Differential Protein Expression in the Pilocarpine Model of Status Epilepticus in Rat Hippocampus

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Abstract

Epilepsy is the most common neurological condition. In those patients who require surgery for the treatment of their epilepsy, mesial temporal sclerosis (MTS) is the most common lesion identified. Up to 50% of these patients have a history of prolonged febrile convulsion in childhood, but the pathogenic mechanisms are still unknown. In this study a pilocarpine model of status epilepticus was used to study the events involved in hippocampal injury associated with status epilepticus. Magnetic resonance imaging (MRI) was carried out prior to hippocampal extraction. To obtain a global view of the protein changes involved in MTS, a proteomic approach was used to characterise the protein changes in the hippocampus of the treated animals when compared with corresponding control animals. A protein map of the hippocampus ensured that all proteins which were expressed in only one group, those that were modified (post translational modifications), and those in which their quantities were altered could be identified. Using PDQuest software, quantitative analysis of the proteins separated by 2-dimensional electrophoresis (2DE) on polyacrylamide gels showed that eleven proteins species were up-regulated in the treated hippocampus compared with those from control animals. Qualitative analysis showed that two proteins spots were only expressed in the treated group. Mass spectrometric (MS) analysis of one of the spots identified it as heat shock protein beta_1 (Hsp27). This result represents a new finding in the pilocarpine model of status epilepticus. This differential expression of HSP27 is in line with findings from the kainic acid model of epilepsy as well as resected temporal tissues of epilepsy patients. This study therefore provides data to support the view that the use of proteomics may be important in the understanding of the pathological processes following status epilepticus.

Abbreviations

BSA	Bovine serum albumin
CHAPS	3,3'-Cholamidopropyl-demethyammonio-1-propanesulphonate
CSE	Convulsive status epilepticus
ddH$_2$0	double distilled water
DTT	Dithiothreitol
GRP78	Glucose regulated protein 78
HS	Hippocampal sclerosis
HSPs	Heat shock proteins
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
LC-MS/MS	Liquid Chromatography/ Mass Spectrometry/Mass Spectrometry
Mr	Molecular weight
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MTE	Mesial temporal epilepsy
MTS	Mesial Temporal Sclerosis
PFC	Prolonged febrile convulsion
pI	Isoelectric point
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Status epilepticus
SPD	Sprague Dawley

SSP NO   Standard spot number

TLE   Temporal lobe epilepsy

ID   One dimension

2DE   2-dimensional electrophoresis

CHAPTER 1   INTRODUCTION

'A seizure is to the brain as a cough is to the lung- the outward sign of an internal tissue irritation' (Mendel, 1995). Epilepsy is not a disease but a variety of disorders that may result from different causes. Epilepsy is characterized by an enduring tendency to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition (The International League against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE) consensus definitions, Fisher et al, 2005). Epilepsy is a brain disorder which has been afflicting man since the dawn of our species. Early medical writings such as Hippocrates' in the 400 BC described it as a brain disorder refuting the idea that it's neither a curse nor a prophetic power. Today, it is still the most common serious neurological condition. In the United Kingdom alone, it has been estimated that there about 160,000 people with epilepsy who require continuing hospital based treatment, based on a prevalence rate of 1/100. Incidence figures have been quoted to be around 50 cases per 100,000 persons per year in the developed countries, and about 100-190/100,000/year in the developing countries (Macdonald et al, 2000). However, these figures vary widely in different studies.

1-1   Prolonged Febrile Convulsion and Mesial Temporal Epilepsy

This section describes the terms prolonged febrile convulsion, mesial temporal sclerosis (MTE) and the long standing debate about their association. The National Institute of Health (NIH) defines prolonged febrile convulsion (PFC) as a seizure lasting at least 30 minutes, which is associated with a fever not of CNS origin, in a neurologically normal child between 6 months and 5 years of age. PFC is a form of convulsive status epilepticus (CSE). "CSE is defined as a disorder in which epileptic activity lasts for more than 30 minutes or more, causing a wide spectrum of clinical symptoms, and with a highly variable pathophysiological anatomical, and aetiological basis" (Shorvon S, 1994). Mesial temporal epilepsy (MTE) is a form of epilepsy arising from a focus in the temporal lobe usually from sclerosis of the hippocampus. Febrile convulsion affects 3% of the population, and 5-10% of these will be prolonged, leading to several thousand children developing prolonged febrile convulsion every year(Scott et al, 1998). However, the most controversial issue in epilepsy research has been whether prolonged febrile convulsions can cause mesial temporal sclerosis (MTS), a condition that involves damage to the hippocampus (section 1-2). MTS is the most common lesion in patients who require surgery for the treatment of epilepsy and of this figure, only about 70% will be expected to be seizure free, suggesting that prevention of the development of MTS will be extremely beneficial to temporal lobe epilepsy (TLE) patients. In the following paragraphs I will outline the research to date that investigates the relationship between PFC and MTS.

Several retrospective studies from epilepsy surgery centres have reported that as many as 40-50% of patients with intractable temporal lobe epilepsy with mesial temporal

sclerosis also have a history of prolonged febrile convulsion in childhood (Williamson PD and Engel J Jr, 1997). A prominent study by Falconer and colleagues in 1964 showed mesial temporal sclerosis in about half of the resected temporal lobes of 100 adults, and 40% of these patients had a history of infantile convulsions. While the figures may suggest a causal relationship, the nature of the association cannot be determined by these studies.

Another study described neuronal necrosis in post-mortem results of children with prolonged febrile convulsion. A report of a child followed up after death described bilateral necrosis in CA1, CA3 and in the hilus subregions of the hippocampus similar to that observed in animal models (Strafstrom et al, 1996). However, these isolated reports have been argued to be the extreme cases that are far from typical. A more recent neuropathological study supported the other line of argument which says that the hippocampal sclerosis and TLE observed had it origin in early life, commonly from cortical dysplasia (Porter et al, 2003). More data from pathological cases is required to investigate the likely cause of hippocampal damage.

Animal models of epilepsy strongly suggest that insults may induce seizures and that after a period of latency can develop into epilepsy. The different experimental paradigms used in inducing seizures in models result in similar damage to the hippocampus, suggesting that the damage is caused by status epilepticus itself and is not a direct consequence of the method used. Convulsive status epilepticus can be induced in animal models by use of various chemicals or by electrical kindling (Scott et al, 1998) as summarized below;

- Anti-GABA drugs: bicucculine in adolescent baboons results in neuronal loss in the hippocampus, neocortex, amygdale, thalamus, and cerebellum. The result is similar to that observed in humans.

- Glutamatergic drugs: intraperitoneal, intra-amygdala, or intraventricular kainic acid leads to hippocampal damage after CSE in rats. The resulting damage is analogous to that seen in humans who have died during CSE.

- Cholinergic drugs: pilocarpine and dipiperidionethane are able to cause CSE in rats with resultant damage in the neocortex, thalamus, amygdala, and hippocampus. The pilocarpine model was used to induce the seizures in this study.

- Electrical stimulation: these models show neuronal death in the hippocampus if continuous stimulation is carried out over time.

Population and family studies: the evidences from population based studies is conflicting with studies from the National Collaborative Perinatal Project (NCCP) claiming that history of febrile convulsion in a parent or prior-born sibling was associated with a threefold increase in the rate of subsequent epilepsy (Nelson KB and Ellenberg JH, 1978), others claim the association is quite weak (Annergers JF et al., 1987). While these evidences from prospective and controlled population studies have shown that the risk is quite low, larger and longer studies that span the time period of the average latency (8-9 years) between PFC and onset of TLE will shed more light on the debate.

Imaging studies: In general, the timing of imaging and selection of children varies between studies. A radiological study showed brain swelling followed by atrophy in

children after episodes of status epilepticus (Alcardi J and Chevrie JJ, 1983). A more specific and recent study that used magnetic resonance imaging (Scott R C et al., 2003) as a research tool was able to show evidence of oedema within 48 hours of a PFC (Scott et al, 2002). A follow up study later showed a reduction in hippocampal volume 4-8 months later with oedema and change in hippocampal asymmetry consistent with injury and neuronal loss associated with PFC (Scott et al, 2003). However an MRI study by Tarkka et al (2003) showed that the occurrence of MTS after PFC was uncommon.

1-2   The Hippocampus and Mesial Temporal Sclerosis

The hippocampus, a horseshoe-shaped structure in the mesial temporal lobe of the brain, is implicated in the consolidation of new memories, emotions, navigation and spatial orientation. It lies above the subiculum and medial parahippocampal gyrus, forming a curved elevation along the floor of the inferior horn of the lateral ventricle. It is a trilaminar archicortex with a single pyramidal cell layer. The hippocampus can be divided into three distinct fields, CA1, CA2 and CA3. Field CA3, ten cells thick, is found between the hilus of the dentate gyrus and field CA2 at the other end. The pyramidal cells in the CA3 field are largest cells in the hippocampus and they receive the mossy fibre input from dentate granule cells on their proximal dendrites. CA2 field has the most compact layer of pyramidal cells. While it lacks a mossy fibre input from dentate granule cells, it receives a major input from the supramamillary region of the hypothalamus. CA1 is described as the most complex of the subdivisions with an appearance that varies along its transverse and rostrocaudal axes. It thickness

varies from ten cells to more than thirty cells. Approximately 10 % of neurons in the field CA1 are interneurons.

