IN VITRO SMALL INTESTINAL MOTILITY IN HORSES WITH AND WITHOUT EQUINE GRASS SICKNESS

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Thesis presented for the degree of
Doctor of Philosophy
The University of Edinburgh
July 1995

DECLARATION

I hereby certify that this thesis and the work it describes was written and performed by myself. Some of the material discussed in this thesis has been presented at meetings and/or published, reprints of which are included in an appendix to the thesis.

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ACKNOWLEDGEMENTS

I gratefully acknowledge the financial support of the Animal Diseases Research Association Equine Grass Sickness Fund and Janssen Research Foundation for this study. Firstly, I would like to thank my supervisor, Dr. David Cottrell for his constant advice and enthusiasm throughout my project. I am also indebted to my co-supervisors, Dr. Elspeth Milne and Dr. Geoff Pearson for their continual encouragement, help and support. Also to Dr. David Doxey, coordinator of the Edinburgh Grass Sickness Research Group. I am especially grateful to Marie Woodman for help in obtaining clinical material and introducing me to the joys of the *post mortem* room!

I would also like to thank Nicola Wallis, Tara Phillis, Sarah Tothill, Judith Shelly, Zoe Davies, Dr. Bruce McGorum, Andy McDiarmid, and Claire Henshaw for supplying me with clinical material and bleeping me regardless of the hour day or night!

I am grateful to Dr. N. Kitamura for help with translation.

I am very grateful to all the staff in the Department of Preclinical Veterinary Science for being so helpful and friendly throughout my time here. The technical assistance has been excellent, but special thanks must go to Andy Macrae (electronics workshop), Colin Warwick (photography), Gordon Goodall and Judith Watson (histology), Jimmy Brown and Graham Moodie (mechanical workshop), Ian Stuart (computing), Suzanne Sellar and Lucy Mitchell. I am indebted to my office mates, Katharine Leach, Kristin Leus and John Reilly for sharing the ups and downs of PhD life, and always having time to listen (even to the adventures of Morris). My sincere appreciation to Katharine for allowing me to use her computer for writing my thesis.

I should like to express gratitude to my parents for their moral support and the occasional food parcel throughout my time in Edinburgh. Finally I would like to thank Dave for his encouragement and understanding over the last three years, and Morris, my horse, alias New Age Traveller for his ability to keep a smile on my face throughout it all!

ABSTRACT

This thesis describes investigations of small intestinal motility in horses with and without equine grass sickness using an *in vitro* technique. Equine grass sickness is a disease of horses of unknown aetiology, characterised by dysfunction of the autonomic nervous system, primarily affecting the alimentary tract. Examination of autonomic ganglia taken from horses affected with grass sickness shows evidence of neuronal degeneration and ultimately depletion of cell numbers.

The *in vitro* technique implemented used strips of intestinal smooth muscle cut parallel to the longitudinal muscle layer from the duodenum and ileum. Tissue was taken from control horses and those affected with the three clinical forms of grass sickness (acute [AGS], subacute [SAGS] and chronic grass sickness [CGS]). Motility patterns were measured isometrically using strain gauge transducers and recorded onto a Washington ink writing oscillograph. Contraction rate and amplitude, alterations in tone (baseline) and the latency before a response to pharmacological agents were recorded.

The characteristics of the background contractions were established. In the control group the duodenal preparations had a significantly higher contractile rate than ileal preparations ($P<0.05$). There was no significant difference in contractile amplitude between the two regions. The contractile rate was reduced in grass sickness cases, although not always significantly. The effect of storage for 24 hours at 4°C was investigated to determine whether stored control tissue would subsequently behave like fresh grass sickness tissue: it was concluded that this was not the case.

Physostigmine was used to establish the viability of enteric cholinergic neurones and to test their capacity to release endogenous acetylcholine. All muscle strips from both control horses and those affected with grass sickness showed significant increases in the rate of contractions following physostigmine addition ($P<0.05$ or less). The latency before a response to physostigmine in the AGS and SAGS groups was significantly larger than for the control groups in both regions of the gut ($P<0.001$ for duodenal tissue, $P<0.05$ for ileal tissue). The effect of storage on responses to physostigmine confirmed that neuronal cell death takes place during this

storage period. Grass sickness tissue could no longer produce a significant increase in contractile rate following physostigmine. Dose response curves to bethanecol were constructed for control, AGS and CGS tissue. For the AGS group there was a leftward shift of the dose response curve and a reduced ED_{50} value, which suggested that the tissue has become supersensitive (denervation hypersensitivity). However, there were no significant differences between the tissue sensitivity of the CGS and the control groups.

Cisapride is a prokinetic drug which has been used with some clinical success in the treatment of selected chronic grass sickness cases. Experiments using cisapride indicated there was an increase in the contractile rate in control and CGS duodenal muscle strips ($P<0.06$) and in control, AGS and CGS groups in ileal regions ($P<0.06$). Cisapride also caused an increase in the amplitude of contractions in ileal muscle strips taken from control horses ($P<0.06$).

In agreement with other workers, it was found that *in vitro* equine small intestine contracts on addition of noradrenaline or adrenaline. This contractile response was found to be due to excitatory $\alpha 2$ receptors on the smooth muscle membrane. Grass sickness affected tissue responded similarly to control tissue in the duodenal region, however, in certain AGS and SAGS cases no contractile response to noradrenaline could be achieved until tissue was pretreated with the β antagonist propranolol. As cold storage had no significant effect on the contractile response to noradrenaline it would suggest the response was independent of nervous elements.

A subjective histological scoring method was applied on sections of small intestine adjacent to those used in pharmacological experiments, to investigate neuronal cell number, size and the proportion of abnormal cells in the myenteric and submucous plexuses. There was a significantly lower score for neuronal cell number for the AGS and CGS groups compared with control values ($P<0.05$). The evidence suggested that in CGS cases the largest enteric neurones were preferentially affected by grass sickness ($P<0.05$). In the ileal AGS group, the proportion of abnormal cells was significantly greater than both the control and CGS ileal groups ($P<0.05$). In the duodenum there was no significant difference between the proportion of abnormal cells between the AGS and CGS groups, although they were both awarded significantly higher scores than the control group ($P<0.05$).

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ABBREVIATIONS

AGS	Acute grass sickness
ATP	Adenosine triphosphate
CGS	Chronic grass sickness
Con.	Contractions
Duo.	Duodenum
ESP	External submucous plexus
g	Gram
Ile.	Ileum
ISA	Irregular spiking activity
ISP	Internal submucous plexus
min	Minute
mM	Millimolar
MMC	Migrating motor complex
MP	Myenteric plexus
mths	Months old
n	Number
NANC	Nonadrenergic noncholinergic
NS	Not significant
NSA	None spiking activity
P	Probability
physos.	Physostigmine
RSA	Regular spiking activity
s	Second
SAGS	Subacute grass sickness
SEM	Standard error of the mean
SP	Submucous plexus
SSB	Short spike burst
TB	Thoroughbred
TTX	Tetrodotoxin
UK	United Kingdom
VIP	Vasoactive intestinal polypeptide
yrs	Years old

CHAPTER 1

LITERATURE REVIEW

1.1 Equine grass sickness

1.1.1 History

Equine grass sickness, formerly known as 'grass disease', was first reported at a military training camp at Barry, Forfarshire, UK, in 1907, although odd cases may have been seen prior to this (Pool, 1928; Pool and Greig, 1928). The disease subsequently progressed through other regions of Scotland and by the 1940's had been recorded in Angus, Perthshire, Kincardinshire, Aberdeenshire, Fife, Inverness-shire, Lothians, Lanarkshire, Renfrewshire, Ayrshire, Wigtown, Kircudbright and the Tweed Valley. In 1939 the Agricultural Research Council carried out a survey which reported that several hundred cases had also occurred in different parts of England in the previous years (Greig, 1942). To date there have been no confirmed cases of grass sickness in Ireland. Grass sickness has however, been recognised in Sweden, Denmark, Germany, Belgium, France and Switzerland (Gilmour, 1989). There has also been an isolated case in the Falkland Islands (Wood and Gilmour, 1991). Similarities with the disease 'mal seco' in Argentina and Chile have lead to the suggestion that this is the same disease (Uzal, Robles and Olaechea, 1992; Uzal and Robles, 1993). Today the prevalence of grass sickness remains highest in Scotland, but the incidence varies widely from district to district.

In the early 1980's clinical conditions with virtually identical pathological changes to grass sickness were recognised in cats (Key and Gaskell, 1982) and dogs (Rochlitz and Bennett, 1983). Despite almost epidemic outbreaks of the feline disease at this time, the condition has now become uncommon. Similarities between these diseases (collectively classified as dysautonomias) were extensively discussed at the Waltham Symposium in 1984 (Edney, Gaskell and Sharp, 1987).

More recently, similar neuropathy to that seen in grass sickness has also been recognised in wild hares (Whitwell, 1991; Griffiths and Whitwell, 1993). In man there are no diseases recognised with identical pathological changes to those seen in grass sickness. Primary autonomic failure and autonomic failure combined with Parkinson's

disease, or associated with other neurological abnormalities, such as multiple system atrophy, have been reported (Price, 1988). Hirschsprung's disease is a congenital disease in children associated with an area of abnormal intrinsic innervation of the large intestine (Trounce and Nightingale, 1960; Heaton, Garrett and Howard, 1988).

1.1.2 Epidemiology

Although principally a disease of horses and ponies, grass sickness has occasionally been recorded in donkeys and once in a zebra (Ashton, Jones and Gilmour, 1977; Gilmour, 1989). All breeds of horse appear to be equally susceptible and whether animals are homebred does not appear to protect them from the disease (Doxey, Gilmour and Milne, 1991a). Grass sickness appears to be rare during the first year of life, and has only once been recorded in a suckling foal, which was 4 months old (Milne, E.M., personal communication). There is evidence that the causal agent of grass sickness does not cross the placenta. In pregnant mares which were euthanased due to the disease, none of the foetuses examined exhibited the characteristic histological changes seen in grass sickness (Whitwell, 1992). However, Gilmour (1973b) reported that a full-term foal born by caesarean section to a mare with acute grass sickness, had some of the lesions associated with grass sickness on histological examination, following its death 24 hours after its birth.

Following surveys undertaken in 1971 and 1972, Gilmour and Jolly (1974) concluded that grass sickness is more frequently encountered in animals between 2 and 7 years of age. No significant differences have been recorded in association with the sex of the animals (Greig, 1942; Gilmour and Jolly, 1974; Doxey *et al.*,1991a), with the exception of a preliminary study by Wood, Doxey and Milne (1994) which suggests that mares are at less risk than male horses.

Grass sickness occurs most frequently in horses at pasture. Cases of grass sickness in stabled horses given a diet free from green grass are rare. Gilmour and Jolly (1974) reported that only 2% of grass sickness cases were stabled full time, in their survey of 90 cases. Wood *et al.* (1994) however, reported that no horse with grass sickness was stabled full time in their survey. Greig (1942) recorded that grass sickness had frequently been observed in horses shortly after they had been moved

from one pasture to another. Later, Gilmour and Jolly (1974) confirmed this, stating that horses which had been on the premises for less than two months experienced grass sickness more frequently than those kept there for longer periods ($P<0.01$). Obel (1955) reported that at a Swedish remount station, animals acquired since the previous spring were those commonly affected by grass sickness. Grass sickness is also more likely to occur on premises previously affected than on those which have never experienced the disease (Doxey *et al.*, 1991a).

Grass sickness has a seasonal incidence, although cases are seen throughout the year. Pool (1928) reported that 60% of cases occurred in June and that the majority of these cases were of the acute type. As the season advanced, the disease was reported to become more chronic in its manifestations. It should be noted that at that time horses were turned out 'full-time' around 28th May. Doxey *et al.* (1991a) reported that the greatest number of cases occurred in May (27.5%) and very few cases occurred in August, January or February. August is often a very wet month in Scotland (Doxey, Gilmour and Milne 1991b). However, cases are currently seen throughout the year (Milne, E.M., personal communication). Pool (1928) remarked that cases became most numerous 10-14 days after most horses were put out at night. However, Gilmour and Jolly (1974) found only a low proportion of affected animals had been turned-out within four weeks before the start of the illness.

As early as the 1920's it was known that fine, dry, warm weather with frosty nights appeared to be a predisposing factor for the disease, while it stopped after rain. This has been confirmed by more recent epidemiological studies. Doxey *et al.* (1991b) found an association between grass sickness and mean temperatures of 7-11°C in the ten days preceding outbreaks of the disease on premises in Scotland. Ground frosts are also often reported prior to the occurrence of the disease. Doxey *et al.* (1991b) reported that in the 14 days preceding the first case of grass sickness during March, April or May there was a mean number of seven frosty mornings.

Grass sickness is associated with varied topographical conditions and has been encountered at all elevations from sea level up to 1200 feet (Greig, 1942). No significant differences in the association of grass sickness and the nature of the pasture has been found (Gilmour and Jolly, 1974; Doxey *et al.*, 1991a). It occurs on new

grass leys, hill and moorland, rough grass and permanent pasture. Doxey *et al.* (1991a) found that horses in good condition were more likely to get grass sickness than would have been expected from their reference population ($P<0.005$).

Doxey *et al.* (1991a) also reported that (in the grass sickness population) there was a high percentage of horses subjected to a stress such as foaling, castration or breaking, although no statistical analysis was carried out on these data. With regard to supplementary feeding, Gilmour and Jolly (1974) found that a higher proportion of horses suffering from grass sickness received no supplementary feeding or only had concentrates, compared with the group receiving hay with concentrates. However, this was not confirmed by subsequent surveys (Doxey *et al.*, 1991a; Wood *et al.*, 1994).

The mortality rate of grass sickness is high, approaching 90%. Most cases are euthanased on humane grounds (Pogson, Doxey, Gilmour, Milne and Chisholm, 1992). There is no evidence that an attack of the disease confers immunity. The disease does not appear to be contagious with only certain horses being affected on a particular premises (Gilmour and Jolly, 1974).

1.1.3 Clinical signs

There are a number of publications which describe the clinical signs of grass sickness in detail (Greig, 1942; Pinsent, 1989; Doxey, Milne, Gilmour and Pogson, 1991; Milne, 1991; Milne, Woodman and Doxey, 1994). The clinical signs vary with the type of case and the stage of the disease. Grass sickness occurs in three overlapping clinical forms; acute (AGS), subacute (SAGS) and chronic (CGS). Horses with AGS show signs of depression. Nasogastric reflux is often present in these cases, although spontaneous reflux is less common than reflux induced by nasogastric intubation. Acute cases usually have dysphagia indicated by difficulty in swallowing food and water, excessive salivation, and intestinal stasis with an absence of gut sounds. Salivation is often periodically very profuse but there is some doubt whether there is an absolute increase in salivary secretion, or whether it results from accumulation of saliva in the mouth due to dysphagia. Horses usually appear to want

to drink but are unable to do so in AGS. Signs of dehydration develop rapidly. Acute cases die or require euthanasia within 48 hours of the onset of the clinical signs.

In SAGS and CGS the clinical signs are less intense but weight loss is a marked feature. Muscle tremors and intermittent sweating are often present in these cases and there is a tendency for tachycardia. The horse often chews slowly, food drops out of the mouth and oesophageal choking may also occur. Horses frequently adopt a huddled posture. All cases have a reduced appetite. Breathing is usually normal in rate, but may sometimes be accompanied by a snoring sound from the nasal cavity. Milne *et al.* (1994) reported that rhinitis was common in chronic cases and was more severe in nonsurvivors.

In AGS and SAGS there may be secondary impaction of the large colon and a few pellets of dry sticky faeces may be passed in the early stages of the disease. A retention of urine has also been recorded. In geldings the penis is usually pendulous (Pool, 1928). In SAGS cases, which live more than a few days after the onset of clinical signs, there is an exceedingly rapid loss of condition, with the abdomen being tucked up to an exaggerated degree. Most horses with SAGS die or require euthanasia within two to seven days of the onset of the disease. A few cases progress to the chronic form, classified as lasting eight days or more. Subclinical cases of grass sickness are suspected to occur (Whitwell, K.E. discussion at the Waltham Symposium, 1984).

There is no definite *ante mortem* test for grass sickness diagnosis at present other than histopathological examination of ileal biopsies obtained at laparotomy (Scholes, Vaillant, Peacock, Edwards and Kelly, 1993b). Confirmation otherwise relies on *post mortem* examination of autonomic ganglia which show characteristic lesions (see Chapter 1.1.5). The clinical signs of AGS and SAGS can be very similar to certain types of equine colic. Analysis of peritoneal fluid demonstrates that grass sickness cases have a higher specific gravity and protein content than cases of medical colic, although the appearance of the peritoneal fluid is similar. Cases of surgical colic associated with gut ischaemia tend to have bloodstained peritoneal fluid, and a higher alkaline phosphatase activity than grass sickness cases (Milne, Doxey and Gilmour,

1990). However, there is considerable overlap between the peritoneal fluid profile obtained from grass sickness and colic cases.

1.1.4 Pathological Features

Gross *post mortem* findings tend to confirm the clinical observations. However, the gross pathological findings are largely non-specific and cannot be considered to be of a primary nature (Pollin and Griffiths, 1992). Linear erosions in the lower oesophagus are often present (Pinsent, 1989). Acute cases show dilation of the stomach and small intestine with greenish foul-smelling fluid (Milne, 1991). The small intestine may also be found to be empty. Usually there are no gross lesions found in the small intestine (Gilmour, 1987). The large colon and proximal small colon show impaction of ingesta, which often has a characteristic black coating of changed blood where it is in contact with the mucosa. However, gross mucosal lesions, including haemorrhage, are not usually seen (Gilmour, 1987). Mucous coating of faecal balls in the small colon in AGS and SAGS cases is sometimes seen (Milne, 1991). In SAGS and CGS cases there are few gross abnormalities, although the gastrointestinal tract is generally empty and in chronic cases the carcase varies from thin to emaciated (Milne,1991). In chronic cases the spleen is often enlarged (Greig, 1942).

