nemoto Y, De Camilli P (2001). Differential expression of endophilin 1 and 2 dimers at central nervous system synapses. *J Biol Chem.* **276(44)**: 40424-30.

Ritter B, Blondeau F, Denisov AY, Gehring K, McPherson PS (2004). Molecular mechanisms in clathrin-mediated membrane budding revealed through subcellular proteomics. *Biochem Soc Trans.* **32(Pt 5)**: 769-73.

Rivera S, Tremblay E, Timsit S, Canals O, Ben-Ari Y, Khrestchatisky M (1997). Tissue inhibitor of metalloproteinases-1 (TIMP-1) is differentially induced in neurons and astrocytes after seizures: evidence for developmental, immediate early gene, and lesion response. *J Neurosci.* **17(11)**: 4223-35.

Roberson ED & Sweatt JD (1996). Transient activation of cyclic AMP-dependent protein kinase during hippocampal long-term potentiation. *J Biol Chem.* **271(48)**: 30436-41.

Roberts LA, Higgins MJ, O'Shaughnessy CT, Stone TW, Morris BJ (1996). Changes in hippocampal gene expression associated with the induction of long-term potentiation. *Brain Res Mol Brain Res.* **42(1)**: 123-7.

Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD, Wenthold RJ (2001). Molecular determinants of NMDA receptor internalization. *Nat Neurosci.* **4(8)**: 794-802.

Roder S, Danober L, Pozza MF, Lingenhoehl K, Wiederhold KH, Olpe HR (2003). Electrophysiological studies on the hippocampus and prefrontal cortex assessing the effects of amyloidosis in amyloid precursor protein 23 transgenic mice. *Neuroscience.* **120(3)**: 705-20.

Roehrs C, Garrido-Sanabria ER, Da Silva AC, Faria LC, Sinhorin VD, Marques RH, Priel MR, Rubin MA, Cavalheiro EA, Mello CF (2004). Succinate increases neuronal post-synaptic excitatory potentials in vitro and induces convulsive behavior through N-methyl-d-aspartate-mediated mechanisms. *Neuroscience*. **125(4)**: 965-71.

Rommelaere H, Van Troys M, Gao Y, Melki R, Cowan NJ, Vandekerckhove J, Ampe C (1993). Eukaryotic cytosolic chaperonin contains t-complex polypeptide 1 and seven related subunits. *Proc Natl Acad Sci U S A*. **90(24)**: 11975-9.

Rosen LB, Ginty DD, Weber MJ, Greenberg ME (1994). Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron*. **12(6)**: 1207-21.

Rosenblum K, Futter M, Voss K, Erent M, Skehel PA, French P, Obosi L, Jones MW, Bliss TV (2002). The role of extracellular regulated kinases I/II in late-phase long-term potentiation. *J Neurosci*. **22(13)**: 5432-41.

Rotenberg A, Mayford M, Hawkins RD, Kandel ER, Muller RU (1996). Mice expressing activated CaMKII lack low frequency LTP and do not form stable place cells in the CA1 region of the hippocampus. *Cell*. **87(7)**: 1351-61.

Rothman JE (1994). Intracellular membrane fusion. Adv Second Messenger *Phosphoprotein Res*. **29**: 81-96.

Routtenberg A & Lovinger DM (1985). Selective increase in phosphorylation of a 47-kDa protein (F1) directly related to long-term potentiation. *Behav Neural Biol*. **43(1)**: 3-11.

Routtenberg A, Cantallops I, Zaffuto S, Serrano P, Namgung U (2000). Enhanced learning after genetic overexpression of a brain growth protein. *Proc Natl Acad Sci U S A*. **97(13)**: 7657-62.

S

Sabatini BL & Svoboda K (2000). Analysis of calcium channels in single spines using optical fluctuation analysis. *Nature.* **408(6812)**: 589-93.

Sabatini BL, Oertner TG, Svoboda K (2002). The life cycle of Ca(2+) ions in dendritic spines. *Neuron.* **33(3)**: 439-52.

Sanchez C, Ulloa L, Montoro RJ, Lopez-Barneo J, Avila J (1997). NMDA-glutamate receptors regulate phosphorylation of dendritic cytoskeletal proteins in the hippocampus. *Brain Res.* **765(1)**: 141-8.

Sanchez Martin C, Diaz-Nido J, Avila J (1998). Regulation of a site-specific phosphorylation of the microtubule-associated protein 2 during the development of cultured neurons. *Neuroscience.* **87(4)**: 861-70.

Sandberg R, Yasuda R, Pankratz DG, Carter TA, Del Rio JA, Wodicka L, Mayford M, Lockhart DJ, Barlow C (2000). Regional and strain-specific gene expression mapping in the adult mouse brain. *Proc Natl Acad Sci U S A.* **97(20)**: 11038-43.

Sandoval ME (1980). Sodium-dependent efflux of [3H]GABA from synaptosomes probably related to mitochondrial calcium mobilization. *J Neurochem* **35(4)**: 915-21.

Satoh M, Kaneko S (1994). Involvement of postsynaptic G-proteins in hippocampal long-term potentiation. *Rev Neurosci.* **5(1)**: 1-9.

Sazuka T, Keta S, Shiratake K, Yamaki S, Shibata D (2004). A proteomic approach to identification of transmembrane proteins and membrane-anchored proteins of Arabidopsis thaliana by peptide sequencing. *DNA Res.* **11(2)**: 101-13.

Schiavo G, Stenbeck G, Rothman JE, Sollner TH (1997). Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can

explain docked vesicles at neurotoxin-treated synapses. *Proc Natl Acad Sci U S A.* **94(3)**: 997-1001.

Schilhab TS & Christoffersen GR (1996). Role of protein synthesis in the transition from synaptic short-term to long-term depression in neurons of Helix pomatia. *Neuroscience.* **73(4)**: 999-1007.