The hippocampus is implicated in many diseases including Alzheimer's disease and Huntington's chorea. More than 50% of all partial epilepsies originate from foci in the temporal lobe. The hippocampus is heavily implicated in the development of seizures in both humans and animal models due to its high susceptibility to seizure-induced injury as evidenced by histopathological changes observed in the brain (Junker et al, 2005; Darrell, 1999). Hippocampal sclerosis also known as Ammon's horn sclerosis or mesial temporal sclerosis is the commonest pathology found in TLE patients, without an underlying structural lesion, undergoing anterior temporal lobectomy (up to 80% of these cases, Daviese t al, 1996). This has been linked to the low seizure threshold described in many of the parts of the hippocampus. The lowest threshold for seizures was found in the dorsal corsa ammonis (CA1) of the hippocampus compared with areas like the motor cortex which has a threshold that is fivefold to sevenfold higher (Burnham W M, 2002).

The nature of the pathology observed in the hippocampus following epilepsy is characterized by neuronal loss and gliosis of areas CA1, CA3 and dentate gyrus of the hippocampal formation. It is generally described using the 'hippocampal grading system' (Davies et al, 1996). The grading system, shown to be consistent and reproducible by different pathologists, is divided into the following categories;

- Grade 0: no hippocampal damage present

- Grade 1: mild mesial temporal damage. Gliosis with slight (<10%) or no neuronal dropout

- Grade 2: moderate mesial temporal damage. Gliosis with moderate (10-50%) neuronal dropout.

- Grade 3: moderate to marked mesial temporal damage (HS or 'classical' Ammon's horn sclerosis). Gliosis with >50% neuronal dropout.

- Grade 4: marked mesial temporal damage (HS or 'total Ammon's horn sclerosis). Gliosis with >50% neuronal dropout.

(Modified from Davies et al, 1996)

The influence of mesial temporal sclerosis on the association between prolonged febrile convulsion and epilepsy of temporal lobe origin is an important one, since the onset of the pathology could either be a consequence of the convulsions or it could precede it as an underlying cause of both the febrile convulsions and mesial temporal epilepsy. Studies have widely supported the idea that the pathology observed is a consequence of the prolonged convulsions. This is especially seen in animal models in which different strategies and species used in inducing seizures bring about the same pathology in a previously normal hippocampus (Abdelmalik et al, 2005).

1.3   Proteomic Separation of proteins using 2-dimensional gel electrophoresis (2DE)

In the post genomic era, one inevitable question posed by the genome sequencing projects is 'what is the function of all the proteins' (Palzkill T, 2001)? The conventional method for investigating an alteration in gene expression is to investigate an individual gene. While this mRNA based screening is very powerful, it is not sufficient to elucidate biological function as a cell is dependent upon a multitude of metabolic and regulatory pathways for its survival. Moreover, a poor correlation between mRNA and protein abundance may exist as observed in some mammalian systems (Greene et al, 2002). In addition, post-translational modifications may not be apparent from the DNA sequence. Nevertheless, individual investigations have helped in the discovery of important proteins e.g. protein huntington for Huntington's chorea, and apoliprotein A and β-amyloid in the pathogenesis of Alzheimer's disease (Price D L et al., 1998).

Proteomics is a branch of functional genomics defined as the large scale study of protein properties such as expression levels, post-translational modifications and interactions with other molecules to obtain a global view of cellular processes at the protein level (Palzkill T, 2001). This direct screening of the protein profile involves the use of two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Although proteomics has its limitations, several successful applications have appeared in the literature, for instance, psoariasin, a putative urinary marker was discovered and can now be used for follow-up of patients with bladder squamous cell carcinoma (Celis et al, ...). Psoariasin was identified when Celis and colleagues

compared the protein profile of secreted proteins from normal tissue with that from cancerous tissue. Proteome maps of human plasma, red blood cells, lymphoma, cerebrospinal fluid, platelet, breast, heart, kidney and placenta are now available, against which pathological proteome maps can be compared.

Two-dimensional gel electrophoresis was first introduced by O'Farrell and Klose in two separate descriptions (O'Farrell, 1975; Klose, 1975). The original technique involved a first-dimension separation in carrier ampholyte-containing polyacrylamide gels in narrow tubes. The sample was applied to one end of each tube gel and separated at high voltages. The gel rods were then removed from their tubes, equilibrated in SDS sample buffer containing a reducing agent (DTT) followed by an alkylating agent (Iodoacetamide) before been placed on vertical SDS-polyacrylamide gels for the second dimension.

The use of this method has a lot of limitations in that sensitivity, reproducibility and its inability to detect some proteins (e.g. membrane proteins) poses problems. Furthermore, the use of carrier ampholytes prevented the widespread application of 2D gel electrophoresis. The carrier ampholytes are mixed polymers that are not well characterized, they suffer from batch-to-batch variations, have unstable pH gradients with a tendency to drift and in turn reduce the reproducibility of the first dimension separation. The power of this technique (recognised since it inception) is only now been utilized due to a number of developments described by Berkelman and Stenstedt (1998) and Gorg (2004).

1.4   Improvements in two-dimensional Electrophoresis

The application of 2-D electrophoresis has become significant due to various improvements in recent years;

✓ The replacement of the carrier ampholyte-generated pH gradients with immobilized pH gradients (IPG) and tube gels with gel strips supported by a plastic film backing. These improvements help generate 2-D maps that are superior in terms of resolution and reproducibility.

✓ Following separation, mass spectroscopic techniques have been developed that can analyse very small quantities of peptides and proteins.

✓ 2-D patterns can now be easily analysed using the improved, more powerful, less expensive computers and software.

✓ The entire genome or at least large parts of it for many organisms are now available allowing rapid identification of the gene encoding a protein separated by 2-D electrophoresis.

✓ Access to spot pattern databases for the comparison of electrophoresis results and to genome sequence databases are now available on the World Wide Web.

(Berkelman T and Stenstedt T, 1998)

1-5 Background to the two-dimensional analysis of the 'PROTEin' complement expressed by a 'genOME'.

The separation of proteins based on 2DE analysis is divided into three parts; sample preparation, separation by charge followed by mass separation.

1-5-1 Sample Preparation

The first step that dictates the successful separation of a protein in a 2DE analysis is the solubilization, denaturation and reduction to completely break the interactions between the proteins and to remove non-protein components (Herbert B, 1999). A major advantage that the 2-D analysis has over the 1-D analysis is it ability to separate complex mixtures such as a rat brain resolving up to 8000 proteins (Leung K et al., 2001). However, the 1-D analysis which can resolve ≤ 100 proteins has the advantage that nearly all proteins are soluble in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10,000-25,000 molecular weight proteins as well as extremely basic or acidic proteins). In order to harness the full advantage of the 2-D analysis, it is important that all translated products are solubilised in the lysis solution. It is now apparent that some proteins cannot be properly solubilized in the solution developed by O'Farrell (Fichmann J and Westermeier R, 1975). In general sample preparation solution must be able to solubilize all proteins, prevent protein aggregation and hydrophobic interactions, remove or thoroughly digest any RNA or DNA and prevent artifactual oxidation, carbamylation, proteolytic degradation or conformational alteration (Fichmann, 1999). There is no universal protocol for the lysis solution used, but a combination of chaotropes, surfactants and reducing agents

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will be present. For example a soluble protein sample which can be easily taken up onto IEF strips would use a sample solution of 8-9.8 M urea (denaturant), a non-ionic detergent (solubilizing agent), dithiothreitol (DTT, reducing agent), and possibly protease inhibitors and protease-free nucleases. In this project, the detergent concentrations were varied to obtain optimal conditions.

1-5-2 Separation on the basis of charge

The next step is the 2-D separation which involves first separating proteins based on their isoelectric points (pI) using isoelectric focusing (IEF). The isoelectric point is the pH at which there is no net charge on a protein. Proteins are amphoteric, they carry positive, negative or zero net charge depending on the pH of their surrounding. They will assume a positive charge at pH values below their pI and a negative charge at pH values above their pI. The presence of a pH gradient is therefore crucial to the IEF technique. Applying an electric field will ensure that a protein moves to the position in the gradient where its net charge is zero. Proteins with positive net charge will move towards the cathode and vice versa. The degree of resolution is a function of the slope of the pH gradient as well as the electric field strength; hence IEF is performed in high voltage (excess of 1000V).

IEF of the protein mixture is followed by immobilized pH gradient (IPG) strip equilibration in an SDS buffer system containing urea, glycerol, reductant, SDS, buffer and dye. The reductant, usually DTT, is replaced by iodoacetamide in a second optional equilibration step.

1-5-3 Separation on the basis of mass

The second step involves the use of Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is an electrophoretic method that separates polypeptides according to their molecular weight (Mw). The SDS in the polyacrylamide gels and sample ensure that the intrinsic electrical charge of the proteins does not play a part in the separation. Treating the sample proteins with SDS as well as with reducing agent (DTT) therefore ensures that separation occurs exclusively by molecular weight.