1.1.5 Histological Findings

Obel (1955) was first to describe the characteristic changes in the paravertebral and prevertebral ganglia of grass sickness cases. She described chromatolytic ganglia, pyknotic nuclei and vaculation of cytoplasm. Other degenerative changes have since been recorded, such as loss of Nissl substance, foamy cytoplasm, and eosinophilic bodies (Mahaffey, 1959; Gilmour, 1975a; Hodson, Wright, Edwards, Mescall, Suswillo, Evans and Jeffries, 1984). Obel reported similar changes in the submucous plexus, but only slight changes in the myenteric plexus in acute cases of grass sickness. However, the specificity of these changes to grass sickness alone has been questioned (Brownlee,1959; 1965; Schuffler, 1990; Valentine, De Lahunta, George, Summers, Cummings, Divers and Mohammed, 1994).

Barlow (1969) observed that the severity of the lesions in grass sickness cases appeared to be directly proportional to the duration of the clinical illness i.e. CGS cases showed extensive chromatolysis. Gilmour (1973a) examined the autonomic ganglia, as well as certain brain stem nuclei and the intermedio-lateral nucleus of the spinal cord from 35 cases of grass sickness. He found that ganglionic lesions were related to the clinical severity of the disease rather than the duration of the illness. He suggested that chromatolysis of autonomic neurones may occur coincidentally with, or in advance of alimentary dysfunction, rather than as a consequence of it. Doxey, Pogson, Milne, Gilmour and Chisholm (1992) found no connection between the severity of the clinical signs of AGS and SAGS and the amount of neuronal damage in the superior cervical, stellate and coeliaco-mesenteric ganglia. A higher proportion of normal neurones were found in chronic cases than acute cases (60% normal neurones were observed in CGS compared with 26% for AGS, 25% for SAGS and 100% normal neurones in the control group). Only neuronal damage in the submucous plexus of the jejunum correlated with clinical severity in their study. Scholes, Vaillant, Peacock, Edwards and Kelly (1993a) reported that enteric neuropathy was widespread in acute cases but was localised to the distal small intestine in chronic cases.

Electron microscopical studies of ultrastructural changes that occur in grass sickness have been undertaken. Gilmour (1975a) first described the appearance of neuronal nuclei in grass sickness affected ganglia which were crenated, sited eccentrically in the perikaryon and had increased electron density of the nucleoplasm. Eosinophilic bodies were also observed which resembled dystrophic axons (Gilmour, 1975b). Pollin and Griffiths (1992) suggest that the lesions seen in grass sickness primarily involve the glycoprotein biosynthetic pathway of neurones and state that numerous toxins are known to have an effect on the organelles associated with protein synthesis in neurones.

The neuropathy of the enteric nervous system is discussed further in Chapter 7. The possible mechanism(s) for the neuronal damage which occurs in grass sickness is discussed in Chapter 1.3.

1.1.6 Possible aetiology

Despite intensive investigation, the cause of grass sickness is still a mystery. Greig (1928) first noted the similarity of the clinical signs of grass sickness with those of sympathicotonia. The one exception was the pupil which is rarely dilated in grass sickness. However, Greig (1928) and others found no appreciable effect upon the pupil following intravenous injection of adrenaline in the horse, which could explain this exception.

In 1942, Greig reviewed the early hypotheses of aetiology which included plant poisoning, a dietary deficiency, anaphylaxis, digestive disturbances (from poisonous products of intestinal origin), a toxic chemical substance developed in the pasture and living infective agents (invasive bacteria, protozoae parasites, fungal toxins, a toxin-producing bacterium and a filtrable virus). Tocher (1924) put forward a theory that grass sickness was caused by *Bacillus botulinus*, now known as *Clostridium botulinium*. There are some similarities between the clinical signs of grass sickness and botulism, however, botulism is usually associated with paralysis of the tongue and jaw which are not seen in grass sickness.

An association between *Clostridium perfringens* Type A enterotoxin and 'grass sickness' seen in Columbia was suggested (Ochoa and de Velandia, 1978). Gilmour, Brown and Johnson (1981) repeated the seroneutralisation tests carried out by Ochoa and de Velandia but their results suggested that there was no association between *Clostridium perfringens* Type A enterotoxin and grass sickness in Scotland. They therefore proposed that grass sickness in Europe and the equine disease in Columbia have a different aetiology.

The epidemiology, and particularly the association with grazing, or a change in pasture, suggests that grass sickness is caused by a toxin ingested with the herbage. Doxey, Robb, Milne and Gilmour (1990) discussed the possibility that grass sickness was due to a fungal toxin; grass sickness occurs in spring and early summer when fungal activity in pasture is high. They also suggested that the histological damage present in grass sickness was similar to that associated with a toxic process. However, the fungi isolated exclusively from horses with grass sickness in their study were unlikely to be toxin producers. It is possible that common fungi present in all horses

require specific conditions for toxin elaboration, or that there is a specific sensitivity of individual animals to specific fungal toxins.

Gilmour (1973b) and Gilmour and Mould (1977) experimentally reproduced the neurological lesions of grass sickness by injecting normal ponies with serum and plasma from acute cases, confirming that a neurotoxin is present. However, subsequent work has failed to identify this neurotoxin (Johnson, Dawson and Mould,1983; Johnson, 1985; Pemberton, Hodgson, Gilmour and Doxey, 1990). Griffiths, Smith, Doxey, Whitwell and Love (1994) injected serum from cases of grass sickness into the parotid salivary gland of experimental ponies and after a week, pathological changes similar to those found in the natural disease were found in the cranial cervical ganglion. This suggests there was a specific effect on the post ganglionic neurone supplying the tissue which was injected and that there was retrograde axonal transport to the cell body.

1.2 Gastrointestinal motility regulation

1.2.1 Introduction

To understand the pathophysiology of equine grass sickness a detailed knowledge of gastrointestinal motility in the horse is required. Despite the fact that disturbances of the gastrointestinal tract account for a large proportion of clinical disorders in the horse, there has been limited research into the regulatory mechanisms involved in the control of gastrointestinal motility (Davies and Gerring, 1983; Adams, Lamar and Masty, 1984; Sellars, Lowe and Rendano, 1984; Gerring and Hunt, 1986; Lester, Bolton and Thurgate, 1992). In addition to these studies, the following account summarizes the structural and functional features of the gastrointestinal tract using equine references wherever possible.

1.2.2 Structure of the gastrointestinal tract

The structure of the gastrointestinal tract varies throughout its length, but there are common features in its overall organization. The general layered structure of the wall of the gastrointestinal tract is shown in Figure 1.1.

The serosa is the outermost layer and consists mainly of connective tissue covered with a layer of squamous mesothelial cells. The muscularis externa consists of two layers of smooth muscle, an outer longitudinal layer and an inner circular layer. Between these muscle layers lies the myenteric plexus (Auerbach's plexus). The submucosa consists of loose connective tissue with collagen and elastin fibres. Within the submucosa there is a network of small ganglia and connecting strands which form the submucous plexus. In the horse this plexus can be divided further into the internal submucous plexus (ISP), which lies closest to the mucosa, and the external submucous plexus (ESP), which is closer to the circular smooth muscle (Pearson and Woodman, 1992; Pearson, 1994). The mucosa is made up of an epithelium, the lamina propria and the muscularis mucosae (Guilford, 1990; Herdt, 1992).

In the enteric nervous system of the pig, which has a similar structure to the horse, it is suggested that there are different physiological functions for the ISP and ESP, the former being involved with mucosal events and the latter with circular muscle control (Gunn, 1968).

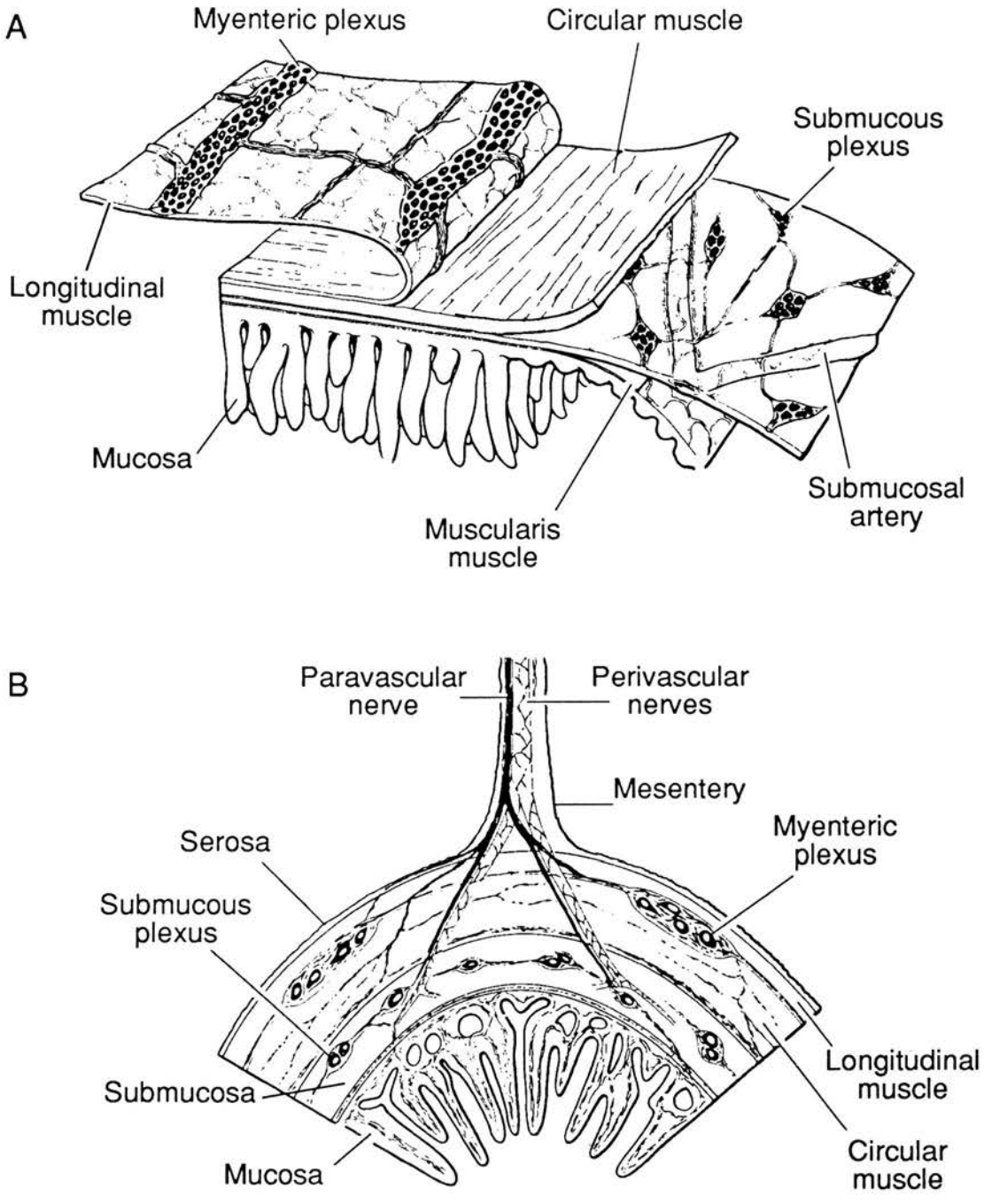


Figure 1.1. Diagramatic representation of the structure of the wall of the gastrointestinal tract, A) in whole mount , B) in transverse section, (modified from Furness & Costa, 1980, Neuroscience 5, 1-20).

1.2.2.1 Overview of gastrointestinal control mechanisms

The gastrointestinal tract is regulated at two levels. One level of control is by the extrinsic autonomic nervous system and endocrine systems, the second is by intrinsic nerves (the enteric nervous system) and endocrine components located within the gut. This intrinsic level of control allows the gut to regulate its function autonomously based on local conditions, such as distension of the gut wall and chemical stimuli. Control of the gut by the central nervous system is mainly secondary to the enteric mechanisms, although there are regional differences along the length of the gastrointestinal tract (Thompson, 1991). The small intestine is heavily dependent on the enteric nervous system whereas the stomach relies on extrinsic nerves such as the vagus for some of its reflexes (Grundy, 1985; Kutchai, 1993).

The extrinsic autonomic nervous system of the gastrointestinal tract can be divided into the parasympathetic nervous system, which generally increases gastrointestinal motility and the sympathetic nervous system, which opposes motility. Preganglionic parasympathetic nerves are always excitatory but they can synapse with excitatory or inhibitory enteric neurones. Preganglionic sympathetic nerves synapse in the paravertebral or prevertebral ganglia. Postganglionic sympathetic fibres either directly inhibit the smooth muscle or have an inhibitory effect on excitatory or inhibitory enteric neurones (Guyton, 1991; Kutchai, 1993).

The pharmacological receptors involved in parasympathetic and sympathetic gastrointestinal motility control can be divided into cholinoceptors and adrenoceptors respectively. Cholinoceptors are further divided into nicotinic and muscarinic receptors and adrenoceptors can be divided into alpha and beta adrenoceptors (Furness and Costa, 1987). Nonadrenergic noncholinergic (NANC) inhibitory nerves also innervate the gastrointestinal tract. These nerves contribute to propulsion of material, the reflex opening of sphincters, the receptive relaxation of the stomach, and descending inhibition during peristalsis (Kutchai, 1993). Several substances are believed to be transmitters in NANC nerves such as adenosine triphosphate (ATP) (Ruckebusch, Paneuf and Dunlop, 1991) and nitric oxide (Maczka, Thor, Lorens and Konturek, 1993; Wiklund, Leone, Gustafsson and Moncada, 1993). The NANC mechanisms are not discussed further within this thesis.

Certain hormones have important physiological roles in the control of gastrointestinal motility (for review see Walsh, 1987). Hormones are signalling molecules that are transmitted around the body via the blood stream. Many hormones may also act as neurotransmitters. Gastrointestinal hormones are all peptides, thus the term neuropeptide may be used to describe hormones such as gastrin and cholecystokinin (CCK).

1.2.3 Properties of intestinal smooth muscle

Intestinal smooth muscle possesses a mechanism for the self generation of rhythmic changes in excitability. These recurrent depolarizations of the muscle are referred to as slow waves, basic electrical rhythm, electrical control activity, or pacesetter potentials (Grundy, 1985). Intestinal motility depends on the superimposition of the action of nerves and hormones on this underlying rhythm.

While it is clear that slow waves are generated by muscle (or at least not by nerves), the ionic basis for the oscillations in membrane potential is not known. There is controversy as to whether the slow waves are due to oscillations in ionic permeability (increased sodium permeability being involved in the depolarizing phase; increased potassium permeability in repolarization) or to oscillation in a metabolically driven sodium pump (Daniel and Sarna, 1978; Benham and Bolton, 1983; Furness and Costa, 1987). Grundy (1985) presents good evidence for slow waves being due to the oscillatory activity of the electrogenic sodium/potassium pump. However, the ionic mechanism may not necessarily be the same in gastrointestinal smooth muscle of the horse.

When the membrane potential reaches a particular threshold, voltage dependent channels in the membrane are opened, spikes (or action potentials) are generated and the muscle contracts. In alimentary smooth muscle, spiking activity depends on the inward movement of calcium ions and, therefore, unlike action potentials in nerves and skeletal muscle, they are resistant to treatment with tetrodotoxin (Wood, 1972). Slow waves have a major role in setting the frequency of muscle contraction. The activity of nerves determine the amplitude of contraction associated with each slow wave (Furness and Costa, 1987).

The intrinsic frequency of slow waves (cycles/min) and velocity of propagation along the intestine (cm/min) varies with species and region of the gastrointestinal tract. Slow wave activity and spike bursts (contractile activity) have been recorded from the stomach, small intestine, caecum and colonic pelvic flexure of horses using electromyography. Davies and Gerring (1983) reported that the frequency of slow waves varies inversely with the distance from the duodenum in the horse. The frequency of slow waves measured ranged from 11.9-15.5 per minute in their study. Rates of slow waves of 5/min for the stomach, 15/min for the duodenum, 10/min for the ileum and 12-13/min for the pelvic flexure have also been reported in the horse (Ruckebusch, 1973: Adams *et al.*, 1984; Lester *et al.*, 1992).

Smooth muscle cells are arranged in bundles and are electrically coupled to each other. The occurrence of slow waves in one area therefore influences those in the next. In the equine gastrointestinal tract, there are thought to be at least three pacemaker regions. One is located in the gastric antrum and responsible for the progression of the migrating motor complex (phase III, see next paragraph) along the small intestine. This pacemaker also coordinates activity between the antral region of the stomach and duodenum to permit gastric emptying (Gerring, 1991). The second pacemaker is near the pelvic flexure and it is responsible for the bidirectional contraction waves needed both to retain digesta within the right ventral colon for microbial fermentation and to transport the digesta distally once this process is completed (Sellars, Lowe, Drost, Rendano, Georgi and Roberts, 1982; Sellars and Lowe, 1986; Ross, Donawick, Sellars and Lowe, 1986). The third pacemaker has been described near the apex of the caecal body, which is thought to be associated with the origin of a progressive series of coordinated spike bursts, which conduct through the caecocolic orifice and continue into the right ventral colon (Ross, Rutkowski, and Cullen, 1989). Jessen and Thuneberg (1991) suggested that the origin of the pacemakers in the guinea pig is the interstitial cells, a thin layer of which are present at the border between the circular and longitudinal smooth muscle layers. Phaneuf and Ruckebusch (1983) reported that in the horse stomach and small intestine, slow waves originate from the longitudinal smooth muscle layer.