Schipper HM, Agarwal-Mawal A, Paudel HK (1999). The topography and subcellular distribution of mitogen-activated protein kinase kinase1 (MEK1) in adult rat brain and differentiating PC12 cells. *Neuroscience.* **93(2)**: 585-95.

Schmidt W, Reymann KG (2002). Proliferating cells differentiate into neurons in the hippocampal CA1 region of gerbils after global cerebral ischemia. *Neurosci Lett.* **334(3)**: 153-6.

Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, *et al* (1987). Sequence and functional expression of the GABA A receptor shows a ligand-gated receptor super-family. *Nature.* **328(6127)**: 221-7.

Schreiber V, Moog-Lutz C, Regnier CH, Chenard MP, Boeuf H, Vonesch JL, Tomasetto C, Rio MC (1998). Lasp-1, a novel type of actin-binding protein accumulating in cell membrane extensions. *Mol Med.* **4(10)**: 675-87.

Schulenberg B, Aggeler R, Beechem JM, Capaldi RA, Patton WF (2003). Analysis of steady-state protein phosphorylation in mitochondria using a novel fluorescent phosphosensor dye. *J Biol Chem.* **278(29)**: 27251-5.

Schullery DS, Ostrowski J, Denisenko ON, Stempka L, Shnyreva M, Suzuki H, Gschwendt M, Bomsztyk K (1999). Regulated interaction of protein kinase Cdelta with the heterogeneous nuclear ribonucleoprotein K protein. *J Biol Chem.* **274(21)**: 15101-9.

Schulman H (2004). Activity-dependent regulation of calcium/calmodulin-dependent protein kinase II localization. *J Neurosci.* **24(39)**: 8399-403.

Schurov IL, McNulty S, Best JD, Sloper PJ, Hastings MH (1999). Glutamatergic induction of CREB phosphorylation and Fos expression in primary cultures of the suprachiasmatic hypothalamus in vitro is mediated by co-ordinate activity of NMDA and non-NMDA receptors. *J Neuroendocrinol.* **11(1)**: 43-51.

Schulz PE, Cook EP, Johnston D (1994). Changes in paired-pulse facilitation suggest presynaptic involvement in long-term potentiation. *J Neurosci.* **14(9)**: 5325-37.

Schuman EM & Madison DV (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. Science. **254(5037)**: 1503-6.

Schwartzkroin PA & Wester K (1975). Long-lasting facilitation of a synaptic potential following tetanization in the in vitro hippocampal slice. *Brain Res.* **89(1)**: 107-19.

Scoville WB & Milner B (1957). Loss of recent memory after bilateral hippocampal lesions. *J Neurochem.* **20(1)**: 11-21

Segal M (1995). Dendritic spines for neuroprotection: a hypothesis. *Trends Neurosci.* **18(11)**: 468-71.

Seike M, Kondo T, Fujii K, Yamada T, Gemma A, Kudoh S, Hirohashi S (2004). Proteomic signature of human cancer cells. *Proteomics.* **4(9)**: 2776-88.

Seki T (2002). Expression patterns of immature neuronal markers PSA-NCAM, CRMP-4 and NeuroD in the hippocampus of young adult and aged rodents. *J Neurosci Res.* **70(3)**: 327-34.

Sensenbrenner M, Lucas M, Deloulme JC (1997). Expression of two neuronal markers, growth-associated protein 43 and neuron-specific enolase, in rat glial cells. *J Mol Med.* **75(9)**: 653-63.

Sergueeva OA, Fedorov NB, Reymann KG (1993). An antagonist of glutamate metabotropic receptors, (RS)-alpha-methyl-4-carboxyphenylglycine, prevents the LTP-related increase in postsynaptic AMPA sensitivity in hippocampal slices. *Neuropharmacology*. **32(9)**: 933-5.

Seress L & Pokorny J (1981). Structure of the granular layer of the rat dentate gyrus. A light microscopic and Golgi study. *J Anat.* **133(Pt 2)**: 181-95.

Seubert P, Larson J, Oliver M, Jung MW, Baudry M, Lynch G (1988). Stimulation of NMDA receptors induces proteolysis of spectrin in hippocampus. *Brain Res.* **460(1)**: 189-94.

Shalin SC, Zirrgiebel U, Honsa KJ, Julien JP, Miller FD, Kaplan DR, Sweatt JD (2004). Neuronal MEK is important for normal fear conditioning in mice. *J Neurosci Res.* **75(6)**: 760-70.

Shang H, Lang D, Jean-Marc B, Kaelin-Lang A (2004). Localization of DJ-1 mRNA in the mouse brain. *Neurosci Lett.* **367(3)**: 273-7.

Shankar S, Teyler TJ, Robbins N (1998). Aging differentially alters forms of long-term potentiation in rat hippocampal area CA1. *J Neurophysiol.* **79(1)**: 334-41.

Sharp FR, Massa SM, Swanson RA (1999). Heat-shock protein protection. *Trends Neurosci.* **22(3)**: 97-9.

Sharp FR, Liu J, Bernabeu R (2002). Neurogenesis following brain ischemia. *Brain Res Dev Brain Res.* **134(1-2)**: 23-30.

Shaw J, Rowlinson R, Nickson J, Stone T, Sweet A, Williams K, Tonge R (2003). Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. *Proteomics.* **3(7)**: 1181-95.

Shen H, Tong L, Balazs R, Cotman CW (2001). Physical activity elicits sustained activation of the cyclic AMP response element-binding protein and mitogen-activated protein kinase in the rat hippocampus. *Neuroscience*. **107(2)**: 219-29.

Shendelman S, Jonason A, Martinat C, Leete T, Abeliovich A (2004). DJ-1 is a redox-dependent molecular chaperone that inhibits alpha-synuclein aggregate formation. *PLoS Biol*. **2(11)**:e362

Sheng ZH, Rettig J, Takahashi M, Catterall WA (1994). Identification of a syntaxin-binding site on N-type calcium channels. *Neuron*. **13(6)**: 1303-13.