1.6 Protein Visualisation in 2DE gels

There are many techniques that can be used for visualisation of protein spots on 2DE gels and most methods that work with SDS gels can be applied. The most sensitive are those that employ radiolabeled proteins in vivo using either ^{35}S, ^{14}C, ^{3}H, or in the case of phosphoproteins. ^{32}P. Autoradiographic detection and fluorography can than be used to visualize the proteins.

More commonly used methods are nonradioactive and include silver staining and Coomassie staining. Silver staining is very sensitive nonradioactive method; hence it is ideal for the detection of trace components within a protein sample and for the analysis of protein samples available in limited amount. However high-purity reagents and precise timing are required for reproducible and high quality results. Fluorescent stains have now been developed with greater sensitivity than silver staining (Leung K et al., 2001). Coomassie staining is 50-fold less sensitive, simpler and more

quantitative than silver staining. It is based on binding of the dye coomassie brilliant

blue G-250, which binds non-specifically to all proteins.

1-7   Identification and characterisation of proteins

Following improvements in the identification of proteins, identification of very

minute quantities of proteins to high degree of accuracy is now possible by the use of

mass spectrometric techniques like tandem mass spectrometry (MS/MS) and matrix-

assisted laser desorption ionization in conjunction with time of flight detection ''.

These are extensively reviewed by Pandey and Mann (Pandey A and Mann M, 2000).

1-8   Aims of the study

This study was carried out to develop the protocol for the use of 2-dimensional

electrophoresis (proteomics) in the characterisation of the changes in the rat model of

status epilepticus.

Changes in the pilocarpine model of status epilepticus will be used to answer

following biological questions:

- To obtain a global protein map of proteins expressed in the normal

  hippocampus and in rat model of mesial temporal sclerosis (MTS)

- Identify those proteins which are expressed in either the treated and control animal hippocampus only

- Identify proteins in which a change in abundance was observed between the treated and control animals

- To lay the foundation for future proteomics studies into the pathogenesis of MTS.

These findings may lead to the generation of hypothesis relating to the mechanism of injury and epileptogenesis following status epilepticus.

CHAPTER 2   Materials and Methods

This chapter describes (i) the steps taken to optimise the procedure by the use of embryo samples as well as (ii) running of the actual experimental gels.

2-1   Materials Used

Insight Biotechnology Limited supplied the Owl Silver staining Kit. The scanner for imaging the gels; GS-800 Calibrated Densitometer and the PDQUEST software (version 7.0) were both from Bio-Rad, Richmond, CA, USA. Silver Staining and all other solutions were made with MilliQ water (18MΩcm) from Millipore, Bedford, MA. Amersham Biosciences, Sweden, supplied all other equipments including the multiTemp III for cooling the units, Ettan DALT six electrophoresis unit for the second dimension step, electrophoresis Power supply-EPS 3501 XL, the Drystrip Aligner, Immobiline ^{TM} DryStrip pH 4-7 18cm were all from Amersham Biosciences, Sweden. Amersham also supplied the Immobiline Drystrip Reswelling Tray and the Multiphor II for the focussing step.

2-2   Tissue Samples

The hippocampi sections were derived from the lithium-pilocarpine model of limbic status epilepticus (n=3) and control (n=2) rats.

We investigated adult male Sprague Dawley (SPD) rats (200-220g) which were pre-treated with *scopolamine* (1mg/Kg), a systemic muscarinic antagonist to inhibit the systemic effect of *pilocarpine*. Pilocarpine was then administered 20-30 minutes later at 30mg/kg. Pilocarpine is a muscarinic agonist with its effects starting about ten minutes after intraperitoneal injection. *Scopolamine* was again injected about 60 minutes later, then *diazepam* (10mg/kg) 90 minutes after status epilepticus to terminate the effects of the drug. The procedure was monitored with magnetic resonance imaging (MRI) before and at 24 and 48 hours interval after injection. 48 hours after pilocarpine injection animals were sacrificed and their hippocampi removed and stored at -70°C until the start of the proteomics study. MRI imaging of two control rats (injected with saline as placebo) were also carried out before extracting their hippocampi. The result of the MRI changes is seen in figure 3-1.
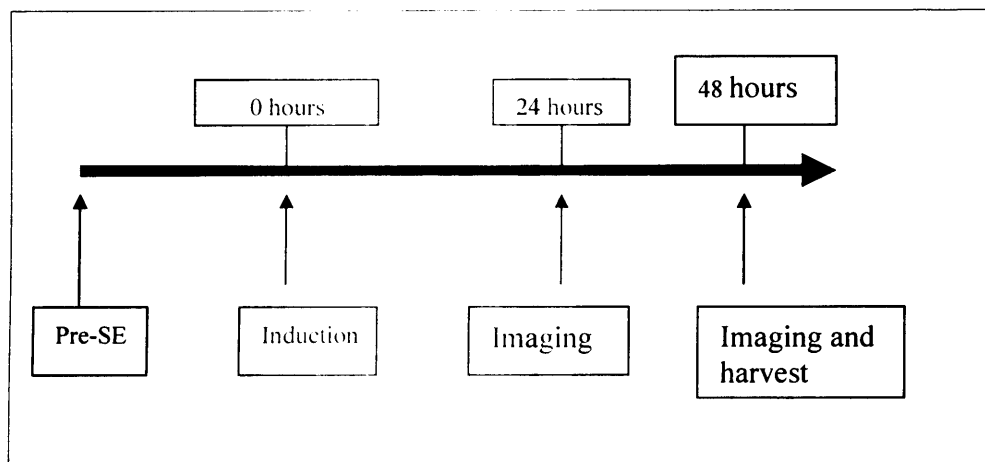
Figure 2-1 schematic process from induction of status epilepticus in rat to the confirmation of hippocampal changes to the harvest of the hippocampus.

2-3   <u>Running of Embryo Samples</u>

A series of 2D gels were run using embryo samples to develop and optimise the procedure by testing different conditions, reagents and buffers. The different approaches, methods and reagents tested are described in section 2-4-9. The detailed procedure for the hippocampi samples are described below.

2-4   <u>Running of Analytical Experimental Gels</u>

- Sample preparation
- IPG strip rehydration
- IEF
- IPG strip equilibration
- SDS-PAGE
- Visualisation
- Analysis

2-4-1 <u>Sample Preparation and Protein solubilization for 2-DE</u>

This was carried out to denature, disaggregate, reduce and solubilize the sample in order to achieve complete disruption of molecular interactions and to ensure that each spot represents an individual polypeptide. All operations were performed in a sterile condition free from contamination by constant use of gloves rinsed with 70% IMS. Three treated and one control hippocampi sections were immediately stored at $-70^{\circ}C$. The operations were also carried out on ice as much as possible. Harvested samples were defrosted (not exceeding $36^{\circ}C$) from $-70^{\circ}C$ before adding 1 ml of lysis buffer (table 2-1). The tissue was partially broken down with an injection needle before subjecting it to sonication (approximately 10 x 1 second at 15μm). The samples were then aliquoted into 200μl tubes and kept frozen at $-70^{\circ}C$ to prevent repeated thawing which might lead to protein degradation.

Table 2-1   Composition of the lysis buffer used

Reagent		Final Concentration
Urea	30g	9.5M
CHAPS	1g	2%
DTT	0.5g	1%
Protease Inhibitors	5 tabs	1x
Phosphatase Inhibitors 1	05ml	1x
Phosphatase Inhibitors 2	0.5ml	1x

2-4-1-1   Protein Quantification

Protein quantification was carried to determine the concentration of the hippocampal

tissue dissolved in the lysis buffer. Using a modified Bradford assay, Bovine serum

albumin (BSA) (1mg/ml in milliQ H_2O) was used as the standard protein to prepare a

standard curve for calibration (table 2-2).

Table 2-2 Composition of the assay for standard curve preparation

Protein (µg/ml)	0	1	2	4	6	8
BSA stock solution (µl)	0	1	2	4	6	8
Lysis Buffer (µl)	2	2	2	2	2	2
MilliQ H_2O (µl)	8	7	6	4	2	0
0.1N HCl:H_2O (1:8) (µl)	25	25	25	25	25	25

The sample solution was diluted with the MilliQ H_2O in dilutions of 1:1; 1:2; 1:5;

1:10; and 1:100 to determine which will fall within the range of the calibration curve.

2ml of each of the samples in duplicate was then added to 8 µl of MilliQ H_2O before

adding 25 µl of the 0.1N HCl to all the samples and the standards. The working

reagent which was the BIO-RAD protein assay solution (1ml) diluted 1 in 4, was then

added to each standard and sample. The solutions were then left for about 10 minutes

before the optical density was read at 595 nm, and the unknown protein concentrations determined using linear regression analysis.