The migrating motor complex (MMC) is the pattern of myoelectrical activity which propagates aborally along the intestine and occurs at regular intervals (Wingate, 1981). The spiking activity relates to contraction of the muscle. The MMC can be divided into 3 phases; phase I is slow waves with non-spiking activity (NSA), phase II is irregular spiking activity superimposed on the slow waves (ISA) and phase III is regular spiking activity (RSA) (Gerring, 1991). ISA is associated with peristaltic activity whereas RSA corresponds to rhythmic segmentation (Phaneuf and Ruckebusch, 1983). Adams *et al.* (1984) recorded periods of NSA, ISA and RSA in the horse, the mean interval from one RSA period to the next was 65.5 minutes. In the fasted horse, the MMC occurs about 20 times a day (Davies and Gerring, 1983). The velocity of MMC propagation is usually greater in the more proximal regions of the gut. A mean value of 7.3cm/min was recorded in the distal jejunum in the horse (Davies and Gerring, 1983). They estimated that the mean velocity throughout the whole small intestine was 32cm/min, which implies that at other regions the velocity of propagation is much higher. The mechanisms which initiate, drive and terminate the MMC are not fully understood. Ruckebusch (1981) suggested that the origin of the MMC was the antral region of the stomach, although in some species, such as sheep, the complex originates in the duodenum. It is suggested that the MMC is triggered by the release of a hormone or hormones. Duodenal alkalisation, which occurs after eating, has been found to stimulate the release of the hormone motilin. Injecting motilin induces premature MMC-like activity in the dog (Lee, Chey, Tai and Yajima, 1978; Walsh, 1987). Thus hormonal release may be related to, but not necessarily be the causal factor of MMC initiation. The initiation of the MMC may be due to some 'neural clock' in the enteric nervous system which gradually alters the activity of neurones (Furness and Costa, 1987). The propagation of the MMC depends on the enteric nervous sytem, as complete extrinsic denervation does not interrupt the MMC progression (Bueno, Pradduade and Ruckebusch, 1979; Oliveira, Meneghelli, Godoy, Dantas and Padovan, 1989). In certain species (e.g. dog, cat, and human), but not in the horse, interruption of the MMC occurs after feeding. This is probably due to a number of factors, such as chemical stimulation of the mucosa by nutrients (Furness and Costa, 1987).

The MMC is not a feature of the large intestine. Analysis of electromyographs from this region in the horse is based on the duration of the bursts of spiking activity. Short spike bursts (SSB) last less than 5 seconds and are usually localised or unpropagated, associated with mixing the digesta. Long spike bursts (LSB) last 10-20 seconds and are spread aborally and/or orally from a pacemaker in the pelvic flexure. LSB are associated with powerful contractions and the propulsion of digesta (Phaneuf and Ruckebusch, 1983). The relative percentages of SSB and LSB activity determine the retention time and alterations from normal may result in constipation or diarrhoea (Adams, 1987).

1.2.4 The enteric nervous system

The enteric nervous system can be defined as the system of neurones and their supporting cells which is found within the walls of the gastrointestinal tract, including the neurones within the pancreas and gall bladder (Furness and Costa, 1987). Numerous descriptions of the morphology and arrangements of the enteric ganglia and plexuses are available, although most studies have been carried out in laboratory species (Dogiel, 1899; Gabella, 1976; Furness and Costa, 1987; Wood, 1987; Furness, Bornstein and Trussell, 1988). Extrapolation of observations from the guinea pig to larger animals may not be justifiable. For example the submucous plexus of the horse (Pearson, 1994), humans (Hoyle and Burnstock, 1989) and pigs (Gunn, 1968; Scheurmann, Stach, Timmermans and Adriaensen, 1989) can be divided further into two plexuses, unlike the rat and guinea pig (Furness and Costa, 1987).

Functional studies have shown that there are several physiologically distinct neuronal cell types in the enteric nervous system. These include both excitatory and inhibitory motor neurones to the muscle, vasomotor neurones, secretomotor neurones, interneurones and sensory neurones (Hirst, Holman and Spence, 1974; Erde, Sherman and Gershon, 1985; Katayama, Lees and Pearson, 1986). The main physiological functions of the enteric nervous system are the control of gastrointestinal motility and blood flow, together with intestinal fluid and electrolyte secretion. Most neurones influencing motility are situated in the myenteric plexus

(Furness and Costa, 1987; Wood, 1987). The submucous plexus is involved in the control of secretion and absorption.

In the last 20 years, it has been found that a number of different substances, some of which may be neurotransmitters, are released in the gastrointestinal tract. These include acetylcholine, noradrenaline, substance P, vasoactive intestinal polypeptide (VIP), dopamine, ATP, enkephalin, somatostatin, dynorphins, 5-hydroxytryptamine, neuropeptide Y, neurotension, galanin, calcitonin gene-related peptide, angiotension, peptide HI, endorphins, gastrin releasing peptide, substance K, CCK and gamma-aminobutyric acid (Furness and Costa, 1987; Wood, 1987). The physiological roles of many of these substances with regards to control of gastrointestinal motility have been difficult to elucidate because they belong to groups of molecules with wide-ranging hormonal functions, in addition to any actions as neurotransmitters (Dockray, 1987).

Acetylcholine is the dominant excitatory neurotransmitter (Grundy, 1985). Substance P is released by nerve stimulation and is probably also an excitatory transmitter (Leander, Hakanson, Rosell, Folkers, Sundler and Tomquist, 1981). It also probably contributes a non-cholinergic excitatory component in the peristaltic reflex. There is evidence that VIP is an inhibitory transmitter to intestinal muscle, a vasodilator and a stimulant of mucosal water and electrolyte secretion (Furness and Costa, 1987). Dopamine is thought to be a neurotransmitter for interneurones and is the probable mechanism by which the sympathetic system effects some inhibitory control (Gerring, 1991). ATP is postulated as a transmitter from enteric inhibitory neurones but there is no adequate pharmacological antagonist to confirm this hypothesis (Grundy, 1985). In the horse, very little work has been carried out on possible functions of neuropeptides. Prostaglandins, such as prostaglandin E1, can increase motility in the gastrointestinal tract in ponies (Hunt and Gerring, 1985).

Immunohistochemical methods have allowed more detailed studies of the enteric neuronal circuitry. The distributions of nerve cell bodies and fibres showing immunoreactivity for putative neurotransmitters (mostly peptides) have been studied in detail for certain species, mainly the guinea pig (Costa and Furness, 1983; Ekblad, Ekman, Hakanson and Sundler, 1984). It is now generally accepted that each enteric

neurone contains more than one neuropeptide i.e. that colocalisation occurs (Furness and Costa, 1987). There have been some recent immunohistochemical studies carried out in the horse (Sabate, Stephenson, Bishop, Probert, Cole, Hodson, Yeats, Bloom and Polak, 1983; Merighi, Kar, Gibson, Ghidella, Gobetto, Peirone, Polak, 1990; Burns and Cummings, 1991 and 1993; Pearson and Woodman, 1992; Pearson, 1994).

Pearson (1994) found immunoreactivities for galanin, VIP, and neuropeptide Y in each of the enteric plexuses of the horse. Substance P was found in the enteric nerve fibres of the horse, but reactivity was weak in the cell bodies of the submucous neurones and less common in the cell bodies of the myenteric plexus. To date, there are no published studies on colocalization in the horse, although neuropeptide Y and galanin have been found to be colocalised in equine enteric neurones (Pearson, G.T., unpublished observations).

1.2.4.1 Reflexes in the enteric nervous system

Bayliss and Starling (1899) proposed the Law of the Intestine of ascending excitation and descending inhibition in response to a distension of the gut by a bolus, now commonly known as peristalsis or the peristaltic reflex. Langley and Magnus (1905) established that this reflex was due to intrinsic reflex circuits from *in vitro* experiments using rabbit gut. Even though this reflex was identified almost 100 years ago, there are still a number of questions that remain unanswered about its neural circuitry, including the nature of the chemical transmitters, type and location of sensory structures involved, and the type of stimuli that initiate the physiological response. After distension of the gut, the increase in amplitude of contractions oral to the distension is due to an increase in the number and frequency of spikes superimposed on every slow wave. Usually there is also a slight increase in slow wave frequency in the excited area. Furness and Costa (1987) suggest that the appearance of this new pacemaker area, associated with distension, was induced by acetylcholine being released from enteric cholinergic nerves, which are the final neurones of this reflex pathway. If the distension is more marked, the spikes may occur between, as well as on top of, the slow waves, resulting in a stationary ring of tonic contraction.

On the aboral side of the distension the existing contraction of the circular muscle ceases almost instantly. The corresponding spikes disappear although the slow waves can still be recorded. Two mechanisms contribute to descending inhibition; the actions of inhibitory motor neurones that cause inhibitory junctional potentials in the muscle (Hirst and McKirdy, 1974) and the inhibition of the excitatory cholinergic motor neurones. The latter has been shown to be by a presynaptic mechanism and is a consequence of a reduction in neurotransmitter release (Grundy, 1985).

Conclusions from the work to date are as follows: that interruption of the myenteric plexus abolishes the peristaltic reflex; the ascending excitation seen in the peristaltic reflex is blocked by muscarinic antagonists such as atropine or hyosine; there is also a non-cholinergic component of the reflex which is blocked by nicotinic antagonists such as tubocuarine, by substance P receptor desensitization or by substance P receptor antagonists; descending inhibition is mediated by a NANC transmitter, possibly VIP (Wood, 1987).

1.2.4.2 Reflexes involving the extrinsic nervous system

In the horse, if there is an obstruction in the gut the circular muscle proximal to the obstruction contracts. Should this persist, repeated vascular compression and increased metabolic demands may damage the intestinal mucosa and musculature. The intestino-intestinal inhibitory reflex acts to prevent this damage and results in the inhibition of the whole gastrointestinal tract (MacHarg, Adams, Lamar and Becht, 1986). This reflex involves the extrinsic nervous system.

1.2.5 Mechanisms of action of drugs enhancing gastrointestinal motility

Drugs are chemical substances which interact with biological systems and change them in some way. They are rarely selective and therefore have side actions as well as their main pharmacological action. There are a number of different mechanisms by which drugs work, with many acting by more than one mechanism (Adams, 1987). A number of drugs have an effect on the gastrointestinal tract.

Anticholinesterase drugs (physostigmine, neostigmine) combine reversibly with endogenous cholinesterase thereby promoting accumulation of endogenous

acetylcholine in tissues to produce both muscarinic and nicotinic effects. Neostigmine is used clinically in the horse but may cause pain associated with intestinal spasm (Adams *et al.*, 1984).

Other drugs act directly on the smooth muscle e.g. bethanecol, a muscarinic cholinergic receptor agonist, which is unaffected by cholinesterases (Kilbinger and Weihrauch, 1982). Bethanecol causes nonspecific cholinergic-like stimulation of the gastrointestinal smooth muscle, but it lacks the ability to coordinate intestinal motor activity. Its clinical use is therefore limited.

Cholinergic drugs are rarely administered to horses because of their unwanted side effects (Adams, 1987). Anticholinergic drugs e.g. atropine and glycopyrrolate are administered to horses for their cardiovascular effects. These drugs decrease motility and their effects may last beyond the duration of an anaesthetic (Roberts and Argenzio, 1986).

There is a group of prokinetic drugs e.g. metoclopramide, domperidone and cisapride, which have the ability to increase coordinated motor activity resulting in a net aboral movement of digesta. Metoclopramide increases tone in the lower oesphageal sphincter, increases the force and frequency of gastric antral contractions, relaxes the pyloric sphincter, promotes peristalsis in the duodenum and jejunum, resulting in accelerated gastric emptying and upper intestinal transit (Pinder, Brogden, Sauyer, Speight and Avery, 1976; Burrows, 1983). Metoclopramide improved transit time and restored coordination of gastric and small intestinal activity in an experimental model of post operative ileus (POI) in ponies (Gerring and Hunt, 1986). The mechanism of action is poorly understood, even though it is clearly a dopaminergic antagonist. The gastrointestinal effects of the drug can be blocked by the local or systemic administration of dopaminergic agonists. Albibi and McCallum (1983) suggest its action is due to either its effects on cholinergic nerve terminals and/or its antidopaminergic properties.

Domperidone possesses both prokinetic and antiemetic properties. It is also a dopaminergic antagonist whose effects closely resemble those of metoclopramide (Laduron and Leysen, 1979). Gerring and King (1989) reported domperidone is effective in restoring electrical and mechanical activity, coordination between gastric

and small intestinal activity cycles and the stomach to anus transit time in POI models in experimental ponies. Cisapride is discussed in further detail in Chapter 5.3.

Adrenergic agonists e.g. noradrenaline, dopamine and isoproterenol are not currently used clinically to modify intestinal motility, but are commonly used in general anaesthesia. They inhibit gut motility by direct inhibition of the smooth muscle or by decreasing the release of acetylcholine from enteric neurones (Adams, 1987). Adrenergic antagonists such as yohimbine (an alpha antagonist) produce a slight increase in propulsive motion in the intestines of ponies with experimental ileus (Gerring and Hunt, 1986).

1.2.6 Conclusion

The control of gastrointestinal motility can be divided into myogenic, neural and humoral components. This thesis is concerned with the enteric nervous system. To date only very limited investigation into the morphology, histology, physiology and transmitter neurochemistry of the enteric nervous system of the horse have been performed. There is considerable evidence of pathological changes in the enteric nervous system that are associated with disorders of the gastrointestinal tract (Smith, 1972). In the horse, enteric neuropathy has been reported in cases of grass sickness (Doxey *et al.*, 1992; Scholes *et al.*, 1993; Doxey, D.L., Milne, E.M., Woodman, M.P., Gilmour, J.S. and Chisholm, H.K. submitted for publication). An improved knowledge of enteric neuropathy in the horse and the possible mechanisms for denervation in grass sickness would therefore be useful.

1.3 Enteric neuropathology

1.3.1 Introduction

The enteric nervous system may be affected by a wide variety of pathological processes which include congenital, degenerative, inflammatory, metabolic and parasitic disease. The aetiology of grass sickness is unknown but there is evidence to suggest that a neurotoxin may be implicated (Gilmour, 1973b; Gilmour and Mould, 1977). The route by which the putative neurotoxin reaches susceptible neurones is unknown.

Although a great deal is known about the histological, ultrastructural and chemical effects of chronic denervation on skeletal muscle, little is known about denervation in alimentary smooth muscle. However, the end result of damage to intrinsic nerves is loss of coordinated peristalsis, smooth muscle hypertrophy and dilation of the lumen (Smith, 1972; Heaton, *et al.*, 1988).

This section includes discussion of mechanisms of neuronal cell death, the phenomenon of neuronal cell depletion with age and some congenital enteric diseases.

1.3.2 Mechanisms of cell death

The mechanism of neuronal cell death which occurs in cases of grass sickness is unknown. In cell degeneration there may be a loss of cellular material (atrophic) or an accumulation of cellular constituents (dystrophic), both of which result in cellular necrosis (Agid and Blin, 1987). Necrotic cells have leaky membranes through which intracellular constituents escape and normally excluded extracellular molecules, such as calcium, enter (Schanne, Kane, Young and Farber, 1979; Nayler, 1981). The mode of action of calcium in cell death is uncertain. It is known to inhibit the membrane sodium/potassium ATPase and thus calcium in abnormally high concentrations could abruptly amplify the intracellular sodium gain and potassium loss. However, it appears more likely that the primary site of action of the incoming calcium is on membrane lipids, activating phospholipase and thus initiating the dissolution of membranes (Wyllie, Duvall and Blow, 1984). It is possible that the uncontrolled swelling of the injured cell, resulting from fluid influx secondary to the failure of membrane pumps,

itself promotes irreversible changes through physical disruption of the membranes (Ganole, Worstell, Iannotti and Kaltenbach, 1977).

Lysosomes appear to play little part in the initiation of necrosis, although their contents become dispersed in the later phases and probably contribute to the terminal intracellular disruption (Wyllie *et al.*, 1984). It is thought that lysosomal hydrolases, leaking from necrotic cells, may also cause secondary effects on adjacent tissue cells, including the initiation of inflammatory and reparative responses, however the evidence for this is not conclusive (Wyllie *et al.*, 1984). Instead, products of membrane phospholipids such as platelet activating factor may be responsible (Camussi, Pawlowski, Tetta, Forrinello, Alberton, Brentjens and Andres, 1983).

Some workers (Gilmour, 1975b and 1976; Hodson and Wright, 1987) have proposed that in cases of grass sickness the axonal lesions are primary and that the changes to the neuronal cell body represent a retrograde axonal reaction, such as that seen after axonotomy. However, Pollin and Griffiths (1992) suggested that the primary effect is on the neuronal cell body and that axonal changes are secondary. Most recently Griffiths, *et al.* (1994) suggested that the putative neurotoxin gains access to the axon terminal and is then transported retrogradely along the axon to the neuronal cell body where it exerts its toxic effect.

There are numerous examples of agents such as viruses, lectins and neurotoxins being transported retrogradely along axons to neuronal cell bodies (Grafstein and Forman, 1980; Harper, Gonatas, Mizutani and Gonatas, 1980; Wiley, Blessing and Reis, 1982; Wiley, Donohue-Rolfe and Keusch, 1985).
Grafstein and Forman (1980) reviewed intracellular transport in neurones and reported that the rates of retrograde axonal transport vary from about 75 to 500mm per day. If similar rates of transport occurred in the horse, neurones with short processes such as those found in the enteric nervous system would be damaged within one or two days of exposure to the toxin in the gut lumen.

Other routes for the 'neurotoxin' spread in grass sickness cases such as cell to cell transfer between Schwann cells or passage along the connective tissue sheaths of the nerve are less probable (Griffiths *et al.*, 1994).

1.3.3 Effects of ageing on the enteric nervous system

In view of the relationship between age and incidence of grass sickness it is interesting that ageing has an effect on the nervous system. A growing number of studies have been concerned with changes in autonomic nerves in development and ageing (Collins, Exton-Smith, Jones and Oliver, 1980; Baker and Santer, 1988; Willis and Douglas, 1988; Burnstock, 1990). Many of these studies concern the innervation of blood vessels. Gabella (1989) found that the number of myenteric neurones in the small intestine of ageing guinea pigs is about half that found in young adult guinea pigs. Santer and Baker (1988) agreed with this in their experiments using rats. Gabella also reported that degenerating neurones were occasionally seen in apparently healthy enteric ganglia, especially in older animals. A study is needed to determine whether neuronal cell reduction occurs with advancing age in the horse. G.F. Schusser and N.A. White (1994 unpublished data) found that foals had a greater number of myenteric plexuses and neurones than adult horses in specific segments of the large and transverse colon. The lower number of neurones seen in adults may be due to the neurones present at birth being stretched apart during the growth in length and circumference of the gastrointestinal tract (Schusser and White, 1994).