Shepherd GM, Harris KM (1998). Three-dimensional structure and composition of CA3-->CA1 axons in rat hippocampal slices: implications for presynaptic connectivity and compartmentalization. *J Neurosci*. **18(20)**: 8300-10.

Shibuki K, Gomi H, Chen L, Bao S, Kim JJ, Wakatsuki H, Fujisaki T, Fujimoto K, Katoh A, Ikeda T, Chen C, Thompson RF, Itohara S (1996). Deficient cerebellar long-term depression, impaired eyeblink conditioning, and normal motor coordination in GFAP mutant mice. *Neuron*. **16(3)**: 587-99.

Shirogane T, Fukada T, Muller JM, Shima DT, Hibi M, Hirano T (1999). Synergistic roles for Pim-1 and c-Myc in STAT3-mediated cell cycle progression and antiapoptosis. *Immunity*. **11(6)**: 709-19.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002). Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus*. **12(5)**: 578-84.

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992a). Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. *Science*. **257(5067)**: 206-11.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992b). Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. *Science.* **257(5067)**: 201-6.

Siman R, Reaume AG, Savage MJ, Trusko S, Lin YG, Scott RW, d DG (2000). Presenilin-1 P264L knock-in mutation: differential effects on abeta production, amyloid deposition, and neuronal vulnerability. *J Neurosci.* **20(23)**: 8717-26.

Siman R, McIntosh TK, Soltesz KM, Chen Z, Neumar RW, Roberts VL (2004). Proteins released from degenerating neurons are surrogate markers for acute brain damage. *Neurobiol Dis.* **16(2)**: 311-20.

Simsek-Duran F, Linden DJ, Lonart G (2004). Adapter protein 14-3-3 is required for a presynaptic form of LTP in the cerebellum. *Nat Neurosci.* **1296-8.**

Slack JR & Pockett S (1991). Cyclic AMP induces long-term increase in synaptic efficacy in CA1 region of rat hippocampus. *Neurosci Lett.* **130(1)**: 69-72.

Slack JR & Walsh C (1995). Effects of a cAMP analogue simulate the distinct components of long-term potentiation in CA1 region of rat hippocampus. *Neurosci Lett.* **201(1)**: 25-8.

Sokolov MV, Rossokhin AV, Astrelin AV, Frey JU, Voronin LL (2002). Quantal analysis suggests strong involvement of presynaptic mechanisms during the initial 3 h maintenance of long-term potentiation in rat hippocampal CA1 area in vitro. *Brain Res.* **957(1)**: 61-75.

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature.* **362(6418)**: 318-24.

Somiari RI, Sullivan A, Russell S, Somiari S, Hu H, Jordan R, George A, Katenhusen R, Buchowiecka A, Arciero C, Brzeski H, Hooke J, Shriver C (2003).

High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast. *Proteomics*. **3(10)**: 1863-73.

Somiari RI, Somiari S, Russell S, Shriver CD (2005). Proteomics of breast carcinoma. *J Chromatogr B Analyt Technol Biomed Life Sci*. **815(1-2)**: 215-25.

Spacek J & Hartmann M (1983). Three-dimensional analysis of dendritic spines. I. Quantitative observations related to dendritic spine and synaptic morphology in cerebral and cerebellar cortices. *Anat Embryol (Berl)*. **167(2)**: 289-310.

Spires TL, Grote HE, Garry S, Cordery PM, Van Dellen A, Blakemore C, Hannan AJ (2004). Dendritic spine pathology and deficits in experience-dependent dendritic plasticity in R6/1 Huntington's disease transgenic mice. *Eur J Neurosci*. **19(10)**: 2799-807.

Spillane DM, Rosahl TW, Sudhof TC, Malenka RC (1995). Long-term potentiation in mice lacking synapsins. *Neuropharmacology*. **34(11)**: 1573-9.

Spurzheim JC. The Physiognomical System of Drs. Gall and Spurzheim (1815). London, 1815

Stamm S, Casper D, Lees-Miller JP, Helfman DM (1993). Brain-specific tropomyosins TMBr-1 and TMBr-3 have distinct patterns of expression during development and in adult brain. *Proc Natl Acad Sci U S A*. **90(21)**: 9857-61.

Stanton PK and Sarvey JM (1984). Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. *J Neurosci*. **4(12)**: 3080-3088.

Steinberg TH, Agnew BJ, Gee KR, Leung WY, Goodman T, Schulenberg B, Hendrickson J, Beechem JM, Haugland RP, Patton WF (2003). Global quantitative phosphoprotein analysis using Multiplexed Proteomics technology. *Proteomics*. **3(7)**: 1128-44.

Steiner B, Kronenberg G, Jessberger S, Brandt MD, Reuter K, Kempermann G (2004). Differential regulation of gliogenesis in the context of adult hippocampal neurogenesis in mice. Glia. **46(1)**: 41-52.

Sternberger LA & Sternberger NH (1983). Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. *Proc Natl Acad Sci U S A*. **80(19)**: 6126-30.

Stevens SM Jr, Zharikova AD, Prokai L (2003). Proteomic analysis of the synaptic plasma membrane fraction isolated from rat forebrain. *Brain Res Mol Brain Res*. **117(2)**: 116-28.

Steward O & Reeves TM (1988). Protein-synthetic machinery beneath postsynaptic sites on CNS neurons: association between polyribosomes and other organelles at the synaptic site. *Neurosci*. **8(1)**: 176-84

Storm-Mathisen J, Leknes AK, Bore AT, Vaaland JL, Edminson P, Haug FM, Ottersen OP (1983). First visualization of glutamate and GABA in neurones by immunocytochemistry. Nature. **301(5900)**: 517-20.