2-4-2   IPG strip Rehydration

Immobilised pH gradient (IPG) strips were used for separation of proteins on the basis of isoelectric point. Samples were loaded by sample in-gel rehydration, in which the samples are already dissolved in rehydration buffer prior to adding the buffer. For the 18 cm IPG strips, 300 µg of protein sample was added to 8 µl Pharmalyte (final concentration of 2%) made up to 400 µl by the addition of the rehydration buffer. The volume of protein sample used was derived from the assay of the stock solution

The rehydration solution was spread evenly over the groove of the reswelling tray and the IPG strip immediately laid over the solution with the gel side down as soon as they are removed from the -20°C fridge. The strip is set such that it is movable and not sticking to the sides of the grooves, this way an even reswelling of the gels is achieved. This was carried out for each of the other strips and the strip identification numbers corresponding to each hippocampi noted. The set up was covered with DryStrip cover oil and left overnight at controlled room temperature for approximately 16 hours to avoid the risk of protein carbamylation from higher temperatures (> 37°C) and urea crystallization on the strips from lower temperature (<10°C) (Gorge et al, 2004).

Table 2-3   Reswelling buffer recipes for 30 mls.

Solution	Recipe for 30 mls	Optimised recipe
Urea (8M)	19.3g	19.3g
AmberliteMB-1	0.5g	0.5g
DTT (in full) (0.2%)	60 mg	6omg
CHAPS (0.5%)	150 mg	300mg (1%)
Pharmalyte (pH 3-10)	150 μl	150 μl
MilliQ H₂0	25.6mls	25.6 mls

The urea was initially dissolved in the deionised water before adding 0.5 g of the Amberlite. The product was stirred for 10 minutes before filtration. DTT, CHAPS and Pharmalyte were then added. The solution was divided into aliquots and stored at -20°C until ready for use.

2-4-3   First Dimension- Isoelectric focusing

On completion of the rehydration step, the IPG strips pH 4-7 were immediately transferred to the multiphor II unit (Amersham, Sweden) for the isoelectric focussing. Prior to this, the isoelectric focussing equipment had been prepared by setting the

temperature to 20°C using the cooling water bath and the strip holder container was set in place with cover fluid underneath to make a seal. Cover fluid (10 ml) was poured into the container and the strip holder placed on top, removing bubbles. The rehydrated strips were then placed in their groves, gel side up, the acid end towards the anode. The strips were covered with DryStrip cover oil and the leads connected to power supply.

At 20°C for 20 hours, the isoelectric focussing was performed in a Multiphor II focussing unit (Amersham Pharmacia Biotech, Bucks, UK) for 65kV h. In a linear gradient form, the conditions were 0-500 V for the first step (1Vh), 500-3500 V for the second step (700Vh) and 3500-3500 V for the last step until 65kVh were achieved. The focussed strips were frozen at -70°C until ready for the second dimension or immediately used for this.

2-4-4   IPG strip equilibration

This step saturates the IPG strip with the SDS buffer system required for the second dimension separation. An aliquot (20ml) of equilibration buffer (table 2-4) was thawed and 1% (w/v) DTT (0.2g) was added. The strips were then removed from the freezer and placed gel side up in the rehydration tray on a shaker in a fume cupboard.

Each strip was overlaid with at least 2 ml of equilibration buffer and left shaking for about 15 minutes.

In the second step, iodoacetamide was added to the second aliquot (20ml) of the SDS-equilibration buffer to 2.5% (w/v). This was then used to replace the DTT SDS-buffer and left to shake for about 25 minutes.

Table 2-4   Equilibration Buffer recipe for a 500ml stock

Reagent		Final Concentration
Urea	180g	6M
Glycerol	150g	30%
SDS	10g	2%
Tris pH 8.8	16.7ml	1.5M
MilliQ H$_2$O	made up to 500ml with MilliQ H$_2$0	

The solution was made up to 500mls with MilliQ H$_2$0 and dissolved before the addition of few grains of Bromophenol blue. It was then divided into aliquots and stored at -20°C.

2-4-5 Second Dimension- SDS PAGE

This procedure was for 1.5 mm thick gels prepared and stored at -4°C until the next day. Although the procedure can all be set up in a day, it was carried out over two days for convenience.

Day 1: Preparation of 12% Acrylamide gels and SDS-electrophoresis buffer

In order to prepare six gels, 2g of Amberlite was added to 180 ml of Acrylamide (40%) and stirred with a magnetic shaker for about 20 minutes. The mixture was then filtered into a 1 litre duran bottle using a 0.45μm circular filter. After filtration, 149 ml of 1.5M Tris pH 8.8 and 260 ml of MilliQ dH$_2$O was added and the solution was de-gassed for 1 hour with stirring (table 2-5).

The gel cassettes to be used were thoroughly cleaned with MilliQ H$_2$O and then 70% IMS before air drying. The cassettes were arranged in the casting chamber with plastic sheets in between to prevent them sticking together on polymerisation of the acrylamide. The chamber was covered, tightened with screws and clamps used to fasten the lid. A mixture of 10% SDS (6ml), freshly prepared 10% APS (4.5ml), and TEMED (130μl) were added to the de-gassed solution. The whole mixture (about 600ml) was immediately poured into the casting chamber until it reaches within 2cm of the top which is just above the level where the loading channel widens. The gels were immediately layered with water saturated butanol, covered with cling film and allowed to polymerise for about 2 hours. After setting the chamber was dismantled and the butanol replaced with milli-Q dH$_2$O. Gel cassettes were then wrapped

between damp tissues and the whole stack wrapped in cling film before been stored at 4°C until the next day.

Table 2-5   **Recipes for 12% Acrylamide Gels**

Solution	Gel for 6 cassettes (600 ml, 12.5%)
Acrylamide (40%)	180 ml
Amberlite	2 g
1.5M Tris-HCl pH 8.8	149 ml
MilliQ H_2O	260 ml
SDS (10% w/v)	6 ml
APS (10% w/v)	4.5 ml
TEMED	130 µl

Preparation of 10x SDS-electrophoresis buffer

The SDS-electrophoresis buffer was made by adding 30.25g of Tris, 144.1g of glycine and 10g of SDS in a 1 litre duran bottle and making it up to 1 litre by the addition of MilliQ dH_2O. It was left stirring on a magnetic mixer until fully dissolved, then stored at 4°C until the next day. In order to optimise the results for the hippocampi protein spots, the amount of SDS used had to be increased slowly from 10g to 12g, 15g and even 50g until the best results was observed at 15g.

Day 2: Running of Second Dimension Gels

In preparation for the running of the electrophoresis, the second dimension gels were removed from storage and allowed to return to room temperature before use.The temperature of the MultiTemp water bath was set at 10°C then connected to the Ettan 6 tank. The buffers were then prepared from the 10x SDS stock and Milli-Q dH_2O in the following dilutions.

- 4 litres of 1x buffer(400 ml 10x buffer + 3600 ml MilliQ water)
- 1 litre of 1x buffer (100ml of 10x + 900 ml MilliQ water)
- 800 ml of 3x buffer (240 ml of 10x + 560 ml MilliQ water)

To prepare the mix for the agarose sealing solution, 0.25g of agarose was added to 50 ml of the 1x SDS-electrophoresis buffer. A few grains of bromophenol blue was then added.

As soon as the IPG strips are equilibrated as described in the section above they were immediately transferred into the grove at the top of the gels in the cassettes. The protein markers were placed at the end of the strips and the warmed agarose sealing solution applied to seal the strips in place, making sure the back of the strip was touching the cassette while also ensuring that no air bubbles were trapped under the strips. The cassettes were arranged into the Ettan tank and the top layer filled with the 3x buffer and the side with the 1x buffer. The electrodes were then connected and the electrophoresis carried out at a low power of 2w per gel overnight.

The power supply was switched off when the dye front had travelled approximately 18 mm down the gel. The cassettes were opened and the gels transferred into the fixing solution described in the following section.

2-4-6 Detection of proteins after PAGE

Silver Staining: The gels were stained in large glass dishes with constant agitation to ensure adequate surface contact with gel. Protein gels were visualised using an adapted form of the PlusOne silver staining procedure (PlusOne reagent kit from Amersham Biosciences, UK). The procedure was originally based on that of Heukeshoven and Dernick (1996). At room temperature, 250 ml of all solutions were used except the washing solutions that were changed to 300 ml of MilliQ dH_2O. The gels were stored in plastic bags moistened with water and stored at $4^{o}C$.

2-4-7 Scanning and Image Analysis

The gels were scanned using the GS-800 calibrated densitometer (Bio-Rad) and subsequently analysed using the PDQuest software version 7.0 (Bio-Rad)which has less human intervention, although spot were still edited to generate spots which are unique to each group and have specific Mr and pI. One of the gels (with the largest number of spots) was selected and a reference (master) gel was created from it, with spots from other gels automatically matched to it. Further details are as described by

Leung and colleagues (2001). Quantitative and qualitative comparisons were carried out between replicate groups comprising of three gels from the control, three from a treated and one of each from two other treated hippocampi tissues. This was followed by student's T-Test (confidence level 0.05) to compare spots with more than 2-fold difference as well as Boolean analysis which compare spots in two other analysis set.

2-4-8 Mass Spectrometry

Spots of interest were excised from the gel using a clean scapel and placed into 1.5 ml eppendorf tubes prior to analysis. The spots detected were analysed by in-gel digestion with trypsin and Liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) as described by Leung and colleagues (2001). This step was carried out by our external collaborator, Dr Robin Wait at Imperial College, London.