Morphological and histochemical evidence suggests that the influence of the sympathetic innervation on intestinal function declines with age. Baker, Watson and Santer (1990) suggested that with age there is either a decrease in the beta-adrenoceptor number in relation to the muscarinic receptor density or a decrease in the coupling of beta-adrenylate cyclase.

Many reports describe changes in peptide populations with age. Burnstock (1990) reported a decrease in the expression of putative vasoconstrictor cerebrovascular neurotransmitters (noradrenaline and 5-hydroxytryptamine) in ageing rats, but an increase in putative vasodilator neurotransmitters (VIP and calcitonin gene-related peptide). These findings are interesting in relation to the increased incidence of cerebrovascular disorders in elderly humans. There is also increasing evidence that the expression of autonomic transmitters can alter in diseases such as Hirschsprung's disease (Hamanda, Bishop, Federici, Rivosecchi, Talbot and Polak, 1987). There is, as yet, no evidence for an age-related effect of peptide population in

the horse (Burns and Cummings, 1991). There have been a number of studies which report reductions in neuropeptide populations in the enteric nervous system in cases of grass sickness (Sabate, Stephenson, Bishop, Probert, Cole, Hodson, Edwards, Yeats, Bloom and Polak, 1983; Bishop, Hodson, Major, Probert, Yeats, Edwards, Wright, Bloom and Polak, 1984; Vaillant, 1991).

1.3.4 Intestinal congenital aganglionosis in the horse

A congential absence of enteric neurones may also occur. Aganglionosis is the absence of enteric ganglia, the effect of which is to produce a region of atony in the gut. Very few accounts of intestinal aganglionosis in the horse have been reported which do not involve the Overo breed (Anderson, King and Rotwell, 1987; Murray, Parker and White, 1988). The mating of two Overo horses (a type of spotting pattern) occasionally results in the production of a white foal which appears clinically normal at birth. However, the foal fails to pass meconium, quickly develops colic and dies between 23 and 132 hours later (Hultgren, 1982). The syndrome is commonly called 'lethal white foal' in the spotted horse industry. Overo horses are numerous in the United States but are uncommon in Europe and other parts of the world. Microscopically, the myenteric and submucous neuronal plexuses are found to be absent throughout the large intestine and in extensive portions of the small intestine. These findings are consistent with the primary diagnosis of ileocolonic aganglionosis. The only other significant finding is the lack of melanin in the skin (Vonderfecht, Trommershausen Bowling and Cohen, 1983). The mode of inheritance of this congential disease is undetermined. Male as well as female foals are affected.

1.3.4.1 Intestinal aganglionosis in other species

Syndromes of congenital intestinal aganglionosis have been reported in other species. A colonic aganglionosis has been described in a strain of piebald mice (Webster, 1974; Brann, 1977). In this strain of mice congenital megacolon results from the expression of a simple recessive gene. Hirschsprung's disease in man is a congenital defect characterised by a region of aganglionic large intestine (Trounce and Nightingale, 1960; Sane and Girdany, 1973; Schuffler, 1990).

CHAPTER 2

THE SCOPE OF THE THESIS

The aims of this work were to increase our knowledge about the nature and extent of damage that occurs to the enteric nervous system in cases of grass sickness and improve our understanding of the relationship between gut motility and muscle and neuronal excitability in the control and grass sickness affected horse. In order to achieve this, the following procedures were developed:-

1) Apparatus was designed to investigate the mechanical activity of horse small intestine, using strips of intestinal smooth muscle cut parallel to the longitudinal muscle layer, organ baths and isometric strain gauge transducers.

2) A method for transporting horse gut to the laboratory in a viable state was devised, involving an iced, oxygenated, modified Krebs solution.

3) The characteristics of background motility in the control horse was established and the differences for the three clinical forms of grass sickness (acute, subacute and chronic) recorded.

4) The responses of control horse small intestine to selected cholinergic and adrenergic pharmacological agents were established.

5) The reports in the literature that *in vitro* horse small intestine contracts in response to the addition of catecholamines was investigated and the mechanism of action determined using adrenergic antagonists.

6) Responses of small intestine taken from horses affected by grass sickness to pharmacological agents were compared with control values and with the clinical severity of the disease.

7) All the results were analysed for statistical significance using the computer statistical package Minitab.

8) The extent of histological damage to the enteric nervous system was investigated using a subjective scoring system and these results were compared with selected pharmacological data.

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection of tissue

All tissue was collected from the Royal (Dick) School of Veterinary Studies, Veterinary Field Station, Bush Estate, Edinburgh. Horses and ponies (100-600kg) of either sex were killed by intravenous (jugular) injection of 50-250ml of a 200mg/ml solution of sodium phentobarbitone (Euthatal(R), Rhone Merieux, Harlow, UK) depending on body weight and hydration status. Following euthanasia, they were immediately exsanguinated from the jugular veins.

Control horses were defined as those euthanased for diseases unrelated to grass sickness such as orthopaedic cases (details of control horses are given in the appendices). The clinical cases of grass sickness were assessed to be acute, subacute or chronic depending on the severity of the clinical signs and the duration of the illness (see Chapter 1.1.3). Horses with AGS showed signs of severe depression, dysphagia and intestinal stasis and were euthanased within 48 hours of the onset of clinical signs. Horses with CGS had less severe clinical signs and survived for eight days or more before euthanasia. Horses with SAGS showed intermediary clinical signs and survived for two to seven days before euthanasia. Diagnosis of grass sickness was confirmed *post mortem* by histological examination of autonomic ganglia (by the staff of the Department of Veterinary Pathology) which identified characteristic lesions (Gilmour, 1973; Howell, Baker and Ritchie, 1974, Chapter 1.1.5).

Samples of small intestine (ten cm lengths) were removed within 15 minutes of death from two sites; the caudal flexure of the duodenum and the terminal ileum proximal to the ileocaecal fold. Figure 3.1 is a diagrammatic representation of the sample sites. The two areas were chosen to determine if grass sickness affected two widely spaced regions of the small intestine to different degrees. Tissue was immediately placed in chilled, oxygenated modified krebs solution which was in a container surrounded by ice. The tissue was continually aerated with 95% oxygen and 5% carbon dioxide using filled balloons attached to three-way taps and cannulas which passed into the modified Krebs solution. The tissue was then transported to the

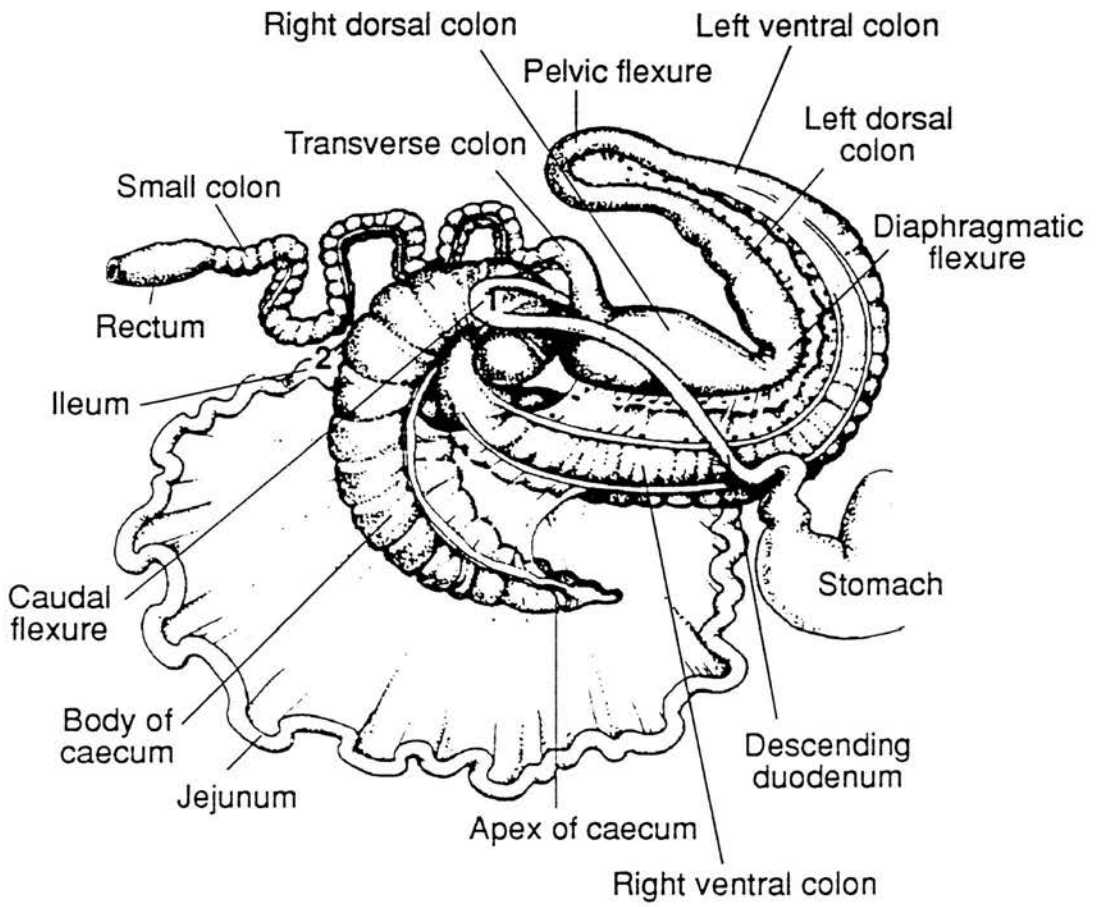


Figure 3.1. The equine gastrointestinal tract showing the sampling sites, 1) the caudal flexure of the duodenum, 2) the terminal ileum. Adapted from; Lehrbuch der Anatomie der Haustiere. Nickel, R., Schummer, A. and Seiferle, E. (1975). Verlag Paul Parey, Berlin and Hamburg, Germany.

laboratory, which took approximately 15 minutes. Pharmacological investigations were performed either immediately (within one hour) or after storage for 24 hours at 4°C. Additional samples were fixed in Bouins solution for histological purposes.

3.2 Pharmacological experiments

On arriving in the laboratory the tissue was transferred into fresh modified Krebs which had been kept on ice and continuously aerated with 95% oxygen 5% carbon dioxide while the tissue was being collected.

Strips of smooth muscle (3x0.5cm) were cut parallel to the longitudinal muscle layer. The mucosa was carefully removed using forceps and a scalpel blade and the strips were suspended in an organ bath (35ml) using a glass rod as shown in Figure 3.2. For duodenal samples from both control and grass sickness material the initial tension applied to the strip of smooth muscle was 2g. For ileal strips, the circular muscle was very thick especially in larger horses and had a tendency to curl up. The tension used for these strips was therefore sufficient just to straighten the preparation; in some ileal strips from control horses this was as great as 35g.

The modified Krebs solution contained mM/litre; sodium chloride 119.8, potassium chloride 3.4, calcium chloride 3.0, magnesium chloride 0.6, sodium hydrogen carbonate 26.2, sodium hydrogen phosphate 1.3 and glucose 6.7. Chemicals were standard laboratory chemicals of Analar grade. This provided salts within the range of normal horse serum and had a pH of 6.8. The modified Krebs solution was maintained at 37°C and aerated with 95% oxygen and 5% carbon dioxide, with the use of a needle into the silicon tubing which attached the organ bath within the water bath. The supply of oxygen was kept constant with the use of a metal tap. Figure 3.2 shows a flow diagram of the apparatus.

Motility was measured isometrically using a Washington Type D strain gauge transducer (Washington Ltd., Kent, UK) and recorded on a Washington 400 MD2 ink writing oscillograph pen recorder (Washington Ltd.). For each experiment there were four sets of apparatus which were set up in the corners of a single perspex water bath (Figure 3.3). Each pen recorder had a range of sensitivities which could be chosen

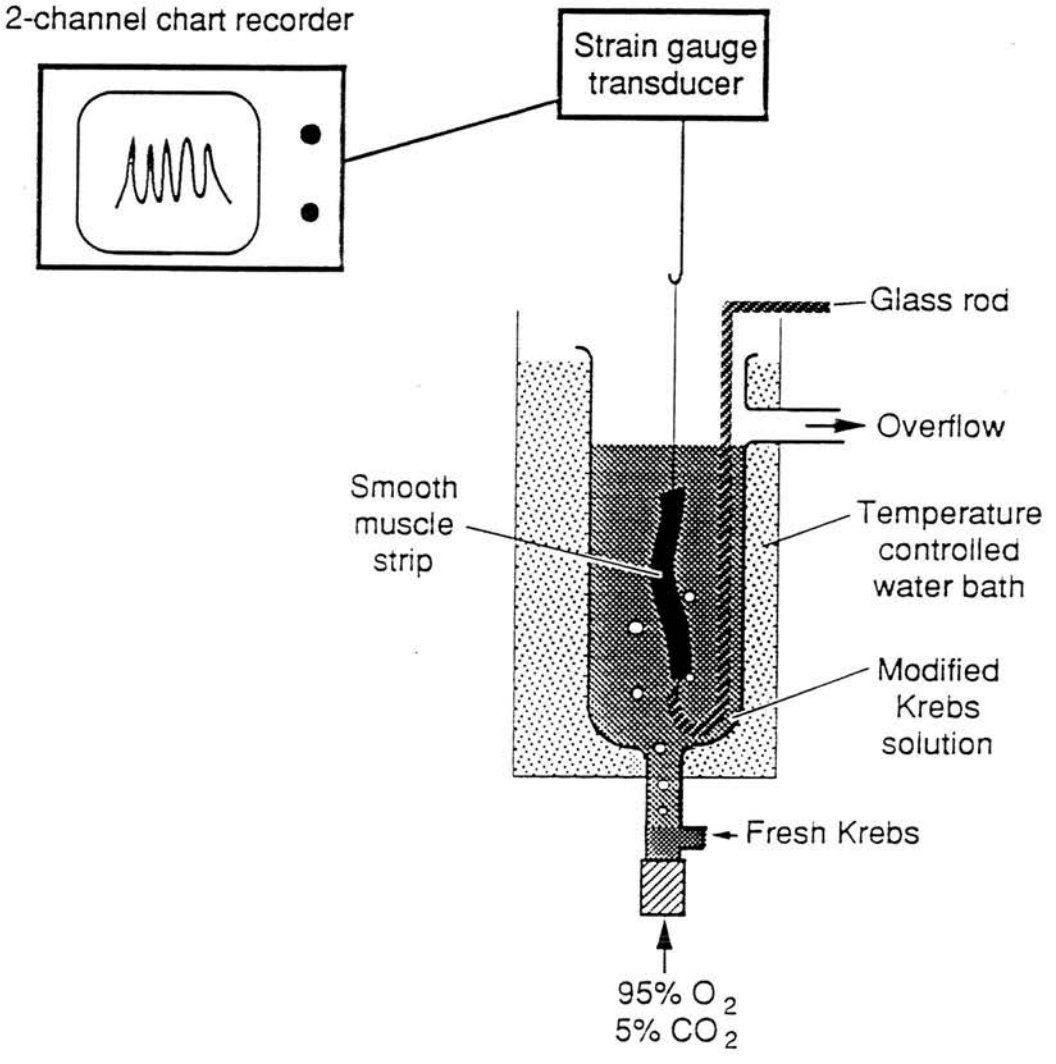


Figure 3.2. Flow diagram of *in vitro* pharmacological apparatus.

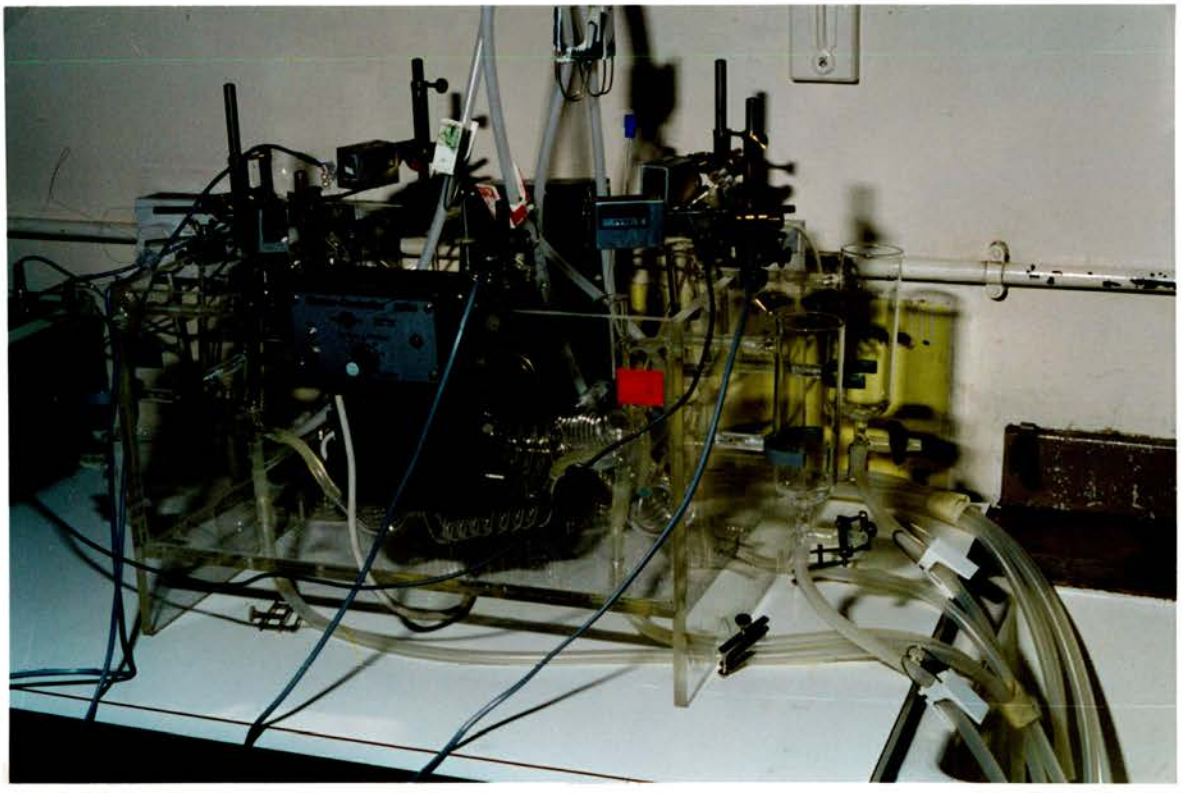


Figure 3.3. Apparatus showing perspex temperature controlled water bath. It contains one organ bath in each corner. Glass coils heat up fresh modified Krebs solution to 37 °C. Strain gauge transducers measure motility isometrically which is recorded on pen recorders.

depending on the amplitude of the background contractions and the amplitude of the responses being recorded. For each sensitivity the strain gauge transducers were calibrated using standard metal weights. Measuring the pen amplifier deflections with a ruler allowed calibration curves to be calculated. Initially calibrations were carried out monthly but as there were no significant differences subsequent measurements were made less frequently.