Stringer JL (1996). Repeated seizures increase GFAP and vimentin in the hippocampus. *Brain Res*. **717(1-2)**: 147-53.

Strittmatter SM, Fankhauser C, Huang PL, Mashimo H, Fishman MC (1995). Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. *Cell*. **80(3)**: 445-52.

Sudhof TC (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature*. **375(6533)**: 645-53.

Sudhof TC & Rizo J (1996). Synaptotagmins: C2-domain proteins that regulate membrane traffic. *Neuron*. **17(3)**: 379-88.

Sugita S, Hata Y, Sudhof TC (1996). Distinct Ca(2+)-dependent properties of the first and second C2-domains of synaptotagmin I. *J Biol Chem.* **271(3)**: 1262-5.

Sugiyama H, Ito I, Hirono C (1987). A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature.* **325(6104)**: 531-3.

Sumi T, Matsumoto K, Nakamura T (2001). Specific activation of LIM kinase 2 via phosphorylation of threonine 505 by ROCK, a Rho-dependent protein kinase. *J Biol Chem.* **276(1)**: 670-6.

Sun MK & Alkon DL (2002). Impairment of hippocampal CA1 heterosynaptic transformation and spatial memory by beta-amyloid(25-35). *J Neurophysiol.* **87(5)**: 2441-9.

Surkova IN & Grishin EV (1991). Presynaptic alpha-latrotoxin receptor components interact with protein p65 of synaptic vesicle membranes. *Biomed Sci.* **2(4)**: 417-20.

Suzuki T, Mitake S, Okumura-Noji K, Shimizu H, Tada T, Fujii T (1997). Excitable membranes and synaptic transmission: postsynaptic mechanisms. Localization of alpha-internexin in the postsynaptic density of the rat brain. *Brain Res.* **765(1)**: 74-80.

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 Psychiatry.* **9(2)**: 128-43.

Sweatt JD (2001). The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem.* **76(1)**: 1-10.

Szklarczyk A, Lapinska J, Rylski M, McKay RD, Kaczmarek L (2002). Matrix metalloproteinase-9 undergoes expression and activation during dendritic remodeling in adult hippocampus. *J Neurosci.* **22(3)**: 920-30.

T

Takahashi T, Momiyama A (1993). Different types of calcium channels mediate central synaptic transmission. *Nature*. **366(6451):** 156-8.

Takahashi S, Ujihara H, Huang GZ, Yagyu KI, Sanbo M, Kaba H, Yagi T (1999). Reduced hippocampal LTP in mice lacking a presynaptic protein: complexin II. *Eur J Neurosci*. **11(7)**: 2359-66.

Takayama S, Xie Z, Reed JC (1999). An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. *J Biol Chem*. **274(2)**: 781-6.

Tan SE, Wenthold RJ, Soderling TR (1994). Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. *J Neurosci*. **14(3 Pt 1)**: 1123-9.

Tanaka H, Katoh A, Oguro K, Shimazaki K, Gomi H, Itohara S, Masuzawa T, Kawai N (2002). Disturbance of hippocampal long-term potentiation after transient ischemia in GFAP deficient mice. *J Neurosci Res*. **67(1)**: 11-20.

Tang Y, Zucker RS (1997). Mitochondrial involvement in post-tetanic potentiation of synaptic transmission. *Neuron*. **18(3)**: 483-91.

Taylor CM, Pfeiffer SE (2003). Enhanced resolution of glycosylphosphatidylinositol-anchored and transmembrane proteins from the lipid-rich myelin membrane by two-dimensional gel electrophoresis. *Proteomics*. **3(7)**: 1303-12.

Tekkok S, Krnjevic K (1995). Long-term potentiation in hippocampal slices induced by temporary suppression of glycolysis. *J Neurophysiol*. **74(6)**: 2763-6.

Terrian DM, Johnston D, Claiborne BJ, Ansah-Yiadom R, Strittmatter WJ, Rea MA (1988). Glutamate and dynorphin release from a subcellular fraction enriched in hippocampal mossy fiber synaptosomes. *Brain Res Bull.* **21(3)**: 343-51.

Terrian DM, Dorman RV, Gannon RL (1990). Characterization of the presynaptic calcium channels involved in glutamate exocytosis from rat hippocampal mossy fiber synaptosomes. *Neurosci Lett.* **119(2)**: 211-4.

Thibault O, Joly M, Muller D, Schottler F, Dudek S, Lynch G (1989). Long-lasting physiological effects of bath applied N-methyl-D-aspartate. *Brain Res.* **476(1)**: 170-3.

Thomas MJ & Malenka RC (2003). Synaptic plasticity in the mesolimbic dopamine system. *Philos Trans R Soc Lond B Biol Sci.* **358(1432)**: 815-9

Thompson KJ, Orfila JE, Achanta P, Martinez JL Jr (2003). Gene expression associated with in vivo induction of early phase-long-term potentiation (LTP) in the hippocampal mossy fiber-Cornus Ammonis (CA)3 pathway. *Cell Mol Biol (Noisy-le-grand).* **49(8)**: 1281-7.

Thuault SJ, Davies CH, Randall AD, Collingridge GL (2002). Group I mGluRs modulate the pattern of non-synaptic epileptiform activity in the hippocampus. *Neuropharmacology.* **43(2)**: 141-6.

Tian N, Petersen C, Kash S, Baekkeskov S, Copenhagen D, Nicoll R (1999). The role of the synthetic enzyme GAD65 in the control of neuronal gamma-aminobutyric acid release. *Proc Natl Acad Sci U S A.* **96(22)**: 12911-6.

Tilleman K, Stevens I, Spittaels K, Haute CV, Clerens S, Van Den Bergh G, Geerts H, Van Leuven F, Vandesande F, Moens L (2002a). Differential expression of brain proteins in glycogen synthase kinase-3 transgenic mice: a proteomics point of view. *Proteomics.* **2(1)**: 94-104.