2-4-9 Alternative Steps considered

The reagents used were chosen for optimum results by trying alternative reagents. Using the embryo samples trials, one-dimensional gels were used to test the effect of using two different staining methods: the Plus One silver staining kit was compared with the Owl silver staining kit. The results of the staining gave a better resolution

with the PlusOne suggesting that it is more compatible with the preparation method. Water prepared by different conditions were also tried, for instance, the result of using MilliQ water ($18m\Omega cm$) and ordinarily distilled water were compared, it was also decided that MilliQ water be used.

The rehydration buffer was also chosen based on a comparison of the results of the gels after staining. The in-house rehydration buffer was compared with the destreak rehydration buffer. Both of this gave the same results at the end, hence the buffer prepared in-house was chosen since it is more economical.

CHAPTER 3   RESULTS

3-1   Reagents for successful 2D Separation

Embryo samples, available from previous studies in the laboratory, were used in comparing alternative reagents and water grades for the best results as described in section 2.2.2.10. One dimensional analysis of proteins using SDS-PAGE showed that the Plus one silver staining kit rather than the Owl silver staining kit was more suitable for the protocol, since staining was more reproducibly achieved.

Comparison of the commercially available Destreak rehydration buffer with that produced in the laboratory showed that both were equally suitable as both showed that the sample was well solubilised and resolved. It was then decided that the rehydration buffer produced-in house be used as it was more economical. As described earlier, water from two different sources also gave the same results; hence, MilliQ water (18MΩcm) from Millipore was used for all the steps involving water.

3-2   Hippocampal changes on MRI

The advantage of this study was the fact that experimental animals were monitored by MRI following status epilepticus. Animals were immediately sacrificed after imaging followed by the extraction of the hippocampus. Extraction was carried out at 48 hours when the most obvious changes were seen in the hippocampus of the rats. The control animals showed no changes in the MRI image as expected. The changes observed by MRI following the induction of status epilepticus are shown in figure 3-1.
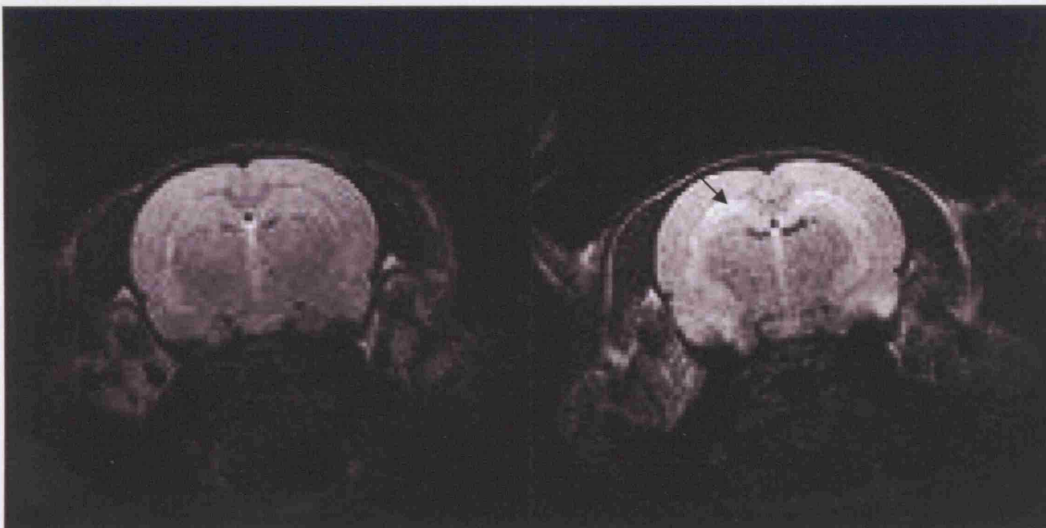

Figure 3-1   Changes to the hippocampus (arrow) of a rat following induction of status epilepticus in a pilocarpine model of status epilepticus using MRI (T2). (First image is control, second is 48 hours after status epilepticus)

3-3   Optimising the Protocol for 2DE Analysis

As the proteomic analysis of hippocampus in a rat model is a novel approach, the methodology had to be developed to obtain the best results. Using the protocol already developed for the mouse embryo, analysis was carried out as explained in chapter 2 and changes were made as the results were observed.

Hippocampal tissue was thawed and 1 ml of lysis buffer added before subjecting it to homogenisation by sonication (described in section 2.2.2.1). This step was not altered throughout the experiment and the composition of the lysis buffer used is given in Table 2-1. This was immediately followed by the protein assay using BSA standards for calibration.

The next step involved in-gel rehydration of the sample in which the IPG strip (pH 4-7) is reswelled and the sample taken up into the gel. This step was modified to improve the pattern of spots observed in the gels since initial experiments showed vertical streaking of spots that may indicate insufficient solubilization of hydrophobic protein. As urea, the chaotrope of choice allows the proteins to unfold and thus expose their hydrophobic cores, the CHAPS (surfactant) in the rehydration buffer solubilizes the hydrophobic residues that are exposed by the chaotrope. Hence, when the protein spots were not well resolved due to vertical streaking on the gels (as shown in figure 3-2), the amount of surfactant used was increased from 0.5 % CHAPS (150mg) to 1% CHAPS (300mg). This modification removed the vertical streaks and lead to a great improvement in the gels that were used for the spot image analysis.

In the 2DE PAGE analysis, the isoelectric focusing was carried out as explained in section 2.2.2.4 without any change in the protocol. At the equilibration stage, the

length of time used for equilibrating with iodoacetamide was increased from 15 minutes to 25 minutes. Iodoacetamide prevents horizontal streaking due to protein reoxidation and other silver staining artefacts.

One of the initial setbacks of this experiment was the streaking observed in the spots on staining, In addition to increasing the amount of CHAPS in the rehydration buffer, modifications was made to the second dimensional analysis by varying the amount of SDS in the electrophoresis buffer. Compared to the initial amount of 10g per litre, the streaking improved as this amount was slowly increased to 12g, 15, and even 50g, the best improvement was at 15g. The final concentration of 15g in the running buffer was thus increased from 0.1% to 0.15%. Hence, the SDS electrophoresis buffer stock used had a composition of 30.25g tris, 144.1g glycine and 15g of SDS made up to 1 litre with MilliQ water. Electrophoresis was carried out at 2w per gel overnight and no other modifications were made to the procedure described in chapter 2.
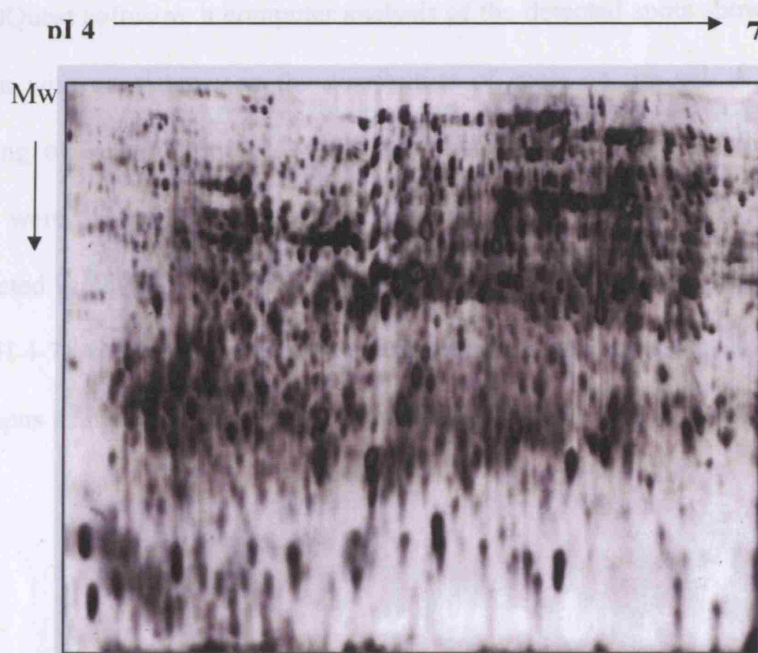
Figure

Figure 3--2   2DE analysis showing protein spots with streaking

After staining with the Plus one silver staining kit, image analysis using the PDQuest software produced spots analysed as described in the next section.

3-4 Analysis of protein Spots on 2D gels

The hippocampi tissue from each rat was labelled R3H, R6H, R18H and R19H respectively. Three gels were selected for the control rat (R6H, R; rat, H; hippocampus); three for treated R6, one gel was selected for each of R18 and R19H. Individual gels were marked with the numbers on their corresponding IPG strips used for the IEF so they could be tracked through the analysis.

Using the PDQuest software, a computer analysis of the detected spots showed good spot detection with consistency in the distribution of spots on the gel. A constant sample loading of 300μg/400μl of lysis buffer also ensured reproducibility when multiple gels were run several times. Figure 3-1 and figure 3-2 shows the pattern of proteins detected in 2D gels following 2D gel electrophoresis using a linear IPG IEF gradient of pH 4-7. An average of four hundred and thirty three spots was detected for the hippocampus using the silver staining Plus One Kit. The details are shown in table 3-1.

Table 3-1   Number of spots detected using the PDQuest software.