The parameters recorded were the rate of contractions (contraction peaks were measured over a ten minute period), the peak amplitude (which was the force in grams of an individual contraction which returned to the baseline), the tone (which was a sustained change in baseline), and the latency (which was the time before a measurable response could be seen following addition of a drug).

A one hour equilibration period was allowed for all fresh tissue, and a two hour period for tissue which had been stored for 24 hours, during which time background contractions developed in all the preparations.

3.2.1 Drugs used

The cholinergic and adrenergic systems of gastrointestinal motility control were investigated using the following drugs: physostigmine sulphate (BDH Chemicals, Poole, UK), bethanecol chloride (Sigma Chemical Co., St. Louis, USA), atropine sulphate (Sigma), tetrodotoxin (Sigma), noradrenaline hydrochloride (Aldrich Chemical Co. Ltd., Dorset, UK), adrenaline hydrogen tartrate (BDH), (S)-propranolol hydrochloride (Sigma), phentolamine hydrochloride (Sigma), prazosin hydrochloride (Sigma), yohimbine hydrochloride (Sigma) and cisapride (Janssen Research Foundation, Wantage, Oxon, UK). The tetrodotoxin was obtained in pure crystalline form and was dissolved in citrate buffer (pH 4.5) before use. Cisapride was dissolved in 0.1ml acetone before being made up to a 1mM solution with fresh modified Krebs solution. All other compounds were prepared daily in modified Krebs solution and kept on ice for the duration of the experiment. Drugs were superfused and washed out by overflow using fresh modified Krebs solution at 37°C. Details of the end bath concentrations used are given in the relevant Chapters.

3.2.2 Statistical analysis

For all statistical tests, probability levels less than or equal to 5% were taken to be significant. Data was analysed using the Minitab computer package, using the non parametric Mann-Whitney U-test and Kruskal-Wallis test for analysing differences between the control and grass sickness groups. The Wilcoxon Signed Rank test was used to measure effects of drugs within a group i.e. for paired data.

3.3 Problems of tissue taken from animals after barbiturate euthanasia

Barbiturates affect neurotransmitter release and ion conductance (Rall, 1990). However, initially an experiment was carried out to see if pentobarbitone could be added *in vitro* to smooth muscle preparations to an estimated lethal dose (0.8mg/g of tissue pentobaritone sodium, 'Lethobarb', Duphar Veterinary Limited, Southampton, UK) and then be washed off again without permanently affecting the rate of background contractions. It was found that pentobarbitone could be washed off after several minutes. Following the initial equilibration period for the muscle strips, therefore, all traces of barbitruate should have been removed. Any concern about residual effects of barbitruate used for euthanasia was therefore dispelled.

3.4 Reasons for 24 hour storage

Tissue was sometimes stored for 24 hours at 4°C in unoxygenated modified Krebs solution in the fridge. This was because early researchers had reported using this method as a technique for causing neuronal death within the enteric nervous system (Vogt, 1943; Kuriyama, Osa and Toida, 1967; Ruckebusch, Grivel and Fargeas, 1971). The aim was to determine whether the neuronal damage that occurred due to storage of control tissue was a suitable model for the neuronal damage known to occur in cases of grass sickness.

3.5 Histological investigations

A light microscopical investigation of the enteric nervous system was carried out on samples of small intestine adjacent to those used for the pharmacological experiments (Chapter 7).

CHAPTER 4

BACKGROUND MOTILITY OF SMALL INTESTINAL SMOOTH MUSCLE STRIPS

4.1 Introduction

Studies of gastrointestinal smooth muscle *in vivo* are limited by such factors as ethical and technical difficulties, by anaesthetic drugs that may be needed, by regulatory effects of extrinsic nerves, by reflexes arising from other parts of the body and by the effects of hormones. These problems do not occur *in vitro* but there are other problems associated with isolation of tissue. There is, for example, no blood supply, so tissue must be kept viable using a warmed oxygenated physiological solution. Also drugs administered into an organ bath may not diffuse into tissue in the normal manner. There may also be changes in tissue sensitivity during the course of an experiment. With these reservations, *in vitro* studies using clinical cases euthanased for reasons unrelated to grass sickness, as well as grass sickness cases, can provide useful information about the mechanisms of equine gut motility, and how it is altered by grass sickness (Murray, Cottrell and Woodman, 1994).

The phenomenon that intestinal smooth muscle undergoes myogenic rhythmic changes in excitability, referred to as slow waves, was discussed above (Chapter 1.2.3). Spikes, or action potentials, are superimposed on these slow waves which lead to contractions of the smooth muscle and it is these background contractions (also referred to as background motility) which can be recorded isometrically *in vitro.*

The aims of this Chapter were to establish the characteristics of the background contractions from the two regions of the small intestine from both control and grass sickness affected horses. Effects of selected pharmacological agents on these background contractions were also investigated.

4.2 Materials and methods

The method of tissue collection, transportation to the laboratory and protocol for setting up intestinal smooth muscle strips into organ baths was discussed above (Chapter 3). Following a one hour equilibration period, the pen recorders were

switched on and the background motility of the equine small intestinal smooth muscle strips recorded isometrically.

The characteristics of the background contractions were calculated for each of the four groups i.e. control, AGS, SAGS and CGS and both regions of the small intestine. Eight horses were used for each group. A minimum of five muscle strips were used to investigate how these background contractions were altered by selected pharmacological agents (see Appendix B for details of horses).

The drugs used were tetrodotoxin (TTX) (Sigma), atropine sulphate (Sigma), (S)-propranolol hydrochloride (Sigma), phentolamine hydrochloride (Sigma), prazosin hydrochloride (Sigma) and yohimbine hydrochloride (Sigma) (see Chapter 3.2.2 for details of the preparation of these drugs).

Samples of duodenum and ileum were examined fresh and after storage at 4°C for 24 hours in unoxygenated modified Krebs. As it took longer for background contractions to develop with stored tissue, a two hour equilibration period was allowed before recordings of the background contractions were taken.

To measure the characteristics of the background contractions, a ten minute recording period was used. The rate of contractions was determined by counting the number of contractions in this period by hand. The amplitude of each individual contraction was measured with a ruler and the mean amplitude calculated. The equivalent isometric force in grams was then calculated using calibration graphs constructed for each transducer, at each sensitivity, for each pen recorder (see Chapter 3.2). The amplitude of contractions was then expressed in terms of grams per gram of wet tissue weight, to take into account some of the variation in tissue thickness between samples.

4.2.1 Statistical analysis

The smooth muscle strips which were used to establish the baseline values for rate and amplitude of background contractions (Tables 4.1 - 4.4) were from different horses to those used for measuring the effects of pharmacological agents on the characteristics of the background contractions, which are described in Chapter 4.3.2. The characteristics of background contractions, following treatment with

pharmacological agents, were therefore analysed for statistical significance using the Mann-Whitney U-test. The same horses were used to study the duodenal and ileal regions, therefore the Wilcoxon Signed Rank test was used to test for significant differences in the characteristics of background contractions between the two regions.

For stored tissue the same horses were used to establish the initial rate and amplitude of the background contractions and the effect of adrenergic antagonists on these characteristics. Therefore any differences due to these pharmacological agents were analysed using the Wilcoxon Signed Rank test. Differences between the two regions of the small intestine were also analysed using the Wilcoxon Signed Rank test. Differences between fresh and stored tissue for the rate and amplitude of background contractions were analysed using the Mann-Whitney U-test. The grass sickness data were also combined into one group and the analysis carried out between the control and grass sickness groups.

4.3 Results

4.3.1 Characteristics of background contractions

4.3.1.1 Duodenal preparations

Within the one hour equilibration period, background contractions developed in all preparations from both control horses and those affected by grass sickness. The tone, i.e. baseline, of all the smooth muscle preparations remained constant following a preliminary relaxation in response to the initial tension applied to the smooth muscle strip (Figure 4.1).

The characteristic pattern of regular background contractions for the duodenal strips of intestinal smooth muscle is shown in Figure 4.2A and 4.2B, for both control tissue and tissue taken from a horse affected by AGS. Table 4.1 shows the analysis of control data compared with all the grass sickness data. From the eight horses analysed for each group, the median rate of contractions for duodenal preparations varied from 3.2 contractions per minute in the AGS and SAGS groups to 6.3 contractions per minute in the control group (Table 4.2). The median amplitude of contractions varied

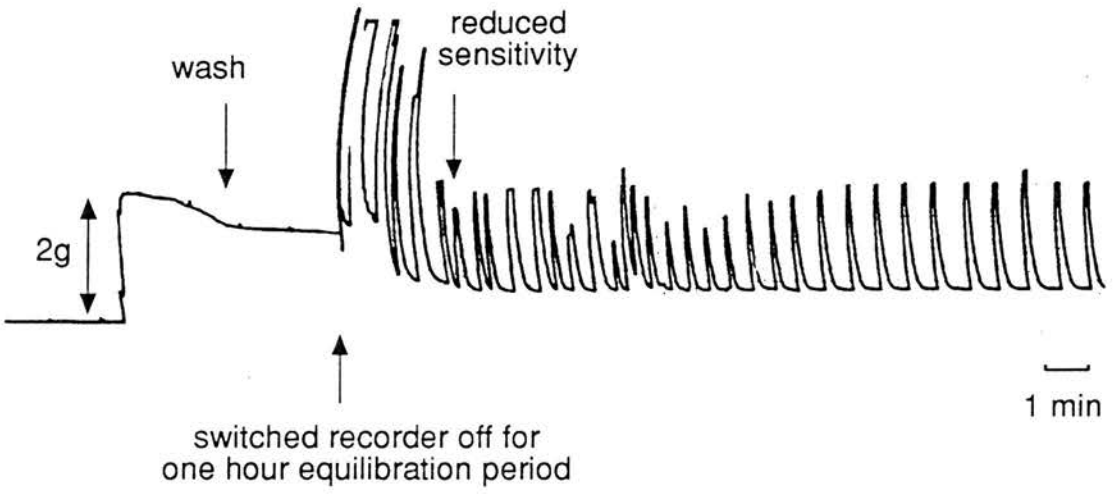


Figure 4.1. Shows the initial tension applied to a duodenal muscle strip which was equivalent to 2g. The tissue relaxed to this tension during the one hour equilibration period. When the pen recorder was switched on, regular contractions could be recorded. Often the sensitivity of the pen recorder needed to be reduced to allow detection of responses to pharmacological agents (see Chapter 3.2).

Figure 4.2A. The characteristic pattern of background contractions for duodenal muscle strips taken from control horses.

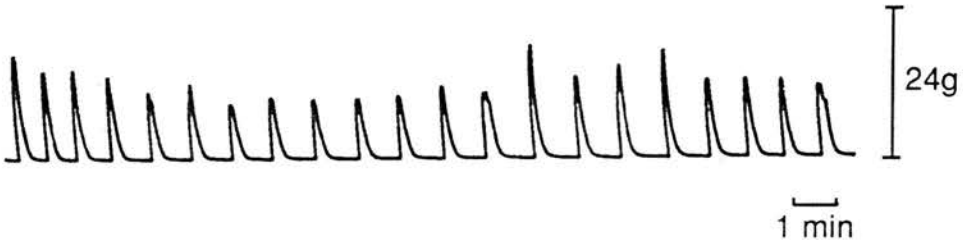


Figure 4.2B. Shows the lower rate of contractions for duodenal muscle strips taken from horses suffering from acute grass sickness (Note the altered transducer calibration).

	n	Rate of background contractions [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	8	6.3 (4.1-8.5)[a]	7.5 (3.3-16.0)[b]
Grass sickness	24	3.4 (0.8-8.4)[a]	3.5 (4.2-25.5)[b]

Table 4.1. Background contractile motility of equine duodenal smooth muscle strips measured isometrically *in vitro*. Values are medians with the range shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test a= $P<0.01$, b= $P<0.05$.

	n	Rate of background contractions [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	8	6.3 (4.1-8.5)[ab]	7.5 (3.3-16.0)[cd]
AGS	8	3.2 (0.8-8.4)[a]	13.8 (4.2-22.4)[c]
SAGS	8	3.2 (2.0-8.0)[b]	17.3 (7.1-22.5)[d]
CGS	8	4.1 (2.6-7.8)	11.0 (6.2-18.2)

Table 4.2 Background contractile motility of equine duodenal smooth muscle strips. Values are medians with the range shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test b= $P<0.01$, a,c,d= $P<0.05$.

	n	Rate of background contractions [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	8	3.2 (1.5-5.4)	10.6 (5.3-27.5)
Grass sickness	24	1.9 (0.8-5.8)	10.6 (1.0-27.8)

Table 4.3 Background contractile motility of equine ileal smooth muscle strips measured isometrically *in vitro*. Values are medians with the range shown in brackets. There were no significant differences between any of the groups.

	n	Rate of background contractions [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	8	3.2 (1.5-5.4)♣	10.6 (5.3-27.5)
AGS	8	1.7 (0.8-3.9)	14.4 (9.5-16.1)
SAGS	8	1.9 (1.5-2.6)♣	9.8 (3.1-27.8)
CGS	8	2.9 (1.2-5.8)	6.9 (1.0-21.0)

Table 4.4 Background contractile motility of equine ileal smooth muscle strips measured isometrically *in vitro*. Values are medians with the range shown in brackets. There were no significant differences between any of the groups. A ♣ symbol indicates there is a significant difference from the corresponding duodenal value when the results were analysed using the Wilcoxon Signed Rank test ($P<0.05$).

from 7.5 g/g of tissue in the control group, to 17.3 g/g of tissue in the SAGS group (Table 4.2).

The AGS and SAGS duodenal grass sickness groups had a significantly lower rate of contractions than the duodenal control group (SAGS $P< 0.01$, AGS $P< 0.05$). There were no significant differences between the contractile rates for any of the grass sickness groups themselves.

The median amplitude of the background contractions was greater in grass sickness affected tissue compared with the control tissue, only the amplitude of the AGS and SAGS groups was significantly higher than the control group ($P<0.05$) (Table 4.2).

4.3.1.2 Ileal preparations

With ileal smooth muscle strips the background contractions occurred in bursts with periods of quiescence between them in both control and grass sickness affected tissue (Figures 4.2C and 4.2D). Occasionally the background contractions were more regular (see Figure 4.2A).

Regardless of the pattern of background activity, the rate of contractions of the control ileal tissue was significantly lower than for control duodenal tissue ($P<0.05$) (Tables 4.3 and 4.4). In the ileal control group the median amplitude of contraction per gram of tissue was larger than for the control duodenal group, however, this was not significant. The rate of contractions for the ileal SAGS group was also significantly lower compared with the duodenal SAGS group ($P<0.05$). There were no significant differences in contractile amplitude for any of the grass sickness groups between the two regions.

When the contractile rates and amplitude for the ileum of control and grass sickness groups were compared between themselves, there were no significant differences between any of the groups (Table 4.3).

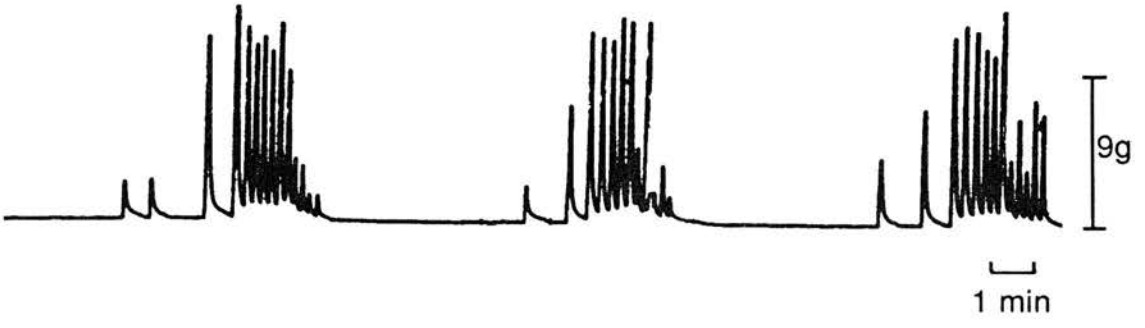


Figure 4.2C. The characteristic pattern of background contractions for ileal muscle strips taken from control horses.

