UNIVERSITY
of
GLASGOW

Analysis of Protein Expression in the Autonomic Ganglia of Horses with Respect to Equine Grass Sickness

Emily Peaston BSc (Hons)

Division of Infection and Immunity
The Joseph Black Building
The University of Glasgow
Glasgow
G12 8QQ

Dept Veterinary Clinical Studies
University of Edinburgh
Easter Bush Veterinary Centre
Easter Bush, Roslin
Midlothian, EH25 9RG

A thesis presented for the degree of Master of Science by Research in the Faculty of Biomedical and Life Sciences, University of Glasgow.

The Royal (Dick) School of Veterinary Studies

I declare that all work within this thesis is my own, unless specifically indicated.

Emily Peaston

Date

Summary

Equine grass sickness (EGS) is essentially a fatal disease of the horse with clinical symptoms that indicate autonomic nervous system (ANS) dysfunction. Such symptoms include varying degrees of colic, intestinal stasis, dysphagia, anorexia, weight loss, tachycardia, excessive salivation, patchy sweating, ptosis (drooping of the eye lids), rhinitis sicca and muscle tremor (Hudson *et al*, 2001). Furthermore, a characteristic loss of stainability (chromatolysis) is commonly seen in neurones of the gut wall plexi, prevertebral and paravertebral ganglia, the intermediolateral tract of the spinal cord and certain brain stem nuclei in EGS cases.

The disease is recognised in three loosely defined clinical categories: acute (disease duration 1-2 days), sub-acute (2-7 days) and chronic (>7 days). It is hypothesised that a neurotoxin is ingested and the extent of the nervous damage is dose dependent. It is also considered that massive neuronal loss likely results in the acute form, whilst less neuronal damage results in less severe signs and the chronic form of the disease (Cottrell *et al*, 1999). The aetiology of EGS is largely unknown. However, the working hypothesis is that it is a toxicoinfection with *Clostridium botulinum* type C, possibly triggered by dietary factors such as cyanide from white clover (*Trifolium repens*).

In this study protein expression in homogenates of cranial cervical ganglia collected from horses with acute EGS (AGS) and from control horses was analysed using two-dimensional electrophoresis (2D-E). Optimal sample preparation and 2D-E techniques were developed for the first time for equine neural tissue. Variations in the protein expression between samples from different locations within the same autonomic ganglia, different autonomic ganglia from the same horse, ganglia from different horses of the same sex and ganglia from horses of both sexes were investigated using the 2D-E method developed. It was concluded that the variations in protein expression between such samples would not affect further comparisons between AGS and control 2D-E tissues and would not interfere with identifying EGS-related proteins. At this point a 2D-E gel was run with sample from control equine autonomic ganglia and sixty spots were subjected to MALDI-MS analysis to construct a preliminary proteome map of equine autonomic ganglia. This will provide a useful reference for future research.

The protein expression on 2D-E gels of AGS and control samples was analysed and any differences were highlighted. Fourteen spots were identified as being up-regulated or only occurring on AGS sample gels and were considered to be AGS related. These proteins were analysed using Matrix-Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS) and tandem MS. After MS analysis the majority of these proteins were recognised as neural proteins (e.g. beta tubulin and gamma enolase) or serum proteins. The presence or up-regulation of some of the proteins in AGS, such as alpha-1-antitrypsin, chaperonin/HSP60, beta tubulin and gamma enolase may well, with further investigation, help reveal the aetiology of EGS by clarifying the nature of the damage that occurs during EGS.

Acknowledgements

First and foremost I would like to thank my parents for their continued encouragement and financial support. Without their help I would not have been able to achieve what I have. I would also like to pay a big tribute to the Equine Grass Sickness Fund, again for financial support and providing a fantastic service to all those unfortunate enough to have been affected by grass sickness. My supervisors Dr Jonathan Wastling and Prof Bruce McGorum deserve a big thank you for guiding me through this project, they have been incredibly encouraging and a huge help. Last but not least, I would like show my appreciation to all those who have given their time and treasured friendship to me over the past year.

List of Contents

Thesis Contents

List of Figures

Page

List of Tables and Graphs

Page

List of Abbreviations

μg – micrograms

μl – microlitres

~ - approximately

+ve – positive

1D – one dimensional gels

2D-E – Two-dimensional gel electrophoresis

ACh – acetylcholine

AGS - acute grass sickness

ANS – autonomic nervous system

Approx – approximately

APS – ammonium persulfate

BCA – bicinchoninic acid

BSA – bovine serum albumin

C1 toxin – *Clostridium botulinum* type C1 neurotoxin

ccg – cranial cervical ganglia

CGS - chronic grass sickness

CHAPS – 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulphate

cm – centimetres

conc^{n} – concentration

CSF – cerebro-spinal fluid

DNAse – deoxyribonuclease

DTT – dithiothreitol

EDTA – ethylenediamin tetra-acetic acid

EGS – equine grass sickness

ESI – electrospray ionisation

EST – expressed sequence tag

g – grams

h – hours

H_2O – Dihydrogen oxide/water

HPLC grade H_2O – high performance liquid chromatography grade H_2O

IAA – iodoacetamide

ID – identity

IEF – isoelectric focusing

IPG – immobilised pH gradient

k – kilo/thousand

kDa kilo Dalton

L – linear

L – litres

Log – logarithm

M – molar

mA – milli-Amps

MADLI – matrix-assisted laser desorption ionisation

MeOH methanol

mg milligrams

min – minutes

ml – millilitres

mm – millimetres

mM – millimolar

mol – molecular

MS – mass spectrometry

MS/MS – tandem mass spectrometry

Na – sodium

NL – non-linear

nm – nanometres

No. – number

°C – degrees centigrade

OD – optical density

PMSF – phenylmethyl sulphonyl fluoride

RNAse – ribonuclease

rpm – revolutions per minute

s – seconds

SAGS – sub-acute grass sickness

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

TEMED – N,N,N',N'-tetramethylethylenediamine

TOF – time of flight

TRIS – tris(hydroxymethyl)aminomethane

V – volts

v/v – volume/volume

-ve – negative

w/v – weight/volume

WR – working reagent

x g – times the force of gravity

I dedicate this work to those loved; past and present.

Chapter One: Background

1.1 Equine grass sickness

1.1.1 Introduction

Equine grass sickness (EGS) is a dysautonomia of unknown aetiology that affects horses. The disease is characterised by the paralysis, and hence dysfunction of the alimentary tract and in the majority of cases is fatal. Other symptoms include varying degrees of colic, intestinal stasis, dysphagia, anorexia, weight loss, tachycardia, excessive salivation, patchy sweating, ptosis (drooping of the eye lids), rhinitis sicca and muscle tremor (Hudson *et al*, 2001). Furthermore, a characteristic loss of Nissl substance (chromatolysis) is commonly seen in neurones of the gut wall plexi, prevertebral and paravertebral ganglia, the intermediolateral tract of the spinal cord and certain brain stem nuclei in EGS cases (Hudson *et al*, 2002).

EGS was first recognised in Scotland around 100 years ago (Tocher, 1924). However, it is now known to be present throughout the UK and much of Northern Europe with isolated cases also being reported in South America and the Falkland Islands (Cottrell *et al*, 1999; Hahn *et al*, 2001). Nevertheless, its highest incidence remains in Scotland and North East England, killing around one in two hundred horses every year (Milne and McGorum, 2000). Epidemiological studies have shown a high incidence during the spring, when horses are turned out to grass. Similar studies also indicate the latency of the disease to be about two weeks (Doxey *et a*,. 1992). Young, usually 2-7 years old, stressed horses and horses that have recently been moved to new pastures appear to be most at risk. However, cases consistently occur in all breeds and types of horses (Doxey *et al*, 1992).

Table 1.1. The clinical presentation of equine grass sickness (from Dr Scott Pirie - Royal (Dick) School of Veterinary Studies, University of Edinburgh, Equine Grass Sickness Fund Website, www.grasssickness.org.uk). Summary of the clinical signs of grass sickness, associated with the three forms of the disease.

ACUTE	SUBACUTE	CHRONIC
• Depression/ somnolence • Distended abdomen • Ileus (absence of gut sounds) • Tachycardia (high heart rate) • Salivation • Gastric reflux • Muscle tremors • Ptosis (drooping eyelids) • Patchy/generalised sweating • Dysphagia (difficulty swallowing) • Small intestinal distension • Colic (occasionally) • Colon impactions (occasionally)	• "Tucked up" abdomen • Weight loss • Dysphagia (difficulty swallowing) • Tachycardia • Colic (as disease progresses) • Gastric reflux (as disease progresses) • Patchy sweating • Ptosis (drooping eyelids) • Muscle tremors • Colon impaction • Reduced gut motility	• Severe weight loss • Markedly "tucked up" abdomen • Base narrow stance • Rhinitis sicca (inflamed nasal passages) • Ptosis (drooping eyelids) • Slightly elevated heart rate (< 60 bpm usually) • Muscle tremors • Patchy sweating • Mild colic • Reduced gut motility

EGS can present in one of three forms: acute, subacute and chronic, each varying in the rapidity of outcome and the severity and duration of the symptoms (table 1.1). In acute EGS, the most severe and invariably fatal form, the symptoms can arise very rapidly and the duration of the disease is typically 24-48 h, ending with the death of the animal - usually due to euthanasia on humane grounds. Subacute EGS is not quite as severe or

as rapid in onset, but is also invariably fatal, and typically lasts for 2-7 days. Chronic EGS, in contrast, can take weeks to develop and may persist for months (Doxey *et al*, 1991b; Hudson *et al*, 2002). At present, approximately 30% of chronic cases can be nursed through the symptoms, with little residual damage and a normal, even full competitive life resuming on recovery (Milne and McGorum, 2000). The cause of EGS is not known, but it is currently thought that the horse may ingest a neurotoxin (Griffiths *et al*, 1994a) and that the degree of nervous damage is dose related, thereby determining the severity of the disease (Barlow, 1969; Pogson *et al* 1992; Doxey *et al*, 1992, 1995a).

1.1.2 EGS and *Clostridium botulinum*

Currently, the working hypothesis is that EGS is a toxicoinfection with *Clostridium botulinum* type C (Hunter *et al*, 1999; Hunter and Poxton, 2001). The botulinum neurotoxins are a family of protein toxins that selectively inhibit the release of acetylcholine (ACh) at the neuromuscular junction and autonomic ganglia (Habermann and Dreyer, 1986; Simpson, 1989; Niessmann *et al*, 1994). Seven immunologically distinct serotypes of botulinum toxin have been identified and designated A-G by the order of their discovery (Habermann and Dreyer, 1986; Simpson, 1989). The dichain protein toxins are produced by the anaerobic bacterium *Clostridium botulinum*. A strong correlation between EGS and the detection of *Clostridium botulinum* type C neurotoxin (C1 toxin) in the ileum and faeces of horses with EGS has been made (Hunter *et al*, 1999).

Professor Ian Poxton of the University of Edinburgh is currently researching the link between *Clostridium botulinum* and EGS (Poxton, http://www.grasssickness.org.uk). He has proposed that, under normal circumstances, most horses at some point have been exposed to the bacterium, but the gut flora can resist the growth of the organism and the production of its toxins. However, an as yet unidentified sudden change or disruption of the normal gut flora (e.g. a change in nutrition/change of pasture) may allow for an abnormally increased production of toxins in the gut. This, in turn, could cause the dysfunction of the autonomic nervous system that characterises EGS.

1.1.3 EGS and oxidative stress

There are two ongoing studies to identify the putative trigger for the overgrowth of *Clostridium botulinum*. The first study investigated a possible link between oxidative stress and excitotoxicity with EGS (McGorum *et al*, 1998). It is hypothesised that plants and grasses which undergo metabolic stress during periods of rapid growth or extreme weather conditions may contain limited amounts of antioxidants and elevated levels of excitotoxic amino acids, e.g. glutamate and aspartate (Bortosz, 1997; Haraguchi, 1997). Ingestion of these metabolites could potentially contribute to EGS (McGorum *et al*, 2000). The second study is investigating the role of cyanide from wild white clover (*Trifolium repens*) as a trigger factor. Pasture improvement schemes using white clover have been related to EGS. The first reports of EGS, in the early 1900s, coincided with the introduction of wild white clover into the grass seed mixtures used to improve agricultural productivity in Scotland (McGorum, 2001). The cyanogenic glycosides in white clover, which have the capability to release cyanide, a known

neurotoxin, could cause the overgrowth of *Clostridium botulinum*. Certain observations support this hypothesis:

- The cyanide content in white clover increases when the plant is rapidly growing, for example during the springtime, which, as previously mentioned, is a peak time for EGS cases (Doxey, 1991).
- The content of cyanide has also been shown to increase following the exposure of the plant to adverse weather conditions, such as drought, frost and snow. EGS has repeatedly been shown to occur following long periods of cold dry weather and overnight frosts (Doxey, 1991).

Current research, by Prof Bruce McGorum, at the Royal (Dick) School of Veterinary Studies, Edinburgh, has shown that white clover collected from fields following a recent outbreak of EGS frequently has elevated levels of cyanogenic glycoside (McGorum, personal communication). Furthermore, it was shown that the blood and urine obtained from exposed horses grazing EGS pastures indicated that these horses had been subjected to increased cyanide exposure. Horses with EGS also showed alterations in blood amino acids resembling those found in humans with chronic cyanide poisoning (McGorum, 2001).

1.1.4 Putative neurotoxins

By the fractionation of plasma, taken from blood from horses with AGS, and using gel filtration and salt precipitation, a >30kDa neurotoxin has been found in the serum of GS

affected horses (Gilmour and Mould, 1977). The preparations were tested for neurotoxic activity by injection into ponies. The resulting neurohistological changes, like those seen in EGS, suggested its association with grass sickness. The neurotoxin was confirmed to be of high molecular weight range and a proteinacious molecule or bound to a high molecular weight protein (Gilmour and Mould, 1977). This protein has also been reported to be capable of inducing autonomic neuropathy, but not the clinical signs of EGS, when administered either intraperitoneally (Gilmour, 1973; Gilmour and Mould, 1977), or directly into the parotid salivary gland (Griffiths *et al*, 1994a). It is believed that the putative neurotoxin affected the neurones by retrograde axonal transport (Griffiths *et al*, 1994a).

However, little else is known about this proteinacious neurotoxin, and it has not yet been characterised, despite previous attempts (Pemberton *et al*, 1990). It is possible that the protein could represent *C. botulinum* toxin, or a *botulinum* toxin associated with a protein carrier such as albumin (Griffiths *et al*, 1994b). Alternatively, the protein may be a neurotoxin unrelated to *C. botulinum* toxin, a metabolite produced as a result of oxidative stress or cyanide poisoning, for example.

1.2 Proteomics

1.2.1 Introduction

Proteins are the main catalysts of biological function and contain many levels of information that collectively indicate the actual rather than the potential functional state. Protein quantity, location and time-point can all be measured to determine this

functional state (Yanagida, 2002). Proteomics is the systematic analysis of protein expression patterns in cells and tissues. It involves the isolation, separation, identification and functional characterisation of the proteins in an organism. Two-dimensional gel electrophoresis (2D-E) offers the greatest protein resolution potential compared to any other currently available separation method. It is also useful for the identification of changes in protein expression (Cohen *et al*, 2002), which would be especially helpful in the investigation of diseased tissues. The term "proteome" was suggested in the mid 1990s as a collective term for the total protein complement of a cell or a tissue. Hence, proteomics has developed as the study of proteomes (Wasinger *et al*, 1995; Wilkens *et al*, 1996).

1.2.2 Two-dimensional electrophoresis

Using two-dimensional (2-D) gel electrophoresis (a combination of isoelectric focusing [IEF] and sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]) proteomes can be analysed with high resolution. Both their molecular weight and isoelectric points can separate thousands of proteins that are expressed in a specific cell or a tissue. This high resolution can help determine which proteins are present in a cell or tissue under specific states (Yanagida, 2002). It is, therefore, possible to identify and isolate differentially expressed proteins in response to changes in the cell or tissue by comparison of various states of the sample. For example, the differential protein expression between diseased and non-diseased cells or tissues can be established.

As an example of the value of proteomic analysis, the differential protein expression between Alzheimer's diseased (AD) brain tissue and control brain tissue was analysed by the use of 2D-E (Tsuji *et al*, 1999). In this study, the brain samples were taken from the temporal cortex of brains at autopsy. Fifteen AD patients and fifteen age-matched (control) patients were studied. Following extraction and solubilisation of proteins from the samples, the proteins were separated using 2D-E along with marker proteins to enable the estimation of the molecular weights and isoelectric points of the unknown proteins. The gels were stained and scanned to digitise them such that comparisons of protein appearance on the gels, and up/down regulation of spots could be performed. A number of interesting observations were made. Five protein spots were identified as being significantly increased (two of which were characterised as glial fibrillary acidic protein) in AD patients and nine spots were found to be present in AD brains only. No proteins were present in controls that were not present in AD brains, but 28 spots (one was identified as glial fibrillary acidic protein) were identified as down regulated.

Such observations were made possible by the high-resolution separation of proteins achieved using 2D-E. The reproducibility of the gels was improved by the use of immobilised pH gradient (IPG) first dimensional strips (Tsuji *et al*, 1999). Furthermore, the database of brain proteins in AD constructed in this study, along with the statistical data of density changes of proteins in AD, provided an invaluable beginning for a comprehensive human brain 2D-E database, facilitating further investigation of pathogenic protein alterations in AD. However, Tsuji *et al* (1999) did still encounter limitations with regards to the separation of proteins. They were unable to demonstrate

proteins of molecular weight exceeding 100kDa. This could have been due to the limited ability of heavy proteins to enter the first dimension IPG gel strip. Furthermore, it is widely accepted that hydrophobic proteins (such as membrane proteins) can be difficult to resolve using 2D-E techniques, due to the charge of the proteins interfering with the IEF separation stage.

1.2.3 Mass Spectrometry and proteomics

The introduction of mass spectromic (MS) techniques greatly improved protein identification strategies. The development of time-of-flight (ToF) mass spectrometers and soft ionisation methods, including matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI), has facilitated the accurate measurement of the molecular mass of biomolecules (Yanagida, 2002). Proteins usually separated by gel electrophoresis are first digested with a sequence-specific endopeptidase such as trypsin, subsequently ionised, and analysed by MS. A mass spectrometer determines the molecular weight of chemical compounds by ionising, separating, and measuring molecular ions according to their mass-to-charge ratio (*m/z*) (Scripps Centre for mass spectrometry, www.masspec.scripps.edu/information/intro/index.html). In MALDI-ToF-MS, a sample (e.g. protein sample) is mixed with a matrix in the presence of a fast evaporating solvent, under extremes of pH, usually achieved by adding triflouroacetic acid (TFA) (Guptasarma, 2001). After mixing, the entire solution is placed on a stage (or platform) and the solvent is allowed to evaporate. This causes the matrix to crystallise, with the sample being non-specifically associated with the crystallising compound. The crystallised mixture is then placed in a vacuum, and a laser beam is

fired on the material. This causes the material to rise in a 'laser-desorbed' cloud of charged molecular ions – both matrix and sample. This part of the procedure is the 'laser desorbtion ionisation,' which is assisted by the presence of the matrix (Guptasarma, 2001).

At this point, a voltage is applied to a nearby grid that causes the charged molecules to rush towards it, in a manner dependant on the charge of the molecule, and the sign and magnitude of the voltage applied (Guptasarma, 2001). The molecules accelerating towards the grid pass through it, entering a high vacuum tube. As the variously sized molecules race through the tube, the time it takes each molecule to reach the end depends on the mass of the molecule. Thus, by recording the time of arrival of ions, the time of flight can be estimated, and from this value the mass can be estimated (Guptasarma, 2001).

However, further fragmentation of the proteins is often required before they can be identified. Tandem mass spectrometry (MS/MS) induces further fragmentation of proteins, achieved by inducing ion/molecule collisions during 'collision-induced dissociation' (CID). The resultant fragments exist as a family of subset ions (e.g. amino acid chains) of the original parent ion (e.g. a whole protein). Computerised analyses of the *m/z* spectrum of these ions are used to obtain a fragment ion spectrum to gain structural information about the parent ion (Lesney, 2001).

Peptide molecular weight measurements are predictive of amino acid structure, and peptide fragmentation information relates to amino acid sequence. Each set of information can be correlated to protein sequences in a database (Yates, 2000). The identity of an unknown protein is determined by comparing the molecular weight map of the unknown protein with the theoretical molecular weights of peptides that are produced by digestion of each of the proteins in a database (Yates, 1996; Yates, 1998).

Sequencing the genome of an organism is now a major stepping-stone to understanding its biology. The data generated by a whole genome sequence has significant benefits in proteomics (Yates, 2000). The genome is the entire genetic make-up of an organism and contains all the information for making specific proteins. Since the genome dictates the proteome, the genome-sequencing projects have played a major role in the advances of protein identification processes.

1.2.4 Proteomics and the nervous system

Proteomics has been used to study protein expression in the nervous system in several studies. One such major on-going project is using proteomics to study learning within the nervous system. For example, early studies, using glass capillary electrophoresis, on protein synthesis at synaptic junctions in the nervous system, with respect to learning, showed that activity in nerve cells could lead to new protein synthesis (Hyden *et al*, 1968; Hyden *et al*, 1969). However, the identity of these synaptic proteins was unknown, and it was not until modern protein sequencing techniques became available that their identity was revealed.

By the late 1980s, 2D gel electrophoresis allowed the simultaneous analysis of many proteins associated with learning. Kandel and colleagues used ganglia from the sea slug (*Aplysia california*) to study proteins that change in level of expression following synaptic activity. They used 2D gels to survey and isolate proteins (Castellucci *et* al, 1988; Sweatt *et* al, 1989). However, there were still limitations with the technique at this time and it was frequently the case that insufficient amounts of proteins were obtained for Edman sequencing. Edman sequencing is a sensitive method involving the sequential cleavage of amino acid derivative, one at a time, from the N-terminus of a protein (Edman and Begg, 1967). Other limitations with Edman sequencing include an upper mass limit and N-terminal blocking. Proteins above 100kDa are seriously limited with the amount of sequence that can be read, due to peptide bond cleavage arising mainly from N to O acyl shifts (Vensel and Tarr, 1995). Moreover, proteins with a chemically inaccessible alpha-amino group cannot be sequenced directly by this procedure and are termed N-terminally blocked (Vensel and Tarr, 1995). It was not until the application of mass spectrometry that the sequencing of such small protein quantities was possible (Grant *et al*, 2001).

Improved quantitative methods are also valuable when investigating the levels of various proteins in complexes obtained from different starting materials, such as different regions of the mammalian nervous system, many of which are very small, especially within the brain. It is imperative to be able to utilise as much of each such sample as possible. Neural tissue often contains a high lipid content (the insulating myelin sheath surrounding nerves contains many lipids), as well as being fibrous due to

the connective tissue (Grays, 34th Ed.). This adds to analytical problems by making solubilisation and protein extraction difficult. Fibrous tissues are physically difficult to homogenise and to extract proteins from, whilst the hydrophobic nature of lipids will resist solubilisation. Such complications may result in undesirable, low protein concentration sample mixtures. However, the advances in mass spectrometry and the ever updated protein databases means there are increasingly higher chances of identifying the proteins in question, despite these limitations.

Additional proteomic studies applied to the nervous system include Jenkins and colleagues' (2002) study of the affect of cortical impact on the hippocampus using 2D-E, Tilleman *et al* (2002) studied neurodegeneration in the brain of tau transgenic mice, also using proteomic methods for the analysis of brain tissue was Brookes *et al* (1990), Preobrazhensky (1993), Dey *et al* (1994), Abe *et al* (1997), Janke *et al* (2000), Oppermann *et al* (2000), Schonberger *et al* (2001), Fonstanze *et al* (2001), Castegna *et al* (2002), and Matsu-ura *et al* (2002). Proteomics was also used by Geschwind *et al* (1996) for the identification and characterisation of novel developmentally regulated proteins in rat spinal cord. Oblinger (1987) used 2D-E to study the characterisation of posttranslational processing neurofilament protein *in vivo* in dorsal root ganglia, Shimoji and colleagues (1999) studied nerve damage following *Mycobacterium leprae* invasion of Schwann cells, also in the peripheral nervous system, using proteomics. Cerebrospinal fluid (CSF) was analysed using proteomics by Sickmann *et al* (2002), Zerr *et al* (1996), and Green *et al* (2001).

There has been very little proteomic research concerning autonomic ganglia and thus, there is limited knowledge either on the protein expression in autonomic ganglia or on the methodology for sample preparations. One of the few examples of such a study was carried out on the trigeminal ganglia of rats during experimentally induced inflammation (Frisco *et al*, 2001). Using high resolution 2D-E and mass spectrometry *in vivo* and *ex vivo*, pro-inflammatory stimulation of rat trigeminal ganglia were studied as a model to analyse nociceptor protein changes during inflammation. A database was compiled containing a list of the proteins identified within rat trigeminal ganglia by MALDI-TOF-MS from the 2D gel (Frisco *et al*, 2001).

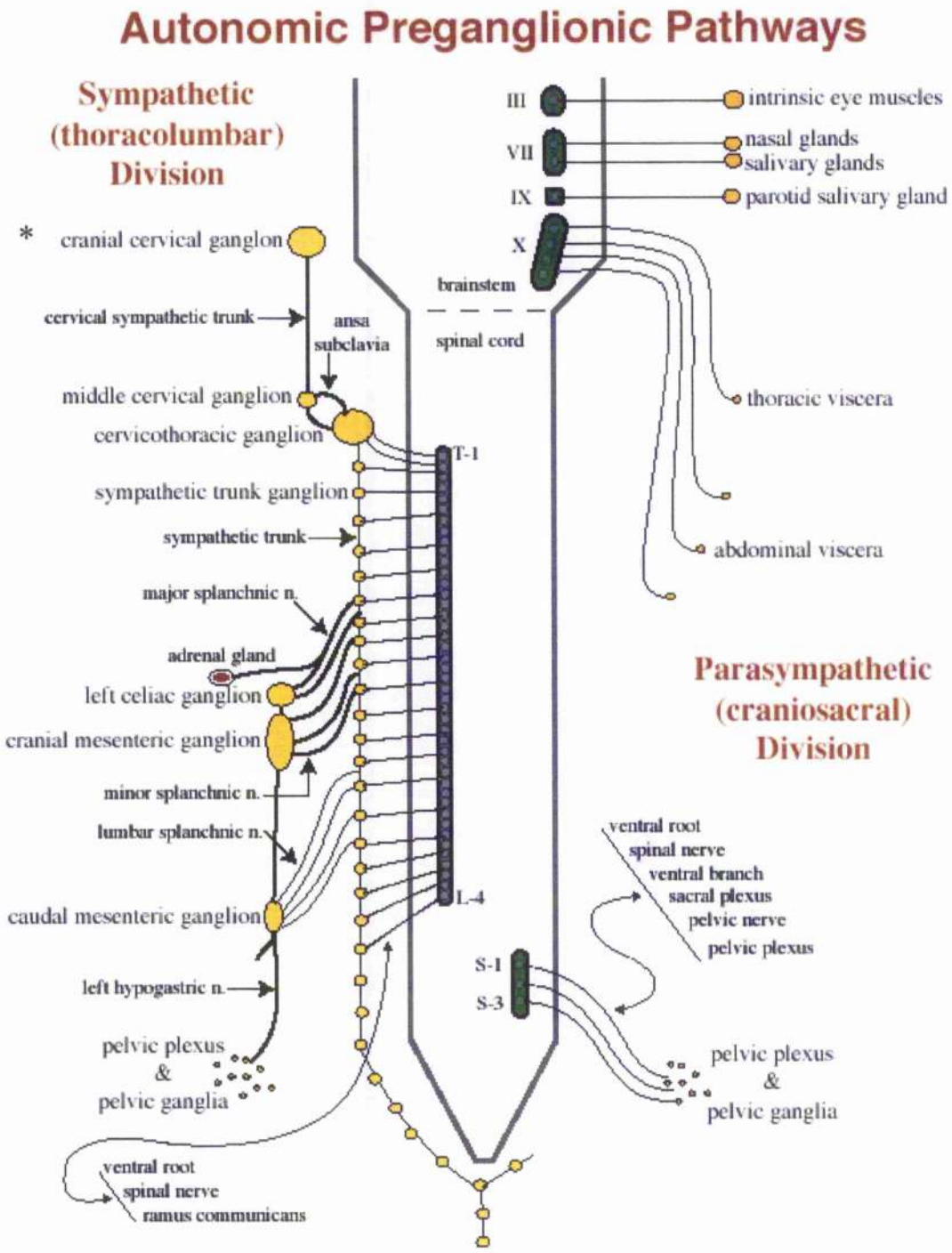
1.3 The autonomic nervous system

1.3.1 Introduction: Structure of the autonomic nervous system

The ANS has previously been described in detail, for a full explanation see Totora, 8th Edition. Briefly, the autonomic nervous system controls involuntary reactions to stimuli, thereby, regulating physiological functions. The autonomic nervous system (fig.1.1) is divided into two major sections: sympathetic and parasympathetic. The afferent nerves sub-serving both systems convey impulses from sensory organs, muscles, the circulatory system and organs of the body to the controlling centres (medulla, pons and hypothalamus) in the brain. From these centres the efferent nerves convey impulses to all parts of the body by the parasympathetic and sympathetic systems (Goldstein and Smith, http://www.ndrf.org/ans.html). Generally, the two systems have opposing effects on given bodily organs, one causing excitation and the

other inhibition. The systems use different neurotransmitters and different neurotransmitter receptors; this is the basis for dual innervation.

Figure 1.1. Diagrammatic structure of the autonomic nervous system. (From http://vanat.cvm.umn.edu/TFFlectPDFs/LectANS.pdf) The diagram shows the location of the cranial cervical ganglion (marked *) which was used in this study.



1.3.2 Autonomic ganglia

Autonomic ganglia are discrete collections of postganglionic neurons of the parasympathetic and sympathetic systems. Autonomic ganglia are generally divided into three groups: two of which are components of the sympathetic division and one group is a component of the parasympathetic division. The neural cell bodies are centralised within the bulk of the ganglia, whilst the nerve fibres (axons/dendrites) are arranged in parallel bundles entering or leaving the body of the ganglion, where it narrows (Fig. 1.2,1.3). The ganglion is wrapped in a protective connective tissue sheath, or endoneurium (BhamBurkar and Prakash, 1992). Strands from the sheath extend into the parenchyma of the ganglion (BhamBurkar and Prakash, 1992).

Figure 1.2. External structure of a ganglion.