Tilleman K, Van den Haute C, Geerts H, van Leuven F, Esmans EL, Moens L (2002b). Proteomics analysis of the neurodegeneration in the brain of tau transgenic mice. *Proteomics*. **2(6)**: 656-65.

Tobaben S, Thakur P, Fernandez-Chacon R, Sudhof TC, Rettig J, Stahl B (2001). A trimeric protein complex functions as a synaptic chaperone machine. *Neuron*. **31(6)**: 987-99.

Tomasetto C, Regnier C, Moog-Lutz C, Mattei MG, Chenard MP, Lidereau R, Basset P, Rio MC (1995). Identification of four novel human genes amplified and overexpressed in breast carcinoma and localized to the q11-q21.3 region of chromosome 17. *Genomics*. **28(3)**: 367-76.

Tonegawa S, Tsien JZ, McHugh TJ, Huerta P, Blum KI, Wilson MA (1996). Hippocampal CA1-region-restricted knockout of NMDAR1 gene disrupts synaptic plasticity, place fields, and spatial learning. *Cold Spring Harb Symp Quant Biol*. **61**: 225-38.

Tong L, Shen H, Perreau VM, Balazs R, Cotman CW (2001). Effects of exercise on gene-expression profile in the rat hippocampus. *Neurobiol Dis*. **8(6)**: 1046-56.

Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M (2001). Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics*. **1(3)**: 377-96.

Toni N, Buchs PA, Nikonenko I, Bron CR, Muller D (1999). LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. *Nature*. **402(6760)**: 421-5.

Toni N, Buchs PA, Nikonenko I, Povilaitite P, Parisi L, Muller D (2001). Remodeling of synaptic membranes after induction of long-term potentiation. *J Neurosci*. **21(16)**: 6245-51.

Torasdotter M, Metsis M, Henriksson BG, Winblad B, Mohammed AH (1998). Environmental enrichment results in higher levels of nerve growth factor mRNA in the rat visual cortex and hippocampus. *Behav Brain Res.* **93(1-2)**: 83-90.

Torres-Munoz JE, Van Waveren C, Keegan MG, Bookman RJ, Petito CK (2004). Gene expression profiles in microdissected neurons from human hippocampal subregions. *Brain Res Mol Brain Res.* **127(1-2)**: 105-14.

Traub RD, Knowles WD, Miles R & Wong RK (1987). Models of the cellular mechanism underlying propagation of epileptiform activity in the CA2-CA3 region of the hippocampal slice. *Neuroscience.* **21(2)**: 457-70.

Tsokas P, Grace EA, Chan P, Ma T, Sealfon SC, Iyengar R, Landau EM, Blitzer RD (2005). Local protein synthesis mediates a rapid increase in dendritic elongation factor 1A after induction of late long-term potentiation. *J Neurosci.* **25(24)**: 5833-43.

Tsugita A, Kawakami T, Uchida T, Sakai T, Kamo M, Matsui T, Watanabe Y, Morimasa T, Hosokawa K, Toda T (2000). Proteome analysis of mouse brain: two-dimensional electrophoresis profiles of tissue proteins during the course of aging. *Electrophoresis.* **21(9)**: 1853-71.

Tsuji T, Shiozaki A, Kohno R, Yoshizato K, Shimohama S (2002). Proteomic profiling and neurodegeneration in Alzheimer's disease. *Neurochem Res.* **27(10)**: 1245-53.

Turner AM & Greenough WT (1985). Differential rearing effects on rat visual cortex synapses. I. Synaptic and neuronal density and synapses per neuron. *Brain Res.* **329(1-2)**: 195-203.

Turner CA, Lewis MH, King MA (2003). Environmental enrichment: effects on stereotyped behavior and dendritic morphology. *Dev Psychobiol.* **43(1)**: 20-7.

Tyler WJ, Alonso M, Bramham CR, Pozzo-Miller LD (2002). From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. *Learn Mem.* **9(5)**: 224-37.

U

Uenishi N, Shors TJ, Finch CE, Nichols NR, Thompson RF (1991). Increased synthesis of two polypeptides in area CA1 of the hippocampus in response to repetitive electrical stimulation. *Brain Res.* **567(2)**: 248-52.

Ulas J, Monaghan DT, Cotman CW (1990). Plastic response of hippocampal excitatory amino acid receptors to deafferentation and reinnervation. *Neuroscience.* **34(1)**: 9-17.

Ullian EM, Christopherson KS, Barres BA (2004). Role for glia in synaptogenesis. *Glia.* **47(3)**: 209-16.

Ullrich B, Li C, Zhang JZ, McMahon H, Anderson RG, Geppert M, Sudhof TC (1994). Functional properties of multiple synaptotagmins in brain. *Neuron.* **13(6)**: 1281-91.

Unlu M, Morgan ME, Minden JS (1997). Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis.* **18(11)**: 2071-7.

Ursic D, Sedbrook JC, Himmel KL, Culbertson MR (1994). The essential yeast Tcp1 protein affects actin and microtubules. *Mol Biol Cell.* **5(10)**: 1065-80.

V

Van den Bergh G, Clerens S, Cnops L, Vandesande F, Arckens L (2003a). Fluorescent two-dimensional difference gel electrophoresis and mass spectrometry identify age-related protein expression differences for the primary visual cortex of kitten and adult cat. *J Neurochem.* **85(1)**: 193-205.

Van den Bergh G, Clerens S, Vandesande F, Arckens L (2003b). Reversed-phase high-performance liquid chromatography prefractionation prior to two-dimensional difference gel electrophoresis and mass spectrometry identifies new differentially expressed proteins between striate cortex of kitten and adult cat. *Electrophoresis* **24(9)**: 1471-81.