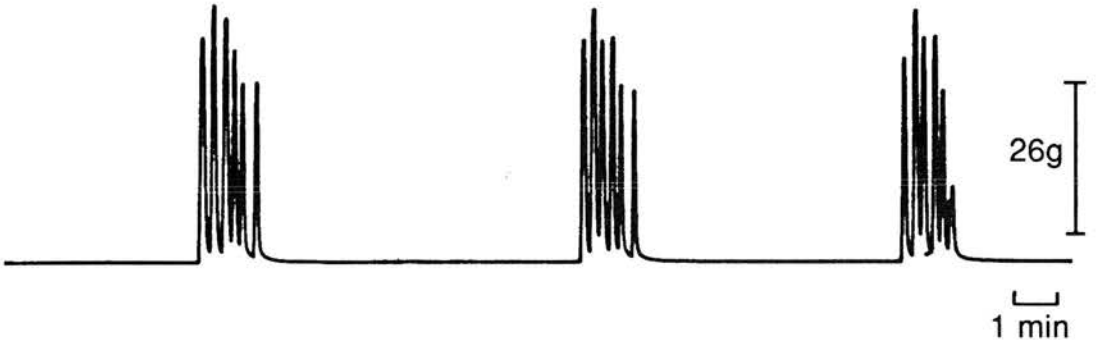


Figure 4.2D. Shows the lower rate of contractions but higher amplitude of contractions for ileal muscle strips taken from horses suffering from acute grass sickness.

4.3.2 Effect of pharmacological agents on background contractions of duodenal and ileal smooth muscle strips

A standard concentration was used for each drug investigated, this was chosen from experiments involving other species where no data was available with horse tissue (Perry, 1968). All concentrations given are final bath concentrations.

4.3.2.1 Tetrodotoxin (TTX)

The effect of TTX ($4.5x10^{-6}M$) on background contractile activity was investigated using tissue taken from five control horses (Table 4.5). There was no significant effect of TTX on either contractile rate or contractile amplitude when compared with those in Tables 4.1 and 4.3 (Figures 4.3A and B). There was also no significant difference before and after TTX treatment within each group. When the results were compared between the two regions, using the Wilcoxon Signed Rank test, for contractile rate $P<0.06$ which means the difference approached significance. It may be that with larger experimental numbers this would indeed be the case. There was no significant difference between the contractile amplitude between the two regions. TTX was not used on grass sickness affected tissue.

	n	Contractile rate following TTX addition [$4.5x10^{-6}M$]	Contractile amplitude following TTX addition [$4.5x10^{-6}M$]
Duodenum	5	7.9 (5.6-9.5)	6.2 (1.6-6.8)
Ileum	5	3.0 (1.9-4.2)♣	4.1 (2.5-12.9)

Table 4.5 Background contractile motility of equine smooth muscle strips taken from control horses following addition of TTX ($4.5x10^{-6}M$). Values are medians with the range of values shown in brackets. A ♣ indicates there may be a difference of biological importance between the duodenal and ileal values when the results were analysed using the Wilcoxon Signed Rank test ($P<0.06$).

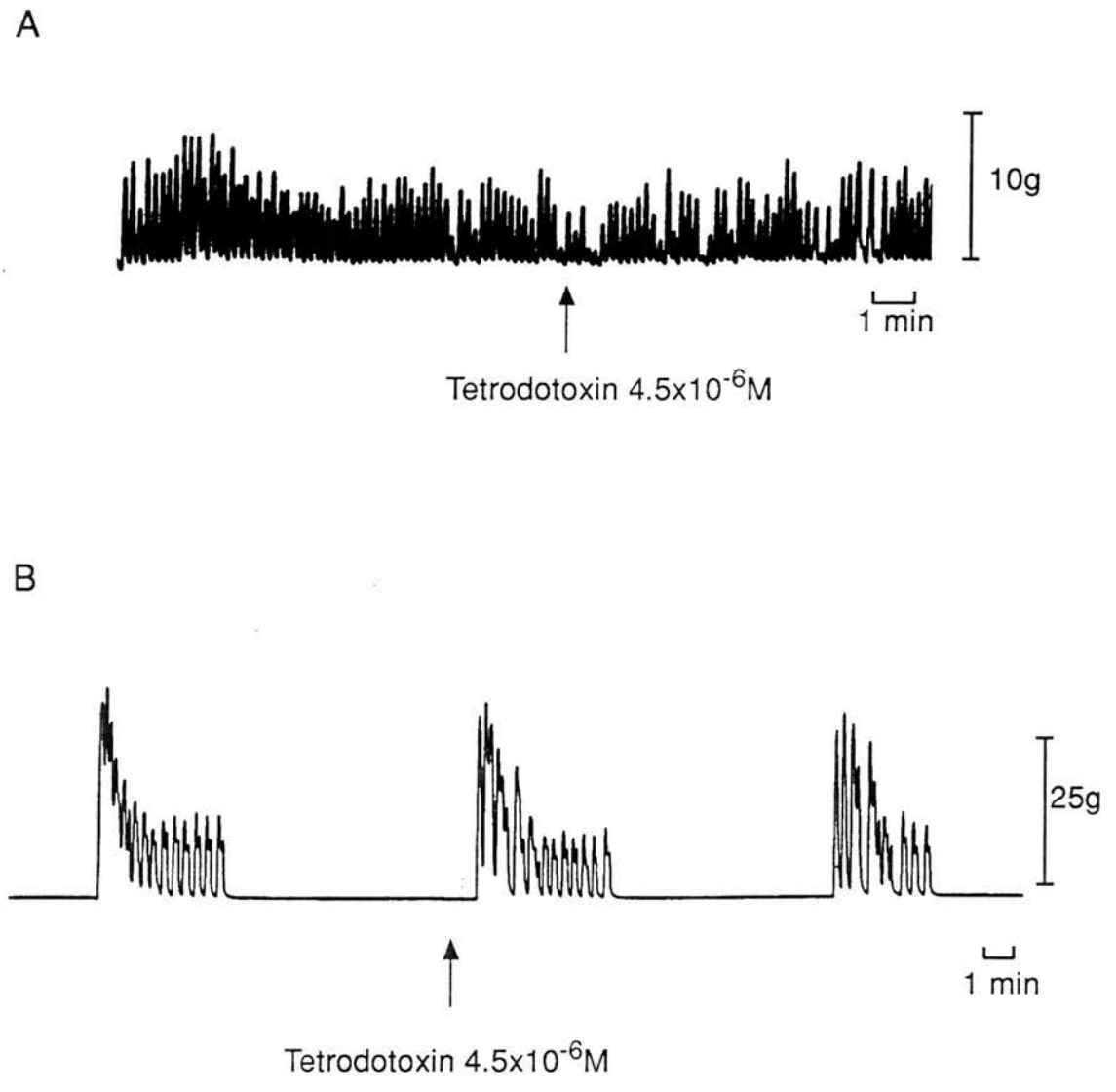


Figure 4.3. Tetrodotoxin addition ($4.5x10^{-6}M$) had no significant effect on the rate or amplitude of background contractions for both A) duodenal, and B) ileal smooth muscle strips taken from control horses.

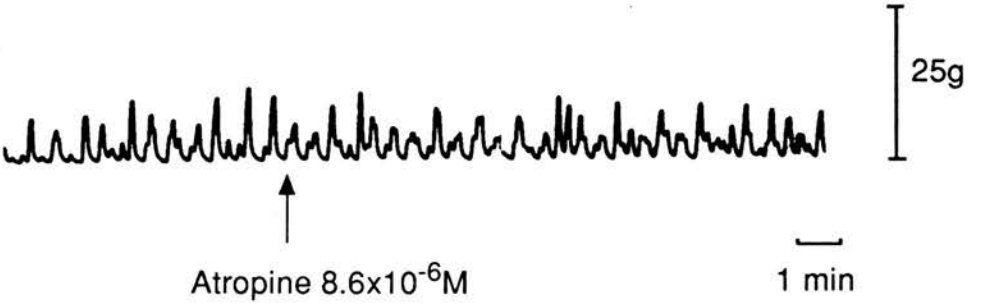


Figure 4.4. Addition of atropine ($8.6x10^{-6}$M) did not affect the rate or amplitude of background contractions. This trace was from a duodenal strip from a horse suffering from subacute grass sickness.

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	4.8 (4.5-6.6)	16.2 (4.9-19.6)
Grass Sickness	15	2.8 (1.2-8.8)	13.3 (5.3-30.9)

Table 4.6 Background contractile motility of equine duodenal smooth muscle strips following addition of propranolol ($1x10^{-4}$M). Values are medians with the range of values shown in brackets.

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	4.8 (4.5-6.6)[a]	16.2 (4.9-19.6)
AGS	5	2.0 (1.2-6.6)	13.3 (9.0-21.1)
SAGS	5	4.8 (2.2-8.8)	12.8 (8.0-30.9)
CGS	5	3.9 (2.2-4.1)[a]	14.3 (5.3-15.5)

Table 4.7. Background contractile motility of equine duodenal smooth muscle strips following addition of propranolol ($1x10^{-4}$M). In this Table the grass sickness data is divided into AGS, SAGS and CGS groups. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	2.6 (2.4-3.1)[a]	16.5 (11.2-22.8)[b]
Grass Sickness	15	1.8 (0.6-3.8)[a]	9.2 (1.8-28.6)[b]

Table 4.8. Background contractile motility of equine ileal smooth muscle strips following the addition of propranolol ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	2.6 (2.4-3.1)[a]♣	16.5 (11.2-22.8)
AGS	5	0.9 (0.6-2.8)	9.2 (2.7-13.2)♦
SAGS	5	1.4 (0.8-2.6)[a]♣	5.7 (2.2-28.6)
CGS	5	2.2 (1.3-3.8)	9.7 (1.8-15.3)

Table 4.9. Background contractile motility of equine ileal smooth muscle strips following the addition of propranolol ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).
A ♦ symbol indicates there was a significant difference between this value and the initial values before pretreatment with propranolol (i.e. Table 4.4) ($P<0.05$, this was analysed using the Mann-Whitney U-test as it involved different horses).
A ♣ symbol indicates there may be a difference of biological importance from the corresponding duodenal value (i.e. Table 4.7) when the results were analysed using the Wilcoxon Signed Rank test ($P<0.06$).

4.3.2.2 Atropine

Atropine ($8.6x10^{-6}M$) appeared to have no effect on background motility in control and grass sickness tissue (Figure 4.4), although only three experiments were performed and no statisitcal analysis was carried out.

4.3.2.3 Propranolol

Following the addition of propranolol ($1x10^{-4}M$) there was no significant difference between the rate or amplitude of the background contractions for duodenal muscle strips between any of the groups, with the exception of the contractile rate for the CGS group which was significantly lower than the contractile rate for the control group ($P<0.05$) (Table 4.7). For ileal muscle strips there was no significant difference in the rate or amplitude of the background contractions following the addition of propranolol ($1x10^{-4}M$) (Figures 4.5A and 4.6A), with the exception of the contractile rate for the SAGS group which was significantly lower than for the control group ($P<0.05$) (Table 4.9).

For the investigation of the effect of propranolol the same horses were used for the duodenal and ileal regions, therefore comparisons between the two regions were carried out using the Wilcoxon Signed Rank test. There was no statistically significant difference between any of the groups, however, the probability of a significant difference between the two regions for the control and SAGS rates was $P<0.06$ so this may be of some biological importance. Figures 4.5A and 4.6A shows an example of the lack of effect of propranolol on background motility.

When these results were compared with those obtained for background contractions alone (Tables 4.2 and 4.4) only the amplitude for the ileal AGS group was significantly lower ($P<0.05$).

4.3.2.4 Phentolamine

Following the addition of phentolamine ($1x10^{-4}M$) there was no significant difference between the rate or amplitude of the background contractions for duodenal muscle strips between any of the groups, with the exception of the contractile rate for the AGS group which was significantly lower than the contractile rate for the control

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	5.9 (4.2-9.6)	8.4 (7.0-18.9)
Grass Sickness	15	4.3 (1.8-10.4)	12.0 (1.0-17.1)

Table 4.10. Background contractile motility of equine duodenal smooth muscle strips following addition of phentolamine ($1x10^{-4}$M). Values are medians with the range of values shown in brackets.

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	5.9 (4.2-9.6)[a]	8.4 (7.0-18.9)
AGS	5	3.3 (1.8-5.8)[a]	13.0 (8.7-14.2)
SAGS	5	8.2 (3.0-8.7)	12.3 (4.7-17.1)
CGS	5	5.8 (2.4-10.4)	6.1 (1.0-14.3)

Table 4.11. Background contractile motility of equine duodenal smooth muscle strips following addition of phentolamine ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. In this Table the grass sickness data are divided into AGS,SAGS and CGS groups. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test ($P<0.05$).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	3.6 (1.3-6.0)	15.2 (6.2-21.4)[a]
Grass Sickness	15	1.6 (0.5-6.8)	5.4 (3.0-15.7)[a]

Table 4.12. Background contractile motility of equine ileal smooth muscle strips following the addition of phentolamine ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test ($P<0.05$).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	3.6 (1.3-6.0)	15.2 (6.2-21.4)[a]
AGS	5	1.4 (0.6-2.4)♣	8.0 (3.7-15.7)♦
SAGS	5	1.6 (0.5-2.2)♣	5.8 (3.5-10.4)[a]
CGS	5	3.3 (1.0-6.8)	3.7 (3.0-15.3)

Table 4.13. Background contractile motility of equine ileal smooth muscle strips following the addition of phentolamine ($1x10^{-4}$M). The grass sickness data has been divided into AGS, SAGS and CGS groups. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test ($P<0.05$). A ♦ symbol indicates there was a significant difference for this value and the initial values before pretreatment with phentolamine (i.e. Table 4.4) ($P<0.05$), this was analysed using the Mann-Whitney U-test as it involved different horses. A ♣ symbol indicates there may be a difference of biological importance from the corresponding duodenal value (i.e. Table 4.11) when the results were analysed using the Wilcoxon Signed Rank test ($P<0.06$).

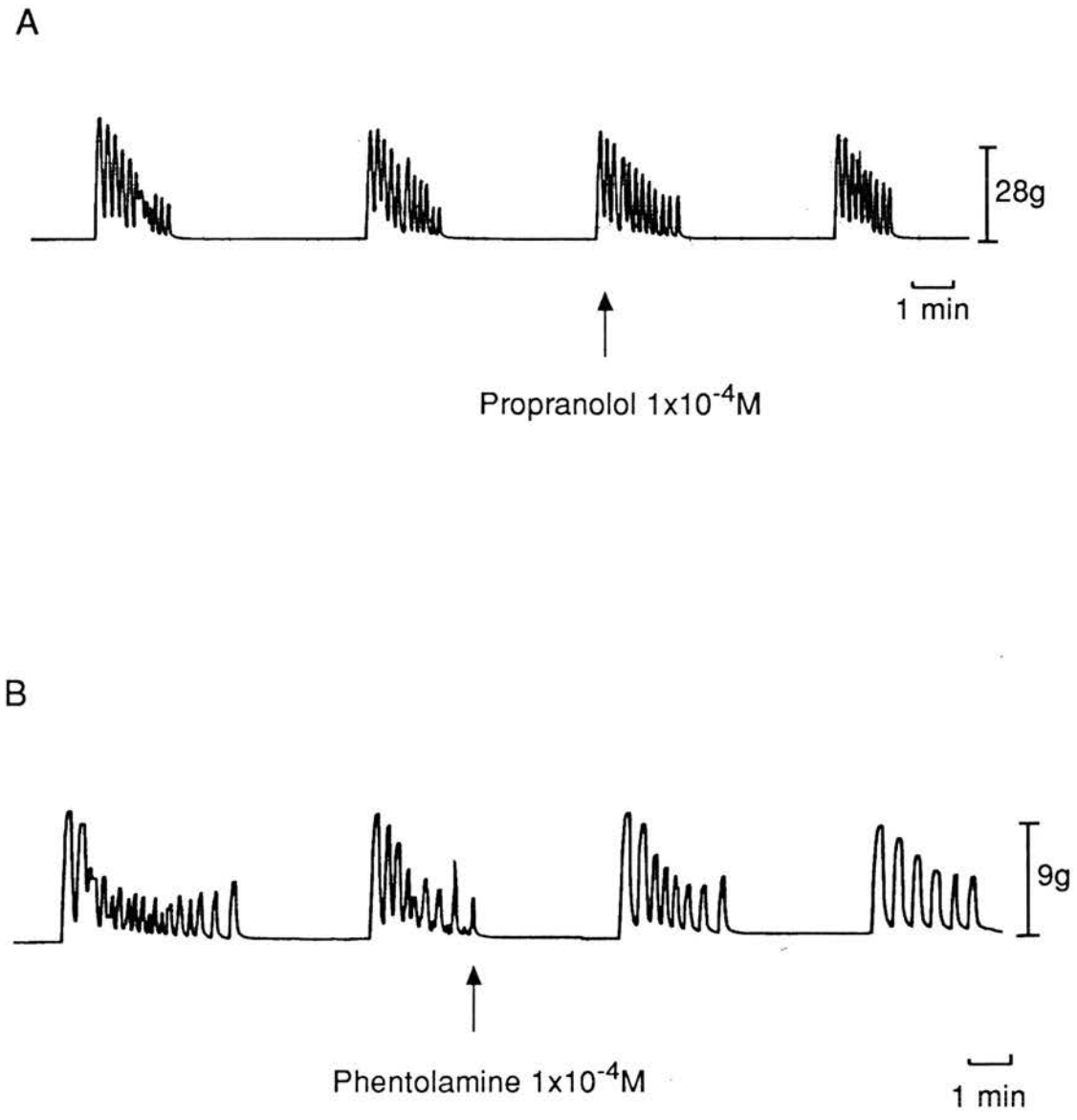


Figure 4.5. With ileal preparations there was no effect on background contractions following A) propranolol, this trace was from an ileal muscle strip taken from a control horse and B) phentolamine, this trace was from an ileal muscle strip taken from a horse suffering from acute grass sickness.