Gel ID	Source	Spots detected	Spots matched	Match rate (%)
R3H 09740	Treated rat	439	430	97
R3H 09739	Treated rat	382	366	95
R3H 09551*	Treated rat	433	433	100
R18H 17695	Treated rat	329	305	92
R19H 17686	Treated rat	348	341	97
R6H 09741	Control rat	382	371	97
R6H 09743	Control rat	339	337	99
R6H 09566	Control rat	323	307	95

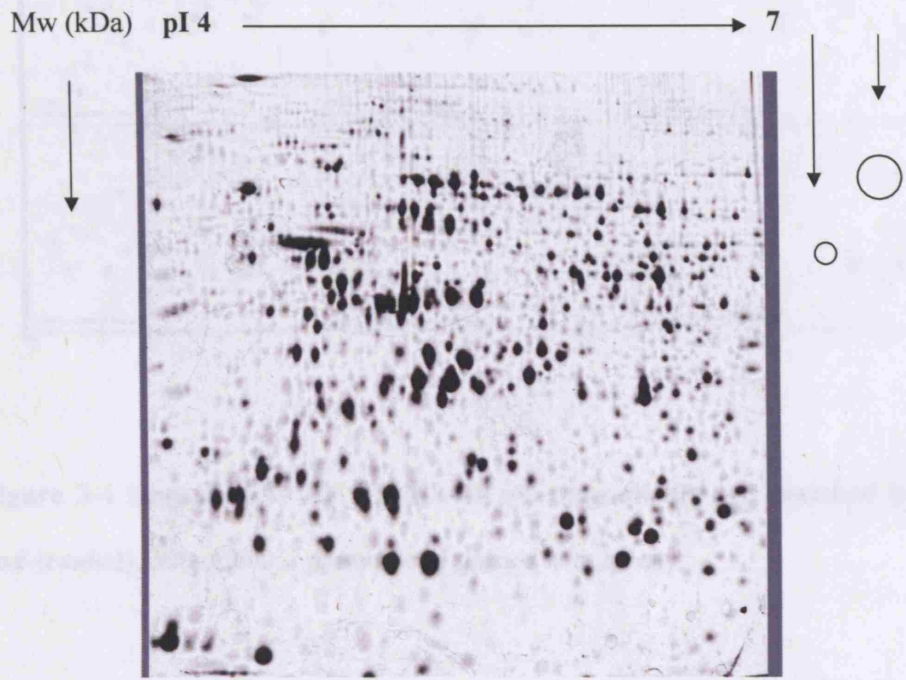R3H 09551 *: the reference gel (master) was created out of this gel.

Figure 3-3.   Large format 2D SDS-PAGE of rat hippocampus (control).
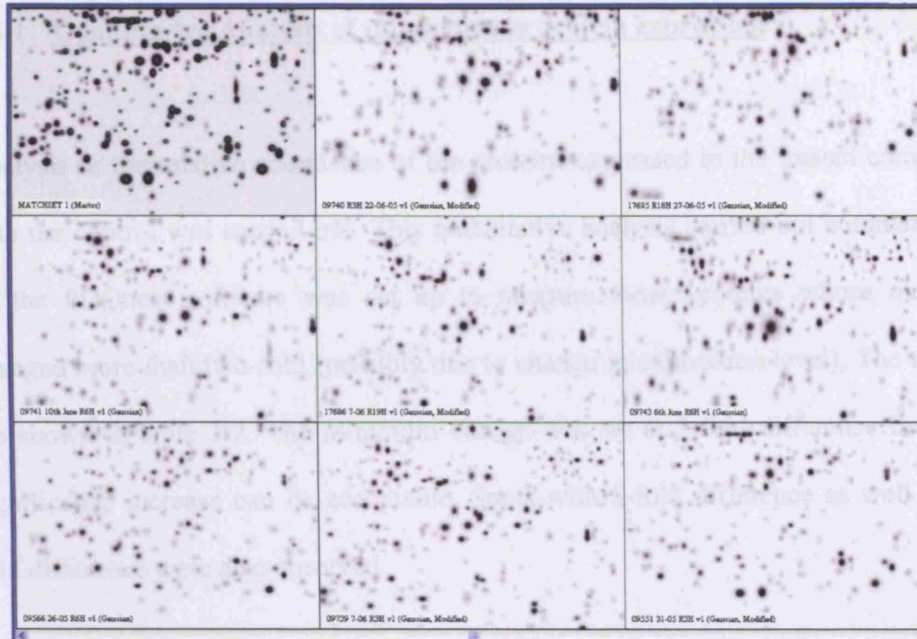   300µg/400µl protein load stained with silver.

Figure 3-4 large format 2D SDS-PAGE showing all the gels matched (control and treated). 300μg/400μl protein load stained with silver.

In order to compare the gels across the group, they were reselected into the group describing the rat from which the hippocampus was harvested, for example the three gels from R3 were selected into the R3 group. The gel from R18 was grouped together with that from R19. This treated group were now compared with the control in turns before the treated were all grouped together. This ensures that a more accurate value can be obtained from the average change.

3-4-1 Quantitative Analysis of the change in protein expression

Analysis of the relative abundance of the proteins expressed in the treated compared with the control was carried out. This quantitative analysis carried out automatically by the PDQuest software was set up to measure those proteins whose quantity changed more than two-fold (possibly due to change in expression level), The results are shown in table 3-2. The minimum change was set at 2-fold difference before a significance increase can be acceptable. Spots with 5-fold difference as well as 10 fold difference were also observed.

Table 3-2 Quantitative Analysis of the change in protein expression

Group ID	Relative Change				total	T-test(0.05)
	10-fold	5-fold	3-fold	2-fold		
R3 vs. R6	1	4	9	27	40	16
R18R19 vs. R6	0	3	12	36	51	27
R3R18R19 vs. R6	1	3	12	12	28	11

The table shows the difference between the groups when proteins turned on in the treated is compared against the control to observe the relative abundance (R3, R18 and R19 are all treated groups while R6 is the control).

A further analysis of the protein spots whose expression levels differ in the section above was carried out. A student's t-test at the 95 percentile was carried out to see if the above changes were statistically significant. For example, comparison of the treated group R3 with the control revealed that among 40 quantitative changes observed, in R3 vs. R6, 16 were statistically significant ($p < 0.05$). Other changes may be statistically significant but would require a greater number of samples to test.

3-4-2 <u>Qualitative analysis</u>

Qualitative analysis to see which protein species are present in one gel but not the other was carried out. All the gels from treated rats were later matched together for a more average comparison with the control. This revealed that two proteins species were expressed in the treated but not found in the control. One of these spots with SSP number 6307 was later identified as described with other spots in the next paragraph. However for other protein spots, a further confirmation of these differences could not be carried out. For example, the protein spots that differ significantly in the treated rats could have been identified to see if the change was constant for all the treated samples as well as a confirmation of their reproducibility on further analysis More work will have to be carried out further to better characterize other proteins in which the expression levels was altered and others which were turned on only in the treated rats.

Table 3-3 comparisons between proteins expressed across the group

Group ID	Source	Qualitative
R3 vs. R6	Treated vs. cont.	7
R18R19 vs. R6	Treated vs. cont.	8
R3R18R19 vs. R6	Treated vs. cont.	2

3-5 Mass-spectrometric identification of excised proteins from rat hippocampus

Eight of the spots were excised and were identified as described in table 4-3. These spots were from multiple gels, for instance, spots 1, 2 and 3 were from the same position on three different gels which suggests that they are the same protein. Indeed these spots were identified to be heat shock protein beta_1 (hspb_1). Spots 2, 5, 6 and 7 were also from the same position on different gels and were all identified to be the same protein i.e. Peroxiredoxin 6. These results confirm the accuracy of the analysis and gel matching procedure using PDQuest software. The theoretical values for the molecular weight as well as the pI of the proteins were obtained from the website www.expasy.org/tools/pi_tool.html. The location of the spots identified is shown on the gel in figure 3-5.

One of the excised portions of the gel contained no spot, which on analysis was identified to be keratin possibly from human handling showing how easily contaminations can occur.