Van Der Kloot WG & Robbins J (1959). The effects of gamma-aminobutyric acid and picrotoxin on the junctional potential and the contraction of crayfish muscle. *Experientia.* **15(1)**: 35-6.

Van Harreveld A, Mendelson M (1996). Glutamate-induced contractions in crustacean muscle. *J Cell Comp Physiol.* **54**: 85-94

van Praag H, Christie BR, Sejnowski TJ, Gage FH (1999). Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc Natl Acad Sci U S A.* **96(23):** 13427-31.

van Praag H, Kempermann G, Gage FH (2000). Neural consequences of environmental enrichment. *Nat Rev Neurosci.* **1(3)**: 191-8.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002). Functional neurogenesis in the adult hippocampus. *Nature* **415(6875)**: 1030-4.

van Rijzingen IM, Gispen WH, Spruijt BM (1997). Postoperative environmental enrichment attenuates fimbria-fornix lesion-induced impairments in Morris maze performance. *Neurobiol Learn Mem.* **67(1)**: 21-8.

Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G, Citron M (1999). Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science.* **286(5440)**: 735-41.

Venable N, Fernandez V, Diaz E, Pinto-Hamuy T (1988). Effects of preweaning environmental enrichment on basilar dendrites of pyramidal neurons in occipital cortex: a Golgi study. *Brain Res Dev Brain Res.* **49(1)**: 140-4.

Vercauteren FG, Clerens S, Roy L, Hamel N, Arckens L, Vandesande F, Alhonen L, Janne J, Szyf M, Cuello AC (2004). Early dysregulation of hippocampal proteins in transgenic rats with Alzheimer's disease-linked mutations in amyloid precursor protein and presenilin 1. *Brain Res Mol Brain Res.* **132(2)**: 241-59.

Vinh DB & Drubin DG (1994). A yeast TCP-1-like protein is required for actin function in vivo. *Proc Natl Acad Sci U S A.* **91(19)**: 9116-20.

Vissel B, Krupp JJ, Heinemann SF, Westbrook GL (2001). A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux. *Nat Neurosci.* **4(6)**: 587-96.

Volkmar FR & Greenough WT (1972). Rearing complexity affects branching of dendrites in the visual cortex of the rat. *Science.* **176(42)**: 1445-7.

Von Eggeling F, Gawriljuk A, Fiedler W, Ernst G, Claussen U, Klose J, Romer I (2001). Fluorescent dual colour 2D-protein gel electrophoresis for rapid detection of differences in protein pattern with standard image analysis software. *Int J Mol Med.* **8(4)**: 373-7.

W

Walsh MJ, Kuruc N (1992). The postsynaptic density: constituent and associated proteins characterized by electrophoresis, immunoblotting, and peptide sequencing. *J Neurochem.* **59(2)**: 667-78.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ (2002). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature*. **416(6880)**: 535-9.

Walz R, Roesler R, Barros DM, de Souza MM, Rodrigues C, Sant'Anna MK, Quevedo J, Choi HK, Neto WP, DeDavid e Silva TL, Medina JH, Izquierdo I (1999). Effects of post-training infusions of a mitogen-activated protein kinase kinase inhibitor into the hippocampus or entorhinal cortex on short- and long-term retention of inhibitory avoidance. *Behav Pharmacol*. **10(8)**: 723-30.

Wang JH & Feng DP (1992). Postsynaptic protein kinase C essential to induction and maintenance of long-term potentiation in the hippocampal CA1 region. *Proc Natl Acad Sci U S A*. **89(7)**: 2576-80.

Wang LH & Strittmatter SM (1996). A family of rat CRMP genes is differentially expressed in the nervous system. *J Neurosci*. **16(19)**: 6197-207.

Wang YT & Linden DJ (2000). Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. *Neuron*. **25(3)**: 635-47.

Wang HW, Pasternak JF, Kuo H, Ristic H, Lambert MP, Chromy B, Viola KL, Klein WL, Stine WB, Krafft GA, Trommer BL (2002). Soluble oligomers of beta amyloid (1-42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus. *Brain Res*. **924(2)**: 133-40.

Wang D, Jensen R, Gendeh G, Williams K, Pallavicini MG (2004a). Proteome and transcriptome analysis of retinoic acid-induced differentiation of human acute promyelocytic leukemia cells, NB4. *J Proteome Res*. **3(3)**: 627-35.

Wang R, Dineley KT, Sweatt JD, Zheng H (2004b). Presenilin 1 familial Alzheimer's disease mutation leads to defective associative learning and impaired adult neurogenesis. *Neuroscience*. **126(2)**: 305-12.

Wang HG, Lu FM, Jin I, Udo H, Kandel ER, de Vente J, Walter U, Lohmann SM, Hawkins RD, Antonova I (2005). Presynaptic and postsynaptic roles of NO, cGK, and RhoA in long-lasting potentiation and aggregation of synaptic proteins. *Neuron.* **45(3)**: 389-403.

Weeber EJ, Levy M, Sampson MJ, Anflous K, Armstrong DL, Brown SE, Sweatt JD, Craigen WJ (2002). The role of mitochondrial porins and the permeability transition pore in learning and synaptic plasticity. *J Biol Chem* **277(21)**: 18891-7

Wehner JM, Sleight S, Upchurch M (1990). Hippocampal protein kinase C activity is reduced in poor spatial learners. *Brain Res.* **523(2)**: 181-7.

Wiedenmann B & Franke WW (1985). Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. *Cell.* **41(3)**: 1017-28.

Weisskopf MG, Bauer EP, LeDoux JE (1999). L-type voltage-gated calcium channels mediate NMDA-independent associative long-term potentiation at thalamic input synapses to the amygdala. *J Neurosci.* **19(23)**: 10512-9.