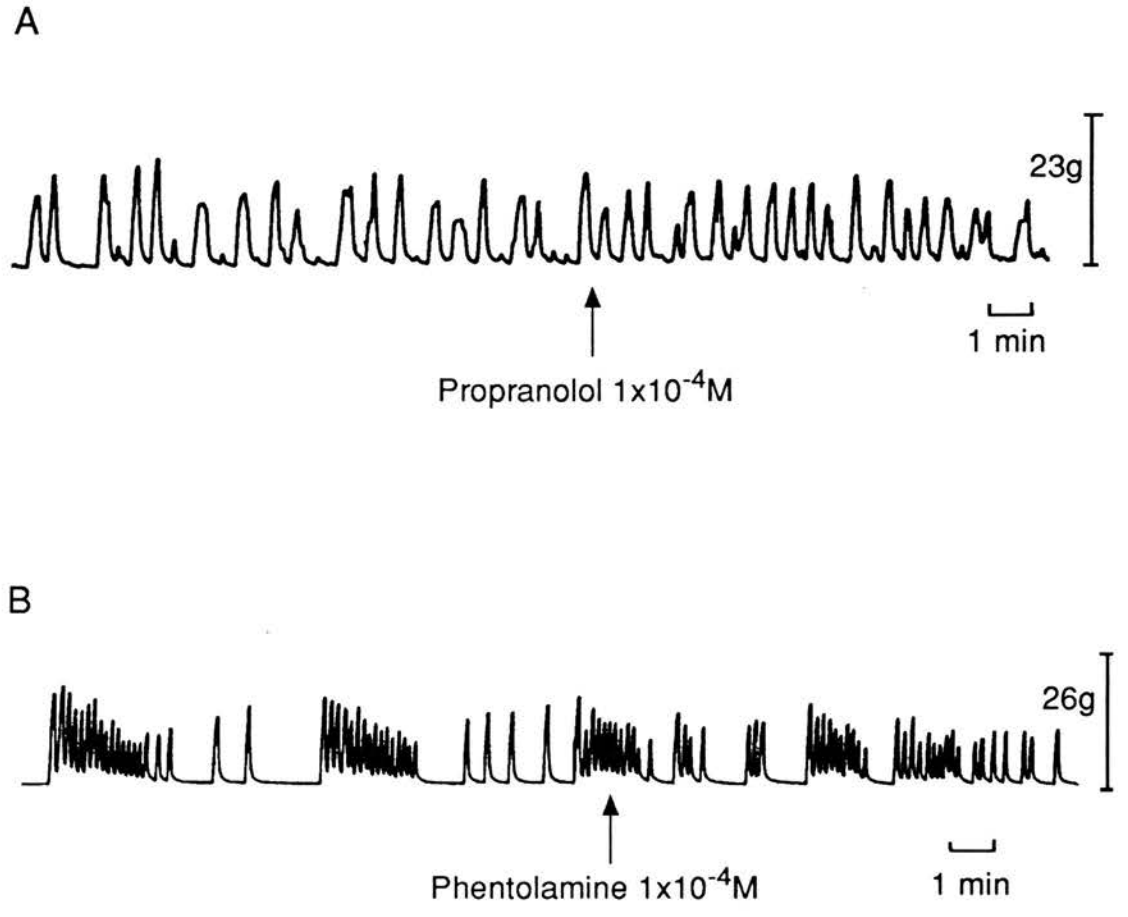


Figure 4.6. With some ileal preparations the background contractile activity was more regular. However, there was still no effect on rate or amplitude of the background contractions following the addition of A) propranolol and B) phentolamine. Both these traces were taken from control horses.

group ($P<0.05$) (Table 4.11). For ileal muscle strips there was no significant difference in the rate or amplitude of the background contractions following phentolamine ($1x10^{-4}M$) (Figures 4.5B and 4.6B), with the exception of the contractile amplitude for the SAGS group which was significantly lower than for the control group ($P<0.05$) (Table 4.13).

When the duodenal and ileal results were compared using the Wilcoxon Signed Rank test there was no statistically significant difference between any of the groups for contractile rate and amplitude, however, the difference between the two regions for the AGS and SAGS rates was $P<0.06$ so although this is not statisically significant, it may be of some biological importance.

When these results were compared with those obtained for background contractions alone (Tables 4.2 and 4.4) only the amplitude for the ileal AGS group was significantly lower ($P<0.05$).

Experiments using more selective alpha antagonists such as prazosin ($\alpha 1$ antagonist) and yohimbine ($\alpha 2$ antagonist) also appeared not to have an effect on the rate or amplitude of background contractions for both control and grass sickness groups and both regions of the small intestine (Figure 4.7).

4.3.3 Effect of storage on characteristics of background contractions

In this section, tissue from the same horses was used to establish the initial rate and amplitude of the background contractions for both regions of the small intestine. This tissue was then used to see if there were any alterations in the characteristic of background contractions in response to selected pharmacological agents. As there were few significant differences found with fresh tissue, only the effects of propranolol and phentolamine were investigated with stored tissue.

4.3.3.1 Duodenal preparations

The median rate of background contractions for the stored control tissue was 4.0 contractions per minute (Table 4.14) compared with 6.3 for fresh tissue (Table 4.1). The only significant difference between the groups of stored duodenal tissue was

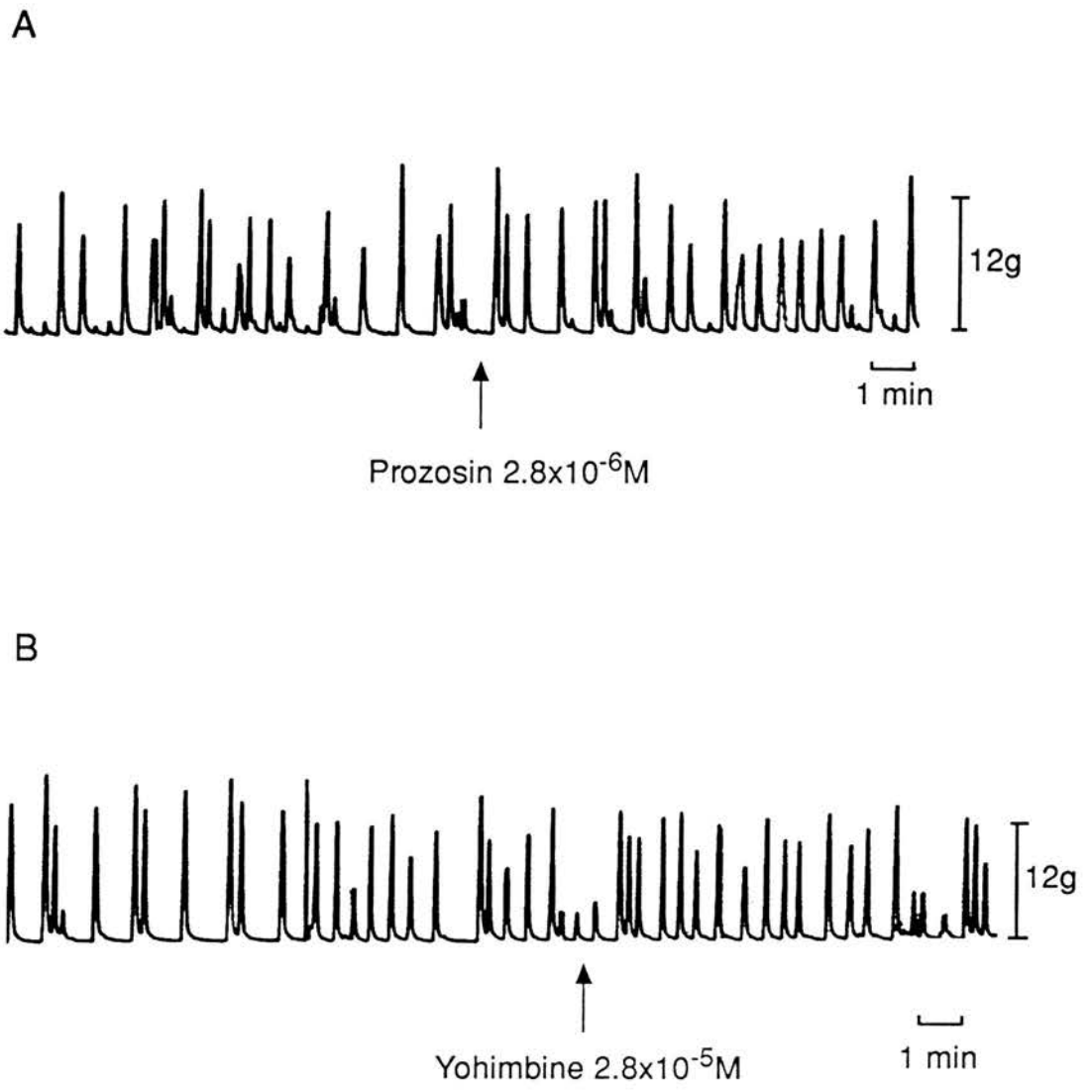


Figure 4.7. Trace from a duodenal muscle strip taken from a horse suffering from chronic grass sickness. It showed that A) the addition of prazosin, an alpha 1 antagonist, and B) the addition of yohimbine, an alpha 2 antagonist had no significant effect on the rate and amplitude of the background contractions.

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	4.0 (3.2-4.8)[a]	22.1 (8.7-34.9)
Grass Sickness	15	1.8 (1.0-6.0)[a]	9.8 (4.9-35.6)

Table 4.14. Background contractile motility of equine duodenal smooth muscle strips following storage for 24 hours at 4°C. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	4.0 (3.2-4.8)[a]♥	22.1 (8.7-34.9)♠
AGS	5	1.8 (1.2-4.4)	8.8 (5.7-30.0)
SAGS	5	2.2 (1.0-6.0)	5.9 (4.9-26.8)
CGS	5	1.8 (1.5-3.2)[a]♥	13.8 (9.8-35.6)

Table 4.15. Background contractile motility of equine duodenal smooth muscle strips following storage for 24 hours at 4°C. The grass sickness data has been divided into AGS,SAGS and CGS. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).
A ♥ or ♠ symbol indicates there was a significant difference for this stored value from the initial fresh values (i.e. Table 4.2) (♥ $P<0.01$, ♠ $P<0.05$, this was analysed using the Mann-Whitney U-test as it involved different horses).

between the contractile rate for the CGS group which was significantly lower than the control group ($P<0.05$) (Table 4.15).

The amplitude of background contractions was largest in the stored tissues of the control group and smallest in the AGS and SAGS grass sickness groups. This was unlike duodenal fresh tissue where the largest median contractile amplitudes were seen in the grass sickness groups. However, there were no significant differences between any of the groups with regard to contractile amplitude (Table 4.15).

The rate of contractions for the control and CGS groups were significantly lower compared with the equivalent fresh tissue groups ($P<0.01$). There was no significant difference between the AGS and SAGS fresh and stored results. The amplitude of contractions for the control tissue significantly increased following storage ($P<0.05$).

The data for the stored control duodenal group were compared individually with those for the fresh AGS, SAGS and CGS groups, to see if the neuronal death which occurred during storage in control tissue caused the tissue to behave like fresh grass sickness affected tissue. There was no significant difference between any of them for both contractile rate and amplitude.

Following the addition of propranolol ($1x10^{-4}M$) to stored tissue the AGS and SAGS groups had significantly lower contractile rates compared with the control group ($P<0.05$) (Table 4.17). There was no significant difference between any of the groups with regard to contractile amplitude.

There was no significant effect on the rate and amplitude of the duodenal background contractions following propranolol addition compared with stored tissue alone (i.e. Table 4.15 compared with Table 4.17). Figure 4.8 shows that both propranolol and phentolamine had no effect on the background contractions of duodenal muscle strips taken from a control horse following storage for 24 hours.

With stored tissue, following the addition of phentolamine ($1x10^{-4}M$), the AGS contractile rate was significantly lower than the control and CGS groups ($P<0.05$). Also the control contractile amplitude was significantly higher than all three grass sickness groups ($P<0.05$) (Table 4.19).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	4.0 (2.2-4.6)[a]	21.3 (12.6-33.2)
Grass Sickness	15	1.8 (0.8-3.8)[a]	8.2 (4.0-33.7)

Table 4.16. Background contractile motility of equine duodenal smooth muscle strips following storage for 24 hours at 4°C and treatment with propranolol ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	4.0 (2.2-4.6)[ab]	21.3 (12.6-33.2)
AGS	5	1.4 (0.8-2.6)[a]	11.3 (5.7-26.2)
SAGS	5	1.9 (1.2-3.8)[b]	6.8 (4.6-29.4)
CGS	5	2.2 (1.3-2.6)	9.5 (4.0-33.7)

Table 4.17. Background contractile motility of equine duodenal smooth muscle strips following storage for 24 hours at 4°C and treatment with propranolol ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	4.4 (3.0-7.8)	30.4 (15.8-31.5)[a]
Grass Sickness	15	2.4 (1.2-4.8)	7.7 (3.3-27.1)[a]

Table 4.18. Background contractile motility of equine duodenal smooth muscle strips following storage for 24 hours at 4°C and treatment with phentolamine ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	4.4 (3.0-7.8)[a]	30.4 (15.8-31.5)[cde]
AGS	5	1.7 (1.2-2.0)[ab]	8.0 (5.9-27.1)[c]
SAGS	5	4.4 (1.2-4.8)	3.9 (3.3-8.0)[d]
CGS	5	3.4 (2.4-4.8)[b]	10.2 (5.2-13.5)[e]

Table 4.19. Background contractile motility of equine duodenal smooth muscle strips following storage for 24 hours at 4°C and treatment with phentolamine ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).

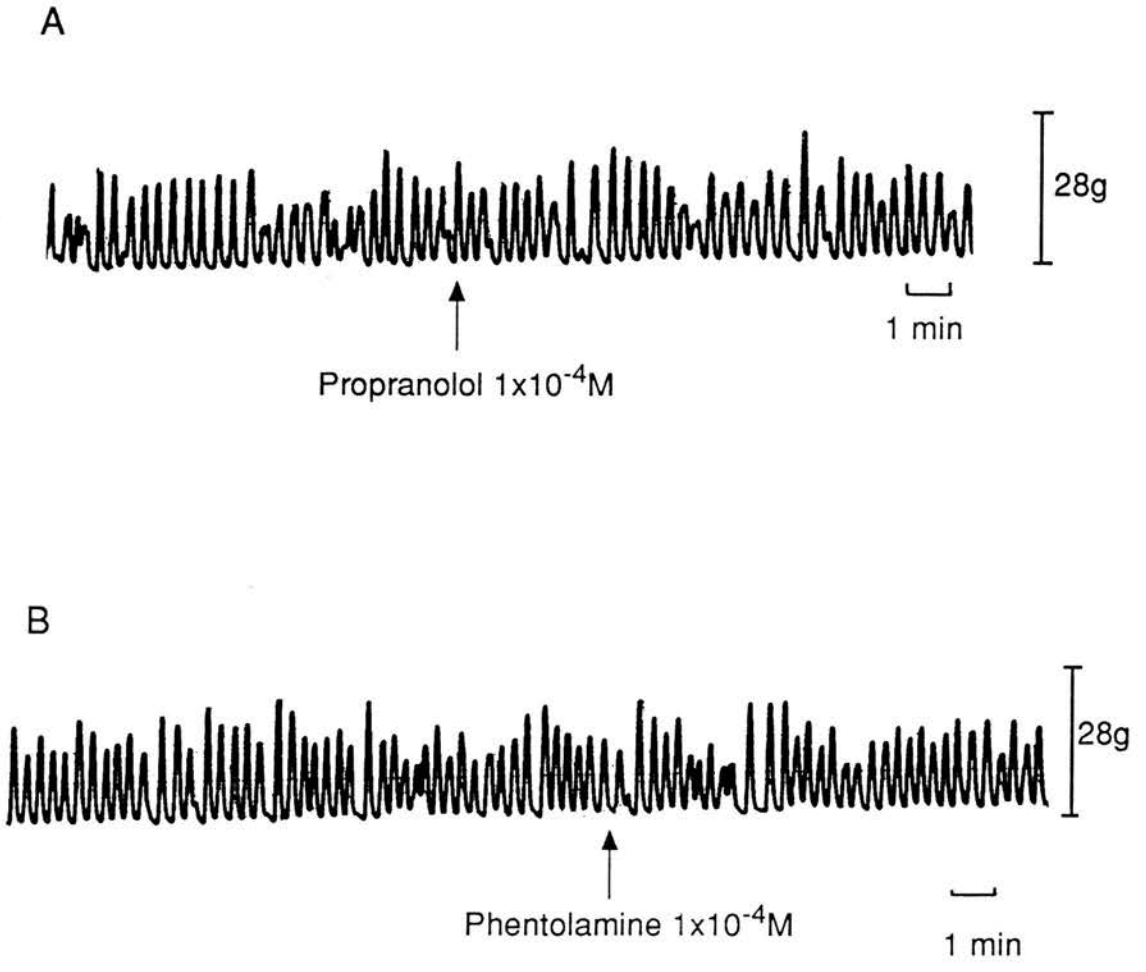


Figure 4.8. Recording from duodenal muscle strip taken from a control horse following storage for 24 hours at 4^{o}C. It shows that both A) propranolol and B) phentolamine had no effect on the rate or amplitude of the background contractions.

There was no significant difference between the rate and amplitude of the background contractions following the addition of phentolamine, compared with stored tissue alone (i.e. Table 4.15 compared with Table 4.19).

4.3.3.2 Ileal preparations

The initial median rate of background contractions for ileal control tissue, after storage for 24 hours at 4°C, was 0.8 contractions per minute (Table 4.20). The CGS group had a significantly larger contractile rate, at 1.9 contractions per minute, than the control group ($P<0.05$) (Table 4.21). There was no significant difference between any of the groups for contractile amplitude.

The rate of contractions was reduced in all four groups following storage. However, only the control and AGS groups showed significant reductions at $P<0.01$ and $P<0.05$ respectively (Table 4.4 compared with Table 4.21). The contractile amplitude of the control group significantly increased in stored tissue compared with fresh tissue ($P<0.05$). For the grass sickness groups there was no significant change in the amplitude of background contractions following storage.

The data for the stored control ileal group were compared with the fresh ileal AGS, SAGS and CGS groups, to see if the neuronal death which occurred during storage in control tissue caused the tissue to behave like fresh grass sickness affected tissue. It was found that the stored contractile rate of control tissue was significantly lower than the fresh grass sickness values ($P<0.05$ for all three groups). The contractile amplitude for the stored ileal group was significantly higher than the fresh grass sickness results ($P<0.01$ for AGS and $P<0.05$ for SAGS and CGS).