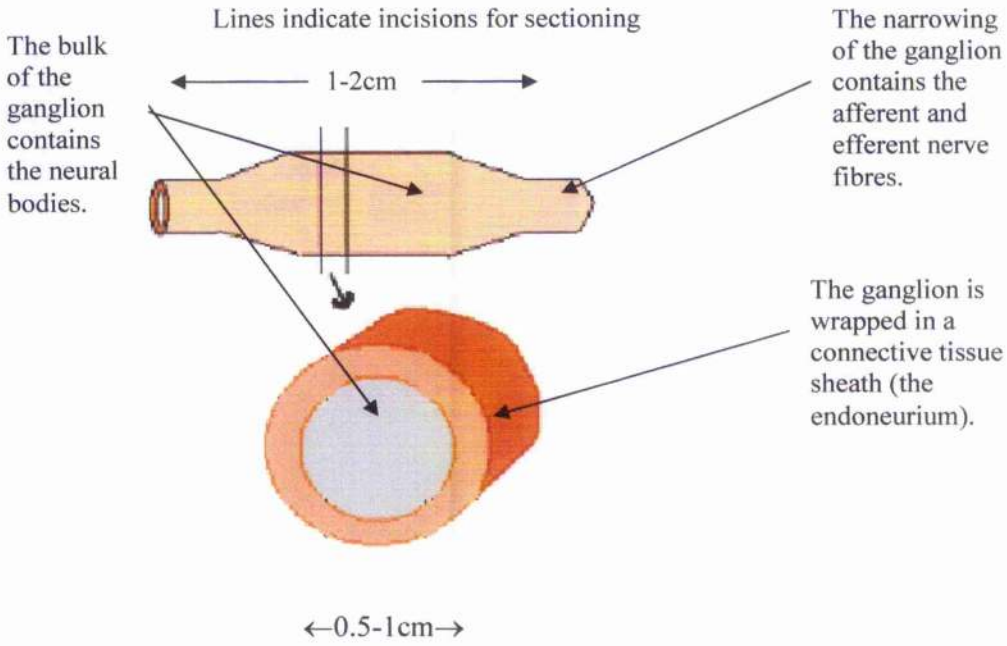
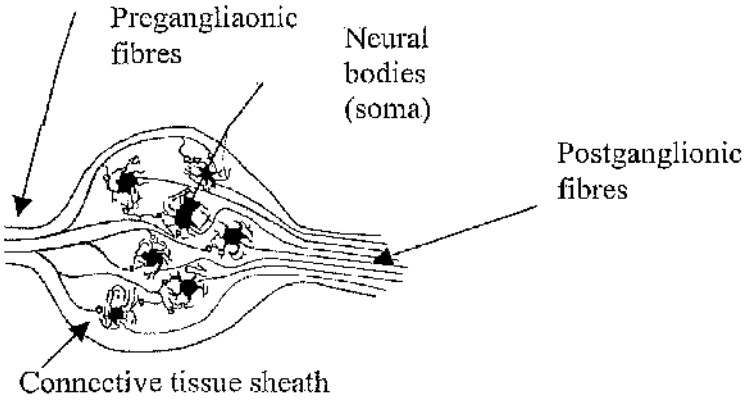


Figure 1.3. Internal organisation of an autonomic ganglion (adapted from Kiernan JA, 2002).



1.3.3 Cranial cervical ganglia

The cranial cervical ganglion (superior cervical ganglion) is the largest of the three ganglia of the cervical portion of the sympathetic trunk. In man, it is of a reddish-grey colour, and usually fusiform in shape, sometimes broad and flattened. Within the ganglion, there are large extracellular spaces that contain collagen (DePace, 1981). The cranial cervical ganglion communicates with the cervical nerves, some thoracic nerves and with a select few cranial nerves.

1.4 Aims and objectives

The aim of this investigation was to further our understanding of EGS by comparing protein expression, using proteomic techniques, in homogenates of cranial cervical ganglia collected from horses with acute EGS and from control horses. It was hoped that proteomic analysis would identify proteins that would contribute to our knowledge

of physiological mechanisms of neuronal death that occurs in EGS and therefore, help determine the aetiology of the disease. The following objectives were met:

1.4.1 Development of sample preparation and optimisation of 2DE for equine autonomic ganglia

Ganglia were homogenised and the component proteins separated by high-resolution 2D-E (Cohen *et al*, 2002). Cranial cervical ganglia where chosen because they consistently display a high proportion of damaged neurones as a result of EGS. Horses with acute EGS were selected in order to increase the chances of identification of the candidate neurotoxin. Since little proteomic work concerning autonomic ganglia had been carried out previously, much of this work was pioneering. The initial objective of the project was to create a method of optimising protein solubilisation and extraction from autonomic ganglia for optimal separation of the proteins. Homogenisation methods, protein amounts to load onto gels and the ideal pH ranges for first dimension separation, were some of the areas that were investigated in order to devise the most reproducible gels and then give the optimal chance of isolating EGS-related proteins.

1.4.2 Eliminating natural variations in ganglia proteome expression

Due to the limited sample numbers and the samples that were obtained coming from animals that were not purpose bred (e.g. transgenic mice), it was considered likely that in this study there would be unavoidable differences in the protein expression between horses of differing breeds, ages and sexes. However, by identifying the proteins associated with these natural variations they could be eliminated from the analysis of

EGS related proteins. Thus, comparisons of samples were made from (1) the same ganglia, (2) different ganglia from the same horse, (3) ganglia from different horses of the same sex, and (4) ganglia from horses of each sex to help eliminate variation unrelated to the development of EGS.

1.4.3 Identification of EGS related proteins

Quantitative comparison of the protein spot patterns on 2D gels of ganglia homogenates from EGS and control horses were carried out to isolate spots that appeared to be EGS related. Protein 'spots' that were consistently up-regulated or down-regulated in all EGS samples were removed from the gels and an attempt to identify them using MALDI mass spectrometry was made in the first instance. The proteins were identified by comparison against the fingerprints of all known protein sequences contained in a database. Considerable difficulty in characterising equine proteins was expected due to the limitations of the equine genome project and hence little information on equine proteins. The identity of candidate proteins for which there is no published sequence was further investigated using tandem mass spectrometry, to provide further information about the sequence of the protein(s) in question.

Chapter Two: Optimisation of 2D-E analysis of equine autonomic ganglia

2.1 Introduction

Sample preparation is considered one of the most important steps for optimal 2D-E analysis. For example, the nature of the sample will determine the method of homogenisation. Frisco and colleagues (2001) used rat trigeminal ganglia for proteomic analysis and used a mechanical glass homogeniser, whilst Sickmann and colleagues (2002) had no need for mechanical homogenisation for their proteomic study using CSF; instead they suspended proteins in a modified reswelling (rehydration) buffer.

The buffers and additives (such as protease inhibitors) will also vary according to the type of sample used. The aim is to extract, solubilise and suspend the greatest quantity of protein from the sample without causing degradation to the proteins. A standard lysis buffer was used by Frisco *et al* (2001) and Jenkins *et al* (2002) to solubilise proteins from rat trigeminal ganglia and rat hippocampal cells, respectively. Geschwind *et al* (1996) homogenised their spinal cord sample in a Tris buffer containing pepstatin A and phenylmethyl sulphonyl fluoride (PMSF).

Therefore, the main aim of the first part of this project was to develop an optimal and reproducible sample preparation method that allowed simple and effective solubilisation of the proteins from equine autonomic ganglia for analysis by 2D-E. There are no published reports of autonomic ganglia from the horse (*Equus caballus*) being analysed by 2D-E gel electrophoresis, therefore, there are no recommended

preparation procedures for the homogenisation of the tissue and solubilisation of the proteins.

The first task was to section the actual ganglia and remove the outer connective tissue sheath. By removing the sheath the possibility of inadvertent contamination from extraneous proteins, such as those present within contaminating blood and serum during post mortem collection would hopefully be eliminated. It was also anticipated that samples taken from each section would be standardised, by sampling a standardised region of the ganglion.

Neural tissue contains high quantities of lipid and fibrous components, especially within the dense collections of neuronal bodies such as ganglia. Such tissue may cause problems during the homogenisation and solubilisation of the proteins from the tissue for proteomic analysis. It was, therefore, proposed that a mechanical homogenisation method would be needed to render the material effectively.

1D mini-gels were run with autonomic ganglia samples to estimate the protein content and to check the efficiency of homogenisation. 1D mini-gels are relatively inexpensive and quick to run, and hence, were ideal for such analyses.

Before a 2D mini-gel was run, a protein assay was performed to assess the protein content in autonomic ganglia preparations. The ganglia sample was then separated by 2D-E on 7cm gels, once the protein content had been calculated and the size of sample needed for a 2D mini-gel had been established. This stage of the development of sample preparation allowed for assessment of the methods for use on a 2D gel, but

without the expense in time and money that would be required for large format 2D-E analysis of protein expression.

The comparisons of sample preparation methods included running gels with DNAse/RNAse, determining the optimal protein-loading amount and comparing the pH ranges of first dimension IEF strips. A further investigation evaluated a cryogenic grinder, in an attempt to improve the extraction of proteins during homogenisation.

Aims

The aims of the work within this chapter were:

- To optimise the sectioning and homogenisation of autonomic ganglia for electrophoretic analysis
- To optimise 1D mini-gels
- To optimise 2D mini-gels
- To assess the protein content of autonomic ganglia preparations
- To optimise large format (24cm) 2D gels
- To optimise the specifics of sample preparation for large format 2D-E by:
    - Evaluating the advantage of DNAse/RNAse treatment
    - Optimising protein loading quantities for 24cm 2D-E gels
    - Determining the optimal pH range for protein analysis
- To explore the value of the cryogenic grinder for sample preparation

2.2 Materials and methods

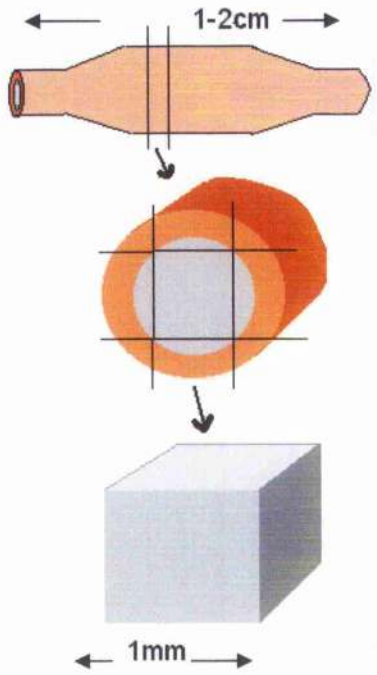
2.2.1 Collection and preparation of cranial cervical ganglia from *Equus caballus*

Cranial cervical ganglia were surgically removed from horses that had died or were euthanised due to EGS on post mortem, at the Royal (Dick) School of Veterinary Studies, Edinburgh. Immediately following collection, the cranial cervical ganglia (ccg) were frozen using liquid nitrogen and sealed in individual zip-top plastic bags, labelled with the ganglion ID and stored at –70°C until needed. The samples were transported to the University of Glasgow on dry ice to maintain their frozen state and preserve the protein composition of the tissue.

2.2.2 Sectioning of cranial cervical ganglia

Figure 2.1 shows how the frozen ganglia were cut and the connective tissue sheath removed, using a scalpel knife, to prevent contamination with blood and serum. By weighing the sectioned ganglion, it was shown that, in general, a 1mm cross section of a whole ganglion would result in approximately 30mg of wet weight tissue suitable for use for proteomic analysis.

Figure 2.1. Sectioning of autonomic ganglia for preparation for 2D-E to remove surface contamination and to standardise the site of sample collection.



a) Whole ganglion showing example incision-lines for sectioning.

b) Transection of ganglion (diameter ~ 0.5cm) showing outer sheath and inner core. The outer sheath is removed.

c) The final product of a section of central ganglion ready to be homogenised for proteomic analysis.

2.2.3 Homogenisation of tissue

Ganglia were placed in eppendorf tubes with lysis buffer and using a scalpel knife were manually rendered, until an almost clear pulp resulted. The suspensions were then subjected to the following treatments:

- frozen in liquid nitrogen
- thawed in a heating block (no hotter than 30 °C)
- vortexed for 1 min.

} repeated 3 times

The resulting solutions were then centrifuged (13,000 x g) to separate any remaining solid material. The supernatants were collected and centrifuged again before being used for 2D-E analysis.

2.2.4 One dimensional SDS-PAGE using mini-gels

1D mini-gels were run with samples from equine autonomic ganglia using the BIO-RAD mini-PROTEAN II electrophoresis cell kit. Acrylamide gels were prepared and poured using a 15% resolving gel and a 5% stacking gel. Promega Broad Range protein marker (catalogue no. P7702S) and samples were loaded into wells in the stacking gel; the electrophoresis chamber was assembled and filled with SDS electrophoresis buffer according to the protocol. The gels were run at 120V, 300mA for ~1.0-1.5 h.

2.2.5 Determination of protein concentration in equine autonomic ganglia

The protein content of sample of equine autonomic ganglia (μg/ml) was estimated using the Pierce BCA Protein Assay kit, following the microplate procedure. Briefly:

1. 25 μl of each BSA concentration standard was pipetted into a microplate well and 25μl of each concentration of sample was pipetted into adjacent wells
2. 200 μl of the working reagent (WR) was added to each well and then thoroughly agitated on a horizontal plate shaker for 30 s
3. The plate was covered and incubated at 37°C for 30 min
4. The plate was cooled to room temperature
5. The absorbency was measured at or near 562 nm on the Titertek Multiscan® MCC/40 microplate reader (Labsystems & Flow Laboratories, Finland)

Table 2.1. Preparation of diluted albumin (BSA) standards. Dilution scheme for standard microplate procedure (Working Range = 20–2,000 µg/ml)

Volume of Diluent		Volume and Source of BSA	Final BSA Concentration
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank

2.2.6 2D mini-gel of equine autonomic ganglia

2D mini-gels provide a good way to practise the relevant steps of running 2D-gels without the expense of the large format gel. 2D mini-gels are also a good indicator of protein content and separation that would be present on the large format gels.

The Amersham Pharmacia Biotech 2-D Electrophoresis protocol was followed to run equine autonomic ganglia sample though 2D SDS-PAGE. Briefly, 125µl of the complete rehydration solution, containing the sample, DTT and IPG buffer, was applied along the strip holder and a 7cm pH4-7 IPG strip was used. The strip was left to run through rehydration in the Amersham IPGphor equipment (a minimum of 10 h), and Isoelectric Focusing (IEF) at about 8000V and very low currents until it had reached 40 000 Volt-hours (V-h), as recommended.

The strips were then equilibrated with SDS buffer and DTT on a horizontal agitator (e.g. the ADLOF KÜHNER AG, SCHWEIZ, Lab-Shaker) at 40min^{-1} for 15 min. The strip was then applied to a Bio-Rad mini-gel (set up prior to equilibration and as

described for 1D mini-gels, but with no need for a stacking gel). The gel was then left to run through second dimension electrophoresis, again as for 1D mini-gels.

2.2.7 Coomassie Brilliant Blue staining for 2D-E gels

- Each gel was placed in a staining tray and completely submerged with Coomassie Brilliant Blue staining solution.
- The staining trays were covered and left on a lab agitator for 1-3 days.
- The background staining was removed by submerging the gels in a de-stain solution (10% acetic acid/ 40% methanol) that was changed every couple of hours, until the desired contrast had been obtained.

2.2.8 Silver-staining for 2D mini-gels

The standard protocol for the Amersham Pharmacia Biotech, protein silver staining kit was followed:

Fixation: 100ml ethanol, 25ml acetic acid and made up to 250ml with deionised H_2O, the gels were covered and left on the Lab-Shaker for 30 min.

Sensitisation: 75ml ethanol, 10ml glutaraldehyde, 10ml sodium thiosulphate, 1 sachet (17g) sodium acetate and made up to 250ml with deionised H_2O, the gels were covered and left for 30 min.

Washing: the gels were rinsed and washed with deionised H_2O 3 times for 5 min.

Silver reaction: 25ml silver nitrate solution, 0.1ml formaldehyde and made up to 250ml with deionised H_2O.

Washing: the gels were rinsed and washed with deionised H_2O 2 times for 1 min.

Developing: 1 sachet (6.25g) sodium carbonate, 0.05ml formaldehyde and made up to 250ml with deionised H_2O. The gels were covered until developed to suitable level (approx. 2-5 min).

Stopping: 1 sachet (3.65g) EDTA-Na_2•2 H_2O and made up to 250ml with deionised H_2O, and added to gels as soon as developing was complete.

Washing: the gels were rinsed and washed 3 times for 5 min with deionised H_2O.

Preserving: 75ml ethanol, 11.5ml glycerol (87%) and made up to 250ml with deionised H_2O. The gels were covered and left for 30 min. Repeat.

2.2.9 Sypro-Ruby staining for 2D-E gels

- The gels were fixed in 10% methanol/7% acetic acid for 30mins whilst on the Lab-Shaker
- The gels were then stained in Ruby Gel stain (10 x volume of gel) for at least 3 h or overnight
- Finally the gels were rinsed in 10% methanol/7% acetic acid for 30-60 min to reduce background fluorescence

2.2.10 Sypro-Orange staining for 2D-E gels

- The proteins were fixed in the gel using 10% MeOH/7.5% acetic acid on the Lab-Shaker for 1 h.
- Probed using 0.05% SDS for 1 h
- Stained with SYPRO-Orange with gentle rocking overnight (making sure that no light gets into the gel.)
- De-stained with 30% methanol/ 10% acetic acid for ~1 min, pre-scan, and repeat de-staining until the desired contrast has been achieved.

2.2.11 Colloidal Coomassie Blue staining for 2D-E gels

- A 5% Coomassie stock (5g/250ml) was made up.
- The colloidal stock was made up (50g ammonium sulphate, 500ml H_2O, 6ml phosphoric acid, 10ml Coomassie stock)
- The stain was made to the following ratio: 1 part methanol, 4 parts colloidal stock
- The gels were fixed for 2 h in 40% ethanol/10%acetic acid
- The gels were then washed twice for ten min in H_2O
- The gels were stained for 1-7 days
- The gels were de-stained by rinsing with H_2O

2.2.12 Large format (24cm) 2D-E gels of autonomic ganglia

The large format 2D gels are based on the same principles and procedures as the 2D-mini-gels. Large gels allow for the separation of proteins over a larger area, therefore, making analysis of protein spots easier. The large format pre-cast, plastic-backed gels (Amersham Pharmacia Biotech) are also compatible with the Ettan Spot-picker robot and subsequent mass spectromic analysis.

Using the Amersham Pharmacia Biotech protocol as a guide, sample solution was loaded into 24cm strip holders and strips placed in each holder. The strips were run at 8000V, requiring a minimum of 70 000 V-h to be reached for optimal focusing of protein spots. During the second equilibration step, pre-cast gels supplied by Amersham Pharmacia Biotech were used. Once electrophoresis was completed the gels were Coomassie brilliant blue stained over night.

2.2.13 Comparisons of sample preparation methods – optimising 2D-E gels of equine cranial cervical ganglia

2D-E gels were run to determine the optimum sample preparation methods and adjust the 2D-E techniques to produce the best quality gels:

- Comparing of gels with sample, with and without DNAse/RNAse treatment
- Comparing gels with different amounts of protein loaded onto IEF strips
- Comparing gels of different IEF strip pH ranges

2.2.14 Novel sample preparation methods - using a cryogenic grinder

Cryogenic grinders have generally only been used for the degradation of hard materials such as hair and nails for forensic analysis. These grinders reduce such materials to a fine powder by freezing them in liquid nitrogen and subsequently hammering them within a small, cylindrical chamber with a pellet at an extremely high frequency. It was, therefore, considered worthwhile to investigate whether the ganglia could be reduced to a smooth liquid pulp in this manner and thus aid the solubilisation of the proteins.

Homogenisation of equine autonomic ganglia with the cryogenic grinder was kindly provided by the Department of Forensics at the University of Glasgow (supervised by Dr Robert Anderson). 45 mg of wet weight sample was loaded into the chamber and the pellet inserted. The chamber was placed in the cryogenic grinder and the entire equipment was filled with liquid nitrogen. The sample was ground at an automated setting, 1 minute of pulping followed by 1 minute of rest/re-freezing and another minute of pulping.

2.3 Results

2.3.1 1D Mini-gel: comparing ganglia with and without sheath

This experiment had two purposes. The first was to determine whether the amount of protein in the size of sample used was adequate for electrophoretic analysis. The second was to examine the extent of inadvertent contamination of the proteins caused by the inclusion of the outer connective tissue sheath of the autonomic ganglia. The ganglia were sectioned as previously described for sample 1. A second sample was also sectioned off, but left as an intact disc of ganglia with the outer connective tissue sheath remaining.

Figure 2.2 shows that the gel contained an adequate amount of protein for electrophoretic analysis, as illustrated by the clear and distinct banding of the separated proteins. It also appears there was little difference between having removed the connective tissue sheath and leaving it intact. However, this could not be guaranteed for each case, and therefore, the sheath was removed from all samples thereafter.

Using the protein marker ladder, the $\log_{10}$ of the molecular weight of the protein bands of the ladder was plotted against the distance of the dye migration, allowing the molecular weights of the protein bands to be estimated. The following relationship was employed (Table 2.2):

Rf = distance of protein migration/distance of dye migration

Table 2.2 Estimation of samples' protein band weights

Weight (kDa)	Log_{10} mol weight	Rf
116	2.064	0.079
66.4	1.822	0.239
42.7	1.63	0.389
26.6	1.425	0.602
14.3	1.155	0.893

Graph 2.1. Log_{10} of molecular weight of marker bands plotted against Rf, used to calculate the relative weights of protein bands.

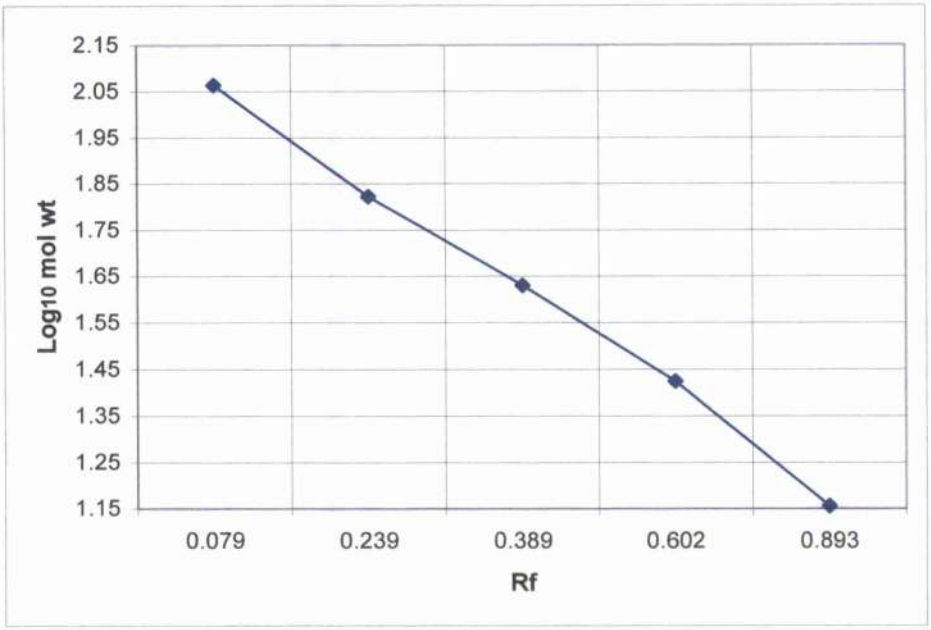
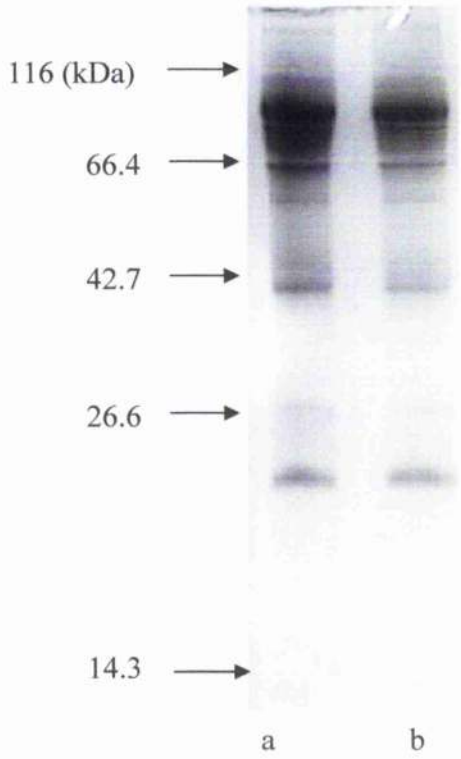


Figure 2.2. 1D Mini-gel of autonomic ganglia homogenate showing the relative weight markers (kDa) calculated from graph 1. (a) 10µl sample without surface connective tissue sheath (b) 10µl sample with surface connective tissue sheath.



2.3.2 Determination of protein concentration in autonomic ganglia

The protein content of a typical sample of equine autonomic ganglia (~30mg wet weight tissue, homogenised in lysis buffer) was estimated using the Pierce BCA Protein Assay kit, a detergent-compatible formulation based on bicinchoninic acid (BCA). Protein concentrations were determined with reference to standards of bovine serum albumin (BSA).

The microplate procedure was used for ease of sample handling with a microplate and the smaller volume (10-25 µl) of protein sample required. Table 2.1 was used as a guide to prepare a set of protein standards. A series of dilutions of known concentration were prepared from the standard protein and assayed in parallel with the unknown (equine autonomic ganglia) samples. The microplate was then covered and incubated at 37°C for 30 min. Once cooled to room temperature, the absorbency was measured at ~562nm using a Titertek Multiscan microplate reader and software. The protein concentration of ~30mg of wet weight equine autonomic ganglia was determined based on a standard curve.

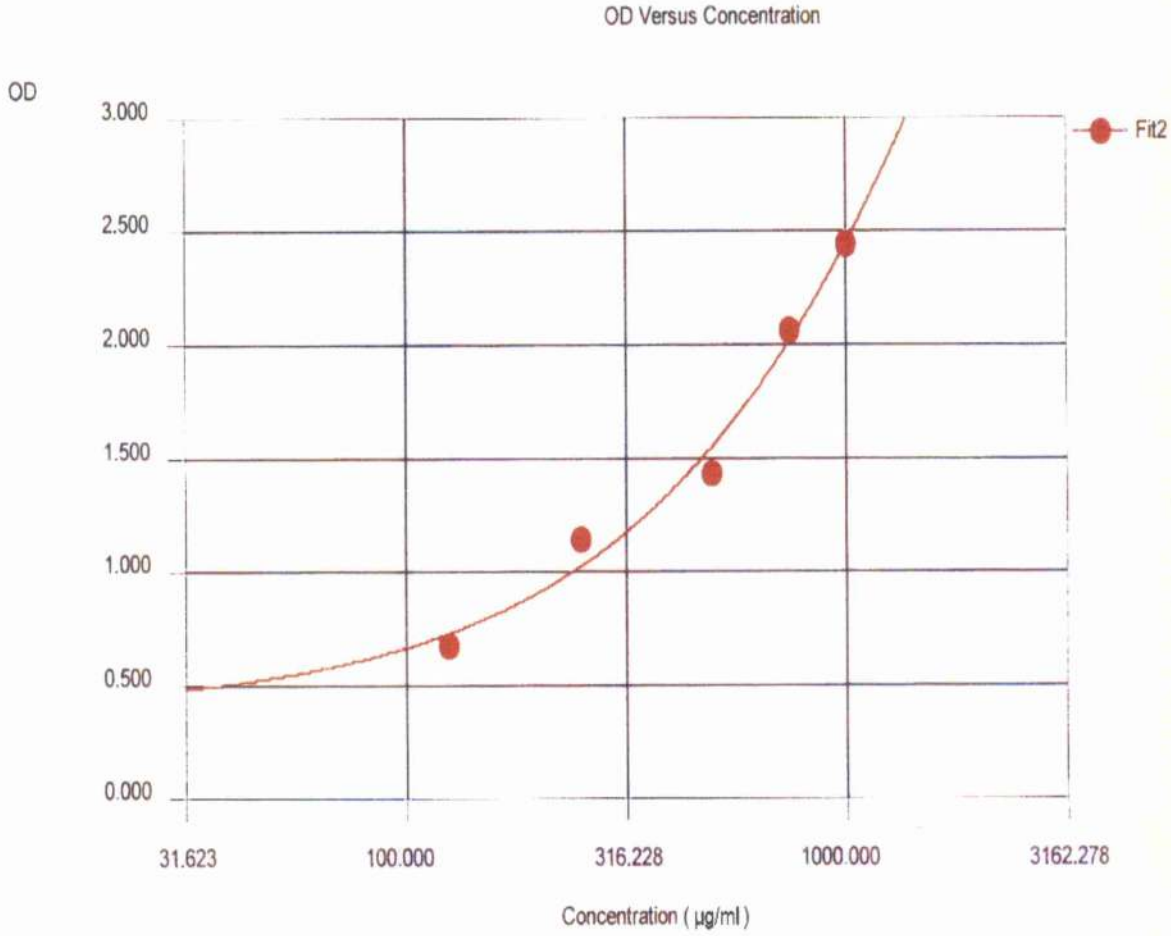
Initially it was found that the protein samples were of too dark a colour to measure the absorbency, indicating that either the protein concentration was too high (exceeding 2000µg/ml) or the sample contained lipids or lipoproteins. The experiment was therefore repeated using samples diluted to 1:5, 1:10 and 1:20. This appeared to solve the problem and good readings of absorbency were obtained and the protein concentration determined.

Table 2.3. Protein assay results and standards.

Sample ID	(OD) Data	(OD) Mean	Concentration (μg/ml)
T1	over	over	*****
T2	1.457	1.457	310
T3	1.048	1.048	150
T4	1.830	1.830	603
S1	over	over	2000
S2	over	over	1500
S3	2.452	2.452	1000
S4	2.078	2.078	750
S5	1.449	1.449	500
S6	1.154	1.154	250
S7	0.682	0.682	125
S8	0.309	0.309	25

Where T = tested sample
S = standard sample
OD = optical density

Graph 2.2 Protein Assay Standard Curve



The protein content of 30mg wet weight tissue was calculated as follows:

25µl of 30mg of ganglia in 200µl of diluted (1:5) lysis buffer = neat protein sample (1:1).

In sample dilutions 1:5 the protein conc^{n} = 602.906µg/ml
1:10 " " = 309.852µg/ml
1:20 " " = 149.275µg/ml

=> The mean protein conc^{n} = $\frac{(602.906x5 + 309.852x10 + 149.275x20)}{3}$

= 3032.85µg/ml

=> In 30mg of ganglia homogenised in 200µl of diluted (1:5) lysis buffer there is a protein concentration of ~ 3033µg/ml

⇨ 606.6µg protein in 200µl of homogenised tissue
⇨ 0.606mg of protein in 30mg of wet weight tissue
⇨ 2µg of protein in 1mg of wet weight tissue.