Mw   pI 4 ⟶ 7

Alpha enolase

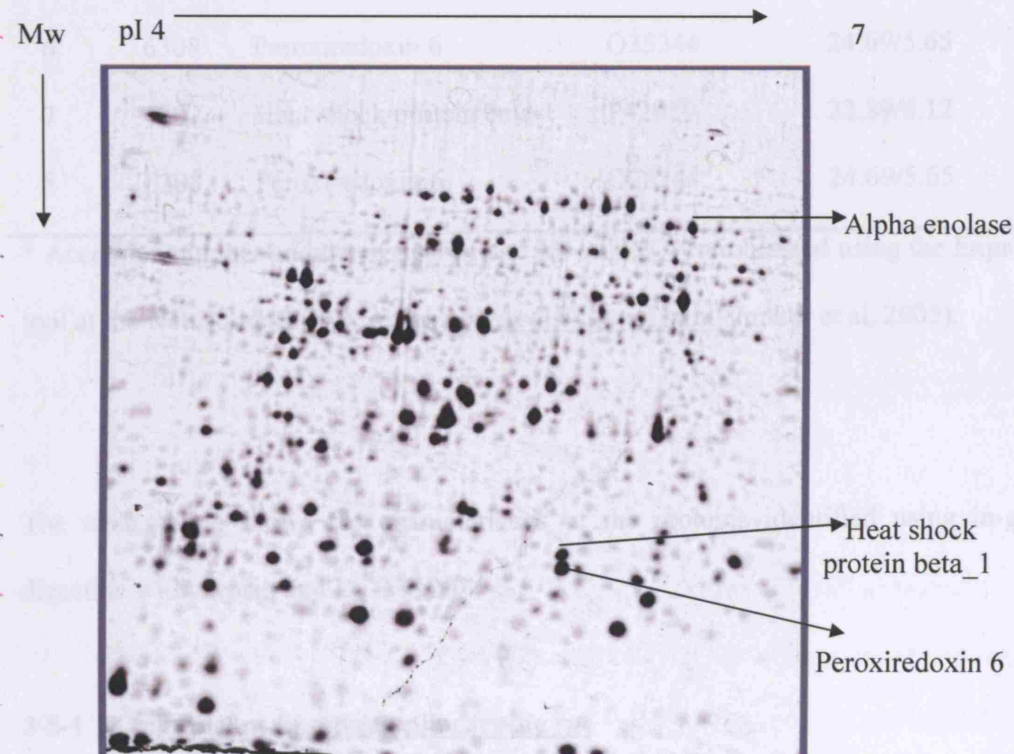Heat shock protein beta_1

Peroxiredoxin 6

Figure 3-5   Position of proteins identified by MS in the 2DE of a rat hippocampus (R3H).

Table 3-4   Identities of proteins excised for identification using MS.

Spot no	SSP no	Protein name	Accession number*	Theoretical Mr/pI*
1	6307	Heat shock protein beta-1	P42930	22.89/6.12
2	6308	Peroxiredoxin 6	O35244	24.69/5.65
3	8705	Alpha enolase	P04764	47.00/6.16
4	6307	Heat shock protein beta-1	P42930	22.89/6.12
5	6308	Peroxiredoxin 6	O35244	24.69/5.65
6	6308	Peroxiredoxin 6	O35244	24.69/5.65
7	6307	Heat shock protein beta-1	P42930	22.89/6.12
8	6308	Peroxiredoxin 6	O35244	24.69/5.65

* Accession number and theoretical pI and Mr values were obtained using the Expasy tool at the web address www.expasy.org/tools/pi_tool.html (Junker et al, 2005).

The table above shows the characteristics of the proteins identified using in-gel digestion with trypsin and LC-MS/MS.

3-5-1   A significant finding in pilocarpine rat

Finding of qualitative difference in the spot with SSP number (standard spot number) 6307 was so obvious that it was detected first by visual comparison. It was later

confirmed by subsequent matchset analysis using qualitative and Boolean methods. This protein with SSP number 6307 was found to be present in the treated hippocampi only and hence makes a significant discovery in the study. Further confirmation of the presence of this protein species in the treated rats was done by carrying out an analysis of a fresh control hippocampus; this protein was again found to be absent. The location of the spot is shown in figure 3-6.

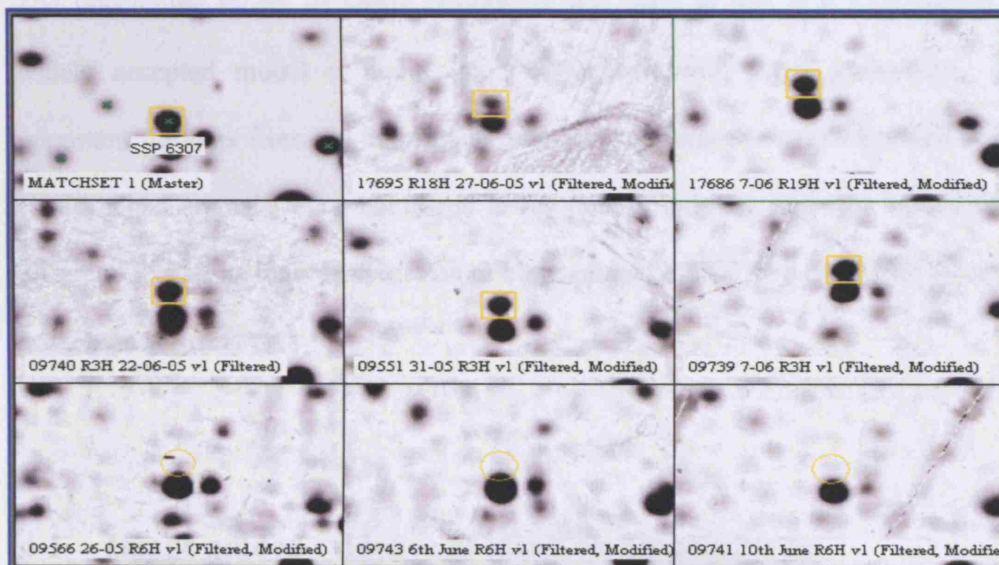**Figure 3-6.** **Portions of the gel from 2DE analysis of hippocampal samples from treated and control rats, with the treated showing the expression of the heat shock protein beta_1 (circled).** The first gel seen is the virtual (master) gel ontaining all the spots present in other gels, all other gels in the first and second row are from the treated hippocampi while the gels on the last row are from the control.

CHAPTER 4   DISCUSSION & CONCLUSION

The aim of this study was to determine whether a proteomic approach is potentially useful in defining the mechanisms of hippocampal injury associated with status epilepticus in an animal model. This is important as clarification of these mechanisms may lead to novel therapies that ultimately improve the outcome of status epilepticus. The pilocarpine model of status epilepticus was used as this is a well described, widely accepted model of brain injury associated with status epilepticus. The important findings from the current study are that differences were identified in the experimental animals when compared to the control animals, and that one of these differences is an increased expression of hippocampal HSP27 two days after the acute neurological insult.

4-1   Image guided evidence of insult on hippocampus

This study used the pilocarpine method for status epilepticus induction, an approach which fulfilled the criteria for seizures in animal models as described by Racine (Racine, 1972). An advantage of my study was that all the experimental animals were imaged using MRI prior to the extraction of the hippocampus for proteomic analysis and therefore it was known that the hippocampi in the experimental animals specifically have an abnormality (in addition to the observed seizures) at the time they were sacrificed. The proteomic studies described in the current dissertation make up

part of a larger study addressing the pathophysiological basis of brain injury and subsequent epileptogenesis using magnetic resonance methodology.

4-2   Novel method of proteomic analysis of rat hippocampus

In recent times, the field of proteomics has developed a great deal not only because of advancements in methodologies for detecting a vast array of proteins and their modifications to a high degree of accuracy but it has come at a time when the majority of the genome is now known. Hence application of the proteomic method to obtain the protein map of a rat model of hippocampal sclerosis is timely. However since the advancement in proteomics is still fairly new, it also means that protein map of many tissues are not yet available.

The current study used the 2D gel approach to characterise the hippocampal proteins following status epilepticus induced by pilocarpine. The approach needed to be developed and therefore several steps in the process had to be tailored for the current study. The amount of surfactant (CHAPS) used to solubilize the hydrophobic residues of the proteins was changed from 0.5% to 1% concentration in the rehydration buffer. Further changes included increasing the equilibration time (using iodoacetamide) from 15 minutes to 25 minutes, and increasing the amount of SDS used in the electrophoresis buffer from 10g to 15g. Different sample reagents were also tried to arrive at a method for analysing the hippocampal tissue so as to obtain a gel map in which proteins were well resolved and devoid of streaking and other anomalies commonly seen in proteomics approach. The result is the gel shown in figure 3-3.

4-3   Protein Analysis and Identification

Proteins were analysed to eliminate various differences in the protein patterns that were not due to the effects of the epilepsy. The use of proteomics is a very sensitive technique in which human contamination can lead to protein contamination (e.g. keratin) being discovered on the gels, as was observed in one of the gels analysed. Differences can be due to gel to gel variation for example a few minutes difference in developing the spots with silver can affect how many spots are visible. Variation can also be due to innate differences in the animals from which the gels were made. In order to correct for all these influences, multiple gels from different animals had to be compared to identify those in which changes were consistently due to the effect of the induced epilepsy. However limitations to this study include the fact that only three treated animals and two control animals were used, also this study was for a short period which meant that running more gels would have given rise to more findings.

Analysis was carried out to check for spots that were present in one group of gels and not the other, for instance, those present in the treated and not in the control (qualitative analysis), and also for those proteins that are present in both groups but their level of abundance was changed in one group and not the other (quantitative analysis).

The latest version of PDQuest software (7.2) was used to analyse the proteins spots captured from the gels to give a highly reproducible result since the software has less human intervention, than previous versions.

4-3-1 Qualitative analysis

A comparison of the gels of the treated groups with the control revealed that some proteins were expressed only in the hippocampus of the treated and not the control rats. This finding was assessed further by visually comparing the gels from the two groups. Gels from rat R3 group also had seven spots which were not seen in the controls gels while R18 and R19 group had seven spots which were absent from controls. However only two of the spots were equally found on both treated groups, and are therefore thought most likely to be associated with epilepsy, and not due to variation between animals.