Wenzel J, Desmond NL, Levy WB (1993). Somatic ribosomal changes induced by long-term potentiation of the perforant path-hippocampal CA1 synapses. *Brain Res.* **619(1-2)**: 331-3.

Wernig A, Muller S, Nanassy A, Cagol E (1995). Laufband therapy based on 'rules of spinal locomotion' is effective in spinal cord injured persons. *Eur J Neurosci.* **7(4)**: 823-9

Wigstrom H, Gustafsson B (1986). Postsynaptic control of hippocampal long-term potentiation. *J Physiol (Paris).* **81(4)**: 228-36.

Williams BM, Luo Y, Ward C, Redd K, Gibson R, Kuczaj SA, McCoy JG (2001). Environmental enrichment: effects on spatial memory and hippocampal CREB immunoreactivity. *Physiol Behav.* **73(4)**: 649-58.

Wilson DA, Willner J, Kurz EM, Nadel L (1986). Early handling increases hippocampal long-term potentiation in young rats. *Behav Brain Res.* **21(3)**: 223-7.

Wong RW, Setou M, Teng J, Takei Y, Hirokawa N (2002). Overexpression of motor protein KIF17 enhances spatial and working memory in transgenic mice. *Proc Natl Acad Sci U S A.* **99(22)**: 14500-5

Woo NH, Duffy SN, Abel T, Nguyen PV (2000). Genetic and pharmacological demonstration of differential recruitment of cAMP-dependent protein kinases by synaptic activity. *J Neurophysiol.* **84(6)**: 2739-45.

Woo NH, Abel T, Nguyen PV (2002). Genetic and pharmacological demonstration of a role for cyclic AMP-dependent protein kinase-mediated suppression of protein phosphatases in gating the expression of late LTP. *Eur J Neurosci.* **16(10)**: 1871-6.

Woo NH, Duffy SN, Abel T, Nguyen PV (2003). Temporal spacing of synaptic stimulation critically modulates the dependence of LTP on cyclic AMP-dependent protein kinase. *Hippocampus.* **13(2)**: 293-300.

Wright JW, Masino AJ, Reichert JR, Turner GD, Meighan SE, Meighan PC, Harding JW (2003). Ethanol-induced impairment of spatial memory and brain matrix metalloproteinases. *Brain Res.* **963(1-2)**: 252-61.

Wu LG, Saggau P (1994a). Presynaptic calcium is increased during normal synaptic transmission and paired-pulse facilitation, but not in long-term potentiation in area CA1 of hippocampus. *J Neurosci.* **14(2)**: 645-54.

Wu LG & Saggau P (1994b). Pharmacological identification of two types of presynaptic voltage-dependent calcium channels at CA3-CA1 synapses of the hippocampus. *J Neurosci.* **14(9)**: 5613-22.

Wu SP, Lu KT, Chang WC, Gean PW (1999). Involvement of mitogen-activated protein kinase in hippocampal long-term potentiation. *J Biomed Sci.* **6(6)**: 409-17.

Wyszynski M, Kharazia V, Shanghvi R, Rao A, Beggs AH, Craig AM, Weinberg R, Sheng M (1998). Differential regional expression and ultrastructural localization of alpha-actinin-2, a putative NMDA receptor-anchoring protein, in rat brain. *J Neurosci.* **18(4)**: 1383-92.

X

Xia W, Zhang J, Ostaszewski BL, Kimberly WT, Seubert P, Koo EH, Shen J, Selkoe DJ (1998a). Presenilin 1 regulates the processing of beta-amyloid precursor protein C-terminal fragments and the generation of amyloid beta-protein in endoplasmic reticulum and Golgi. *Biochemistry.* **37(47)**: 16465-71.

Xia SZ, Feng CH, Guo AK (1998b). Multiple-phase model of memory consolidation confirmed by behavioral and pharmacological analyses of operant conditioning in Drosophila. *Pharmacol Biochem Behav.* **60(4)**: 809-16.

Xia Z, Storm DR (2005). The role of calmodulin as a signal integrator for synaptic plasticity. *Nat Rev Neurosci.* **6(4)**: 267-76.

Xu J, Zhong N, Wang H, Elias JE, Kim CY, Woldman I, Pifl C, Gygi SP, Geula C, Yankner BA (2005). The Parkinson's disease-associated DJ-1 protein is a transcriptional co-activator that protects against neuronal apoptosis. *Hum Mol Genet.* **14(9)**: 1231-41

Xue J, Tsang CW, Gai WP, Malladi CS, Trimble WS, Rostas JA, Robinson PJ (2004). Septin 3 (G-septin) is a developmentally regulated phosphoprotein enriched in presynaptic nerve terminals. *J Neurochem.* **91(3)**: 579-90.

Y

Yamaguchi K, Takada M, Fujimori K, Tsuchimoto Y, Kushima Y, Sanada M, Fujiwara T, Akagawa K (1997). Enhancement of synaptic transmission by HPC-1 antibody in the cultured hippocampal neuron. *Neuroreport.* **8(16)**: 3641-4.

Yamauchi T (2002). Molecular constituents and phosphorylation-dependent regulation of the post-synaptic density. *Mass Spectrom Rev.* **21(4)**: 266-86.

Yano S, Fukunaga K, Ushio Y, Miyamoto E (1994). Activation of Ca2+/calmodulin-dependent protein kinase II and phosphorylation of intermediate filament proteins by stimulation of glutamate receptors in cultured rat cortical astrocytes. *J Biol Chem.* **269(7)**: 5428-39.

Yao WD, Gainetdinov RR, Arbuckle MI, Sotnikova TD, Cyr M, Beaulieu JM, Torres GE, Grant SG, Caron MG (2004). Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioral plasticity. *Neuron.* **41(4)**: 625-38.

Ye Y, Meyer HH, Rapoport TA (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature.* **414(6864)**: 652-6.