2.3.3 2D mini-gel of equine autonomic ganglia

A 2D mini-gel, of homogenate from equine autonomic ganglia 02/852 was run with 125µl of rehydration solution, also containing DTT. The Amersham Pharmacia Biotech protocol was followed, using a 7cm, pH4-7 strip, to rehydrate and run through IEF overnight.

The strip was equilibrated twice in equilibration buffer containing DTT and iodoacetamide (IAA) for 15 min each, in preparation for second dimension electrophoresis. The BIO-RAD mini-gel equipment was set up as previously described. The gel was run through electrophoresis for 1.0-1.5 h until the dye front had reached the bottom of the gel at which point it was removed from the electrophoresis apparatus and placed in a staining tray and Coomassie stained overnight.

This mini-gel was the first 2D-gel performed using equine autonomic ganglia and so it was particularly useful in determining optimum sample preparation methods. The gel did clearly have protein on it, although there was a lack of focusing possibly due to excess protein, poorly homogenised or solubilised sample or poor technique. Data not shown.

2.3.4 2D mini-gel comparing protein spots of samples from ganglia from different horses

Two 7cm strips were run with the aim of comparing two samples of equine autonomic ganglia from 1) a control horse without grass sickness and 2) a horse with sub-acute grass sickness. Strip 1 was run with 125µl of rehydration solution containing DTT and sample from control autonomic ganglia 02/852. Strip 2 was run with 125µl of rehydration solution, also containing DTT, but with a sample from AGS autonomic ganglia 02/431. The Amersham Pharmacia Biotech protocol was followed using 7cm, pH4-7 strips and the strips left to rehydrate and run through IEF overnight.

The strips were equilibrated twice in equilibration buffer containing DTT and iodoacetamide (IAA) for 15 min each. The BIO-RAD mini-gel equipment was used. The gels were run through electrophoresis for 1.0-1.5 h. The gels were then removed from the electrophoresis apparatus and placed in staining trays and Coomassie stained overnight. The gels were subsequently silver stained to improve the visualisation of the protein spots.

The silver stained gels were satisfactorily focused (fig 2.3), with significant quantities of protein present and fairly good focusing of protein spots. However, the stain was slightly over developed and therefore, the images were excessively dark. The gel containing sample from the autonomic ganglia 02/852, was damaged with over-handling during the relatively lengthy silver-staining procedure used (data not shown).

2.3.5 Large format (24cm) 2D-E gel of autonomic ganglia

As for 2D-mini-gels, $1mm^2$ slices of autonomic ganglia were taken, from the control ganglia 02/852 (sample 1) and 02/431 (sample 2), the sheaths removed and the remaining ~30mg of tissue were mechanically homogenised in lysis buffer (200µl), centrifuged and the supernatants collected into individual aliquots. 300µl of rehydration buffer, containing DTT and the appropriate IPG buffer, was added to each sample solution. The strips were left to run through rehydration and IEF over night.

Following IEF, the strips were equilibrated twice, with DTT then IAA, on the Lab-Shaker for 15 min. During the second equilibration step, pre-cast gels supplied by Amersham Pharmacia Biotech were positioned on the gel cassettes as instructed. The strips were then rinsed in electrophoresis buffer and applied to the top of the large gels and sealed in place with 0.5% agrose sealing solution. The gel cassettes complete with gels and strips were placed in the Ettan Dalt II electrophoresis tank and left to run through second dimension over night using the electrophoresis buffer kit supplied by Amersham Pharmacia Biotech.

Once electrophoresis was completed the gels were placed in staining trays and submerged in Coomassie brilliant blue stain over night. The gels were de-stained (30%

methanol/ 10% acetic acid) until an adequate contrast was obtained; at which point the gels were scanned onto computer using the Amersham Pharmacia Biotech Light Scanner and Labscan software.

The gels were very faint with some minor streaking, thought to be caused by insufficient protein loading. Data not shown. This experiment was repeated. The same poor quality gel, with faint markings and slight streaking was obtained (data not shown), suggesting that there was an inadequate amount of protein loaded onto the gels. It was considered that a more sensitive method of staining (e.g. silver staining) might alleviate the problem.

The same experiment was repeated again, this time using ~60mg of wet weight tissue, resulting in far more protein appearing on the gel (Figure 2.4). However, the gel was very streaky and poorly focused. Subsequently, a sample preparation method using DNAse/RNAse was employed in an attempt to alleviate some of the streaking.

2.3.6 Comparison of sample preparation methods – with and without DNAse/RNAse treatment

The previous gels showed adequate protein; however, the gels were very streaky and not properly focused. In an attempt to remove the streaking and improve the focusing of spots, comparative sets of gels were run. One gel was run with sample that was prepared in the usual manner, whilst the other gel was run using sample was run incorporating DNAse/RNAse treatment.

Following completion of both dimensions, the gels were washed in 10% methanol and 7% acetic acid for 30 min, before staining with Sypro-ruby over night. The Sypro-ruby was poured off and the gels were de-stained (10% methanol and 7% acetic acid). The gels were then scanned using the Typhoon scanner.

The gel run with sample containing DNAse/RNAse treated sample was somewhat clearer (figure 2.5, (b)) and more focused than the gel without DNAse/RNAse (figure 2.5, (a)). However, the use of DNAse/RNAse had still not completely solved the problem, as the gels were not focused and contained high levels of streaking and coagulations of protein spots.

2.3.7 Determination of optimal protein loading quantity

Despite the use of DNAse/RNAse the gels were still very streaky and unfocused, possibly due to a problem with the quantity of protein loaded onto the gels. Some gels did not contain enough protein (if ~30mg wet weight tissue used, equating to 600µg protein), whilst others had too much protein loaded (~60mg wet weight tissue used, equating to 1200µg protein) on them. It was, therefore, a logical progression to compare several protein loads to determine the optimal content required for a good gel image with optimal spot intensity and minimal streaking.

Sections of ganglia were collected, as described, from ganglia 02/431. Sample 1 weighed ~45mg wet weight tissue (~900µg protein), and sample 2 ~60mg wet weight tissue (~1200µg protein). Each sample was prepared and the proteins separated by 2D-E as previously described. The gels were then stained with SYPRO-Orange following the standard protocol.

From the images (Figure 2.6) it can be seen that the gels had adequate protein and good focusing. Using pH4-7 strips (as used with the mini-gel system) seemed to make a big difference, as both quantities of protein produced good gels, however, the gel with ~900µg protein (~45mg wet weight tissue) was the clearest gel and showed the least streaking.

The experiment was repeated to check the reproducibility of these gels. This time, 18cm gel strips were used and the sample weights reduced proportionately, otherwise the protocols remained the same. The gels produced (Figure 2.7) were comparable to the previous gels (Figure 2.6) and validated the reproducibility of the system.

2.3.8 Determination of optimal pH range

Having run several 2D gels with little separation of proteins occurring; the pH range used was revised. Gels with a range of pH 3-10 linear (figure 2.8) appeared not to be separating the proteins adequately. Therefore, to establish the most appropriate pH range to use for 2D gels, a comparison between pH4-7 (as successfully used in the 2D mini-gels and previously in 24cm 2D gels) and pH3-10 non-linear was set-up. Non-linear pH strips over-represent the middle pH range in contrast to a linear pH gradient strip. Two ~45mg sections of ganglia (02-889) were collected and prepared for 2DE. pH4-7 strips were used with sample 1 and pH3-10NL strips with sample 2.

For the purposes of this project, using pH4-7 IEF strips was more effective. On 2D-E gels using pH4-7 strips in the first dimension, more spots were seen and better separation of proteins occurred than on gels using the pH3-10 strips. The proteins that

were not separated using pH4-7, i.e. those with isoelectric points of pH3-4 and pH7-10, can be studied using specialised strips that cover the required range if required.

Figure 2.3 A silver-stained, 2D-E mini-gel of equine autonomic ganglia 02-431.

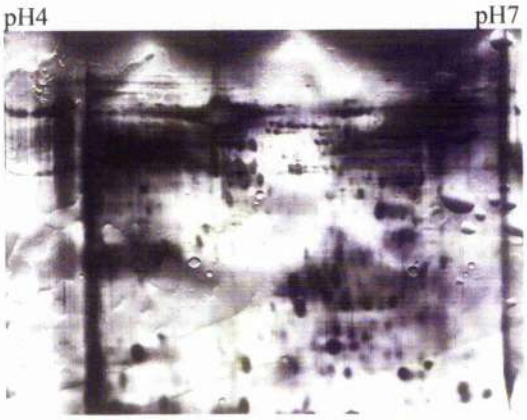


Figure 2.4. A Coomassie Blue stained, 2D-E large format gel of 60mg wet weight autonomic ganglia/1200μg protein, using a pH3-10 linear (L) IEF strip. The arrows highlight overloading of protein/unfocused protein.

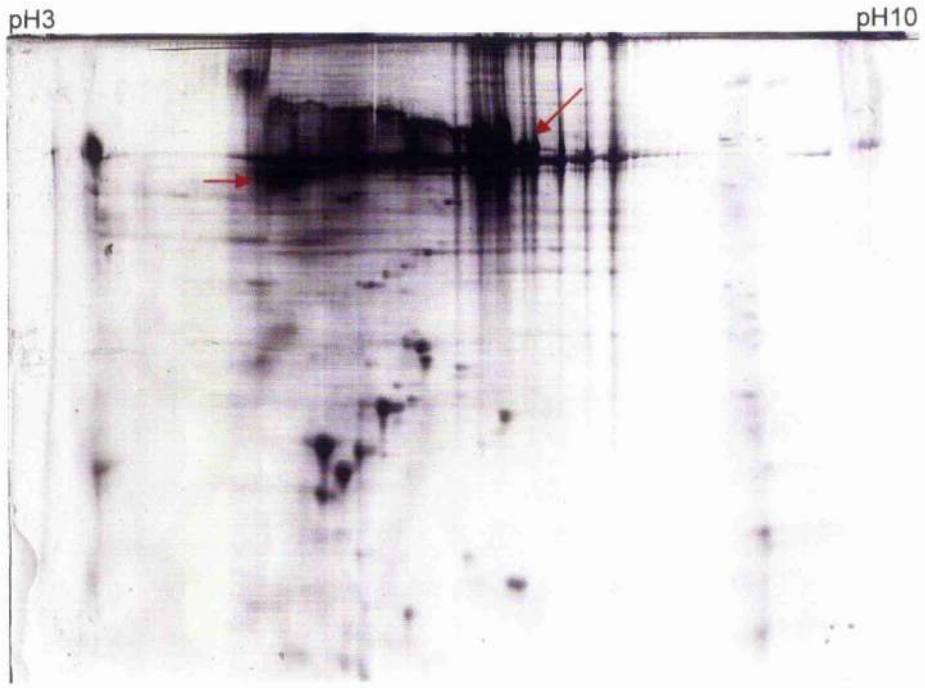
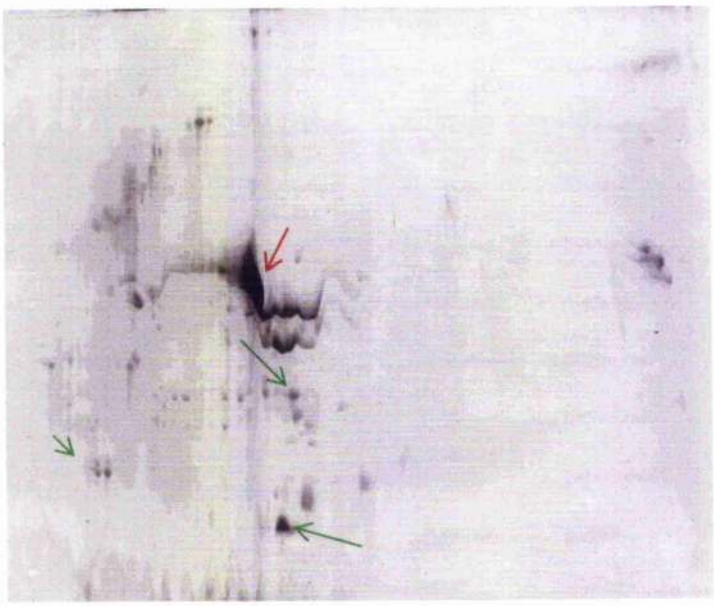


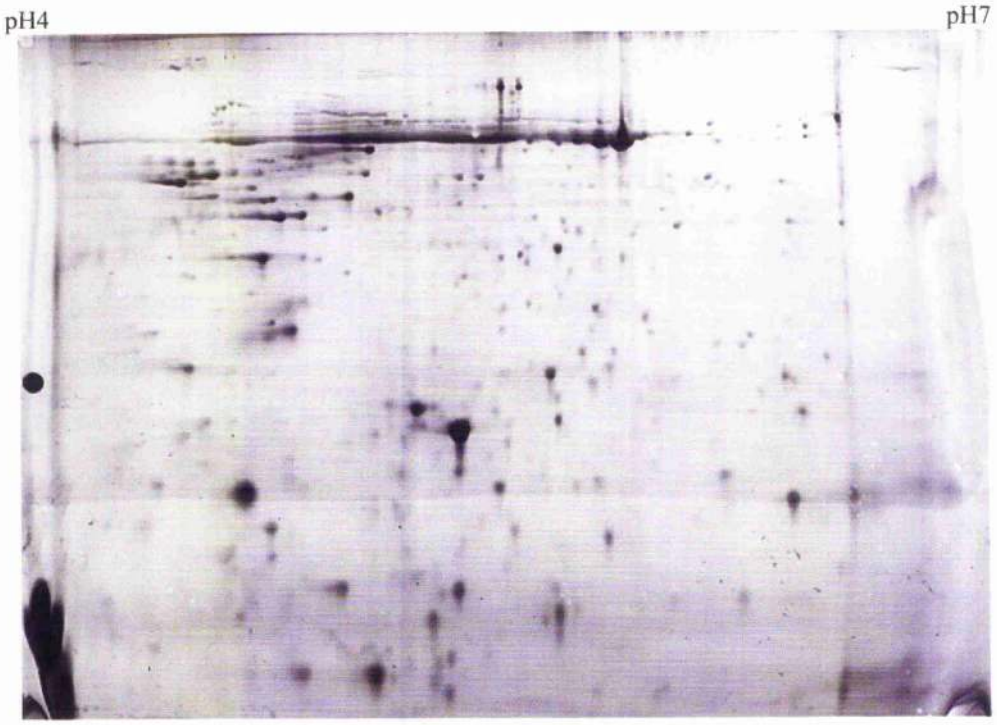
Figure 2.5. A comparison of sample preparation methods for 2D-E analysis of equine autonomic ganglia (b) with and (a) without DNAse/RNAse treatment. These gels were Sypro-ruby stained. The red arrows indicate unfocused protein, green arrows indicate focused protein.

(a) no DNAse/RNAse

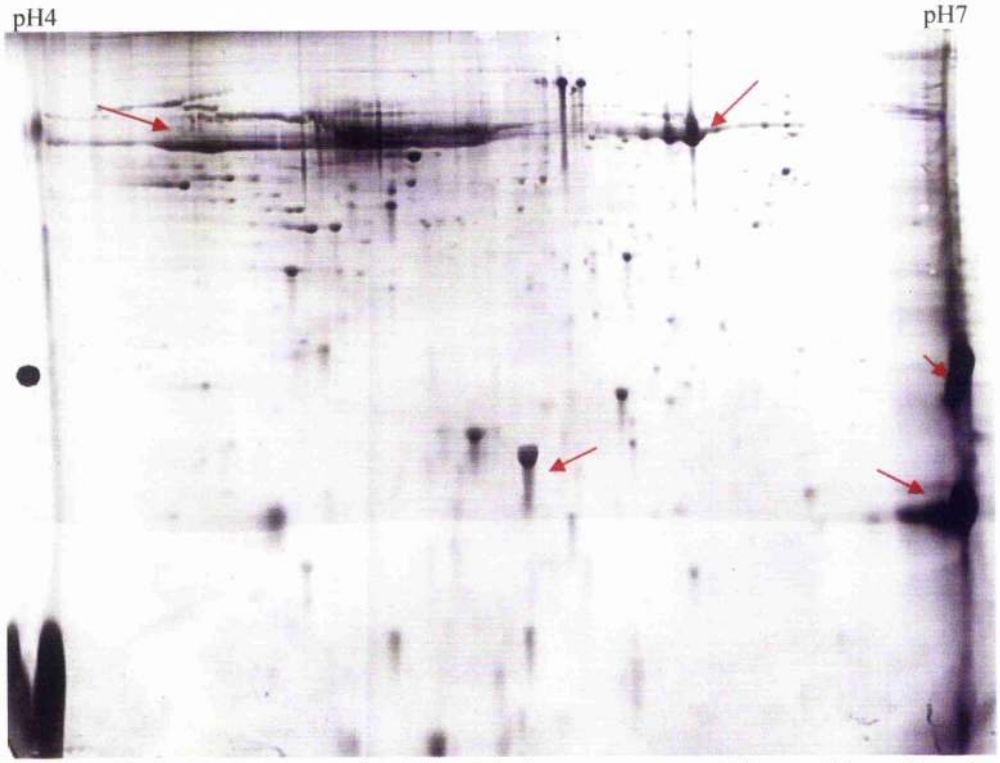


(b) 10µl DNAse/RNAse

Figure 2.6. Two 24cm, pH4-7, Sypro-orange stained, 2D-E gels comparing sample sizes of equine autonomic ganglia to determine the optimal separation and focusing of proteins.



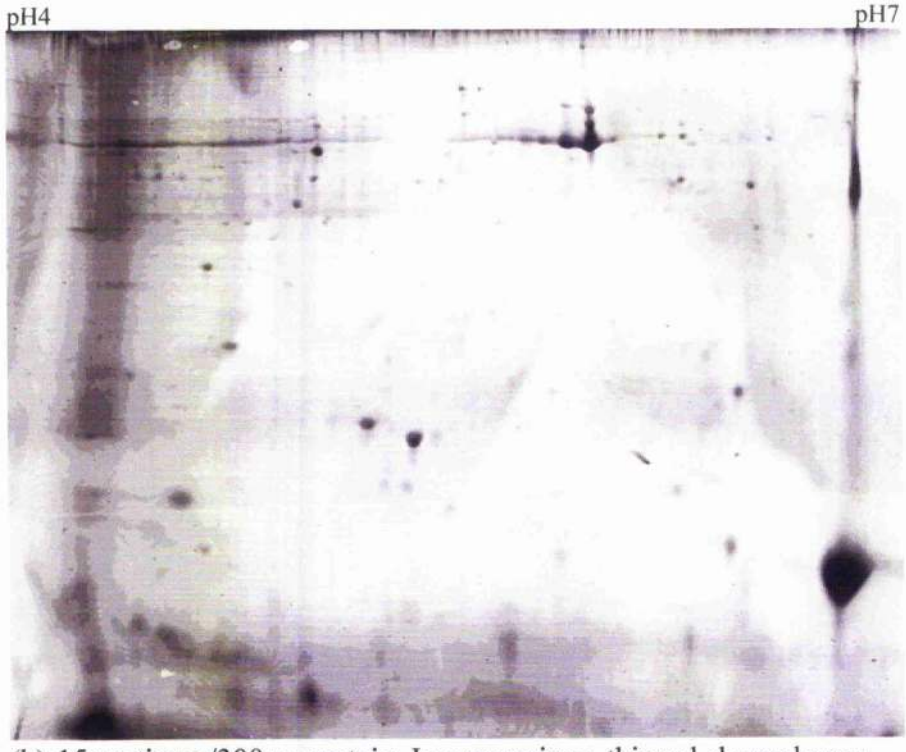
(a) 45mg tissue/900µg protein



(b) 60mg tissue/1200µg protein. This gel shows more streaking and less focusing of protein (arrows) due to overloading with sample.

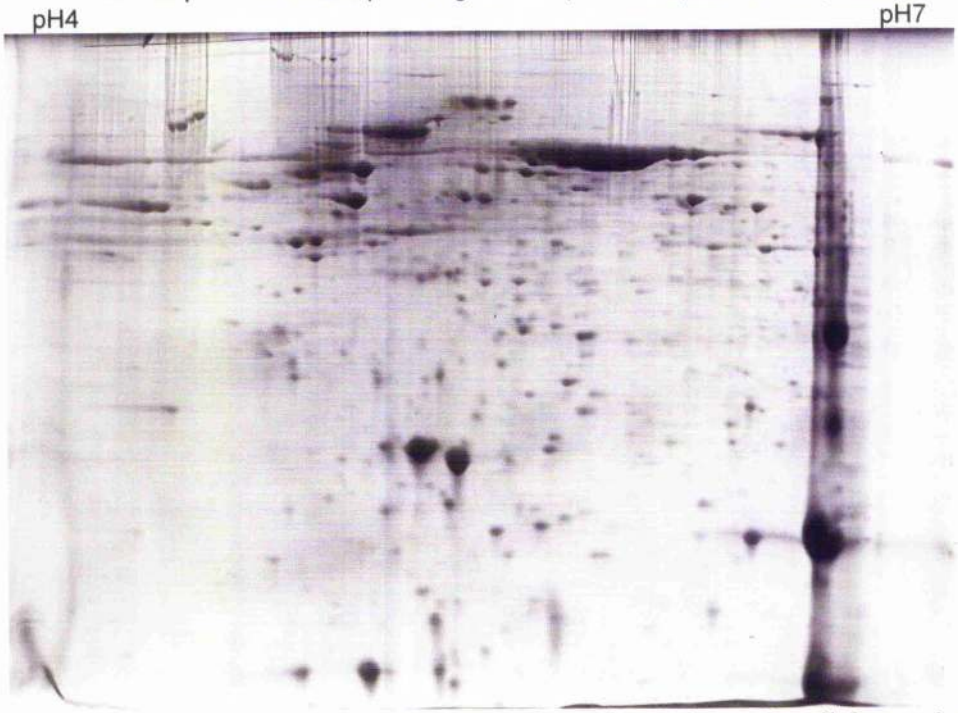
Figure 2.7. Two 18cm, pH4-7, Sypro-orange stained, 2D-E gels assessing the reproducibility of sample preparation methods and optimal sample sizes.

(a) 30mg tissue/600µg protein. This gel shows good focusing and plenty of protein, but some streaking.

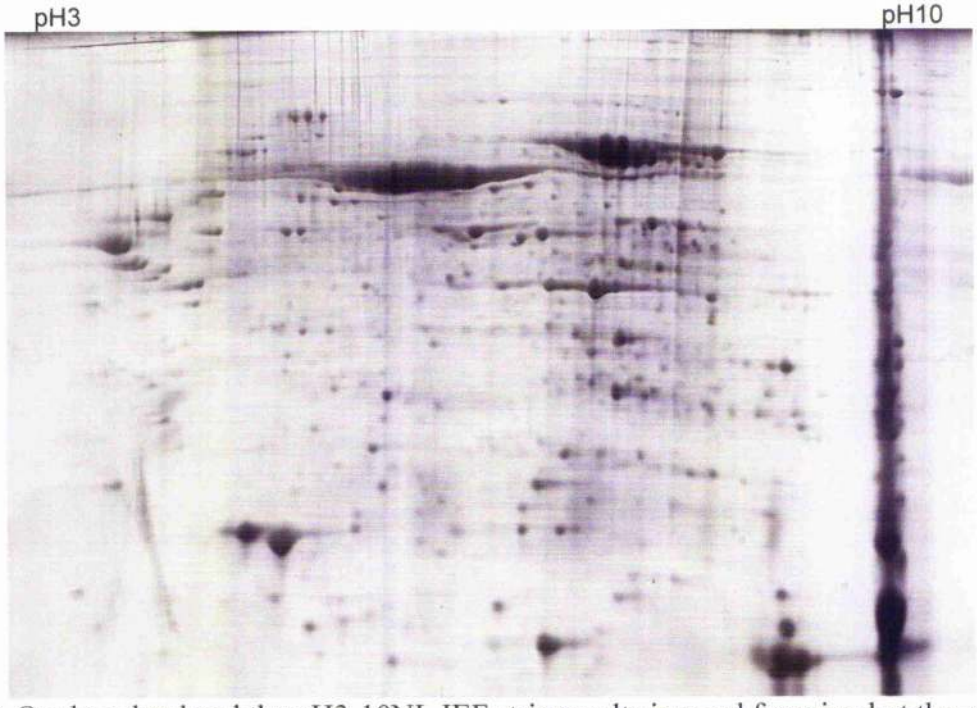


(b) 15mg tissue/300µg protein. In comparison, this gel shows lower numbers of protein spots, but good focusing and little streaking.

Figure 2.8 Two 24cm, Colloidal Coomassie Blue stained, 2D-E gels run to compare IEF strips of different pH ranges for optimal separation of proteins.

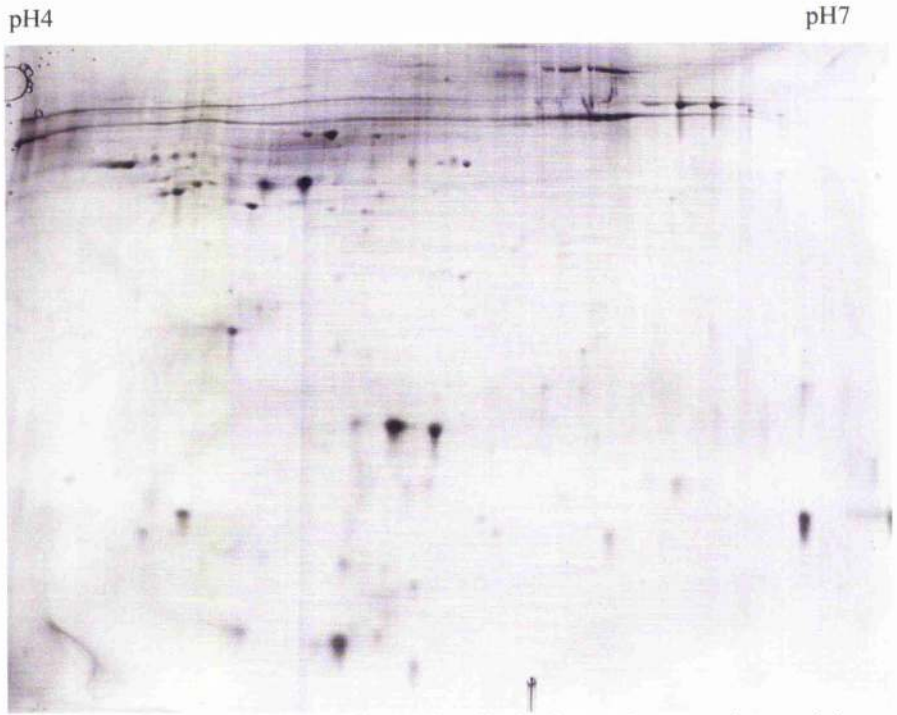


(a) The pH4-7 IEF strip results in good separation of proteins and well focused spots.

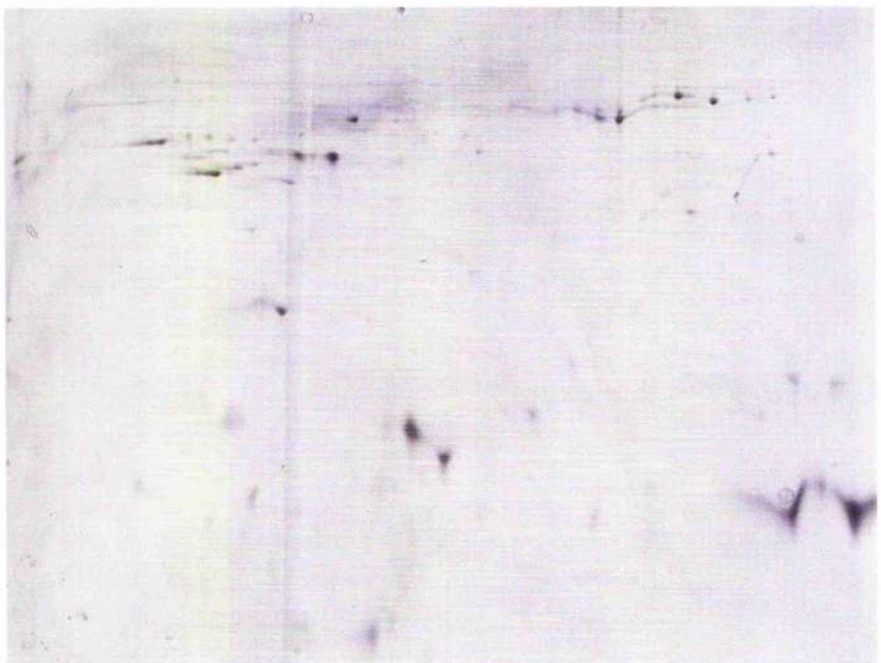


(b) On the other hand the pH3-10NL IEF strip results in good focusing but the proteins are less separated making it more difficult to distinguish individual spots.

Figure 2.9. Two 24cm, pH4-7, colloidal Coomassie Blue stained 2D-E gels run to assess homogenisation methods (a) using cryogenically ground tissue (b) using manually homogenised tissue.



(a) cryogenically ground tissue. This gel has limited protein appearing on it because of the unavoidable dilution of the sample. However, using the cryogenic grinder has extracted more protein than the conventional homogenisation method, see below.



(b) Conventional sample preparation method. This gel is less focused and has poorer extraction of proteins than the gel run with cryogenically ground tissue.

2.3.9 Using a cryogenic grinder

Despite great improvements in gel quality following DNAse/RNAse treatment and optimising protein concentration and pH ranges, there remained the possibility that the tissue was not being adequately homogenised so the use of a cryogenic grinder was investigated.

Samples were processed by the cryogenic grinder as described in materials and methods and the resulting homogenate analysed by 2D-E. Sample 1 contained ~45mg wet weight equine autonomic ganglia that was cryogenically ground and rinsed off the grinder with 200µl lysis buffer. However, there was insufficient liquid to remove all the pulped tissue so water was subsequently used, meaning the final sample solution also contained 1ml of HPLC grade H_2O. Sample 2 contained ~45mg of manually homogenised equine autonomic ganglia, as in previous experiments, in 200µl lysis buffer. 1ml of H_2O was also added to sample 2 to keep the protein concentrations equal. The samples were separated using 2D-E, stained and scanned.

Both gels showed inadequate protein, presumably due to the excessive dilution of the samples. However, it was still possible to see more focusing and less unsolubilised material on the gel containing cryogenically ground sample. To confirm this, the gels were re-stained with colloidal Coomassie blue, a more sensitive stain to aid visualisation, and therefore, analysis (Figure 2.9).

However, due to the need for excessive dilution of samples during collection of rendered sample, it was decided that the manual homogenisation method would be more suitable for the purpose of this study.