Mass Spectrometric (MS) analysis of all these spots will allow confirmation of the differences observed. One of the spots reported in chapter 3 as having SSP number of 6307 was identified by MS as heat shock protein beta_1. This spot was present in every sample of the treated hippocampus and not in the control. Further 2DE gels from a different control animal again showed absence of this protein from the hippocampus, suggesting that up-regulation of heat shock protein beta_1 is related to the pathophysiological processes associated with brain injury following status epilepticus. A full description of this protein is in section 4-5.

4-3-2 Quantitative analysis and Student's t-test

Analysis of the proteins to assess the amount of change in abundance between the treated and control rats showed that some proteins were differentially expressed with up to five-fold difference in abundance. When all the treated samples were matched and compared as one averaged group to the control group, 12 protein species were differentially expressed at two-fold difference while three of the spots showed a greater difference in abundance with a five fold quantitative difference.

Differences in expression levels however could not be confirmed further due to the time limitation of this study. Such confirmation can be obtained by running more 2DE gels to determine whether the same protein spots will be altered in all treated animals. A further analysis may also be carried out to see if the observed protein had a change in abundance or post-translational modification in which an isoform of the protein will be found on mass spectrometric analysis. Instead of using the silver staining method, Coomassie blue staining, although less sensitive than silver staining, is better at determining changes in abundance on gels and hence can be used. Further confirmation can be made by separation of protein samples on 1D polyacrylamide gels and then Western blotting using specific antibodies to the protein of interest.

4-4 Protein spots identified by mass spectrometry

The protein spot that differed in the treated and control rat hippocampi was analysed for identification along with other non-varying protein spots by using LC-MS/MS. The nine spots were from four different proteins which was as expected as the spots

were excised from corresponding positions in different gels. One of the spots was an artefact identified to be keratin from human contamination. Of the three spots identified from the gel, two were cellular metabolic enzymes while the third was a stress induced protein.

4-4-1 <u>Metabolic Proteins</u>:

Peroxiredoxin 6: This protein was one of the most abundant protein spots present in both treated and control samples. It has a theoretical molecular weight (Mr) of 24.69 kDa and an isoelectric point (pI) of 5.65 which was in line with its position in the slight acidic part of the gel and in the lower part of the gel where low molecular proteins migrate, as confirmed by molecular weight markers.

Peroxiredoxin 6 is normally found in the lysosomes of many cells as well as in the secretory organelles of the lungs. This protein is involved in catalytic activities especially in redox regulations of the cell. It helps in the reduction of hydrogen peroxides and is implicated in oxidative injury. The level of peroxiredoxin 6 was found to be significantly increased in a study that investigated the proteomic map of Sprague dawley (SPD) rats brain using kainic acid for inducing status epilepticus. I did not identify an increase in peroxiredoxin 6 in the current study but this may be related to the different timescales from acute insult to sacrifice when the current study is compared to that using kainic acid. Peroxiredoxin 6 is also known as antioxidant protein 2.

Alpha enolase: This is a multifunctional enzyme with a theoretical Mr of 47kDa and pI of 6.12. It plays a role in glycolysis and various other processes like growth, hypoxia, and allergic response. It may function as a receptor on cell surface of leukocytes and neurons, subcellularly, it is found in the cytoplasm. This protein is therefore a common finding in all tissues and in this study was present in both treated hippocampus and controls without any modification to it.

Cellular Function of all Proteins Identified

Table 4-1   Cellular roles of the proteins identified

Protein no	Protein name	Category	Function
1	Heat shock protein beta-1	Protein folding	Involved in stress resistance and actin organisation
2	Peroxiredoxin 6	Energy metabolism	Involved in redox regulation of the cell. Reduce H_2O_2 & short chain fatty acids. Play a role in phospholipid turnover and protection against oxidative injury
3	Alpha enolase	Energy metabolism	Multifunctional, has roles in glycolysis and various processes such as growth control, hypoxia tolerance and allergic

responses. May function in the

intravascular and pericellular

fibrinolytic system on the cell surfaces

of several cell types such as neurons and

leukocytes.

(Adapted from www.expasy.org)

4-4-2 Heat shock Proteins

Heat shock proteins (HSPs) are a group of proteins first described in the salivary

glands of *Drosophilia* by Ritossa in 1962 (Ritossa, 1962). Members of this family

include ubiquitin, HSP27, HSP70, and HSP90. They are characterised by the fact that

their expression level is enhanced by high temperature exposure. In fact this was the

basis of their name, heat shock proteins. However, many of them have been proved to

act as chaperones facilitating correct folding and assembly of nascent polypeptide

chains in normal conditions. They are found in normal conditions as constitutive

proteins involved in cellular transport as well as in apoptosis. Heat shock proteins are

also found to be up-regulated during development. HSP70 was found to be up-

regulated during neurulation where the increase has been attributed to their function

as chaperones (Greene et al, 2002).

HSPs are described on the basis of their elevated expression in response to stress

together with their molecular weight. For example HSP90 is heat inducible and has a

molecular weight of approximately 90kDa, while GRP78 and calreticulin respond to

endoplasmic reticulum stress such as hypoglycaemia.

Focus on these proteins is as a result of their enhanced expression to protect against protein damage and cell survival. They have been observed in animal models of stroke and epilepsy although the full extent of their pathophysiological role is still unknown. Interestingly it has recently been demonstrated that overexpression of HSP2 is neuroprotective in an animal model of stroke (Badin et al, 2005).

Heat shock Protein Beta_1:

Heat shock protein beta_1 is also known as HSP27. It is a protein that has been studied extensively because of its chaperone-like activity and its ability to be induced by diverse stimuli e.g. heat shock, hypertonicity and heavy metals (Bidmon et al, 2004). HSP27 is normally found in many tissues for example, it has been found as a constitutive protein in skeletal and heart muscles, probably due to their constantly exposure to environmental stress (Sugiyama et al; 2000).

HSP27 has been implicated in many neuropathological conditions, where it may not only serve as markers for disease progression but may have protective functions; their anti-apoptotic effects has been studied in transgenic animals where HSP27 was shown to reduce neuronal cell death in the CA3 region of the hippocampus (Akbar et al; 2003, Kalwy et al; 2003). This action has also been reported in glioblastomas, multiple sclerosis, and other neurodegenerative diseases (Hitotsumatsu et al; 1996, Hermisson et al; 2000, Assimakopoulou et al; 2001).

In the investigation of the pathophysiology of epilepsy, both human and animal studies have observed the presence of heat shock proteins. Erdamar described the

presence of HSP27 in the hippocampal formation, lateral and mesial temporal cortices associated with hippocampal sclerosis (Erdamar; 2000, Bidmon et al; 2004).

However, it is not only in humans that HSP27 has been found. It appears to be a common protein involved in the pathogenesis of epilepsy. Studies using different approaches have detected the up-regulation of HSP27 and other heat shock proteins in animal models (Krapfenbauer et al; 2001 and Plumier et al; 1996). The work by Krapfenbauer et al is a study that used proteomics to characterise the proteins whose expression level differ from that of control following kainite induced status epilepticus. Although this study used a different chemical model of epilepsy (kainic acid model) as well as analysed the whole brain, the findings are similar. It also lends ` support to the notion that the expression of HSP27 in the kainic acid model is possibly not because of their hyperthermic effect (Bidmon et al; 2004) but rather their effect in inducing seizures.

Studies using the pilocarpine model of epilepsy have demonstrated up-regulation of HSP70 and HSP72 (Tetich et al; 2005 and Lian et al; 2005).To the best of my knowledge HSP27 has not been detected in the rat pilocarpine model of epilepsy before, which indicates that this is a novel finding that may require further investigation. However, the finding of dramatically increased HSP27 in two different models of status epilepticus suggests that HSP27 is an important component of the cascades of events underlying the brain injury and subsequent development of epilepsy following status epilepticus.

To maximise the potential of proteomics in further studies of pilocarpine model of status epilepticus, prospects will include the use of more animals to obtain more

significant differences and running multiple protein gels to exclude variations (due to methodology and animal differences). Also the use of wider pH strips (pH 3-10) will increase the number of proteins that can be resolved hence enabling the resolution of those proteins whose isoelectric points (pI) lies at the extremes of the pH. Other analyses may involve changing the time point from induction of status epilepticus to tissue extraction to see those proteins which might be expressed at the earlier or much later stage of the insult.

The role of hsp27 in the wider context of events in the pathology of epilepsy has been suggested to involve region specific stimuli in which insults are directed at specific loci in the cortex as evidenced by the different studies in epilepsy surgery as well as animal models (Plumier et al; 1996, Bidmon et al, 2004). The protective actions of HSP27 in viable cells that are faced with pathological conditions means that these proteins are induced in stress responses. They may therefore serve as biological markers in regions in which seizures have initiated a stress response. The role of hsp27 is likely to be important and further studies may lead to alternative therapeutic interventions in the modulation of the development of temporal lobe epilepsy following status epilepticus in humans.

Thus in conclusion the current study provides pilot data supporting the view that proteomic approaches may be useful in understanding pathophysiological processes following status epilepticus. Although HSP27 is the only protein identified so far, identification of the other differentially expressed proteins may lead to the generation of hypothesis relating to mechanisms of injury and epileptogenesis following status epilepticus.

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