Yeh GC, Bonhaus DW, Nadler JV, McNamara JO (1989). N-methyl-D-aspartate receptor plasticity in kindling: quantitative and qualitative alterations in the N-methyl-D-aspartate receptor-channel complex. *Proc Natl Acad Sci U S A.* **86(20)**: 8157-60.

Yin JC, Wallach JS, Del Vecchio M, Wilder EL, Zhou H, Quinn WG, Tully T (1994). Induction of a dominant negative CREB transgene specifically blocks long-term memory in Drosophila. *Cell.* **79(1)**: 49-58.

Yoshida K, Kobayashi K, Miwa Y, Kang CM, Matsunaga M, Yamaguchi H, Tojo S, Yamamoto M, Nishi R, Ogasawara N, Nakayama T, Fujita Y (2001). Combined

transcriptome and proteome analysis as a powerful approach to study genes under glucose repression in Bacillus subtilis. *Nucleic Acids Res.* **29(3)**: 683-92.

Yoshimi K, Ren YR, Seki T, Yamada M, Ooizumi H, Onodera M, Saito Y, Murayama S, Okano H, Mizuno Y, Mochizuki H (2005). Possibility for neurogenesis in substantia nigra of parkinsonian brain. *Ann Neurol.* **May 23**

Yoshimura Y, Yamauchi Y, Shinkawa T, Taoka M, Donai H, Takahashi N, Isobe T, Yamauchi T (2004). Molecular constituents of the postsynaptic density fraction revealed by proteomic analysis using multidimensional liquid chromatography-tandem mass spectrometry. *J Neurochem.* **88(3)**: 759-68.

Young D, Lawlor PA, Leone P, Dragunow M, During MJ (1999). Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. *Nat Med.* **5(4)**: 448-53.

Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L, Tandon A, Song YQ, Rogaeva E, Chen F, Kawarai T, Supala A, Levesque L, Yu H, Yang DS, Holmes E, Milman P, Liang Y, Zhang DM, Xu DH, Sato C, Rogaev E, Smith M, Janus C, Zhang Y, Aebersold R, Farrer LS, Sorbi S, Bruni A, Fraser P, St George-Hyslop P (2000). Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. *Nature.* **407(6800)**: 48-54.

Yu X, Malenka RC (2003). Beta-catenin is critical for dendritic morphogenesis. Nat Neurosci. 2003 Nov;6(11):1169-77

Yuan X & Desiderio DM (2005). Proteomics analysis of prefractionated human lumbar cerebrospinal fluid. *Proteomics.* **5(2)**: 541-50.

Yuste R & Bonhoeffer T (2001). Morphological changes in dendritic spines associated with long-term synaptic plasticity. *Annu Rev Neurosci.* **24**: 1071-89

Z

Zakharenko SS, Zablow L, Siegelbaum SA (2001). Visualization of changes in presynaptic function during long-term synaptic plasticity. *Nat Neurosci.* **4(7)**: 711-7.

Zamanillo D, Sprengel R, Hvalby O, Jensen V, Burnashev N, Rozov A, Kaiser KM, Koster HJ, Borchardt T, Worley P, Lubke J, Frotscher M, Kelly PH, Sommer B, Andersen P, Seeburg PH, Sakmann B (1999). Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. *Science.* **284(5421)**: 1805-11

Zanata SM, Lopes MH, Mercadante AF, Hajj GN, Chiarini LB, Nomizo R, Freitas AR, Cabral AL, Lee KS, Juliano MA, de Oliveira E, Jachieri SG, Burlingame A, Huang L, Linden R, Brentani RR, Martins VR (2002). Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. *EMBO J.* **21(13)**: 3307-16.

Zeiner M, Gebauer M, Gehring U (1997). Mammalian protein RAP46: an interaction partner and modulator of 70 kDa heat shock proteins. *EMBO J.* **16(18)**: 5483-90.

Zhang L, Ashendel CL, Becker GW, Morre DJ (1994). Isolation and characterization of the principal ATPase associated with transitional endoplasmic reticulum of rat liver. *J Cell Biol.* **127(6 Pt 2)**: 1871-83.

Zhang W, Benson DL (2001). Developmentally regulated changes in cellular compartmentation and synaptic distribution of actin in hippocampal neurons. *J Neurosci Res.* **69(4)**: 427-36.

Zhao YT, Tekkok S, Krnjevic K (1997). 2-Deoxy-D-glucose-induced changes in membrane potential, input resistance, and excitatory postsynaptic potentials of CA1 hippocampal neurons. *Can J Physiol Pharmacol.* **75(5)**: 368-74.

Zhao X, Lein ES, He A, Smith SC, Aston C, Gage FH (2001). Transcriptional profiling reveals strict boundaries between hippocampal subregions. *J Comp Neurol.* **441(3)**: 187-96.

Zhou X, Richon VM, Ngo L, Rifkind RA, Marks PA (1999). Cloning of the cDNA encoding phenylalanyl tRNA synthetase regulatory alpha-subunit-like protein whose expression is down-regulated during differentiation. *Gene.* **233(1-2)**: 13-9.

Zhou G, Li H, DeCamp D, Chen S, Shu H, Gong Y, Flaig M, Gillespie JW, Hu N, Taylor PR, Emmert-Buck MR, Liotta LA, Petricoin EF 3rd, Zhao Y (2002). 2D differential in-gel electrophoresis for the identification of esophageal scans cell cancer-specific protein markers. *Mol Cell Proteomics.* **1(2)**: 117-24.

Zhou Q, Homma KJ, Poo MM (2004). Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses. *Neuron.* **44(5)**: 749-57.

Zirlinger M, Kreiman G, Anderson DJ (2001). Amygdala-enriched genes identified by microarray technology are restricted to specific amygdaloid subnuclei. *Proc Natl Acad Sci U S A.* **98(9)**: 5270-5.