2.4 Discussion

There has been no published work covering the proteomic analysis of equine neural tissue. This combined with the fibrous and lipid properties of autonomic ganglia made the homogenisation and extraction of proteins challenging. Thus, a protocol for sample preparation and optimal running conditions for equine cranial cervical ganglia for 2D-E had to be developed. From the comparative experiments conducted throughout this chapter, an optimal protocol for 2D-E was developed for equine cranial cervical ganglia.

Following the protein assay (section 2.3.3) and gels comparing the different sample sizes loaded onto them (section 2.3.8), a section of equine autonomic ganglia weighing approximately 45mg was found to be the optimal weight to allow 900µg of protein to be run on the 24cm 2D-E gel. This also agrees with the protocol supplied by Amersham Pharmacia Biotech, which suggests a maximum of 1mg of protein should be loaded onto 24cm 2D-E gel strips. This optimal loading amount allowed for the greatest intensity of spots with minimum streaking.

The outer connective tissue sheath of the ganglia section was removed to prevent inadvertent contamination from blood and serum picked up during post mortem and surgical collection of the equine autonomic ganglia. Jenkins and colleagues (2002) also considered that contamination may present a problem and consequently washed all hippocampi in cold isotonic saline before preparation for 2D-E analysis. The section of ganglia was then manually homogenised in an eppendorf tube containing 200µl of lysis buffer, using a scalpel knife, interspersed with 30s periods of vortexing until an almost clear pulp resulted. There was nearly always some fibrous material remaining in

suspension due to the fibrous nature of autonomic ganglia. Therefore, another method of homogenisation was investigated (e.g. using a cryogenic grinder, section 2.3.10) but the simplicity of manual homogenisation with the scalpel knife, and complications with collection of homogenised sample from the grinder, meant the original manual method was favoured. If this project were to be followed up, it would be worth investigating the value of a Dounce homogeniser, which was favoured by Geschwind and colleagues (1996) and Preobrazhenzky (1992).

To aid the solubilisation of protein from the ganglia, the homogenised suspension was frozen in liquid nitrogen, thawed on a heating block and then vortexed, this procedure was repeated three times. Geschwind and colleagues (1996) used a similar freeze-thaw method in their sample preparation, during proteomic analysis of rat spinal cord. This freeze-thaw procedure was tried with five repetitions but little improvement was seen after three repetitions. The resultant solution was centrifuged to separate any remaining solid material and the supernatant was collected. To prevent interference with the absorption and separation of proteins into the first dimensional IPG strip, the supernatant was also then centrifuged.

For optimal focusing of proteins in the first dimension, DNAse/RNAse was added to the lysis buffer during homogenisation (section 2.3.7). The use of DNAse/RNAse during the sample preparation is also commonly seen in other proteomic studies of neural tissues, for example Frisco *et al* (2001) and Jenkins *et al* (2002). Thus, 150µl of the supernatant, containing DNAse/RNAse, were added to 300µl of rehydration buffer containing DTT and IPG buffer in all subsequent gel preparations. The pH of the IPG buffer should always correspond to the pH of the IPG strip used, thus, IPG buffer pH4-

7 was always used since it was concluded that an IPG first dimension gel strip of pH4-7 produced optimal separation of proteins on the 2D gel (2.3.9). Therefore, 450µl of a solution containing sample and rehydration buffer, with the necessary additives, was pipetted into the 24cm strip holder and the strip laid on top followed by dry-strip cover solution and the holder lid. The strips were left for approximately 24 h on the IPGphor to rehydrate (10 h) and followed by IEF at 8000V until a minimum of 70 000Vh had been reached. Following IEF, the strips were first rinsed in equilibration tubes containing equilibration buffer with DTT added to saturate the strips with SDS, creating an overall negative charge in the proteins. This eliminates the effect of the charges of the proteins during separation by their molecular weight, and helps the gel front to run evenly. A second equilibration step was performed with equilibration buffer containing IAA. This step allows the gel to be compatible with the Ettan Spot-picker Robot and prevents the proteins from re-oxidising and minimises the reactions of cysteine residues (Cohen, 2002). The strips were briefly rinsed in cathode buffer to remove oils before being applied to the top of the 24cm pre-cast gel and sealed in place with a 0.5% agarose sealing solution. The gels were then run in an Ettan Dalt electrophoresis tank, using the buffer kit system, for approximately 22 h or until the gel front reached the bottom of the gel.

The gels were stained with colloidal coomassie blue (see section 2.2.7) for improved sensitivity and simplicity of staining procedure and scanning of the gel. Coomassie blue was a simple staining procedure; however, it was not nearly as sensitive as colloidal coomassie blue and would not pick up on some of the less concentrated proteins on the gels. Sypro stains were very sensitive, but they are expensive and require the use of a fluorescence scanner, such as the Typhoon scanner, which is time consuming. Silver

spectrometry and, therefore, was not a suitable method if further analysis of the gel was needed. The protocol for silver staining is also very time consuming and quite complex, with many steps. Therefore, colloidal coomassie blue, which is a sensitive stain, compatible with the light scanner, spot-picker and MS, and had a simple procedure, was the most efficient stain used.

By using the established procedure described above, the best and most reproducible gels were obtained: optimal focusing of protein spots, minimisation of streaking, and high resolution of protein content allowing for further analysis of the gel.

Chapter Three: Evaluating natural proteome variations and assessment of MALDI-MS for the characterisation of equine proteins

3.1 Introduction

3.1.1 Equine genome variation

Genetic variation occurs between different individuals and breeds of horse. Since genes encode for specific proteins, it is also likely there will also be a considerable variation in the protein expression between such horses and groups of horses. Blood typing and DNA typing have been used to evaluate intra- and inter-breed genetic diversity in horse breeds (Kelly *et al*, 2002). Kelly and colleagues used variations in blood groups, biochemical polymorphisms and microsatellite loci to characterise Uruguayan Creole horses. They also investigated the phylogenic relationship among Creole breeds, their ancestor breeds and other American horse breeds. Data from this study provided evidence for considerable genetic variation within Uruguayan Creole horses and of a distinctive breed profile. This suggests it could be possible that there is a significant differentiation in protein expression between all horses of the same breeds, for example, if they are of different sexes or ages. Even horses of the same breed, sex and age can display differences in gene maps – if they are different colours. For example, the colour locus, referred to as C in the horse, is linked to microsatellite markers on horse chromosome 21. Alleles of the horse colour locus (C) is thought to control the colour dilutions seen in the body colour of horses (Locke *et al*, 2001). Such genetic variations could make the identification of a specific protein that is disease related difficult, as there could be an extensive variation between the proteomes of horses, even prior to disease related changes.

Another potential variable worth considering is the change in protein expression along the length of each ganglion. The cellular components along the length of the ganglion could vary with position. For example, at the ends of the ganglion are channels of axons and dendrites, whilst neural cell bodies will be located centrally in the ganglion (fig 1.3). These are all factors that had to be recognised throughout this project as there is little that can be done to avoid such variations, especially given the low sample numbers available. This chapter was aimed at investigating these unavoidable genetic variations and how they affect comparative experiments with regards to diseased and non-diseased tissue samples.

3.1.2 Using MALDI-MS for the characterisation of equine proteins

Little work has been carried out on the equine genome and hence there is limited information contained within the protein fingerprint databases (e.g. NCBI). Therefore, the efficiency of MALDI-MS will be limited with respect to identifying equine autonomic ganglia proteins. The only proteins identified, as equine proteins were those that had previously been characterised and their information stored. However, other proteins may well be recognised but matched to those belonging to a different species. Provided there is homology between the species and *Equus caballus* the match could be considered successful.

3.1.3 Aims

To investigate the extent of variation seen on 2D-E gels, between:

- Samples taken from different locations along the length of the same equine autonomic ganglion
- Samples taken from different ganglia from the same horse
- Samples taken from ganglia from different horses of the same sex

- Samples from horses of different sex

- To investigate the efficiency of MALDI-MS for the characterisation of equine autonomic ganglia proteins.

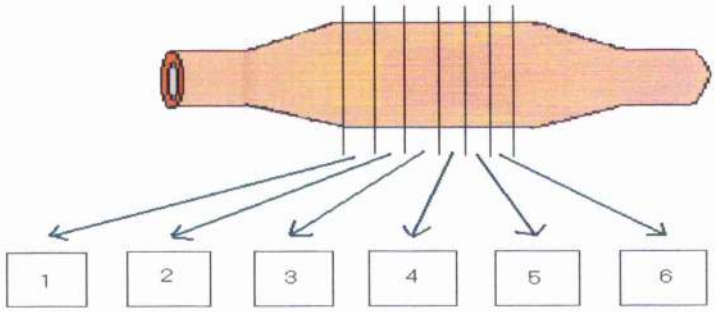
3.2 Materials and methods

3.2.1 Determination of variations in ganglionic protein expression

Sets of 2D-E gels were run, as previously described, to analyse the differences in protein expression between:

- Different sections taken from along the length of the same ganglion (Fig 3.1)
- Different ganglia from the same horse
- Ganglia from different horses of the same sex
- Ganglia from horses of different sexes
- Ganglia from horses with AGS and control horses

Figure 3.1 Sections of ganglia cut to determine whether there is any significant variation in the protein expression along the length of the ganglia.



3.2.2 Evaluating the effectiveness of 2D-E for comparison of EGS affected ganglia and control ganglia

2D-E gels were run, using the optimal method developed in Chapter Two, to compare the protein expression of EGS affected ganglia with that of control ganglia.

3.2.3 Evaluating MALDI-MS for the characterisation of equine proteins

A 2D-E gel was run and a picklist made using the ImageMaster analysis software (Amersham Pharmacia Biotech). The spots picked and an in-gel trypsin digest was carried out by the Ettan Spot Picker Robot. The peptide fragments were then presented to the PerSeptive Biosystems Voyager DE-STR MALDI-MS. The acquired peptide mass fingerprint spectra were compared to known peptide spectra contained within databases to identify unknown proteins.

3.3 Results

3.3.1 Comparison of protein expression along the length of ganglia

To determine the extent of variation of protein expression along the length of the ganglia, a series of sections were cut from a ganglion as shown in Figure 3.1. The proteins in these sections were then separated using 2D-E and the gels analysed in order to identify any major differences in the protein expression.

Six sections of the control autonomic ganglion 01-681 were sliced from one end of the ganglion and labelled 1-6 respectively (fig 3.1). The samples were run simultaneously through 2D-E, as described in the previous chapter, to separate the proteins. On completion of electrophoresis, the gels were stained in Colloidal Coomassie brilliant blue over night and scanned using the Amersham Pharmacia scanner and Labscan software.

From the gels in figure 3.2, it could be seen that there was little significant difference in the protein expression of samples taken from each position along the length of one ganglion. Any differences that were visible were perhaps due to minor differences in sample sizes causing some material to remain unfocused. Also, there were some wavy sections in the gels, through uneven running, that may have been caused by the electrophoresis buffers in the tank not circulating adequately.

3.3.2 Comparing the protein expression of ganglia from the same horse and from different horses.

The variation in protein expression between the two (left and right) cranial cervical ganglia from the same horse and subsequently two autonomic ganglia each from different horses

were determined. Sections of ganglia were cut from both ccgs from the horse 02-56 and one section from ccg 01-681. The proteins in the samples were then separated by 2D-E. The gels were stained in Colloidal Coomassie brilliant blue and scanned using the Amersham Pharmacia scanner and Labscan software.

Figure 3.3 shows gel images of samples from ganglia from the same horse and samples from ganglia from different horses. By careful comparison of the gel images from samples 1 and 2, it was evident that there is also little difference in the protein expression from samples taken from the two ganglia from the same horse. Comparison of sample 2 (and/or 1) with sample 3 highlights limited variation in the protein content of ganglia from different horses.

3.3.3 Sex differences in equine autonomic ganglia proteome

The differences in gene expression between males and females are well known on a genetic level (Kelly *et al*, 2002). These differences will, in turn, influence the protein expression. It was, therefore, important to establish the differences in protein expression in equine autonomic ganglia between the sexes, thus aiding the identification of EGS related proteins by eliminating such differences.

Two sections of autonomic ganglia were cut from the control horses with identity 02-182 (female) and 02-889 (male) and prepared, and the proteins separated by 2D-E. The gels were stained with Colloidal Coomassie brilliant blue and scanned using the Amersham Pharmacia scanner and Labscan software.

The gels were analysed by subjective visual comparison and differences were highlighted (fig. 3.4). The gels in figure 3.4 show there were only a few differences in the protein expression of autonomic ganglia between male and female horses. There are only three or four spots, out of several hundred, that appear more intense on the gel with autonomic ganglia from a female horse than that from a male horse. This could imply that these are more prominent proteins in female horses. None the less, these intensity differences should not affect the comparison of diseased and non-diseased ganglia.

3.3.4 Preliminary comparative of AGS tissue and control tissue

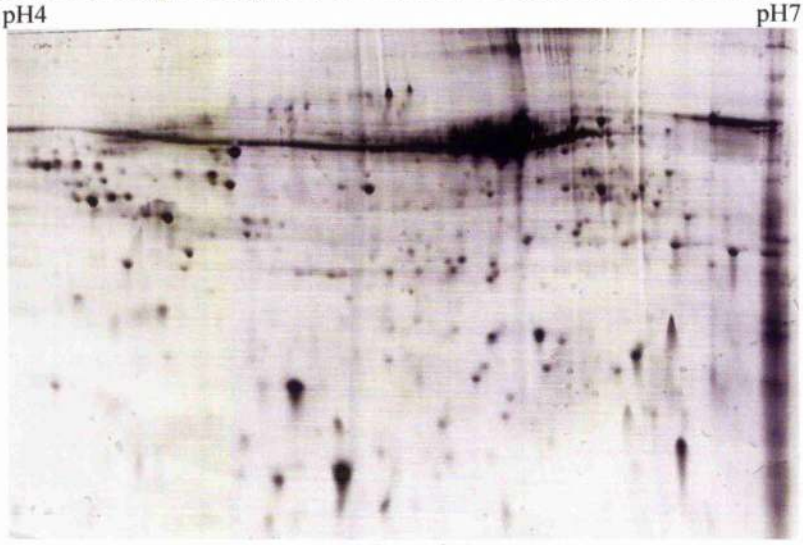
A preliminary comparative investigation between diseased and non-diseased tissue was carried out to give an indication of the protein differences that might be expected and to evaluate the effectiveness of 2D-E analysis for comparing diseased and non-diseased samples.

Two sections were cut from the acute grass sickness (AGS) diseased ccg 02-913 (samples 1 and 2) and two sections from the control ccg 01-681 (samples 3 and 4). The samples were run through 2D-E to separate the proteins. To visualise the gels, they were stained in colloidal Coomassie blue and scanned using the Amersham Pharmacia light scanner and

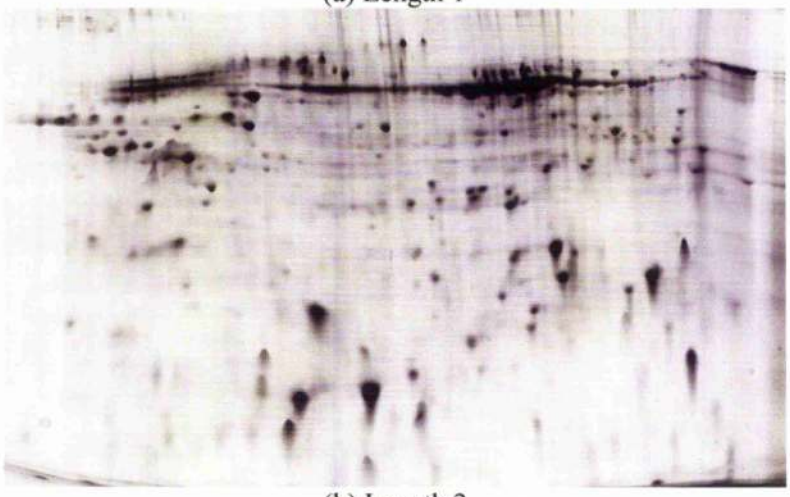
By subjective visual comparison of the gel images (figure 3.5), it was easy to identify some major differences between the diseased and non-diseased tissues. Interestingly, some of the same differences occur between both of the AGS infected samples and their controls, implying that they could be diseased related. These gels confirmed the potential of 2D-E analysis of diseased and non-diseased samples for identifying EGS-related proteins.

Figure 3.2. Analysis of protein expression along the length of the ganglia. Six sections of one ganglion were taken and homogenised, then the proteins separated using 24cm, pH4-7, colloidal coomassie blue stained 2DE gels.

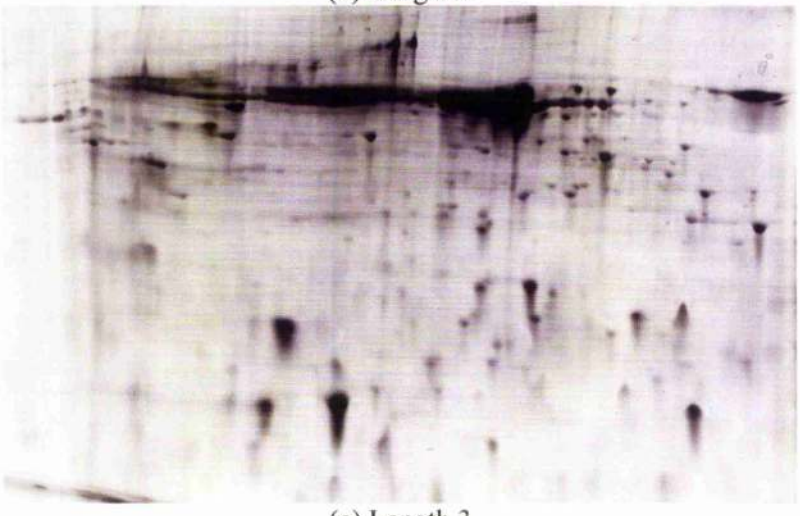
pH4 pH7



(a) Length 1



(b) Length 2

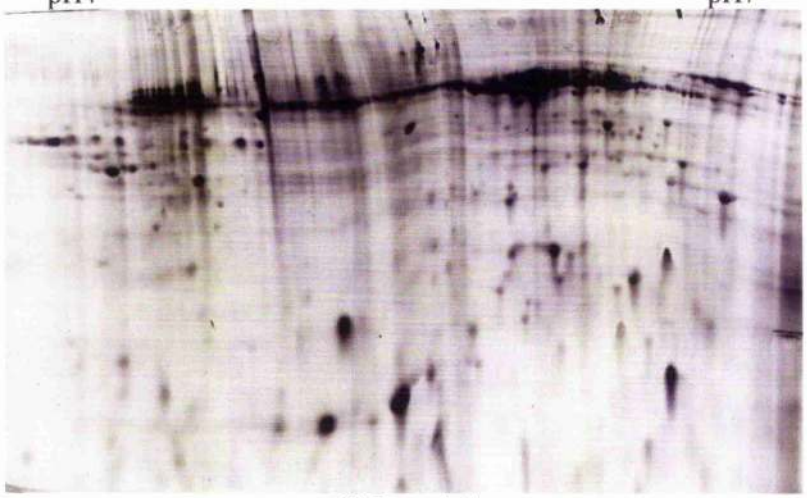


(c) Length 3

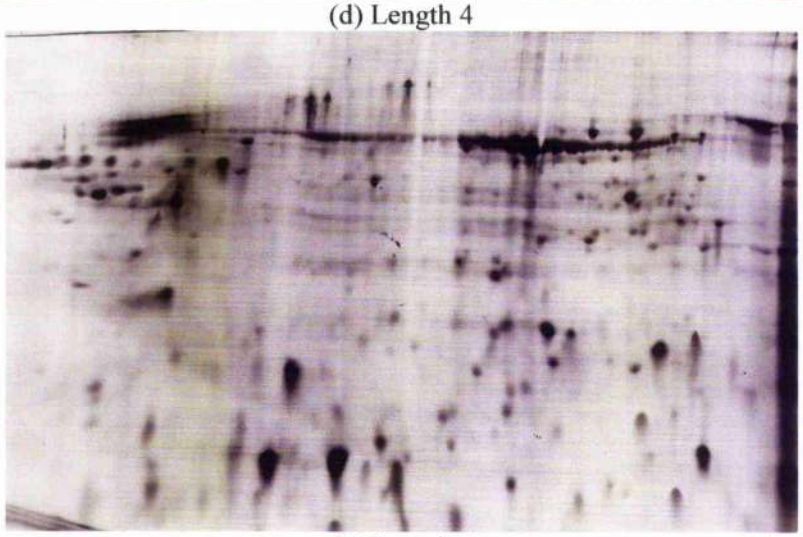
continued...

Figure 3.2. Analysis of protein expression along the length of the ganglion.

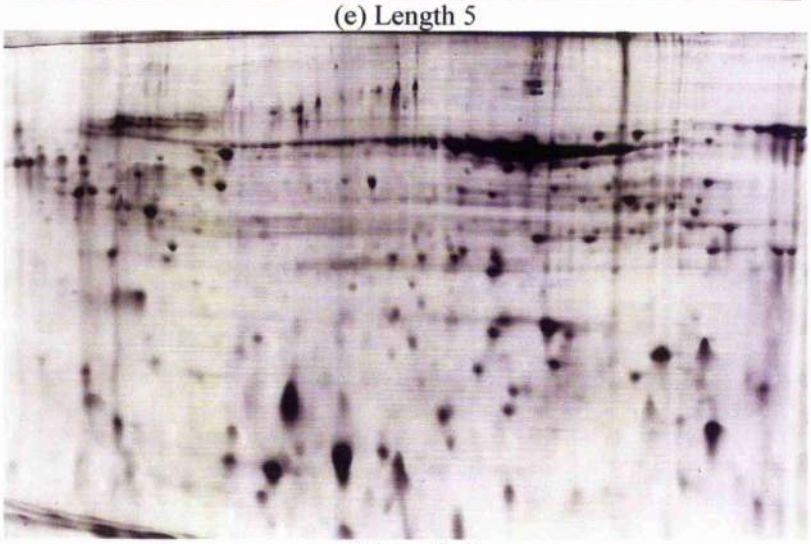
pH4 pH7



(d) Length 4



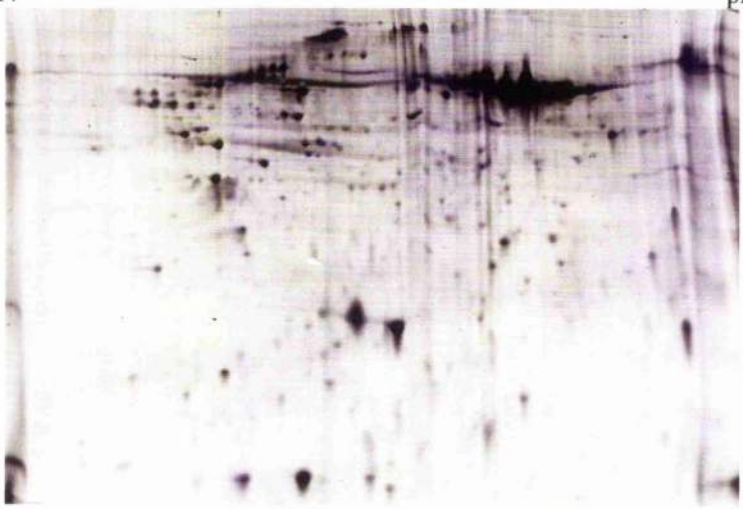
(e) Length 5



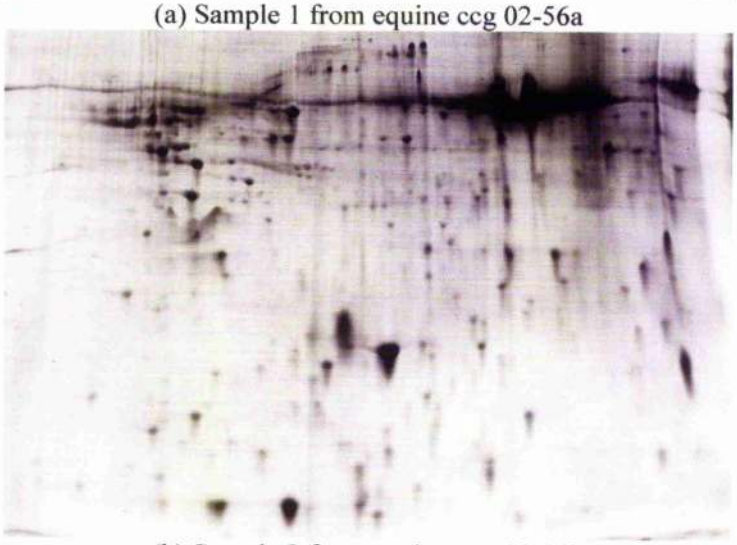
(f) Length 6

Figure 3.3. Analysis of the protein expression in samples from (1) two different ganglia from the same horse (a + b) and (2) ganglia from different horses (a/b + c) using 24cm, pH4-7 colloidal coomassie blue stained 2D-E gels.

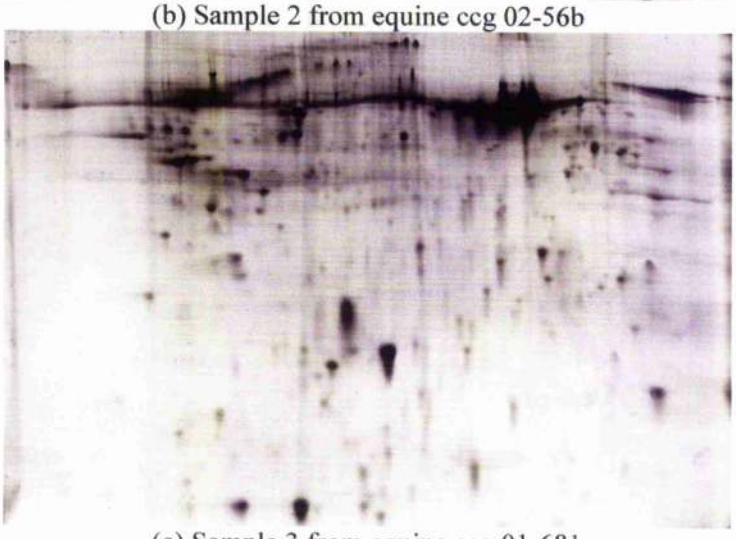
pH4 pH7



(a) Sample 1 from equine ccg 02-56a

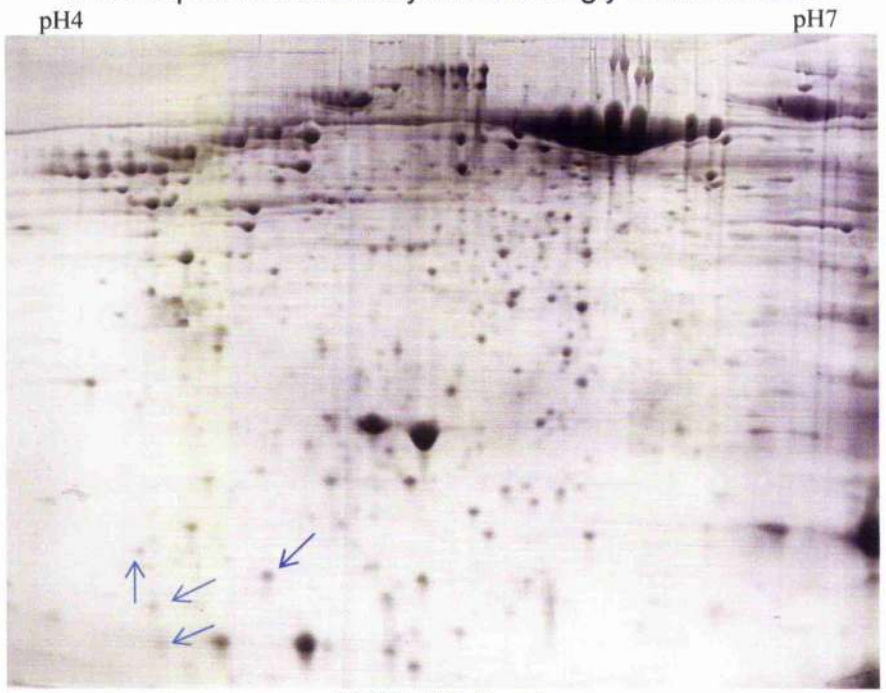


(b) Sample 2 from equine ccg 02-56b

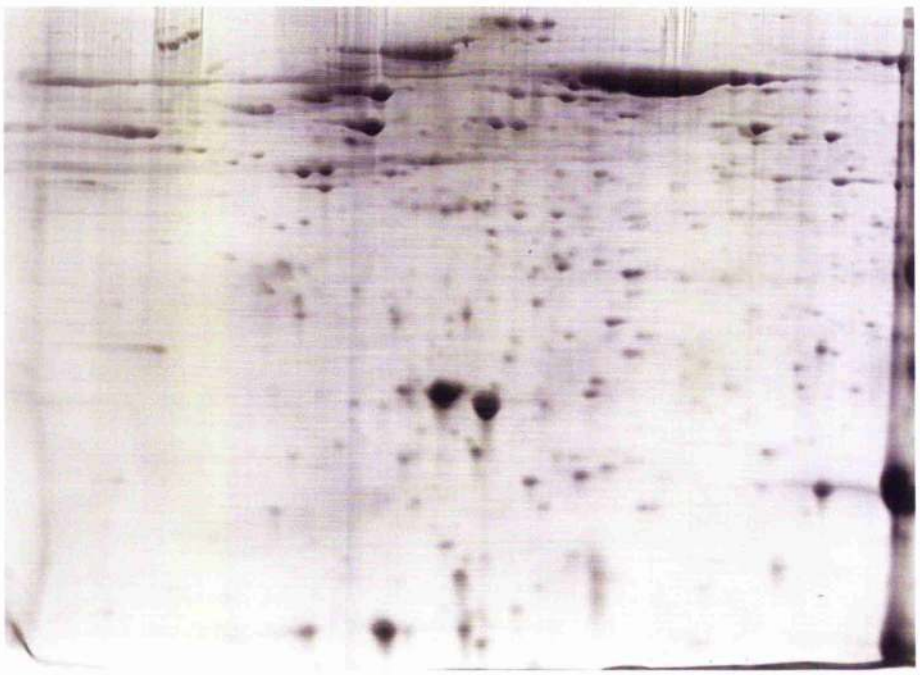


(c) Sample 3 from equine ccg 01-681

Figure 3.4. Analysis of (a) female and (b) male protein expression in equine autonomic ganglia on 24cm, pH4-7, Colloidal Coomassie Blue stained 2D-E gels. The blue arrows indicate protein spots that appear to be expressed relatively more strongly in the female.

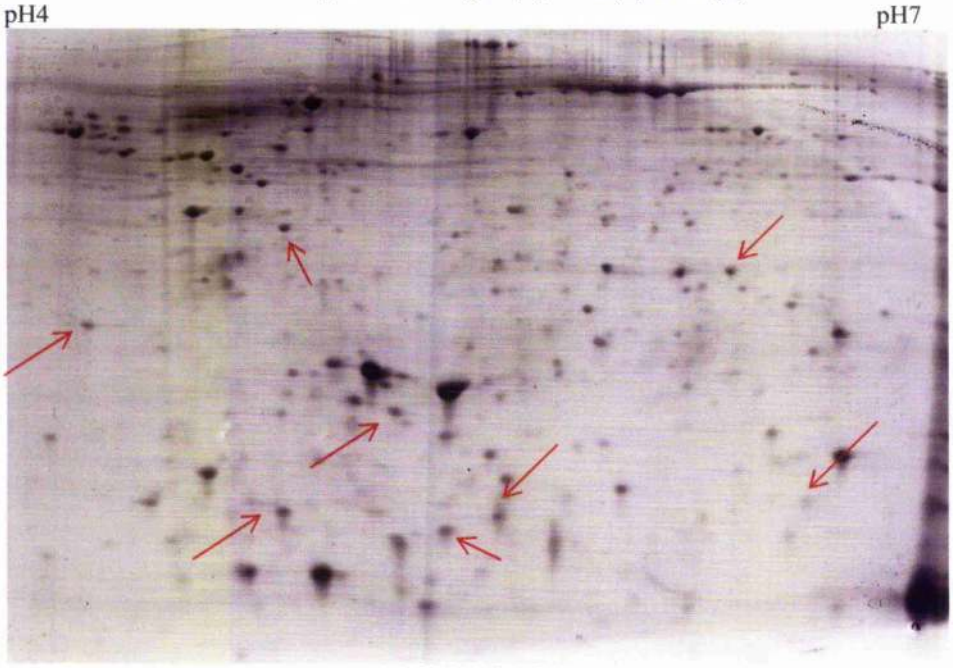


(a) 02-182 female

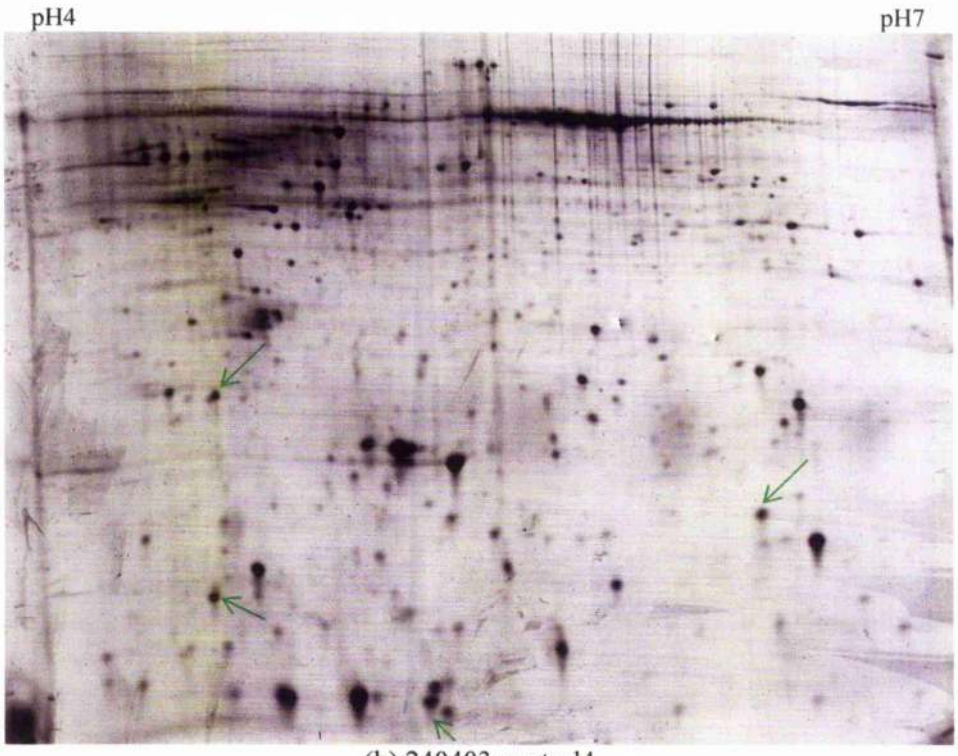


(b) 02-889 male

Figure 3.5. Preliminary analysis of protein expression of samples from AGS (a and c) infected ganglia with control ganglia (b and d) on 24cm, pH4-7, Colloidal Coomassie Blue stained 2D-E gels. Green arrows indicate spots that appear to only occur on or are more strongly expressed on control sample gels than AGS gels, whilst red arrows indicate spots that appear to only occur on or are more strongly expressed on AGS sample gels than control gels. Gel (a) was compared with gel (b) and (c) with (d).



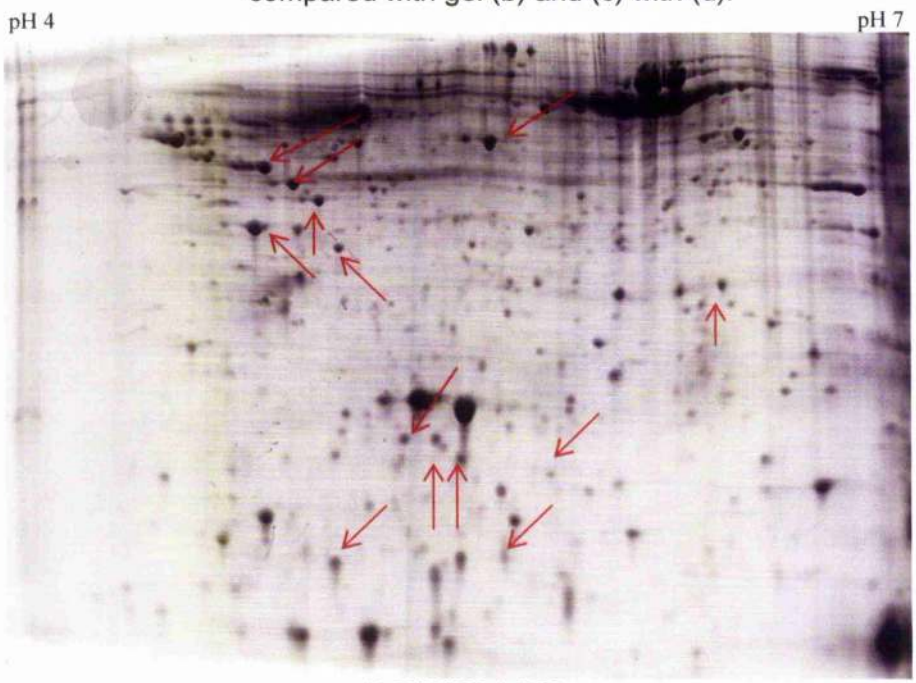
(a) 240403 AGS1



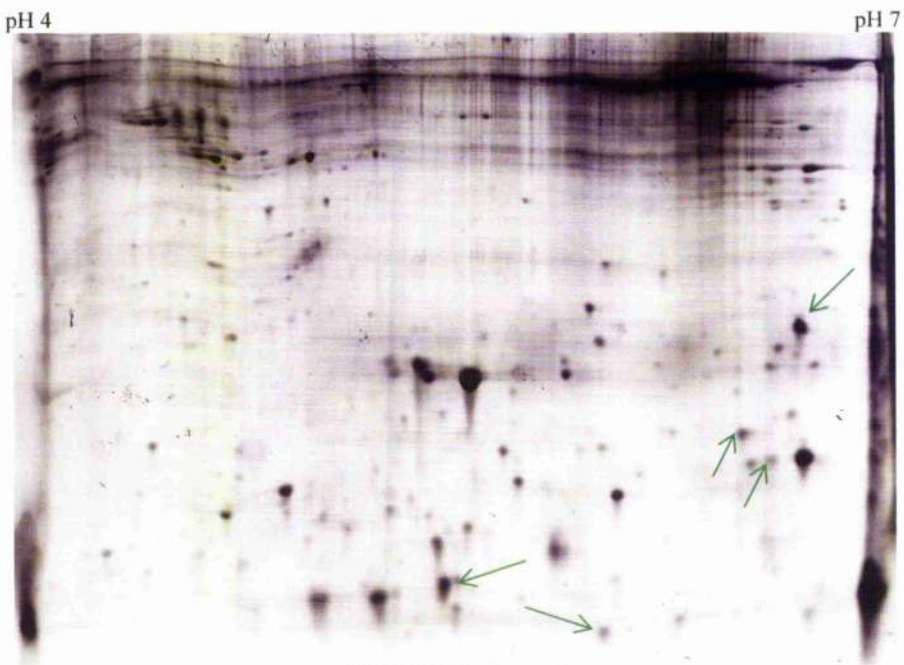
(b) 240403 control4

continued...

Figure 3.5. Preliminary analysis of protein expression of samples from AGS (a and c) infected ganglia with control ganglia (b and d) on 24cm, pH4-7, Colloidal Coomassie Blue stained 2D-E gels. Green arrows indicate spots that appear to only occur on or are more strongly expressed on control sample gels than AGS gels, whilst red arrows indicate spots that appear to only occur on or are more strongly expressed on AGS sample gels than control gels. Gel (a) was compared with gel (b) and (c) with (d).

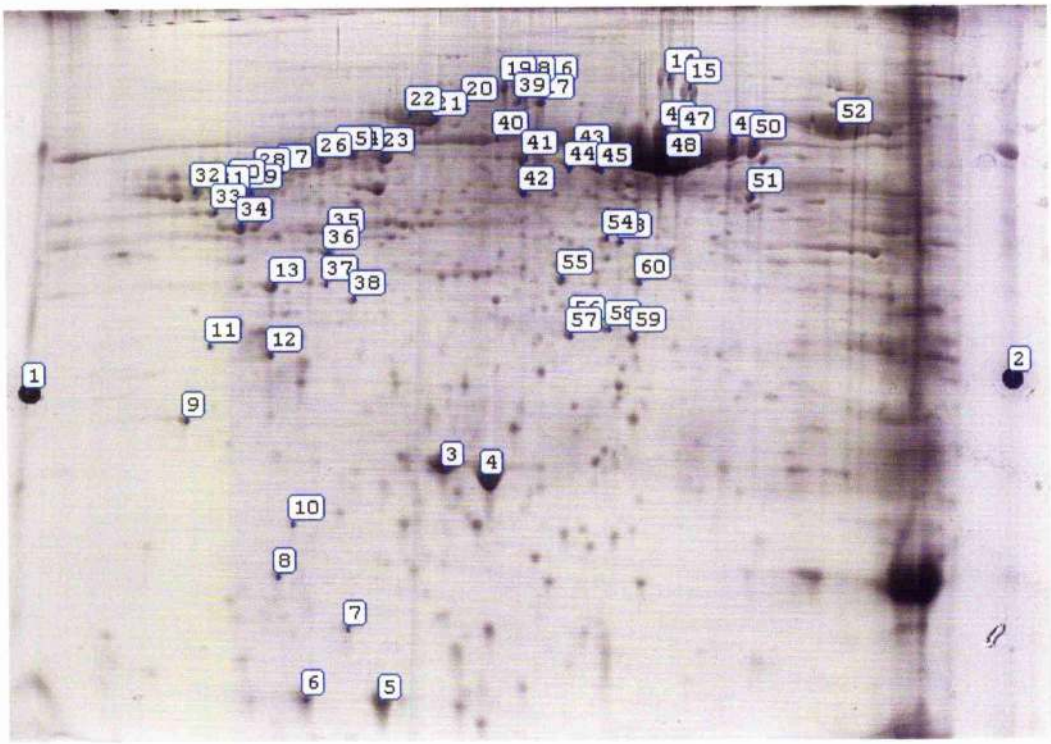


(c) 240403 AGS2



(d) 240403 control3

Figure 3.6. A 24cm, pH4-7, 2D-E gel of control autonomic ganglia showing the spots numbered and used in the pick-list for the spot picker robot.



3.3.5 Evaluating MALDI-MS for the characterisation of equine proteins

The 2D-E gel of control equine autonomic ganglia was used to create a picklist of protein spots (figure 3.6). The spots chosen for the picklist were representative of the most intense and focused spots on the gel. The picklist was transferred to the Ettan Spot Picker Robot, which then automatically picked the spots and performed an in-gel trypsin digest in preparation for MALDI-MS. The resultant peptides were fragmented and mass analysed by the PerSeptive BioSystems Voyager DE-STR MALDI-MS, from which peptide mass finger print spectra were obtained. The BioSystems Voyager Data Explorer software was used alongside the Mascot data search engine to search the NCBI database for protein matches. Table 3.1 shows the proteins that were identified with a significant ($p < 0.05$) hit.

Table 3.1 Equine autonomic ganglia proteins identified using MALDI-MS.
*For a match to be a significant hit it must have a score than the required score. The number of peptides matched, the pI and the sequence coverage (data not shown) all contribute to the score of each hit.

spot no.	protein	GenInfo Identifier	species	score (required*)	peptides matched	mass (Da)	pI
1	N/A	N/A	N/A	N/A	N/A	N/A	
2	N/A	N/A	N/A	N/A	N/A	N/A	
3	neurone cytoplasmic protein/	gi 18202924	*Equus caballus*	53(57)	6	25245	8.39
	spinesin		*Mus musculus*	61(57)	8	34702	5.14
4							
5	DNA-dependant kinase...	gi 17646641	*Equus caballus*	50(57)	10	473807	6.60
6							
7							
8							
9	RNAse	gi 229576	*Camelus sp*	34(57)	3	14183	6.96
10	Tumour protein	gi 4507669	*Homo sapiens*	59(57)	5	19697	4.84
11							
12	tropomyosin beta chain	gi 71604	*Equus caballus*	83(67)	11	28488	4.65
13							
14	serum albumin precursor	gi543794	*Equus caballus*	83(74)	24	70550	5.95
15	serum albumin precursor	gi543794	*Equus caballus*	161(74)	31	70550	5.95
16	peripherin	gi 2253159	*Mus musculus*	94(74)	30	52769	5.36
17	glial fibrillary protein	gi 4503979	*Homo sapiens*	93(74)	31	49907	5.42
18	peripherin	gi 2253159	*Rattus norvegicus*	114(74)	29	54349	5.37
19	peripherin + ser alb precu	gi 2253159+gi543794	*H. sapiens +Eq. c*	88(67)+150(67)	17+22	53732+70550	5.37+5.95
20	serum albumin precursor	gi543794	*Equus caballus*	101(67)	19	70550	5.95
21							
22	vimentin	gi 1403310	*Equus caballus*	98(67)	25	53698	
23	P19 lipocalin		*Equus caballus*	57(57)	4	21019	9.87
24							
25	vimentin	gi 14389299	*Rattus norvegicus*	161(74)	25	53698	5.06
26	vimentin	gi 860908	*Cricetulus griseus*	171(74)	30	53698	5.06
27	vimentin	gi 860908	*Cricetulus griseus*	215(74)	34	53698	5.06
28	vimentin	gi 860908	*Cricetulus griseus*	72(74)	18	53698	5.06

29							
30	vimentin	gi 860908	*Cricetulus griseus*	70(74)	8	44611	4.75
31							
32							
33							
34	Alpha-1antitrypsin	gi 3982489	*Equus caballus*	71(67)	9	47141	5.23
35	Beta tubulin	gi 3745822	*Sus scrofa*	68(57)	7	43819	5.17
36	(77)Gamma enolase	gi 5803011	*Homo sapiens*	101 (67)	16	47581	4.91
37	(77)Beta tubulin	gi 3745822	*Sus scrofa*	69(57)	9	48319	5.17
38	Phosphoglycerate kinase	gi 129921	*Equus caballus*	43(57)	10	44865	8.64
39	GFAP+peripherin	gi 4503979+gi 6981416	*H.sapiens+R.norv*	124(67)+78(67)	23+17	49907+53631	5.42+5.37
40	Ser alb prec + beta tubulin	gi543794+gi7106439	*Eq. c + Bos taurus*	128(67)+88(67)	20+18	70550+50096	5.95+4.78
41	Heat shock protein 70	gi 27805925	*Bos taurus*	156(57)	25	71432	5.49
42		gi 790781	*Equus caballus*	65(57)	5	8099	7.88
43	Serum albumin precursor	gi543794	*Equus caballus*	125(67)	20	70550	5.95
44	Heat shock protein 70	gi 6063529	*Capra hircus*	108(57)	20	70109	5.51
45	Heat shock protein 70	gi 2495339	*Bos taurus*	159(57)	23	70470	5.68
46	Serum albumin precursor	gi543794	*Equus caballus*	224(67)	28	70550	5.95
47	Serum albumin precursor	gi543794	*Equus caballus*	202(67)	27	70550	5.95
48							
49	serum albumin precursor	gi543794	*Equus caballus*	203(67)	27	70550	5.95
50							
51	Serum albumin precursor/	gi543794	*Equus caballus*	50(57)	9	70550	9.95
	Protein disulphide isomerase	gi 1083063	bovine	77(57)	13	55281	6.51
52	Serotransferrin (transferrin)	gi 136190	*Equus caballus*	325(67)	39	80268	6.83
53	RNAse						
54							
55	Elongation protein 4	gi 26329633	*Mus musculus*	72(67)	11	46809	8.98
56	Cysteine-rich secretory 3/	gi 3023562	*Equus caballus*	42(57)	5	28202	5.38
	Eukaryotic translation factor	gi 4503513	*Mus musculus*	68(67)	9	36837	7.42
57	transferrin	gi 136190	*Equus caballus*	45(57)	5	80268	

3.4 Discussion

From the experiments described in this chapter, it can be concluded that any differences in the protein expression between sections of equine autonomic ganglia whether taken from (1) different locations within the same ganglia, (2) different ganglia from the same horse, (3) ganglia from different horses of the same sex or (4) ganglia from horses of different sexes, would not interfere with the identification of EGS-related proteins. The differences in protein expression, in the latter two cases especially, is perhaps a surprising result given that the genetic variation between two horses could be quite substantial especially if they are of different breeds (Kelly *et al*, 2002).

All the gels produced in this chapter showed good consistency regarding the pattern of protein expression. Having compared the patterning throughout several sets of gels it was concluded that later comparative analyses of EGS-affected autonomic ganglia and control autonomic ganglia would be practical.

The preliminary comparative investigation between AGS affected autonomic ganglia and control ganglia provided some useful and very interesting results. It appears from this series of experiments that there may well be several distinct protein differences between ganglia taken from diseased horses and from those from control horses. Further experiments were performed (chapter 4) to determine whether these differences were disease related and if they occurred in all diseased samples.

Following MALDI-MS of protein spots and the analysis of the resulting peptide mass fingerprint spectra by a Mascot database-search of the NCBI database, several proteins were

characterised. Thirty-four out of sixty equine proteins gave significant hits to various proteins such as serum albumin precursor, transferrin, tropomyosin beta chain, vimentin, lipocalin and alpha-1-antitrypsin (Table 3.1). A further seven proteins were matched with hits that were nearly significant. The matches to serum albumin precursor and transferrin are consistent with previous studies of the protein expression of equine autonomic ganglia (Griffiths *et al*, 1994b), which suggest serum proteins are regularly found within ganglia. Vimentin is a neural filament protein and is therefore also an unsurprising match (embl harvester, see Appendix 2). Tropomyosin is most commonly found in muscle tissue; however, it also plays an active role in the transport and assembly of microtubule structures (embl harvester, see Appendix 2).

There were a number of proteins that matched proteins from a variety of other species, ranging from *Homo sapiens* to *Mus musculus* (mouse). These proteins matches are unlikely to represent real matches and demonstrate the limitation of using MALDI-MS data to identify proteins from an organism for which there is limited genome information. Whilst the MALDI-MS analyses in this chapter has enabled a preliminary proteome map of equine autonomic ganglia to be constructed, a full proteome map will have to await the more complete genome sequence of the horse. The limitations of MALDI-MS analysis when using a small genome database can be partially overcome by the use of tandem MS. This method of mass spectrometry generates individual peptide fragmentation data rather than polypeptide mass fingerprints and hence is more useful for identifying proteins via analysis of common peptides conserved between homologous proteins.

In subsequent chapters both MALDI-MS and tandem MS analyses were therefore used for identification of differentially expressed protein samples.

At this stage it is not practical to attempt a complete proteome map of equine tissues. Such analyses will have to await the publication of a genome sequence for the horse.

Chapter Four: Comparison of AGS samples and control samples: Identifying EGS related proteins

4.1 Introduction

The main aim of the work carried out in this chapter was to produce 2D-gels of EGS-affected ganglia and compare them with gels of control samples. The control ganglia came from horses that had died or were euthanased due to causes completely unrelated to EGS, e.g. euthanasia due to extreme lameness. Then the gels were analysed and any consistent differences in the protein content between gels of AGS and control samples were highlighted. Once the protein spots thought to be EGS-related had been isolated and picked using the spot picker robot, they were identified using mass spectrometry and by searching the NCBI database for protein matches. By identifying these EGS-related proteins, it was hoped that clues to the aetiology of the disease might be found.

The proteins were first characterised using MALDI-MS, as described in the previous chapter. However, the degree of efficiency of MALDI-MS was limited due to the lack of current information regarding the equine genome (Chapter 3). Thus tandem mass spectrometry was used in addition to MALDI-MS. Tandem Mass Spectrometry induces further fragmentation of peptides and allows mass analysis of the fragmented ions. Fragmentation is achieved by inducing ion/molecule collisions by a process known as collision-induced dissociation (CID). CID is accomplished by selecting an ion of interest with a mass analyser and inducing that ion into a collision cell (Lesney, 2001). The selected ion then collides with a collision gas (e.g. argon or helium) resulting in fragmentation. The fragments are then analysed to obtain a fragment ion spectrum

(Lesney, 2001). Tandem mass analysis is mainly used to obtain further information about a compounds sequence.

4.1.2 Aims

- To analyse 2D-E gels of EGS-affected autonomic ganglia alongside gels of control autonomic ganglia.
- To identify and extract any proteins that appear to be EGS-related.
- To characterise any EGS-related proteins by mass spectrometry and investigate their possible functional association with EGS.

4.2 Materials and methods

4.2.1 2D-E gel comparison of diseased and non-diseased samples

2D-E gels were run, as previously described, of equine autonomic ganglia to compare EGS-affected samples with control samples (Table 4.1). Spots that appeared most consistently up- or down-regulated were highlighted. A picklist for the Ettan Spotpicker Robot (Amersham Pharmacia Biotech) was created using the ImageMaster® 2DElite software (Amersham Pharmacia Biotech).

Table 4.1 Summary of 2D-E gel-to-gel comparisons relating to each set of gels run.

Group Comparisons	EGS Case Number	Control Case Number
1	03-345	02-889
	03-359	
	03-425	
2	02-248	01-9133
	02-913	
3	02-352	02-56
	02-376	
4	02-302	02-182
	02-349	
5	03-392	02-889
	03-409	
	03-408	
	03-425	
	03-445	

4.2.2 Identification of proteins

Fourteen spots from the gel containing sample from the AGS autonomic ganglia 03-425, chosen for its optimal spot focusing and intensity, (Fig 4.2), were picked and in-gel trypsin-digested using the Ettan Spotpicker Robot (Amersham Pharmacia Biotech). The spots were presented to the PerSeptive BioSystems Voyager DE-STR MALDI-MS. The obtained peptide mass fingerprint spectra were subsequently compared to spectra already in the protein database (NCBI) using the BioSystems Voyager Data Explorer and Mascot software packages to search for protein matches. The peptide samples of the spots, also obtained from the Spotpicker Robot, were run through the BioSystems Q-STAR tandem MS and the spectra were analysed using the Analyst QS software/Bio Analyst software. Protein matches were searched for using the Mascot database search engine.

4.3. Results

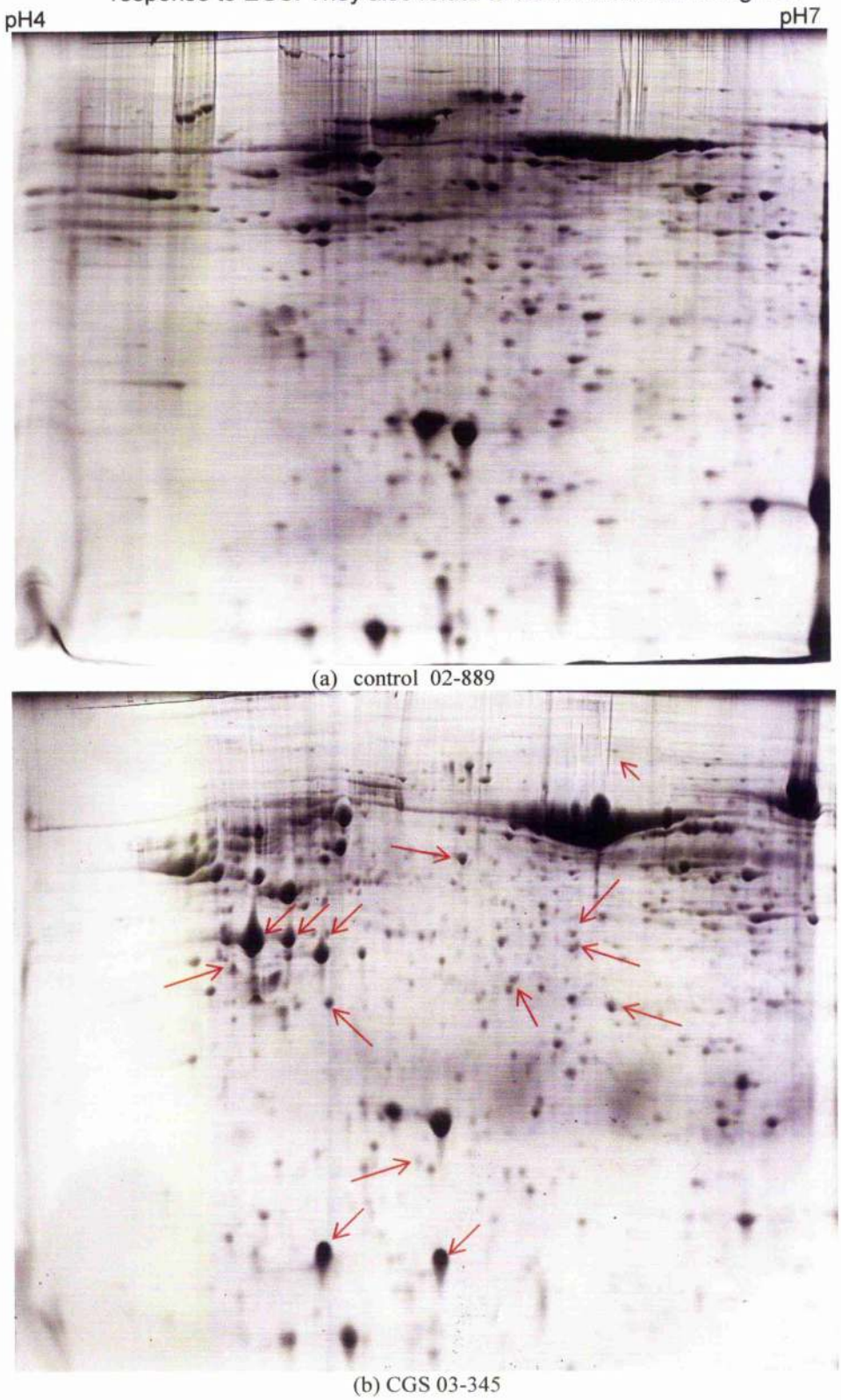
4.3.1 Comparison of AGS sample gels and control sample gels: determination of AGS-related proteins.

The first step in comparing diseased and non-diseased (control) samples was to separate the proteins of all diseased samples on 2D-E gels alongside control samples in order to isolate the protein spots that were most consistently different between diseased and non-diseased ganglia samples. Both chronic grass sickness (CGS) and acute grass sickness (AGS) samples were subjectively analysed and visually compared with controls.

A total of 15 gels were run with sample from EGS affected autonomic ganglia (13 AGS and 2 CGS), as 5 sets of comparative gels. In each set one section of control autonomic ganglia was taken along with several different sections of EGS affected ganglia. Each set of comparative samples was separated using 2D-E using the methods developed in Chapter Two. The gels were then stained in Colloidal Coomassie brilliant blue and scanned using the Amersham Pharmacia light scanner and Labscan software.

Once EGS and control autonomic ganglia gels had been run, each set of gels was compared (Fig 4.1 and Appendix 1; however, it should be noted the gel images included in the text are of poorer resolution to the screen based high-resolution images used during analysis, leaving gel comparisons from figures in the thesis open to error). Throughout the sets there were six consistent absences of protein spots on the majority of control gels compared to the gels run with AGS-affected ganglia. These spots were highlighted throughout the series of sets of comparative gels with arrows (Fig 4.1 and Appendix 1). A further eight spots, also highlighted with arrows, appeared on both EGS gels and control gels but showed consistent up-regulation of their expression on the majority of EGS gels (with the exception of spot 10 on gel (c) Appendix 1, Fig B and spot 13, on gel (a) Fig 4.1 were they are of similar relative intensity to those on comparative gels). Highlighted spots may be relevant to the determination of the disease pathway and were analysed (Fig. 4.2). The two gels of CGS-affected sample showed similar protein expression to those run with AGS sample. Some of the differential proteins on AGS sample gels were more intense on the CGS sample gels.

Figure 4.1. Four 24cm, pH4-7, Colloidal Coomassie blue stained 2D-E gels, analysing CGS, AGS and control autonomic ganglia for differences in protein expression. The red arrows indicate spots that, after a visual analysis of all comparative gels run, most consistently only appear on EGS gels or that most consistently show up -regulation in response to EGS. They also relate to those numbered in Fig 4.2.

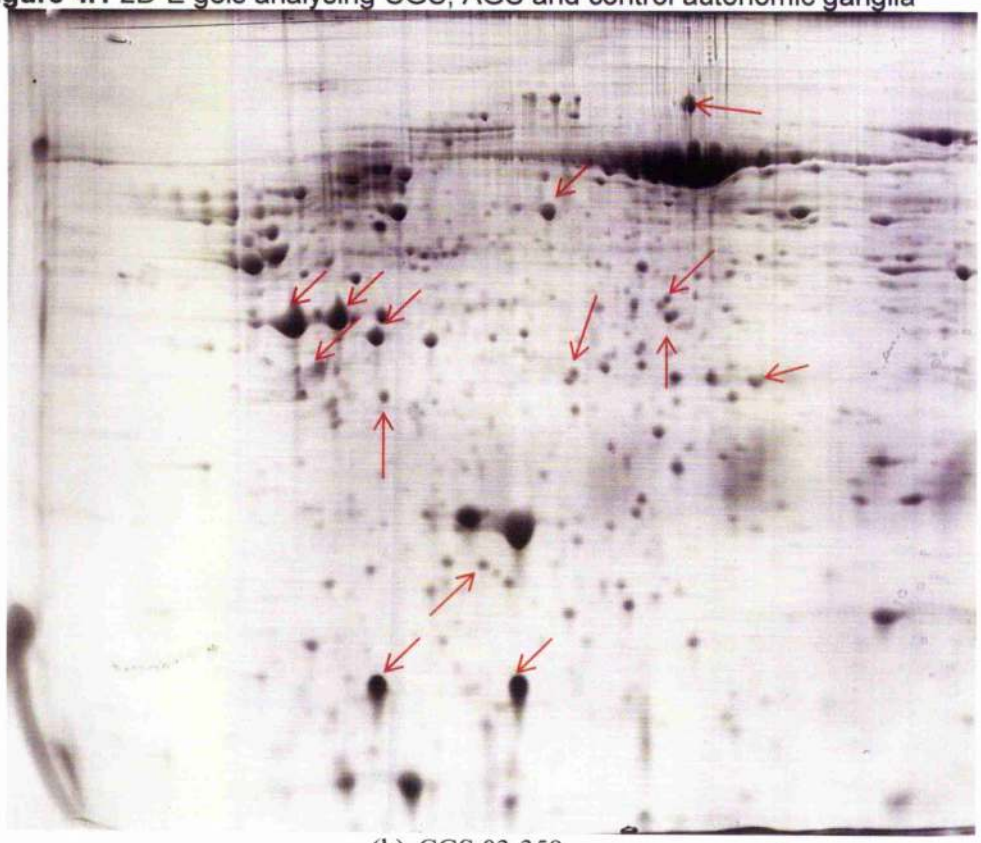


(a) control 02-889

(b) CGS 03-345

Continued…

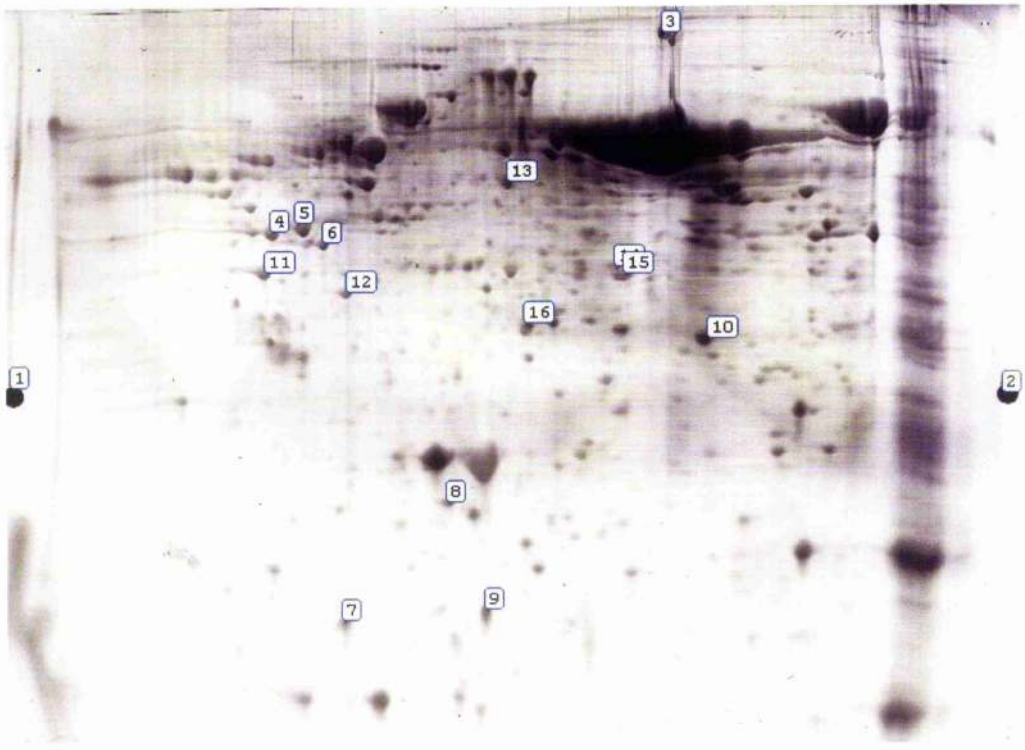
Figure 4.1 2D-E gels analysing CGS, AGS and control autonomic ganglia



(b) CGS 03-359

(d) AGS 03-425

Figure 4.2. A 24cm, pH4-7, Colloidal Coomassie Blue stained 2D-E gel of AGS autonomic ganglia, showing the protein spots that were most consistently up-regulated or most consistently only appearing on EGS gels, after the subjective analysis of all comparative sets of gels run. These spots represent the picklist to be excised by the spot-picker robot and for the MS analysis of proteins thought to be EGS-related.



4.3.2 Identification of proteins by MALDI-MS

The 2D-E gel labelled AGS 03-425 (Fig 4.2) was analysed using the ImageMaster 2D Elite Software and a protein picklist was generated. The fourteen proteins that were differentially expressed in EGS tissue were selected from the gel. The protein picklist was then transferred to the Ettan Spot Picker Robot, which then automatically picked the relevant protein spots, in accordance with the picklist. Self-adhesive markers had been attached to the gel backing prior to scanning to create a link between the electronic image and the actual gel. The Ettan Digester, working alongside the spot picker, automatically tracked samples from spot picking and performed in-gel digestion of proteins, using trypsin and yielded peptide fragments, which were transferred onto a target plate ready for MALDI-ToF analysis.

The target plate, obtained from the spot picker robot, containing the protein spots was inserted into the Biosystems Voyager MALDI-ToF mass spectrometer. Using the computer software, each spot was highlighted, ionised, fired at and then mass analysed. The protein fragmentation patterns were then compared with the patterns predicted for all proteins within a database (e.g. NCBI) and matches were statistically evaluated. Since the presence of Arg and Lys residues are common, trypsin cleavage (specific for Arg and Lys) usually produces a large number of fragments, which in turn offer a high probability of identifying the target protein. However, the success of protein identification by this method relies on the existence of the protein sequence within the database. The following proteins were identified corresponding to the proteins in the picklist as on the gel in Figure 4.2 (Table 4.2):

Table 4.2. Protein identities of spots from a 2D-E gel run (fig 4.1) with AGS autonomic ganglia sample after MALDI-MS.

spot no.	protein	GenInfo Identifier	species	score (*)	peptides matched[1]	mass (Da)
Protein that appeared only on AGS gels						
3	hypothetical protein	gi 23021297	*Clostridium thermocellum*	43(63)	4	28623
4	thiamine phosphate	gi 18310318	*Clostridium perfringens*	44(63)	4	23018
5	alpha-1-antitrypsin/ p-21	gi 3982489 gi 619266	*Equus caballus/* *Clostridium botul...*	59(57) 60(63)	7 5	47141 19571
7	fibroblast growth factor	gi 1807390	*Equus caballus*	43(57)	4	15223
10	serum albumin precursor	gi 543794	*Equus caballus*	86(74)	12	70550
16	beta tubulin	gi 135490	*Sus scrofa*	60(57)	5	50285
Up-regulated proteins						
6	gamma enolase	gi 182118	*Homo sapiens*	111(74)	15	44568
8	serum albumin precursor	gi 543794	*Equus caballus*	49(57)	11	70550
9	transferrin	gi 6176213	*Equus caballus*	39(57)	3	7052
11	fibroblast growth factor	gi 1807390	*Equus caballus*	59(57)	6	15223
12	Phosphoribosyl pyrophosphate synthetase	gi 18310531	*Clostridium perfringens*	62(63)	8	41888
13	heat shock protein 60	gi 1334284	*Rattus norvegicus*	131(74)	21	58061
14	serum albumin precursor	gi 543794	*Equus caballus*	85(74)	23	70550
15	serum albumin precursor	gi 543794	*Equus caballus*	71(57)	15	70550

* Score required for a match of significance $p<0.05$

[1] a recommended minimum of 4 peptides should be matched

For those spots that remained unidentified or needed verification of their identification after MALDI-MS analysis, their digested peptides were then analysed using the BioSystems Q-Star tandem MS to obtain further information regarding their structure.

4.3.3 Identification of proteins by tandem MS

Because of the limited applicability of MALDI-MS analysis for species with limited genome information, tandem mass spectrometry (MS/MS) was employed to reveal the necessary structural information and aid the characterisation of the proteins. The BioSystems Q-Star MS system was used along with Analyst QS software/Bio Analyst software to run the extra sequence information generated through the Mascot database search engine to search for protein matches. Table 4.3 displays the resulting characterisations:

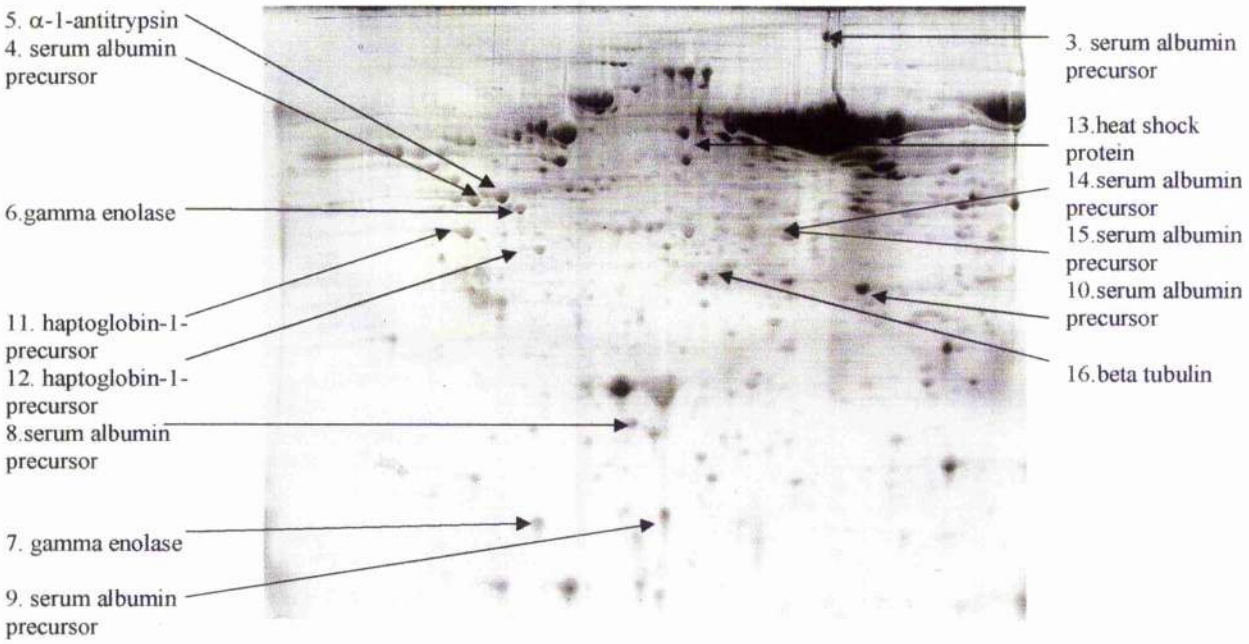
Table 4.3. Protein identities of spots from a 2D-E gel run with AGS autonomic ganglia sample after tandem MS.

spot no.	protein	GenInfo Identifier	species	score (†)	peptides matched	mass (Da)
Proteins that only appear on AGS gels						
3	serum albumin precursor	gi 543794	*Equus caballus*	219	6	68554
4	serum albumin precursor	gi 543794	*Equus caballus*	199	5	68554
5	*alpha-1-antitrypsin	gi 3982489	*Equus caballus*	97	3	46913
7	gamma enolase	gi 182118	*Homo sapiens*	172	4	44168
10	serum albumin precursor	gi 543794	*Equus caballus*	301	11	68554
	beta casein precursor	gi13661026	*Bos taurus*	90	2	25091
16	*beta-tubulin	gi 109432	*chinese hamster*	524	13	49716
	osetoglobin precursor	gi 129077	*Bos taurus*	184	5	34188
	HSP60	gi 129378	*Cricetulus griseus*	110	2	60950
	beta casein precursor	gi 115660	*Bos taurus*	77	1	25091
Up-regulated proteins						
6	*gamma enolase	gi 182118	*Homo sapiens*	876	44	44168
	phosphopyruvate hydratase	gi 1363309	*Rattus norvegicus*	703	16	47096
	beta-tubulin	gi16088719	*Homo sapiens*	169	5	49640
	peripherin	gi 91171	*Mus musculus*	93	4	50679
8	*serum albumin precursor	gi 543794	*Equus caballus*	229	10	68554
9	serum albumin precursor	gi 543794	*Equus caballus*	57	1	68554
11	haptoglobin-1 precursor	gi 123507	*Homo sapiens*	168	5	38427
	beta casein precursor	gi 115660	*Bos taurus*	72	2	25091
	beta actin	gi 28336	*Homo sapiens*	69	2	41786
	serum albumin precursor	gi 543794	*Equus caballus*	60	2	68554
12	haptoglobin-1 precursor	gi 2118092	*chimpanzee*	106	3	38843
	peripherin	gi 730299	*Homo sapiens*	97	3	53846
	beta casein precursor	gi 115660	*Bos taurus*	87	2	25091
13	chaperonin groEL precursor	gi 72957	*Mus musculus*	1372	42	60903
	*chaperonin (HSP60)	gi 306890	*Homo sapiens*	1294	41	60986
	peripherin	gi 730299	*Homo sapiens*	355	7	53846
	phosphoprotein	gi 473912	*Mus cookii*	251	6	50961
	*internexin neural intermediate filament protein	gi14249342	*Homo sapiens*	105	6	55357
	beta casein	gi 115660	*Bos taurus*	79	2	25091
	beta-tubulin	gi 92930	*Rattus norvegicus*	74	1	49905
14	*serum albumin precursor	gi 543794	*Equus caballus*	666	18	68554
	chaperonin(HSP60)	gi 306890	*Homo sapiens*	308	7	60986
	albumin	gi19705431	*Rattus norvegicus*	166	5	68674
	ssDNA-binding protein	gi 346338	*Homo sapiens*	99	3	34648
15	*serum albumin precursor	gi 543794	*Equus caballus*	625	12	68554
	kappa casein precursor	gi 162811	*Bos taurus*	55	1	21212

* denotes hits that had also been identified with MALDI-MS

† the score required for a hit of significance $p<0.05$ is 67

Figure 4.3. 2D-E gel of AGS affected ganglia showing the final identities of proteins thought to be AGS related.



4.4. Discussion

Following MALDI-MS analysis of spots thought to be EGS-related proteins, several spots were significantly matched to proteins from the NCBI database. Gamma enolase, a glycolytic enzyme that is found in nervous tissue, was identified at spot 6 with a score of 108 (74 was needed for a match of significance of $p < 0.05$). Therefore, this was a likely match. Tandem MS, confirmed spot 6 as gamma enolase with a highly significant score of 876. The identification of fibroblast growth factor (FGF) at spot 11 (score 59; 57 needed for significance) and tentatively at spot 7 (score 43; 57 needed for significance) are also likely matches. However, following tandem MS spot 7 was characterised as gamma enolase (with a score of 172). In addition, spot 11 was characterised as haptoglobin-1 precursor (with a score of 168) using tandem-MS and not fibroblast growth factor as suggested by MALDI-MS. The strength of the score for haptoglobin-1 precursor, combined with serum proteins having been isolated from autonomic ganglia before (Griffiths *et al*, 1994) suggests this is the most likely match.

Beta tubulin was identified at spot 16, being matched with proteins from *Bos taurus* (MALDI-MS) and Chinese hamster (tandem-MS), significant hits were achieved on both accounts (60 requiring 57, and 524 needing 67 respectively). Beta tubulin is observed in cells of most tissues and plays a significant role in the assembly of the microtubule support structures found in cells, including neuronal cells. Although beta-tubulin has not yet been sequenced for *Equus caballus*, it was identified by tandem MS probably because of the highly conserved nature of the beta-tubulin gene.

Serum albumin precursor was consistently identified at several spots on EGS gels. It was characterised at spots 8, 10, 14, and 15 after MALDI-MS, these being confirmed following tandem MS. Tandem MS also gave rise to additional characterisations of serum albumin precursor at spots 3, 4 and tentatively at 9. Serum albumin precursor is

usually found in blood. However, its presence within the ganglia is consistent with previous findings that autonomic ganglia contain serum proteins including albumin (Griffiths *et al*, 1994). Alternatively it could result from inadvertent contamination of the ganglion with blood or serum during the post mortem collection of the ganglia. However, this is unlikely given that the contamination would not have penetrated through the ganglion capsule into the central part of the tissue that was used for the analysis. In addition, it would be expected that its presence would be consistent in both EGS and control gels. It is also possible that the protein could be found within the extracellular space, or in the small capillaries supplying the neuronal tissues.

MALDI-MS identified transferrin, as the main, albeit not significant, candidate at spot 9. Transferrin is an iron binding transport protein responsible for the transport of iron from sites of absorption and haeme degradation to those of storage and utilisation. Serum transferrin may also stimulate cell proliferation (embl harvester, see Appendix 2). Again it is possible, although unlikely, that the contamination of the ganglia, during post mortem, could have resulted in transferrin being found within the tissue. Alternatively, transferrin may have been present in the tissue to minimise iron mediated cell damage. It also is possible that spot 9 might contain a mixture of both proteins. Transferrin may have been bound to serum albumin and co-migrated on the 2D-E gel.

Several *Clostridial* proteins were also tentatively recognised in the AGS affected autonomic ganglia by MALDI-MS. A 'hypothetical protein' from *Clostridium thermocellum* was the most significant match at spot 3, a thiamine-phosphate protein from *Clostridia perfringens* at spot 4 and phosphoribosyl also from *Clostridium perfringens* at spot 12. These were particularly interesting finds given the suspected role of *Clostridium botulinum* in EGS (Hunter *et al*, 1999). The proteins may not have been significant hits for the botulinum proteins, but given that *C. botulinum* has not been

fully sequenced yet, it could be possible that these proteins do derive from *C. botulinum*. If this were the case, it might help implicate the role of *C. botulinum* in EGS. However, highly significant hits for serum albumin precursor (at spots 3, 4, and 5) and haptoglobin-1 precursor (at spot 12) were found when these spots were subsequently analysed by tandem MS.

Furthermore, after MALDI-MS, there were conflicting results with a relatively high score at spot 5, but no significant match, for either alpha-1-antitrypsin (*Equus caballus*) or for p21 from *C. botulinum*. However, it was again the case that tandem MS produced a significant hit – for alpha-1-antitrypsin. It could be that these spots (3,4 and 5), at which *Clostridial* proteins were recognised, but no significant hit was made, are the proteins that tandem MS matched but that they had *Clostridial* proteins associated with them. For instance, the *Clostridial* proteins may be bound to serum proteins as carrier molecules.

The chaperonin groEL precursor, characterised at spot 13 by both MALDI-MS and tandem MS, is a heat shock protein. Heat shock proteins are involved with protein folding and transport across membranes. They prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions, such as during disease, within the mitochondrial matrix (Edenhofer *et al*, 1996). Therefore, an up-regulation of a chaperonin protein could be a likely observation during EGS.

In conclusion, the strength of the hits that were generated after tandem MS implies that they are the most likely characterisations for the proteins highlighted as being EGS-related. However, the characterisation results obtained after MALDI-MS should not be discarded as they potentially present some interesting leads for further investigation.

However this analysis further underlies the difficulties of protein identification by mass spectrometry in an organism that has limited genome sequence data. This is underlined by the fact that a few of the top matches for equine proteins hit proteins from another species such as rat and human. Whilst these may reveal some interesting homologies, some of these hits may also prove suspicious or require a more detailed investigation.

Chapter 5: Final discussion and conclusions

For the past century equine grass sickness has puzzled scientists, vets and horse owners alike. Previous attempts to understand EGS have found autonomic dysfunction, especially within the gastrointestinal tract, with neuronal damage and loss in affected areas. The current hypotheses suggest EGS is a toxicoinfection with *Clostridium botulinum* type C (Hunter *et al*, 1999; Hunter and Poxton, 2001). Most horses have been exposed to the bacterium and the normal gut flora can usually resist growth of the organism and any production of its toxins (Poxton, Equine Grass Sickness Fund website). There are two hypotheses for a putative trigger for the overgrowth of *C. botulinum*:

1. Oxidative stress and excitotoxicity of plants during periods of rapid growth or extreme weather conditions (McGorum *et al* 1998; 2000).
2. Cyanide from wild white clover (Doxey, 1991).

The aim of this project was to compare protein expression, using proteomic techniques, of homogenates of cranial cervical ganglia collected from horses that had AGS and those of control horses. An optimal and reproducible method for the 2D-E analysis of autonomic ganglia was developed (Chapter 2) to allow the production of good quality gels with the optimal separation of proteins for the comparative investigations of equine autonomic ganglia that were to follow. A series of comparisons were then run to determine the extent of protein variation between homogenates of ganglia from the same ganglia, the same horse, different horses and horses of different sexes. These experiments were aimed at making the final comparisons of EGS and control samples easier by

eliminating protein differences that were unrelated to EGS, which may occur between samples. The effectiveness of MALDI-MS was also evaluated at this point with respect to characterising equine proteins. Approximately sixty spots from a control (using non-diseased autonomic ganglia) gel were excised, digested and run through MALDI-MS. The acquired mass finger-print spectra were used to identify as many of the proteins as possible by comparing them to those already stored within the NCBI database. Thirty-five spots were characterised at this point, with a significant score indicating they were not random matches. A further eight spots were matched to proteins in the database, but with a score just below the required score to gain a significant score and so there is a chance these hits could be random events. However, given that approximately 30% (17 spots) were not characterised, further information on the acquired peptide sequences/structures would be required to characterise all spots from equine autonomic ganglia. None-the-less, the start of a preliminary proteome map was compiled for equine autonomic ganglia (Chapter 3). This will provide a good reference, and starting point, for others investigating EGS.

2D-E gels were then run to compare the AGS affected autonomic ganglia samples with like samples from control horses (horses that had died or were euthanased due to circumstances unrelated to EGS). The gels were studied and the protein spots that most consistently occurred either only on gels of AGS sample or only on gels of control samples, or were most consistently up or down regulated were highlighted. A typical AGS gel was taken and 14 spots, considered to be EGS related, were digested and excised using the spot picker robot and analysed using MALDI-MS. The spectra obtained

from MALDI-MS were compared against spectra already in the NCBI database using the Mascot database search engine software. Seven of the proteins were significantly identified at this point. Although some of these proteins occur in neurones under normal circumstances, it is the case that some have been shown to be up regulated during neuronal damage or ischaemia. For example, gamma enolase, which was found to be up regulated at spot number 6, is a neurone specific, glycolytic enzyme that catalyses the inter-conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway (Takei *et al*, 1991). Gamma enolase exists in three molecular forms in the human brain: non-neural enolase, neurone-specific enolase and the unstable alpha, gamma- enolase. Interestingly, the neurone specific form is a highly acidic protein of 78kDa, which has been found not only in central and peripheral nervous elements but also in derivative tumours. Cell injury causes the release of the enzyme into the blood and cerebrospinal fluid (CSF). In fact, the measurement of neurone-specific enolase levels in serum and CSF following cerebral ischaemia and traumatic head injuries provides a reliable indicator of the degree of brain cell damage (Takei *et al*. 1991). It could, therefore, be possible that the up regulation/ appearance of gamma enolase seen in AGS affected equine autonomic ganglia could be a direct result of the neuronal damage caused by the disease.

In addition, at spots 7 and 11, fibroblast growth factor (FGF) was initially identified using data obtained from MALDI-MS. FGF, a member of the heparin iron-binding family, is found in neurones and has several roles, but is most commonly involved in nervous system development and function (embl harvester, see Appendix 2). Consistent with their

names it is widely known that most FGFs initiate fibroblast proliferation. However, the general FGF designation is obviously limiting by its description of one target cell and one biological activity. For example, the FGF-2 molecule has been shown to be more than a growth factor. It induces adipocyte differentiation, stimulates astrocyte migration and prolongs neuronal survival (Strutz *et al*, 2002). Again, it could be plausible that the up regulation/appearance of FGF is a consequence of EGS causing neuronal damage.

Furthermore, the up regulation/appearance of beta tubulin at spot 16, could also be a response to EGS. Axonal injury of adult neurones results in several changes that are detectable in the cell body, including changes in the levels of many types of mRNAs. In the peripheral nervous system, an overall increase in the total tubulin mRNA is observed after peripheral nerve injury (Fournier *et al*, 1997). This pattern of beta tubulin expression is seen in neurones undergoing a neurogenic response to either physical or chemical lesions in the adult neuroepithelium (Roskams *et al*, 1998). Under normal circumstances the tubulin proteins assist microtubule assembly during embryonic development. However, it is also considered that beta-III tubulin plays an important role in axonal growth because of its unique neurone-specific pattern of expression and its substantial increase in neurones that have been stimulated to regrow their axons, following axotomy for example (Moskowitz *et al*, 1993). A similar increase in beta-III tubulin may well result following neuronal damage, such as that occurring during the course of EGS.

Another protein that is of potential interest is alpha-1-antitrypsin, which was characterised at spot 5. Alpha-1-antitrypsin is an inhibitor of serine proteases (embl harvester, see appendix 2). It primarily protects elastin fibres and other connective tissue components from degradation by neutrophil elastase (Song *et al*, 1998). Alpha-1-antitrypsin is a major plasma protein (Tardiff and Krauter, 1998) which has been isolated from equine plasma (Laegreid *et al*, 1982), which has a role in tissue repair by stimulating fibroblast proliferation and procollagen production (Dickenson and Alpher 1994). It could play such a role in neural tissue, since a disturbed balance between the proteolytic activity and protease inhibition in the pericellular environment has been implicated to contribute to the development of pathological changes during neurodegenerative disease, such as Alzheimer's disease (Mcins *et al*, 2001). Thus, it could well be the case that the causative agent of EGS may well trigger a cascade of events that results in a disruption in the balance between proteolytic activity and protease inhibition, such as that provided by alpha-1-antitrypsin. The end result would be the neurodegeneration seen in EGS cases. Moreover, alpha-1-antitrypsin is an acute phase protein which is up-regulated in response to cell injury. Milne and colleagues (1994) have shown similar acute phase proteins have been identified in the blood of horses with AGS.

The protein spots that remained unidentified, but were thought possibly to contain small amounts of protein derived from *Clostridia*, were particularly interesting given the suspected role of *Clostridium botulinum* in EGS (Hunter *et al*, 1999). The proteins may not have been significant hits for *Clostridia* botulinum proteins, but given that *C. botulinum* has not been fully sequenced yet; it could be possible that these proteins do

derive from *C. botulinum*. This requires further investigation, for example, by picking and running the corresponding spots from other AGS affected sample gels through MS to see if further hits for *Clostridial* proteins occur. If *Clostridial* proteins appeared in all corresponding spots on all gels, it would help support the role of *C. botulinum* in EGS.

The peptides of the protein spots were run through tandem MS in an attempt to characterise the remaining proteins and confirm the identity of those already characterised by MALDI-MS. The extra structural information provided by tandem MS resulted in all spots being characterised, with the majority having highly significant matches. Spot 5 was confirmed as being alpha-1-antitrypsin, spot 6 as gamma enolase, spot 8, 14 an 15 as serum albumin precursor, spot 13 as a chaperonin protein/heat shock protein and spot 16 as beta tubulin. These confirmations of protein characterisation help validate the efficiency of MALDI-MS with respect to identifying equine proteins, as evaluated in Chapter 3, but only if the corresponding gene sequences are available.

Those proteins that were not significantly matched following MALDI-MS were characterised following tandem MS. For example, spots 3, 4, 5 and 12 that were originally tentatively matched to *Clostridial* proteins were characterised as serum albumin precursor (spots 3 and 4), alpha-1-antitrypsin (5) and haptoglobin-1-precursor (12) by tandem MS. In some respects this was somewhat disappointing because a positive match to *Clostridial* proteins would have supported the link between *Clostridium botulinum* and EGS (Hunter *et al*, 1999). Spot 7, tentatively matched to fibroblast growth factor using MALDI-MS data, was then strongly matched to gamma enolase, whilst spots

9 and 11 (with tentative matches to transferrin and fibroblast growth factor respectively) were then significantly characterised as serum albumin precursor and haptoglobin-1 precursor respectively. By comparing the results of the two formats of MS, it can be concluded that when working with a species with a limited genome project and hence little information concerning characterised proteins, tandem MS is an extremely valuable tool.

The matches to haptoglobin at spots 11 and 12 following tandem MS were also interesting. Haptoglobin-1's usual role is to combine with free plasma haemoglobin to prevent the loss of iron through the kidneys and hence, protect the kidneys from damage by haemoglobin, while at the same time making haemoglobin accessible to degenerative enzymes. The beta chain is related to serine proteases, but displays no enzymatic activity itself (embl harvester, see Appendix 2). The function of haptoglobin-1 precursor in the equine autonomic ganglia is unclear, and there has been no published data of it having been characterised in neural tissue before. Why it was up-regulated during EGS is also unclear.

Serum albumin precursor was characterised at spots 8, 10, 14, and 15 after MALDI-MS and this was confirmed following tandem MS. Tandem MS also showed serum albumin precursor at spots 3, 4 and tentatively at 9 as well. Serum albumin precursor is usually found in blood, but there are previous findings that autonomic ganglia from EGS horses contain serum proteins including albumin (Griffiths *et al*, 1994).

A heat shock protein, Chaperonin groEL precursor, was characterised at spot 13 by both MALDI-MS and tandem MS. Heat shock proteins are involved in the folding and mitochondrial transport of proteins across membranes (embl harvester, see Appendix 2). They are also reported to help prevent misfolding and promote the refolding and correct assembly of unfolded polypeptides generated under stress conditions, such as during disease, within the mitochondrial matrix (Edenhofer *et al*, 1996). Hence, an up-regulation of a chaperonin protein is a likely observation during EGS.

There were also several spots that displayed high scores for several proteins. For example, spot 13 had scores in 4 figures for heat shock proteins, but also a score of 355 for peripherin (an adhesion molecule found in peripheral nervous tissue membrane (embl harvester, see Appendix 2)), a score of 251 for phosphoprotein (inhibits apoptosis and regulates glucose transport in most tissue types (embl harvester, see Appendix 2)) and a score of 105 for a neural intermediate filament protein. All of these hits are very significant and plausible matches, however, the huge scores obtained for heat shock proteins imply that this spot was most likely to be a heat shock protein. It is possible, however, that there could be a mixture of proteins at this position on the gel. Similar situations also occurred at spots 11, 14 and 16.

The repeated appearance of casein precursors, a milk protein, was a surprising result. It was significantly matched at spots 10, 11, 12, 13, 15 and 16. This was obviously an unexpected hit and unexplainable. There is no obvious reason why a protein that, according to literature, occurs exclusively in milk and mammary glands (embl harvester,

see Appendix 2) would occur in autonomic ganglia. It could be that casein has been characterised and studied so much it has numerous mentions within the database and more or less any sequence could match a section of it to a certain degree.

Studying the effect of a disease has its limitations. It confirms the result of the disease; the damage caused to tissues, but may not reveal the cause of the disease. However, in this project, having studied the damage and the changes occurring to the protein expression of autonomic ganglia of horses affected with EGS, it may be possible to determine the route of damage. For instance, by comparing the EGS-related proteins discovered in this project, with proteins that occurred in other diseases and causes of neuronal damage, clues to the route of damage occurring in EGS may be revealed. This in turn could help determine what actually caused the damage, i.e. what caused EGS.

The small sample numbers caused further limiting problems in this project, for instance with respect to the identified differential expression between diseased and healthy tissue warrants further investigation and ideally increased sample sizes. Moreover, animals could not be genetically matched in the same way that model organisms can be in laboratory experiments, meaning that there are many more variables than tissues just being 'diseased' or 'controls'. However, there is no way of avoiding this within the circumstances of grass sickness in horses. Horses are too big, and expensive to keep and have far too long a gestation period to breed for experimental purposes. In this study, as many replicate experiments as possible were run (Chapter 3) to overcome this problem. Repeating the experiments carried out in chapter 3 with further samples would confirm

the consistency of protein expression changes that were highlighted in this project, with respect to breed/sex etc.

A further area of this project that could be investigated more fully would be a study of the proteins out with the pH range 4-7. Due to time and expenses limitations, gels were not optimised for the pH ranges either side of 4-7. However, it may be worth while to consider this as there could be further differences between diseased and non-diseased samples that could have been picked up within this particular project.

The limited genome project for *Equus caballus* was another factor that limited the proteomic analysis in this project. Having a limited gene bank meant that it was difficult to characterise equine proteins. MALDI-MS was especially limited by this factor. As the equine genome project continues and more information becomes stored in the databases, projects continuing this work will be more useful and conclusive.

However, the work carried out in this project will be of a great help to those continuing and embarking upon EGS research. A preliminary proteome map for an equine autonomic ganglion has been characterised which will provide a significant reference point. Several proteins that most consistently appeared during EGS or were up regulated during EGS were characterised. A further investigation into these proteins functions and roles both within autonomic ganglia and during disease may help to reveal some clues regarding the aetiology of EGS. The possible presence of Clostridial proteins, in the autonomic ganglia is especially interesting and deserves further investigation.

References

1. Abe TK, Tanaka H, Iwanaga T, Odani S, and Kuwano R, 1997, The presence of the 50-kDa subunit of dynactin complex in the nerve growth cone, *Biomedical and Biophysical Res Com*, 233, 295-299.
2. Barlow RM, 1969, Neuropathological observations in grass sickness of horses. *J Comp Path.*, 79, 407-411.
3. Brookes SF, Erusalimsky JD, Totty NF, and Rozengurt E, 1990, Purification and internal amino acid sequence of the 80kDa protein kinase C substrate from Swiss 3T3 fibroblasts, *FEBS*, 268, 1, 291-295.
4. Bortosz G., 1997, Oxidative stress in plants. *Acta Physiol. Planta*, 19, 47-64.
5. BhamBurkar VR, Prakash P, 1992, Histological architecture of certain ganglia in goat (carpa hircus). *J. Anat. Soc. of India*, 41, 25-9.
6. Castegna A, Aksenov M, Thongboonkerd V, Klei JB, Pierce WM, Booze R, Markesbery WR and Butterfield DA, 2002, Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrmidinase-related protein 2, γ-enolase and heat shock cognate 71, *J Neurochem*, 82, 1524-1532.
7. Castellucci VF, Kennedy TE, Kandel ER, Goelet P, 1988, A quantitative analysis of 2D gels identifies proteins in which labelling is increased following long-term sensitisation in Aplysia. *Neuron*, 1, 321-328.
8. Cohen AM, Rumpel K, Coombs GH, and Wastling JM, 2002, Characterisation of global protein expression by two-dimensional electrophoresis and mass spectrometry: proteomics of *Toxoplasma gondii*. *Int J Parasitol*, 31, 39-51.

9. Cottrell DF, McGorum BC, Pearson GT, 1999, The neurology and enterology of equine grass sickness: A review of basic mechanisms. *Neurogastroenterol. Mot.*, 11, 79-92.

10. DePace DM, 1981, Morphologic study of the blood vessels of the superior cervical ganglion of the albino rat. *Acta Anat. (Basel)*. 109, 238-46.

11. Dey PM, Graff RD, Lagunowich LA Reuhl KR, 1994, Selective loss of the 180-kDa form of the neural cell adhesion molecule in hippocampus and cerebellum of the adult mouse following trimethyltin administration, *Toxicology and Applied Pharmacology*, 126, 69-74.

12. Dickenson and Alpher, 1994, Children's liver disease foundation website, http://www.childliverdisease.org/aatd.html

13. Doxey DL, Gilmor JS, Milne EM, 1991, The relationship between meteorological features and equine grass sickness (dysautonomia). *Equine Vet J*, 53, 370-373.

14. Doxey DL, Pogson DM, Milne EM, Gilmour JS, Chisholm HK, 1992, Clinical equine dysautonomia an autonomic nerve damage. *Res. in Vet. Sci.*, 53, 106-109.

15. Doxey DL, Pearson GT, Milne EM, Woodman MP, Gilmour JS, Chisholm HK, 1995, Small intestine and small colon neuropathy in equine dysautonomia (grass sickness). *Vet. Res. Com.*, 19, 529-543.

16. Edman P, Begg G., 1967, A protein Sequenator, *Eur J Biochem*, 1, 80-91.

17. embl harvester website: http://www.db.embl.de

18. Edenhofer F, Rieger R, Famulok M, Wendler W, Weiss S, Winnacker EL, 1996, Prion protein PrPc interacts with molecular chaperones of the Hsp60 family, *J Virol*, 70, 4724-4728.

19. Fonstanze F, Winklhofer F, Hartl U, Tatzelt J, 2001, A sensitive filter retention assay for the detection of PrP^{Sc} and the screening of anti-prion compounds, *FEBS Letters*, 503, 41-45.

20. Frisco G, Kaiser L, Raud J, Wikstrom L, 2001, Differential protein expression in rat trigeminal ganglia during inflammation. *Proteomics*, 1, 397-408.

21. Geschwind DH, Kelly GM, Fryer H, Feeser-Bhatt H, and Hockfield S, 1996, Identification characterisation of novel developmentally regulated proteins in rat spinal cord, *Developmental Brain Res*, 97, 62-75.

22. Gilmour JS, 1973, Experimental reproduction of the neurological leisions associated with grass sickness. *Vet. Rec.*, 565-566.

23. Gilmour JS and Mould DL, 1977, Experimental studies of neurotoxic activity in blood fractions from acute cases of grass sickness. *Res. Vet. Sci.*, 22, 1-4.

24. Goldstein D.S., Smith L.J, National Dysautonomia Research Foundation, http://www.ndrf.org/ans.html

25. Grant SGN and Husi H, 2001, Proteomics of multiprotein complexes: answering fundamental questions in neuroscience. *Trends in biotechnology*, 19, 49-54.

26. Green AJE, Thompson EJ, Stewart GE, Zeidler M, McKenzie JM, MacLeod M-A, Ironside JW, Will RG, and Knight RSG, 2001, Use of 14-3-3 and other brain-specific proteins in CSF in the diagnosis of variant Creutzfeldt-Jakob disease, *J Neurol Neurosurg Psychiatry*, 70, 744-748.

27. Griffiths IR, Smith S, Doxey DL, Whitwell K, Love S, 1994a, Evidence that the agent of equine grass sickness may reach neurons by retrograde axonal transport. *Vet. Rec.*, 26, 520-523.

28. Griffiths IR, Smith S, Kyriakides E, Barrie JM, 1994b, Autonomic neurons from horses with grass sickness contain serum proteins. *Vet. Rec. Short Communications*, 135, 90-91.

29. Guptasarma P, 2001, National Workshop on Genomics and Proteomics, imtech.res.in/bic/workshop/nwgp/chap6.html

30. Habermann E and Dreyer F, 1986, *Curr. Top. Microbiol. Immunol.*, 129, 93-179.

31. Hahn CN, Mayhew I, de Lahunta A, 2001, Central neuropathy of equine grass sickness. *Acta Neuropathol.* (Berl), Aug; 102, 153-159.

32. Haraguchi H, 1997, Protection against oxidative stress by plant secondary metabolites. *Plant Physiol.* (Rockville), 114, 232-233.

33. Hudson N, Mayhew I, and Pearson G, 2001, A reduction in the interstitial cells of Cajal in horses with equine dysautonomia (grass sickness). *Autonomic Neuroscience*, 92, 37-44.

34. Hudson N, Mayhew I, and Pearson G, 2002, Presence of in vitro electrical activity in the ileum of horses with enteric nervous system pathology: equine dysautonomia (grass sickness). *Autonomic Neuroscience*, 99, 119-126.

35. Hunter LC, Miller JK, and Poxton IR, 1999, The association of *Clostridium botulinum* type c with equine grass sickness: a toxicoinfection? *Equine Vet J.*, 31, 492-499.

36. Hunter LC, and Poxton IR, 2001, Systemic antibodies to *Clostridium botulinum* type C: do they protect horse from grass sickness (dysautonomia)? *Equine Vet. J.*, 33, 547-553.

37. Hyden H and Lange PW, 1968, Protein synthesis in the hippocampal pyramidal cells of rats during a behavioural test. *Science*. 159, 1370-1373.

38. Hyden H and Lange PW, 1969, Protein synthesis during learning. *Science*. 164, 200-201.

39. Janke C, Beck M, Holzer M, Bigl V, and Arendt T, 2000, Analysis of the molecular heterogeneity of the microtubule-associated protein tau by two-dimensional electrophoresis and RT-PCR, *Brain Res Protocols*, 5, 231-242.

40. Jenkins LW, Peters GW, Dixon CE, Zhang X, Clark RSB, Skinner JC, Marion DW, Adelson PD, and 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, *Journal of Neurotrauma*, 19, 6 715-740.

41. Kelly L, Postiglioni A, De Andres DF, Vega-Pla JL, Gagliardi R, Biagetti R, Franco J, 2002, Genetic characterisation of the Uruguayan Creole hors and analysis of relationships among horse breeds. *Res Vet Sci*, 72, 69-73.

42. Kiernan JA, 2002, Department of Anatomy and Cell Biology, The University of Western Ontario, London, Canada. http://publish.uwo.ca/~jkiernan/autgang.gif

43. Kingsley R.E., Concise text of neuroscience, 2nd Edition, Lippincott Williams and Wilkins, Chpt 13, pp 471-476, 484-486.

44. Laegreid WW, Breeze RG, Counts DF, 1982, Isolation and some properties of equine alpha-1-antitrypsin, *Int J Biochem*, 14, 327-334.

45. Lesney MS, 2001, Proteins on mass (spectrometry, that is). *Todays Chemist at work*, 10, 30-32, 34-35.

46. Locke MM, Ruth LS, Millon LV, Penedo MC, Murray JD, Bowling AT, 2001, The cream dilution gene, responsible for the palomino and buckskin coat colours, maps to

horse chromosome 21. International Society for Animal Genetics *Animal Genetics*, 32, 340-343.

47. Matsu-ura T, Nakadai T, Oda Y, Nagasu T, Mikoshiba K, Tamura T, 2002, Seizure-mediated accumulation of the β subunit of Ca^{2+}/calmodulin-dependant protein kinase II in nuclei of mouse brain cells, *Neurosci Letters*, 332, 149-152.
48. McGorum BC, Pirie RS, Mayhew IG, 1998, Is equine grass sickness (EGS) a consequence of oxidative stress? *Equine Grass Sickness Fund 10th Anniversary Symposium Proceedings*, Edinburgh.
49. McGorum BC, Fry SC, Wallace G, Coenen K, Robb J, Williamson G, Aruoma OI, 2000, Properties of herbage in relation to Equine Dysautonomia: Biochemical composition and antioxidant and prooxidant actions. *J. Agric. Food Chem.*, 2346-2352.
50. McGorum BC, Royal (Dick) School of Veterinary Studies, 2001, Equine Grass Sickness Clover Trial, The Equine Grass Sickness Fund website, www.grasssickness.org.uk
51. Meins M, Piosik P, Schaeren-Wiemers N, Franzoni S, Troncoso E, Kiss JZ, Brosanle C, Scwab ME, Molnar Z, Monard D, 2001, Progressive neuronal and motor dysfunction in mice overexpressing the serine protease inhibitor protease nexin-1 in postmitotic neurones, *The Journal of Neuroscience*, 21, 8830-8841.
52. Milne EM, Pemberton AD, McGorum BC, Dixon PM, Scudamore CL, Miller HR, 1994, Quantitation of alpha-1 proteinase inhibitor in the pulmonary epithelial lining fluid of horses with chronic obstructive pulmonary disease, *Res Vet Sci.* 57, 262-4.
53. Milne EM, McGorum BC, 2000, Grass sickness in horses, The Equine Grass Sickness Fund Website, http://www.grasssickness.org.uk/advice.htm

54. Niessmann H, Blasi J, and Jahn R, 1994, *Trends in Cell Biol.*, 4, 179-185.

55. Oblinger MM, 1987, Characterisation of posttranslational processing of the mammalian high-molecular-weight neurofilament protein *in vivo, J Neurosci*, 7, 8, 2510-2521.

56. Oppermann M, Cols N, Nyman T, Helin J, Saarinen J, Byman I, Toran N, Alaiya AA, Bergman T, Kalkkinen N, Gonzalez-Duarte R, and Jornvall H, 2000, Identification of foetal brain proteins by two-dimensional gel electrophoresis and mass spectrometry, *Eur J Biochem*, 267, 4713-4719.

57. Pemberton AD, Hodgson JC, Gilmour JS, Doxey DL, 1990, Characterisation of compounds isolated from the sera of horses with acute grass sickness. *Res Vet Sci.* 49, 315-8.

58. Poxton I, Role of Clostridium botulinum is EGS, Medical Microbiology, University of Edinburgh Medical School, Equine Grass Sickness Fund Website, http://www.grasssickness.org.uk

59. Pogson DM, Doxey RL, Gilmour JS, Milne EM, Chisholm HK, 1992, Autonomic neurone degeneration in equine dysautonomia (grass sickness). *J. Comp. Pathol.*, 107, 271-283.

60. Preobrazhensky AA, 1993, Electrophoresis of neurochordins, a family of high molecular weight neural tissue glycoproteins, on horizontal submerged agarose gels in the presence of dodecyl sulfate, *Anal Biochem*, 209, 315-317.

61. Schonberger SJ, Edgar PF, Kydd R, Faull RLM, Cooper GJS, 2001, Proteomic analysis of the brain in Alzheimer's disease: Molecular phenotype of a complex disease process, *Proteomics*, 1, 1519-1528.

62. Scripps Centre for mass spectrometry, www.masspec.scripps.edu/information/intro/index.html

63. Semra YK, Scidi OA, Shrief MK, 2002, Heightened intrathecal release of axonal cytoskeletal proteins in multiple sclerosis is associated with progressive disease and clinical disability. *J Neuroimmunol*, 122, 132-139.

64. Shimoji Y, Ng V, Matsumura K, Fischetti VA, and Rambukkana A, 1999, A 21-kDa surface protein of *Mycobacterium lepae* binds peripheral nerve laminin-2 and mediates Schwann cell invasion, *Proc Natl Acad Sci USA*, 96, 9857-9862.

65. Sickmann A, Dormeyer W, Wortelkamp S, Woitalla D, Kuhn W, Meyer HE, 2002, Towards a high resolution separation of human cerebrospinal fluid, *Journal of Chromatography B*, 771, 167-196.

66. Simpson LL, 1989, Botulinum neurotoxin and Tetanus Toxin (Simpson lI, Ed), *Academic Press*, New York, 153-178.

67. Song S, Morgan M, Ellis T, Poirier A, Chestnut K, Wang T, Brantly M, Muzyczka N, Byre BJ, Atkinson M, Flotte TR, 1998, Sustained secretion of human alpha-1-antitrypsin from murine muscle transduced with adeno-associated virus vectors, *Pro. Natl. Acad. Sci. USA*, 95, 14384-14388.

68. Sweatt JD Kennedy TE, Wager-Smith K, Gawinowicz MA, Barzilai A, Karl KA, Kandel ER, 1989, Development of a database of amino acid sequences for proteins identified and isolated on two-dimensional polyacrylamide gels. *Electrophoresis*, 10, 152-157.

69. Tardiff J and Krauter KS, 1998, Divergent expression of α1-protease inhibitor genes in mouse and human. *Nucleic Acids Research*, 26, 16, 3794-3799.

70. Tillemann K, Haute CVD, Geerts H, Leuven FV, Esmans EL, and Moens L, 2002, Proteomic analysis of the neurodegeneration in the brain of tau transgenic mice, *Proteomics*, 2, 656-665.

71. Tocher JF, Brown W, Tocher JW, Buxton JB, 1923, Grass sickness investigation report. *Vet. Rec.* 3, 75-89.

72. Tocher JF, 1924, Grass sickness in horses. *Transact. Royal Highland Agric. Soc.*, 36, 65-83.

73. Tsuji T, Shimohama S, Kamiya S, Sazuka T, Ohara O, 1999, Analysis of brain proteins in Alzheimer's disease using high-resolution two-dimensional gel electrophoresis. *J. Neurol. Sci.*, 166, 100-106.

74. University of Liverpool, 2000, Faculty of Veterinary Science, Grass Sickness in Horses, www.cee.hw.ac.uk/~rose/equine/gs-main.htm.

75. Vensel WH, Tarr GE., 1995, Minimising N-to-O shift in Edman sequencing, Techniques in Protein Chemistry VI, (Crab JW, ed.), Academic press, San diego.

76. Wasinger VC, Cordwell SJ, Cerpa-poljak A, Yan JX, Gooley AA Wilkins MR, Duncan MW, Harris R, Williams KL, Humphery-Smith I, 1995, Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *Electrophoresis*, 16, 1090-4.

77. Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez JC, Yan JX, Gooley AA, Hughes G, Humphery-Smith I, Williams KL, Hochestrasser DF, 1996, Large-scale protein identification by two-dimensional electrophoresis and amino acid analysis, *BioTechnology*, 14, 61–65

78. Yanagida M, 2002, Functional proteomics; current achievements. *J Chromatography*, 771, 89-106.

79. Yates JR, 2000, Mass spectrometry from genomics to proteomics, *TIG* 16, 5-8.

80. Yates JR, 1998, Mass spectrometry and the age of the proteome, *J. Mass Spectrom*, 33, 1-9.

81. Yates JR, McCormack AL, Eng J, 1996, Mining genomes with mass spectrometry. *Anal Chem.* 68, 534-540.

82. Zerr I, Bodemer M, Otto M, Poser S, Windl O, Kretzschmar HA, Gefeller O, 1996, Diagnosis of Creutzfeldt-Jakob disease by two-dimensional gel electrophoresis of cerebrospinal fluid, *The Lancet*, 348, 846-849.

Bibliography

1.Grays H, Anatomy, 34th Edition, Longmans, Green and Co Ltd.

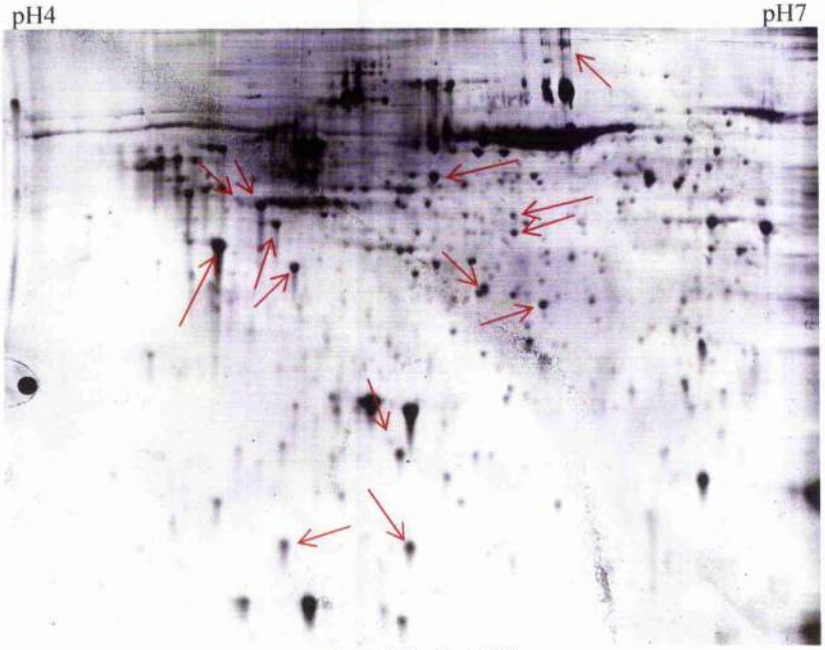
2.Kingsley R.E., Concise text of neuroscience, 2nd Edition, Lippincott Williams and Wilkins.

3.Tortora GJ, Principles of Human Anatomy, 8th Ed., Benjamin/Cummings Science Publishing.

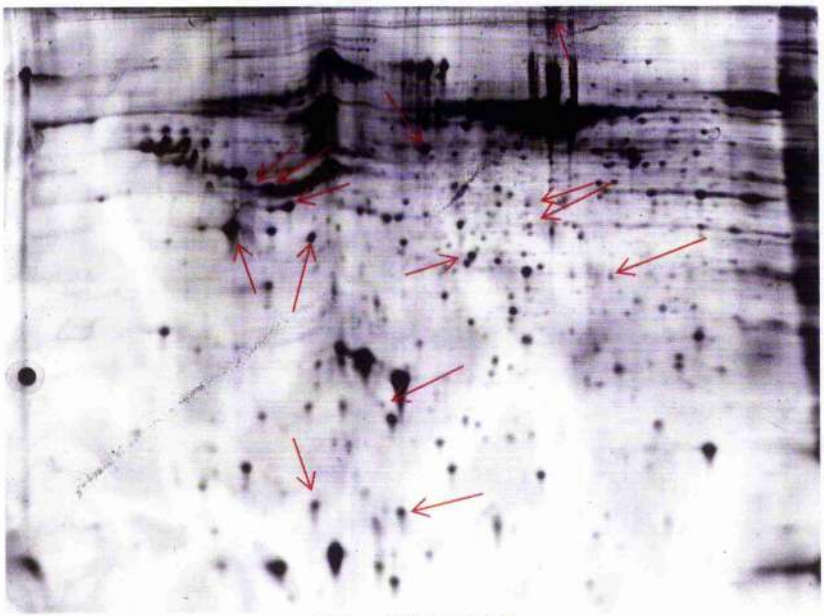
Appendix 1

24cm, pH 4-7, Colloidal Coomassie Blue stained 2D-E gels comparing the protein expression of AGS affected equine autonomic ganglia with control equine autonomic ganglia

Figure A. 2D-E gels comparing the protein expression of EGS affected equine autonomic ganglia 02-913 and 02-248 with control equine autonomic ganglia 01-681. The arrows indicate spots that either consistently only appear in EGS gels or are consistently up-regulated in EGS gels, and correspond to the spots numbered for 'picking' in Fig 4.2.

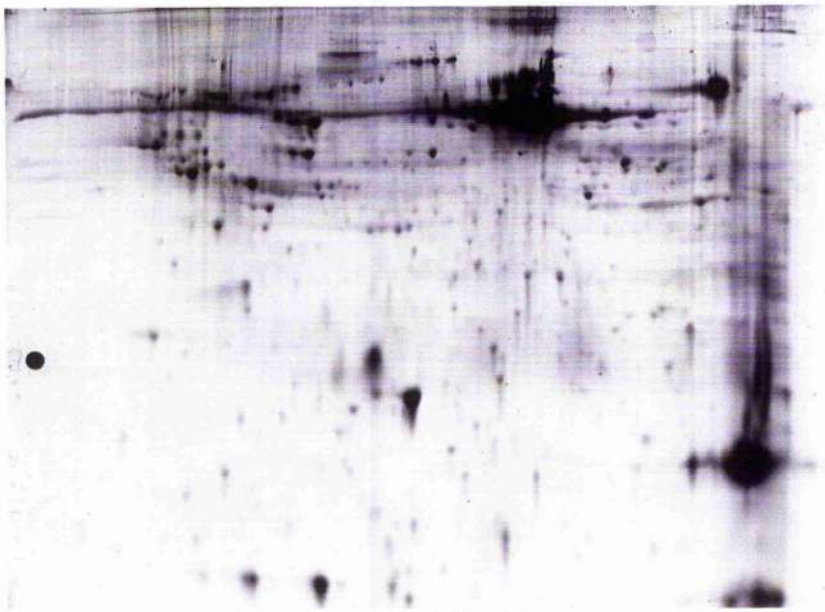


(a) AGS 02-248



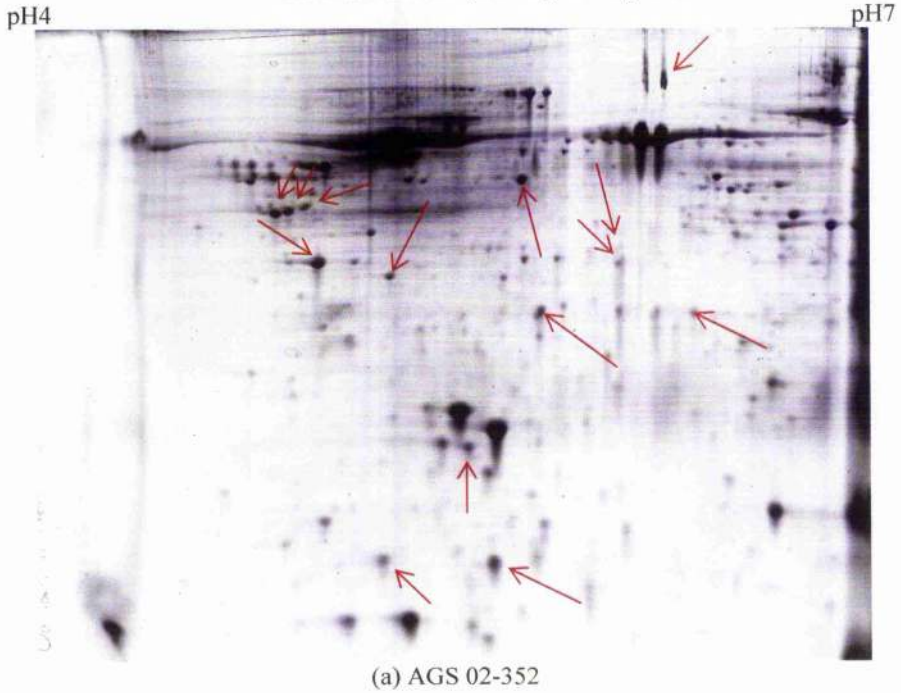
(b) AGS 02-913

continued...



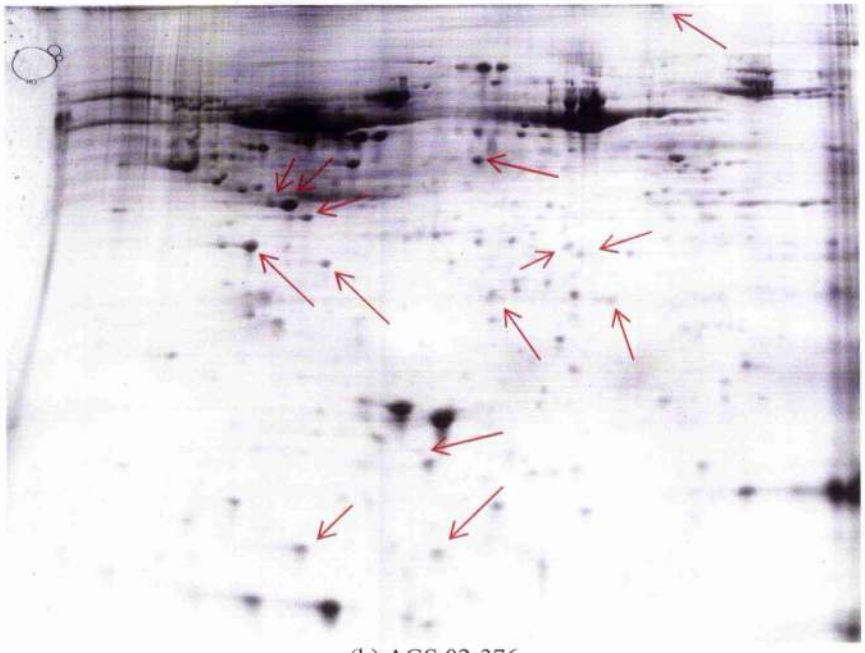
(c) control 01-681

Figure B. 2D-E gels comparing the protein expression of EGS affected equine autonomic ganglia 02-376 and 02-352 with control equine autonomic ganglia 02-56. The arrows indicate spots that either consistently only appear in EGS gels or are consistently up-regulated in EGS gels, and correspond to the spots numbered for 'picking' in Fig 4.2.

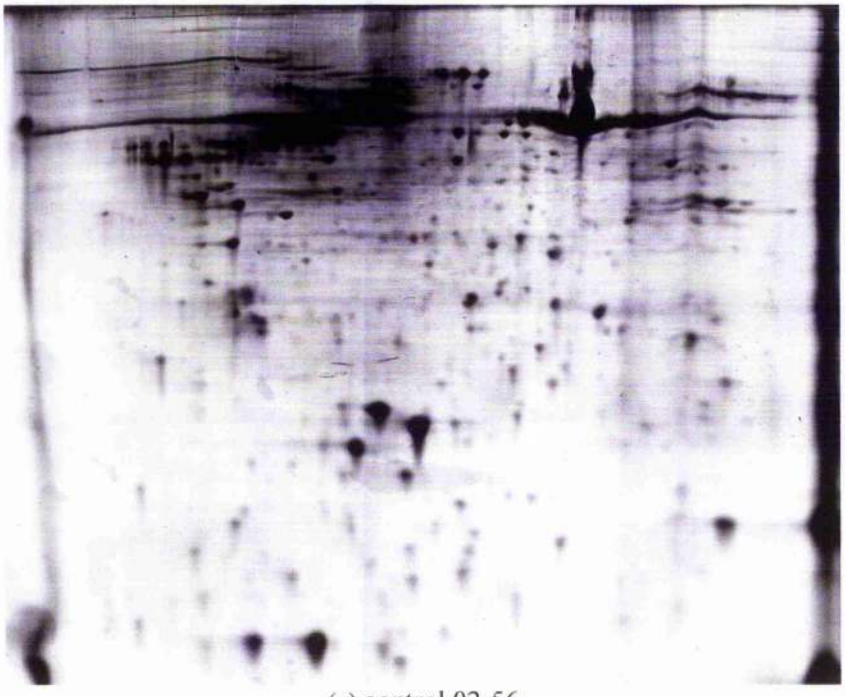


(a) AGS 02-352

continued...

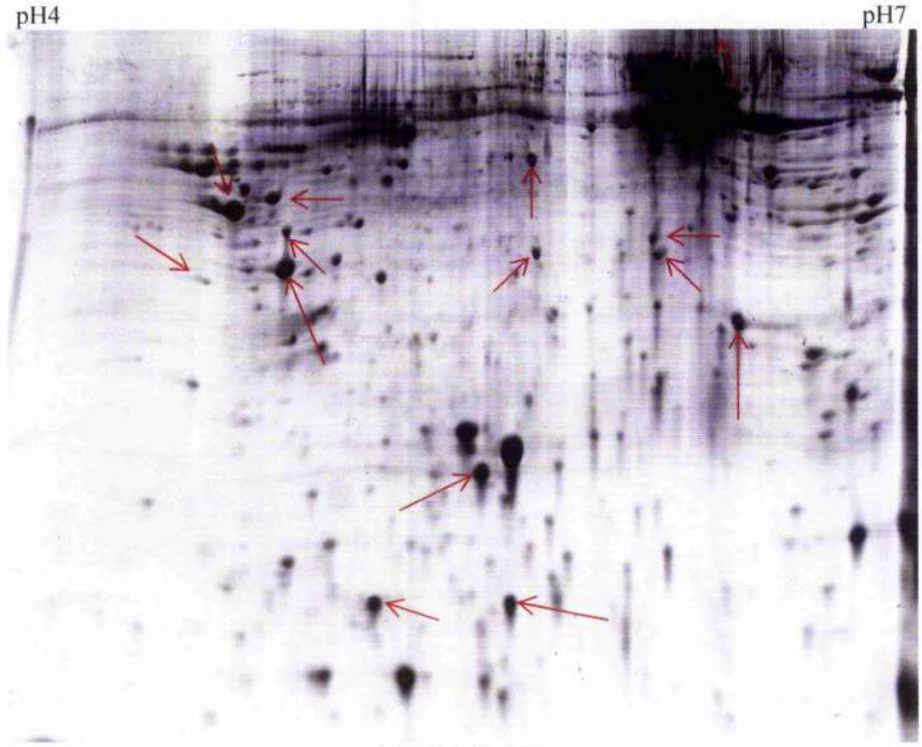


(b) AGS 02-376

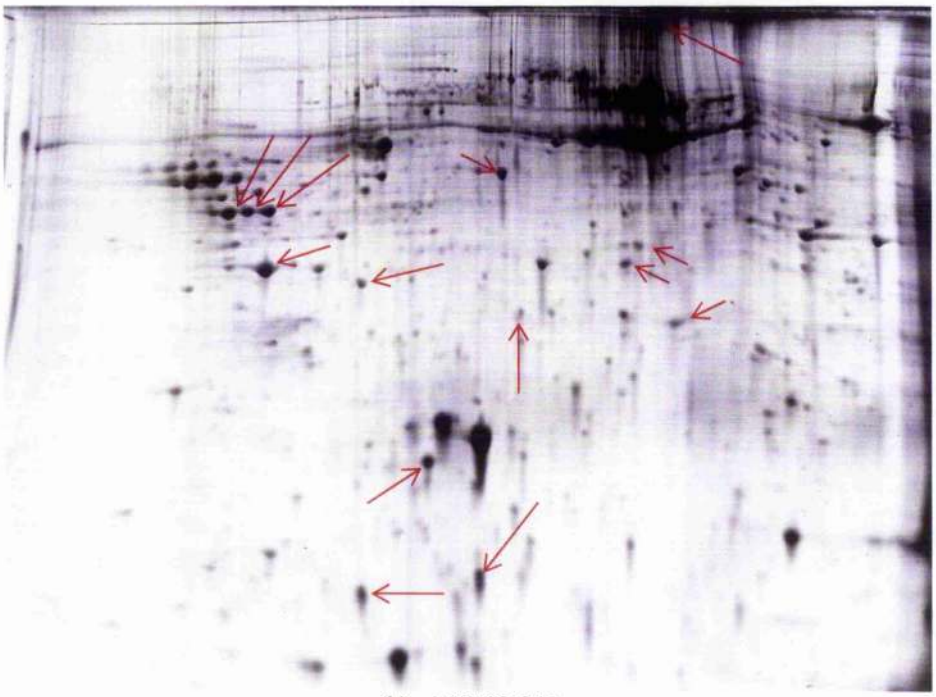


(c) control 02-56

Figure C. 2D-E gels comparing the protein expression of EGS affected equine autonomic ganglia 02-302 and 02-349 with control equine autonomic ganglia 02-182. The arrows indicate spots that either consistently only appear in EGS gels or are consistently up-regulated in EGS gels, and correspond to the spots numbered for 'picking' in Fig 4.2.

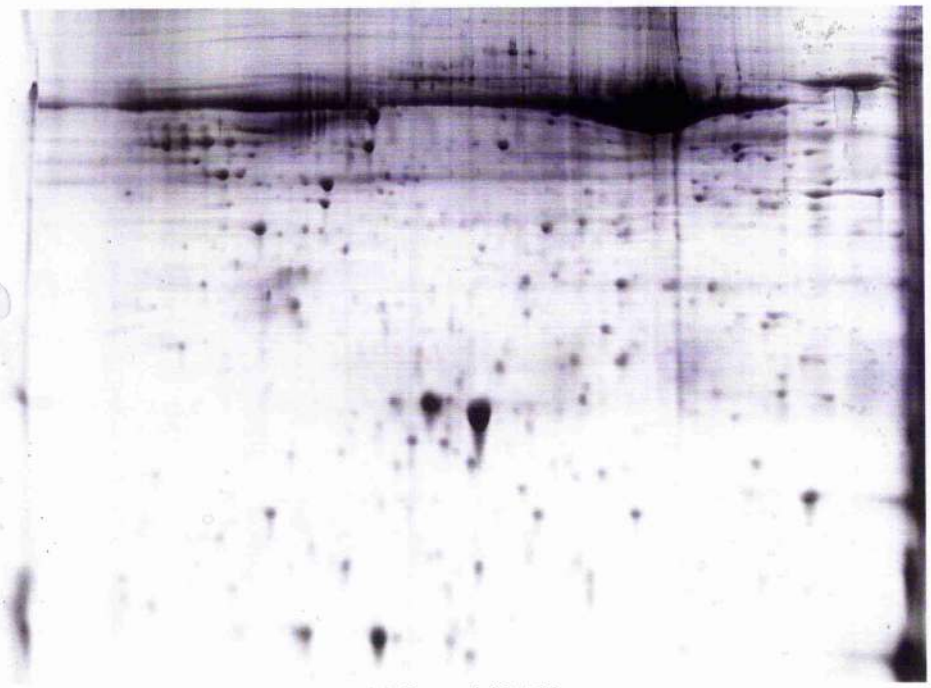


(a) AGS 02-302.



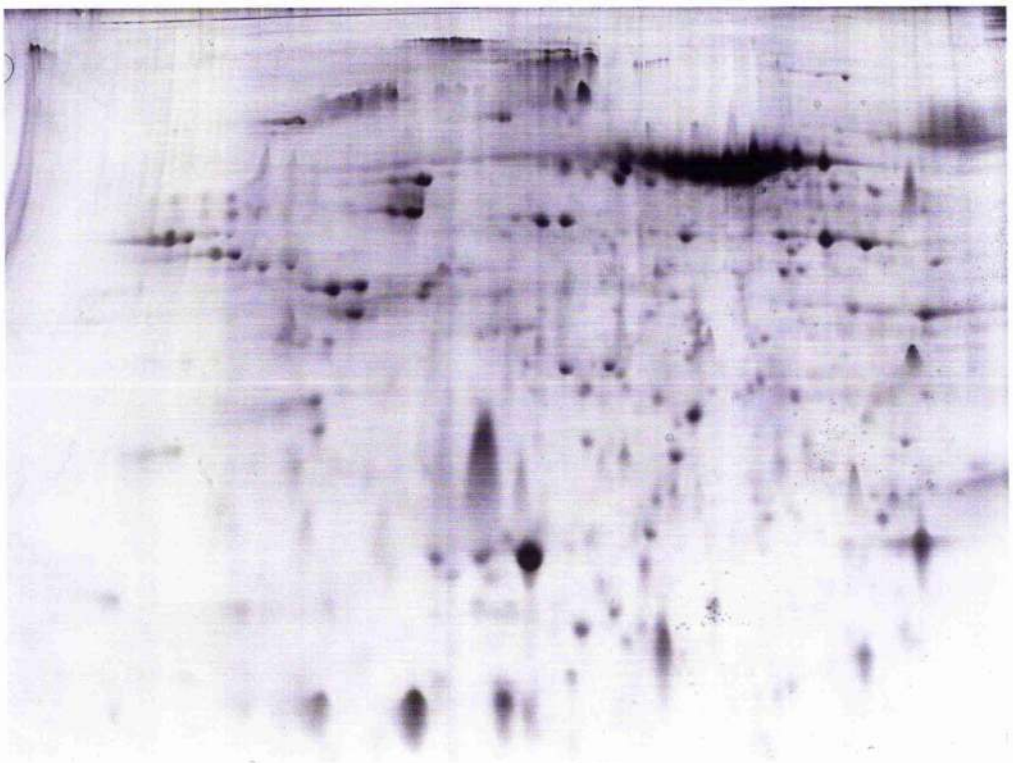
(b) AGS 02-349

continued...



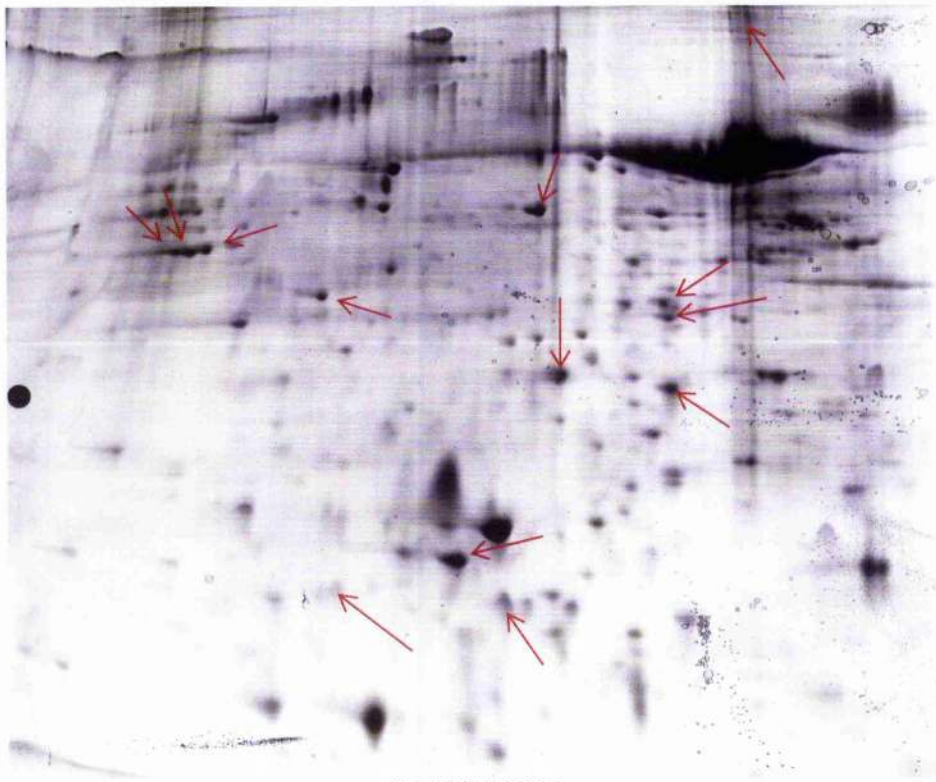
(c) Control 02-182

Figure D. 2D-E gels comparing the protein expression of EGS affected equine autonomic ganglia 03-3392, 03-409, 03-408, 03-425 and 03-445 with control equine autonomic ganglia 02-889. The arrows indicate spots that either consistently only appear in EGS gels or are consistently up-regulated in EGS gels, and correspond to the spots numbered for 'picking' in Fig 4.2.

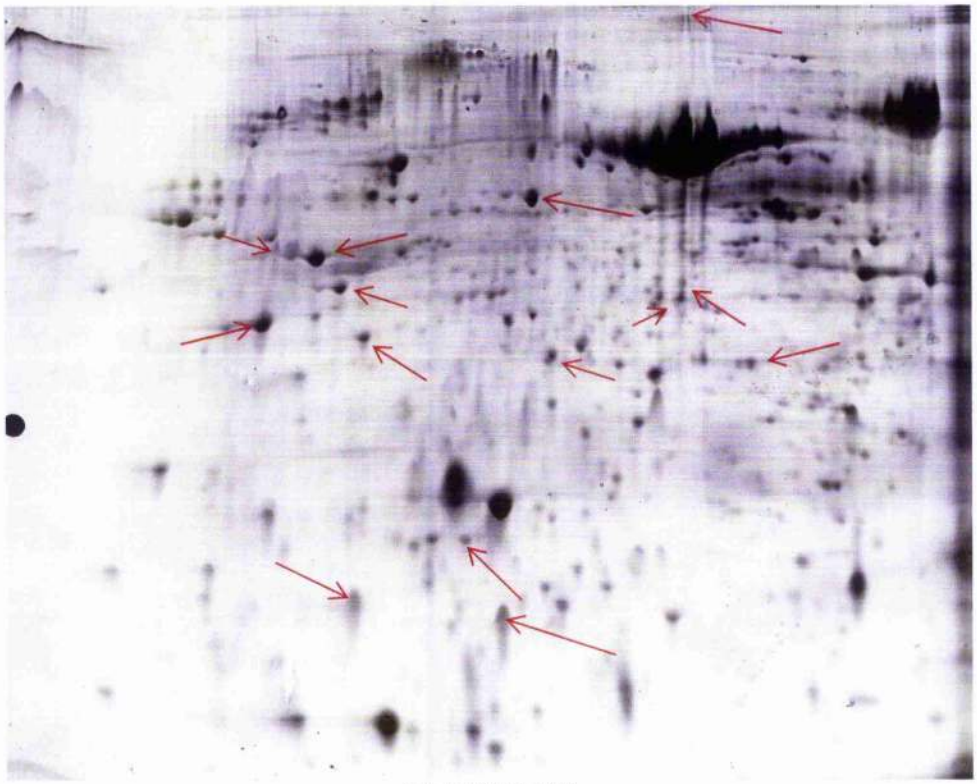


(a) control ccg 02-889

continued...

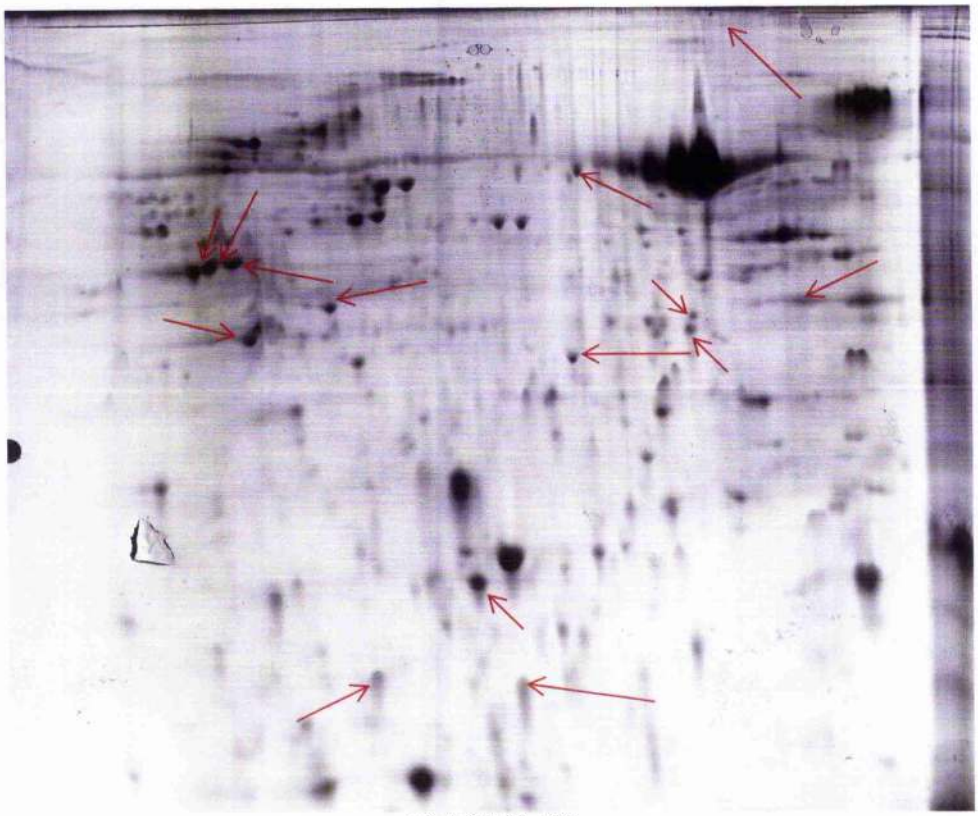


(b) AGS 03-392

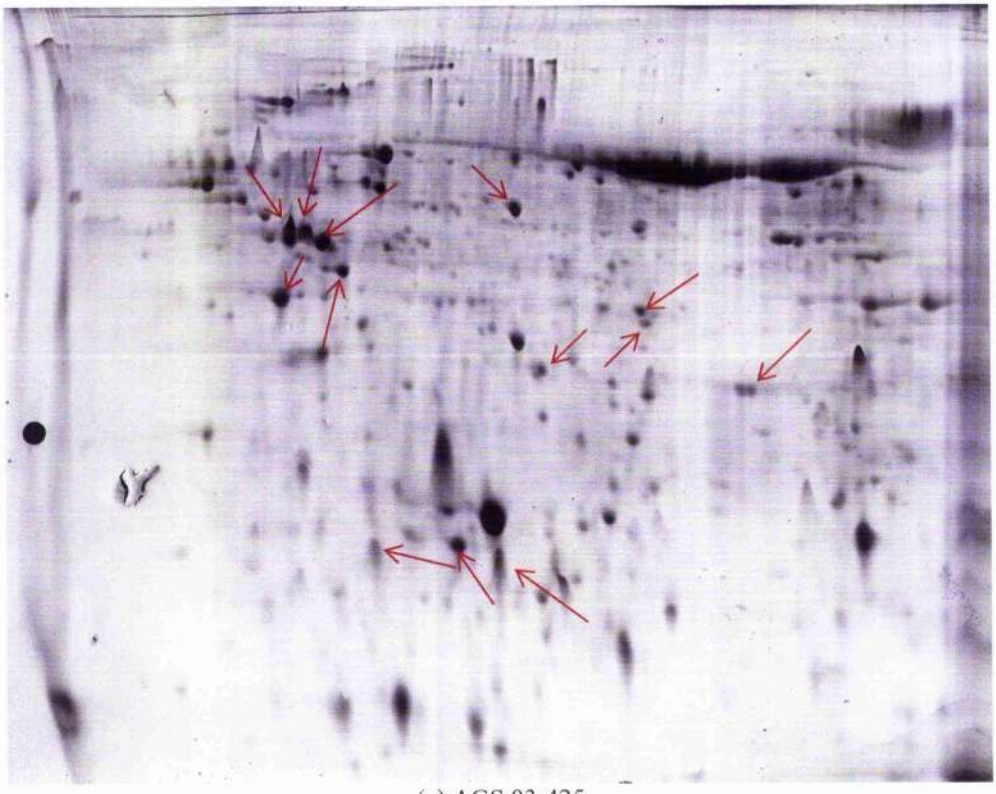


(c) AGS 03-409

continued...

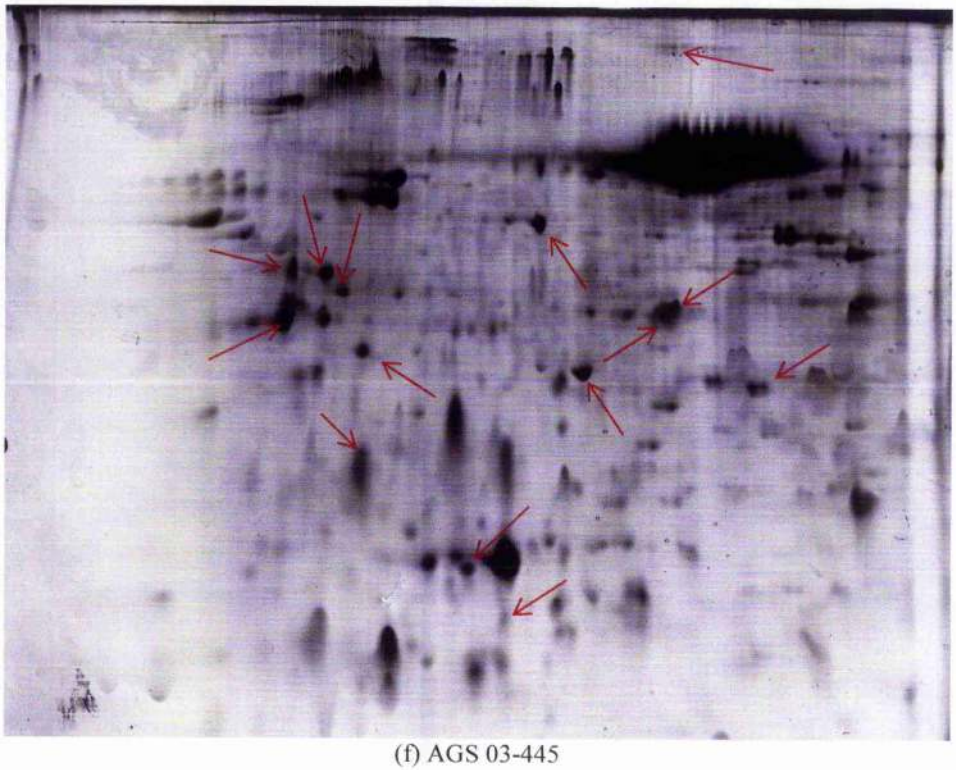


(d) AGS 03-408



(e) AGS 03-425

continued…



(f) AGS 03-445

Table 6. A list of all characterised proteins and some details about them (from the embl harvester at http://harvester.embl.de/) 2DE gels of EGS and control equine autonomic ganglia.

Protein	Function	Tissue/location	Comments
neuron cytoplasmic protein/ spinesin	predominantly expressed in neurones in their axons and at the synapses of moto neurones in the spinal cord	neuronal cytoplasm	type 2 membrane protein serine 5, transmembrane protein
DNA-dependant protein kinase catalytic			
tropomyosin beta chain	binds to actin filaments, plays a central role in association with the troponin complex in the calcium dependant regul ation of striated muscle contraction	muscle and non-muscle cells	belongs to the tropomyosin family
serum albumin precursor albumin	serum albumin is the main protein of plasma, binds water, calcium, sodium, potassium fatty acids, hormones etc main function is the regulation of colloidal osmotic pressure of blood	plasma	addition of 4 heme groups allows it to deliver oxygen to tissues
peripherin	adhesion molecule involved in stabilisation and compaction of other segment disks or in the maintenance of the curva ture of the rim. Essential for disk morphogenesis	integral membrane protein in the PNS	
glial fibrillary acidic protein	class 2 intermediate filament during the development of the CNS. Distinguishes astrocytes from other glial cells	CNS	Belongs to the intermediate filament family

Protein	Function	Tissue/location	Comments
vimentin	class 3 intermediate filament found in various non-epithelial cells phosphoryation is enhanced during cell division resulting in the reorganisation of vimentin filaments	highly expressed in fibroblasts	1 of the most prominent phosphoproteins in cells of mesenchymal origins Belongs to the intermediate filament family
P19 lipocalin	might have a transport function across the blood brain barrier	secreted into CSF, expressed mainly in the choroid plexus, lower expression in other brain areas	belongs to the lipocalin family
alpha-1-anitrypsin	inhibitor of serine proteases primary target is elastase also has a moderate affinity for plasmin and thrombin	extracellular and in plasma	belongs to serpin family deficeincy causes COPD
Trypsin precursor	Encodes a trypsinogen	pancreas and brain	belons to peptide family s1
beta tubulin	major constituent of micro tubules, binds 2 moles GTP polymerises to form micro tubules		belongs to tubulin family
phosphoglycerate kinase	glycolytic enzyme and acts as a polymerase alpha cofactor protein (primer recognition)		belongs to phosphoglycerate kinase family
heat shock protein Chaperonin groEL precursor Chaperonin (HSP60)	mitochondrial protein import and macromolecular assembly May facilitate correct folding of imported proteins and prevent misfolding and promote the refolding and assembly of proteins generated during stress	mitochondrial matrix	chaperonin (HSP60) family

Protein	Function	Tissue/location	Comments
inducible nitric oxide synthase	produces NO in vascular smooth muscle, mediating endothelial growth factor and blood clotting through activation of platlets.Neuro transmitter in brain and PNS	platelets, also ubiquitously expressed in skeletal muscle and brain	belongs to the NOS family
serotransferrin (transferrin)	iron binding transport protein, responsible for the transport of iron from sites of absoption and heme degredation to those of storage and utilisation. Serum transferrin may also stimulate cell proliferation	expressed by liver and secreted in plasma	belongs to the transferrin family
RNAse	digests double stranded RNA	added during sample prep	
elongation protein 4	promotes the GTP dependant binding of aminoacryl tRNA to the ribosome during bio synthesis	cytoplasmic	belongs to the elongation factor family
cysteine-rich secretory protein 3		secreted in neurophils loaclised in specific granuoles	belongs to the CRISP family
gamma enolase/ phophoenolpyruvate	glycolisis pathway	cytoplasmic	three classes of enolases exist, gamma is only found in the nervous system
fibroblast growth factor	involved in nervous system development and function	brain, eye etc	belongs to the herapin iron-binding growth factors family
Beta casein precursor	important role in determination of the surface properties of the casein micelles	extracellular; mammary gland, milk	belongs to the beta-casein family
Kappa casein precursor	stabilises micelle formation preventing casein precipitation in milk	extracellular; mammary gland, milk	belong to the kappa-casein family

Protein	Function	Tissue/location	Comments
Haptoglobin-1 precursor	haptoglobin combines with free plasma hemoglobin preventing loss of iron through the kidneys and protecting kidneys from damage by hemoglobin, while making hemoglobin accessible to degenerative enzymes	extracellular expression in the liver and secreted in plasma	beta chain is related to serine proteases, but no enzymatic activity itself.
Beta actin	highly conserved proteins involved in various types of cell motility	ubiquitously expressed in all eukaryote cells in the cytoplasm	3 groups: alpha (muscle) beta and gamma (co-exist in most cell types as components of cytoskeleton and mediators of cell motility
phosphoprotein	inhibits fas- and tnfrsf1a/ tnfr1-mediated caspase 8 activity and apoptosis. regulates glucose transport	ubiquitously expressed associated with microtubules most abundant in heart, brain, muscle, adipose tissue, which utilise glucose	higher levels in tissues from type2 diabetes sufferers. contains 1 death effector (ded) domain
Internexin intermediate precursor	neuronal intermediate filament that is able to self assemble. Involved in morphpgenesis of neurons	adult CNS. Expressed in brain as early as the 16th week of gestation	belong to the intermediate filament family
Mimecan precursor (osteoglycin)	induces bone formation	bone	belongs to the small leucine rich poteoglycans family

Appendix 3

List of Samples (supplied by the Edinburgh Royal (Dick) School of Veterinary Studies)

Case/ID No.	Date of collection	Diagnosis	Sex	Breed	Age
97-1053	15/11/02	control			
01-681	07/02/03	control	MN		
02-889	14/11/02	control	MN		
02-56	08/05/02	control			
02-182		control	F		
02-642	14/08/02	control	F		
02-913	17/11/02	AGS	F		
02-349	12/05/02	AGS	MN		
02-176		control	MN		
01-537		control	MN		
02-248		AGS	F		
02-376		AGS	MN		
02-352		AGS	MN		
02-302		AGS	MN		
03-094	24/02/03	control	MN	TB	5
03-342	05/05/03	AGS	F	TBx	10
03-392	22/05/03	AGS	MN	TBx	5
03-345	06/05/03	CGS	MN	Cob	9
03-359	27/05/03	CGS	MN	Irish cob	7
03-409	27/05/03	AGS	MN	pony	3
03-408	28/05/03	AGS	F	TB	8
03-425	30/05/03	AGS	MN	TBx	5
03-445	03/06/03	SAGS	F	Highland	3

Appendix 4

Commonly used reagents

Lysis buffer

19.2g 8M Urea,
1.6g 4% (w/v) CHAPS,
0.194g 40mM Tris Base
H_2O to 40ml

SDS sample buffer

62.5mM TRIS-HCl pH6.8
10% (v/v) glycerol
2% (w/v) SDS
5% (v/v) β-mercaptoethanol

Coomassie Brilliant Blue

0.25g of Coomassie Brilliant Blue R
90ml of methanol: H_2O (1:1 v/v)
10ml acetic acid

Colloidal Coomassie Brilliant Blue

5% Coomassie stock (5g Coomassie Brilliant Blue G in 250ml HPLC grade H_2O)
Colloidal stock: 50g ammonium sulphate
500ml H_2O
6ml phosphoric acid
10ml Coomassie stock

The actual stain: 1 part methanol
4 parts colloidal stock

TRIS-glycine electrophoresis buffer

15.1g og TRIS-base
94g of glycine
900ml H_2O
50ml of a 10% (w/v) stock solution of electrophoresis-grade SDS
Adjust the volume, with H_2O, to 1000ml

15% Resolving Gel

4.6ml H_2O

10ml 30%acrylamide
5ml 1.5M TRIS buffer pH8.8
200µl 10% SDS
200µl 10% APS
10µl TEMED

5% Stacking Gel

3.4ml H_2O
830µl 30%acrylamide
630µl 1.5M TRIS buffer pH8.8
50µl 10% SDS
50µl 10% APS
5µl TEMED

Rehydration buffer (stock solution)

12g Urea
0.5g CHAPS
trace bromophenol blue
make up to 25ml H_2O

SDS equilibration buffer

6.7ml 1.5M TRIS-Cl pH8.8
72.07g urea
69ml glycerol (87% v/v)
4g SDS
a few grains of bromophenol blue
make up to 200ml H_2O

10% APS

100mg APS in 1ml H_2O

10% SDS

5g SDS
50ml H_2O