Following the addition of propranolol ($1x10^{-4}M$) there was no significant difference between any of the groups for both contractile rate and amplitude (Table 4.23). There was also no significant difference in contractile rate and amplitude following propranolol addition and stored tissue alone (Table 4.21 compared with Table 4.23).

Following the addition of phentolamine ($1x10^{-4}M$) there was no significant difference in the rate of contractions between the four groups (Table 4.25). The

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	0.8 (0.7-0.8)	23.3 (16.9-36.5)
Grass Sickness	14	1.4 (0.4-3.6)	13.0 (2.5-38.1)

Table 4.20. Background contractile motility of equine ileal smooth muscle strips following storage for 24 hours at 4°C. Values are medians with the range of values shown in brackets.

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	0.8 (0.7-0.8)[a]♥	23.3 (16.9-36.5)♠
AGS	4	1.1 (0.7-1.4)♠	13.0 (10.4-24.8)
SAGS	5	1.3 (0.4-2.4)	26.4 (2.5-38.1)
CGS	5	1.6 (0.8-3.6)[a]	8.4 (4.4-32.2)

Table 4.21. Background contractile motility of equine ileal smooth muscle strips following storage for 24 hours at 4°C. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$). A ♥ or ♠ symbol indicates there was a significant difference for this stored value from initial fresh values (i.e. Table 4.4 (♥ $P<0.01$, ♠ $P<0.05$, this was analysed using the Mann-Whitney U-test as it involved different horses).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	1.8 (0.6-3.6)	20.5 (3.4-33.1)
Grass Sickness	14	1.7 (0.4-4.2)	6.7 (2.5-31.1)

Table 4.22. Background contractile motility of equine ileal smooth muscle strips following storage for 24 hours at 4°C and treatment with propranolol ($1x10^{-4}$M). Values are medians with the range of values shown in brackets.

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	1.8 (0.6-3.6)	20.5 (3.4-33.1)
AGS	4	0.6 (0.4-2.3)	12.4 (3.4-31.1)
SAGS	5	1.0 (0.5-3.8)	6.7 (2.8-20.1)
CGS	5	2.2 (1.9-4.2)	5.0 (2.5-8.0)

Table 4.23. Background contractile motility of equine ileal smooth muscle strips following storage for 24 hours at 4°C and treatment with propranolol ($1x10^{-4}$M). Values are medians with the range of values shown in brackets.

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	2.3 (0.6-4.6)	26.0 (12.2-42.0)[a]
Grass Sickness	14	2.0 (1.0-4.0)	6.9 (1.0-16.0)[a]

Table 4.24. Background contractile motility of equine ileal smooth muscle strips following storage for 24 hours at 4 °C and treatment with phentolamine ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).

	n	Background contractile rate [contractions/min]	Amplitude of background contractions [g/g of tissue]
Control	5	2.3 (0.6-4.6)	26.0 (12.2-42.0)[ab]
AGS	4	1.6 (1.0-2.0)	10.4 (7.5-14.0)[c]
SAGS	5	2.0 (1.0-4.0)	3.3 (1.0-16.0)[a]
CGS	5	2.6 (1.2-3.8)	5.1 (3.2-8.2)[bc]

Table 4.25. Background contractile motility of equine ileal smooth muscle strips following storage for 24 hours at 4 °C and treatment with phentolamine ($1x10^{-4}$M). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed with the Mann-Whitney U-test ($P<0.05$).

control ileal contractile amplitude was significantly higher than the SAGS and CGS groups. The AGS group had a significantly higher contractile amplitude than the CGS group ($P<0.05$). Following the addition of phentolamine there was no significant difference in rate or amplitude for any of the groups compared with their initial values for background contractions following storage (Table 4.21 compared with Table 4.25).

4.4 Discussion

That background contractions could be recorded from all muscle strips validated the collection method and experimental protocol and confirmed that the tissue was arriving at the laboratory in a viable state. However, it cannot be said with certainty that the *in vitro* observations exactly reflect *in vivo* activity.

The horses used to determine the characteristics of the background contractions established that the data was not normally distributed because the median values were not in the middle of the range of values and the upper value was often more than three times the lowest value. Therefore all the results were analysed using non-parametric tests.

As discussed in Chapter 1, intestinal smooth muscle undergoes rhythmic changes in excitability referred to as slow waves. Slow waves bring the resting membrane potential near to the threshold for the generation of action potentials (spikes). When the threshold is reached voltage-dependent channels in the membrane open, leading to the ionic movements responsible for the generation of spikes. The amplitude of the background contractions is determined by the number of spikes superimposed on each slow wave. The rate of background contractions is limited by the slow wave frequency (Grundy, 1985; Read, 1990). It has also been suggested that the interstitial cells of Cajal may have an effect on the rate of background contractions in certain regions of the gut (Thuneberg, 1982; Christensen, 1988).

This study of background motility demonstrated that contractile patterns in the two regions of the small intestine were quite different. Fresh duodenal strips taken from control horses had a significantly higher contractile rate than ileal control strips ($P<0.05$). It has been reported that the intrinsic frequency of slow waves *in vivo* is

greater in the more proximal regions of the equine gastrointestinal tract (Davies and Gerring, 1983), therefore a greater contractile rate in the more proximal regions would be expected. Ruckebusch *et al.* (1971) found the mean frequency of background contractions *in vitro* to be 8.7 and 4.8 in equine duodenum and ileum respectively compared with the median values of 6.3 and 3.2 in these experiments. There were differences between these sample sites and those of Ruckebusch *et al.* (1971).

There was no significant difference in the amplitude of contractions between the two regions in control horses (Tables 4.1 and 4.3). There have been no reports of the amplitude of background contractions in the horse *in vitro*, with the exception of Malone, E.D., Brown, D.R., Trent, A.M. and Turner, T.A. (submitted for publication) who only give absolute values which could therefore not be compared with the results described here.

In both duodenal and ileal regions the difference in contractile rate between the control and grass sickness groups was approximately a 50% reduction. However, for the control and grass sickness groups, there was no significant difference between their contractile rates and amplitudes for ileal tissue (Table 4.4). For the duodenal preparations the AGS and SAGS groups had background contractile rates significantly lower than the control group ($P<0.05$ and $P<0.01$ respectively). For these two groups, the contractile amplitude was also significantly higher than the control group ($P<0.05$). The reason both regions do not show the same differences is unclear. There may be a reduction in slow wave frequency in the duodenal regions in grass sickness cases, however, if this was the case a similar reduction would be expected in the ileal region. Heeckt, Halfer, Schraut, Lee and Bauer (1993) noted a decrease in spontaneous contractile activity whilst studying chronic rejection in intestinal smooth muscle after small intestine transplantation. The mechanism for this was not determined. The frequency of the slow waves was not significantly altered although the amplitude of the slow waves was significantly decreased ($P<0.0001$). Another explanation for the reduced contractile rate could be that the background contractile activity is influenced by spontaneously released chemical transmitters from intrinsic nerves. Kuriyama, Osa and Toida, (1967) concluded in their investigation of smooth

muscle strips from guinea pig jejunum, that normal membrane activities may be influenced by the release of acetylcholine. In cases of grass sickness where neuronal death is known to occur there would be a reduction in this spontaneous neurotransmitter release and therefore this may cause a reduction in the rate of background contractions.

There have been a number of reports of the effect of pharmacological agents on background contractions. A number of authors working with other species have reported that, unlike the situation in nerves and skeletal muscle, the puffer fish venom, TTX, fails to abolish the spikes which occur in smooth muscle (Narahashi, Moore and Scott, 1964; Bulbring and Tomita, 1966; Kuriyama, Osa and Toida, 1966). TTX selectively blocks the voltage-dependent sodium channels. The inward current of the spikes in smooth muscle is therefore not carried by sodium ions , however, it is blocked by interference with calcium entry into the cells. The spikes seen in smooth muscle are therefore mainly, if not exclusively, dependent on the inward movement of calcium ions (Grundy, 1985).

The insensitivity of the smooth muscle strips in control horses to TTX as found in these experiments, indicates that the background motility of the small intestine is independent of nervous elements. However, there have been some conflicting reports in the literature. Furness (1970) reported that the addition of TTX, in the guinea pig colon, caused a reduction in the rate of background contractions suggesting that intrinsic nerves were having an excitatory effect on background contractions. If this was the case in the horse, it would help to explain the lower contractile rates observed in the duodenum from the AGS and SAGS groups, as neuronal activity is depleted in grass sickness cases. However, Wood (1987) and Read (1990) reported that TTX blocks the activity of inhibitory nerves which are supressing the rate of background contractions, therefore in the presence of TTX, the background rate of contractions increases.

The concentration of TTX chosen in these experiments may not have been suitable to show a significant effect on background contractions. Indeed some traces did appear to demonstrate that the TTX was having an effect on background contractions (Figure 4.3A) although the differences were not significant when

analysed. However, Malone, E.D, Brown, D.R., Trent, A.M. and Turner, T.A. (submitted for publication 1995) found that the baseline activity of strips of equine jejunum was unaffected by TTX. They used a similar concentration of TTX to these experiments ($1x10^{-6}$M compared with $4.5x10^{-6}$M).

Atropine appeared to have no effect on the contractile rate or amplitude of control tissue. However, controlled experiments to provide sufficient data for statistical analysis were not carried out. Malone, E.D, Brown, D.R., Trent, A.M. and Turner, T.A. (submitted for publication 1995) reported that atropine (1μm) caused no significant effect on the frequency of contractions, but that there was a significant reduction in the contractile amplitude ($P<0.05$). Adams, Lamar and Masty (1984) found that atropine decreased motility of the distal portion of the jejunum and pelvic flexure in ponies. They found nearly a complete absence of spike potentials on the slow waves. Roberts and Argenzio (1986) also found that increasing doses of atropine reduced and then abolished intestinal sounds, provoked abdominal discomfort and delayed defaecation in healthy ponies. The transit time of soluble markers was also prolonged. It may be that higher concentrations of atropine were needed in the *in vitro* experiments described here and then a reduction in background contractile activity may have been seen.

In these studies propranolol, a β antagonist (Jacob, Brandt, Farkas and Frishman, 1983), had no significant effect on the rate or amplitude of background contractions in either region of the small intestine from both control and grass sickness tissue with one exception (Tables 4.7 and 4.9). The amplitude of the ileal AGS group was significantly reduced ($P<0.05$). Phentolamine also had no significant effect on background contractions in either region of the small intestine with the same exception (Tables 4.11 and 4.13). As observed with propranolol the amplitude of the ileal AGS group was significantly reduced ($P<0.05$) following the addition of phentolamine. An explanation for this reduction in amplitude could not be given.

Adrenergic blockade has not been shown to have a significant effect on gut motility in normal horses either clinically or experimentally (Gerring and Hunt, 1986). Gerring and Hunt (1986) found propranolol was the least effective agent in improving electrical and mechanical activity throughout the gut in an equine post operative ileus

model. Smith, Kelly and Weinshilboum (1977) found combined α and β blockade with propranolol and phentolamine in dogs improved gastric activity but not propulsion. Malone, E.D, Brown, D.R., Trent, A.M. and Turner, T.A. (submitted for publication 1995) also found little response to adrenergic blockade in equine jejunum *in vitro*. They suggested that this indicated minimal baseline sympathetic tone in the equine jejunum. However, in their study they did find that phentolamine caused a significant decrease in the amplitude of background contraction of the longitudinal muscle, but they could not suggest a reason for this effect. When they used more selective α antagonists, however, no reduction in the amplitude was seen.

In Tables 4.4, 4.9 and 4.13 the data for the duodenal and ileal regions were from the same horses and were therefore analysed using the Wilcoxon Signed Rank test. In some cases the P value was less than 0.06 which is not statistically significant. This was probably due to the small numbers which were used and therefore could suggest differences of biological importance.

The effect of storage was investigated to see if enteric neuronal death would take place and produce alterations in motility which may be similar to that known to occur in grass sickness (Chapter 4.3.3.1 and 4.3.3.2). The rate of background contractions for the control duodenal and ileal groups was significantly reduced and the amplitude of contractions was significantly increased following storage ($P<0.05$) (Tables 4.15 and 4.21). This contrasts with equine grass sickness tissue where there was no significant difference in the rate or amplitude of contractions following storage, with the exception of the rates of contractions for the duodenal CGS group ($P<0.01$) and the ileal AGS group ($P<0.05$).

This would suggest that intrinsic nerves must indeed have an excitatory effect on the rate of background contractions, as when denervation occurs to some degree during storage, there is a reduction in contractile rate.

In the duodenal region, there were no significant differences for the control tissue following storage, compared with fresh AGS, SAGS and CGS data. This suggested that this storage period may have some potential for a model of grass sickness. However, when the stored ileal data were compared with fresh AGS, SAGS and CGS data, the rate of contractions was significantly lower and the amplitude

significantly higher. This could suggest the rate of damage during storage was greater in the thicker ileal tissue. It must therefore be concluded that storage for 24 hours would not be a suitable model for the neuronal degeneration which occurs in grass sickness.

With stored tissue, propranolol and phentolamine had no significant effect on the rate or amplitude of background contractions compared with stored tissue alone. This suggested that, as with fresh tissue, the adrenergic system is not involved in the control of background contractions.

In conclusion, these results demonstrate that *in vitro,* the characteristic background contractions for control tissue and grass sickness tissue show some significant differences. The responses to selected pharmacological agents suggest that the background contractile rate and amplitude of the horse small intestine *in vitro,* is mainly due to myogenic mechanisms although there is likely to be some influence from intrinsic nerves, especially in the duodenal region.

The investigation of the characteristics of background contractions provides useful information especially since the literature provides little information about *in vitro* work with equine intestinal tissue. Now it is necessary to investigate some of the intrinsic systems which control motility in more detail and establish how tissue is affected by grass sickness.

CHAPTER 5

CHOLINERGIC SYSTEM

5.0 General introduction

The motor responses of the cholinergic and adrenergic systems, either acting directly on the muscle or via the enteric nervous system, were examined using the *in vitro* technique. The cholinergic system will be discussed first under three main headings; responses to physostigmine (5.1), responses to bethanecol (5.2) and responses to cisapride (5.3). The aims of the work were to investigate the extent of damage to cholinergic neurones in cases of grass sickness and also to see whether cholinergic receptor sensitivity was altered. In addition the prokinetic drug cisapride was investigated in order to assess the potential for any therapeutic effect in the treatment of grass sickness cases.

5.1 RESPONSES TO PHYSOSTIGMINE

5.1.1 Introduction

Physostigmine, also known as eserine, is a naturally occurring anticholinesterase agent obtained from the Calabar bean (Schild, 1980). Anticholinesterases prevent the breakdown of acetylcholine by cholinesterases. When present, acetylcholine therefore accumulates at the cholinergic receptor sites. Consequently anticholinesterases are capable of producing effects equivalent to excessive stimulation of cholinergic receptors throughout the central and peripheral nervous systems. In view of the widespread distribution of cholinergic neurones it is not surprising that anticholinergic agents, as a group, have received extensive application as toxic agents in the form of agricultural insecticides and potential chemical warfare 'nerve gas' (Taylor, 1990b).

There are several mechanisms by which anticholinesterases exert their action. For reversible inhibitors, such as physostigmine, their action is due to the formation of a reversible complex between the cholinesterase enzyme and the inhibitor molecule. As this complex is a carbamyl and not acetyl ester, it takes longer to hydrolyse (Pugh, 1985; Wilson, Hatch and Ginsburg, 1960). The duration of action of

anticholinesterase depends on the rate of dissociation of the enzyme inhibitor complex and the rate at which the free inhibitor is removed from the body by metabolism and excretion. Irreversible inhibitors, such as the organophosphorous compounds, probably exert their action by phosphorylation of the esteratic site of the active enzyme centre of cholinesterase (Rang and Dale, 1991).

Although, due to its anticholinesterase properties, physostigmine increases gut motility, it is not used clinically as it produces nausea and vomiting in man, it also causes a slowing of the heart, a fall in blood pressure, muscular twitching and other central nervous system effects (Schild, 1980). Physostigmine may be used to produce miosis of the pupil and reduce intraocular pressure in the treatment of glaucoma (Einstein, Jones, Knifton and Starner, 1994). Physostigmine is not used therapeutically in the horse. There has however, been one report of the use of physostigmine in grass sickness cases (Masheter, 1922), although they were acute type cases and showed no response to physostigmine.

5.1.2 Materials and methods

The apparatus for these experiments is described in Chapter 3. In this series of experiments 25 control horses, ten horses suffering from AGS, eight horses suffering from SAGS, and 12 horses suffering from CGS were used. Details of the horses are given in Appendix C. Only the control, AGS and CGS groups were used to study the effect of storage for 24 hours at 4°C. The drug used was physostigmine sulphate (BDH Chemicals). When equine intestinal muscle is studied *in vitro* the response to an accumulation of acetylcholine can be measured as an increase in the rate of background contractions, an alteration in the amplitude of contractions, or an increase in the tone of the muscle strip.

A ten minute period prior to the addition of physostigmine was used to measure the initial rate of background contractions and to establish the baseline tone. The latency was the time before any alteration in rate or tone could be observed following the addition of physostigmine. At this point the following seven minutes were used to calculate any enhanced rate of contractions. At the end of this period the increase in tone was measured if applicable.

5.1.2.1 Statistical analysis

Results are presented as medians with the range of values shown in brackets. The data were analysed firstly control tissue compared with all the grass sickness data combined. Then the control data against the three grass sickness groups, AGS, SAGS and CGS which depended on clinical signs and duration of illness. Also in Appendix D the grass sickness data was divided into two groups depending on the time taken before a response to physostigmine was observed. Differences between the groups were analysed for statisical significance using the Mann-Whitney U-test. For differences within a group the Wilcoxon Signed Rank test was used. Differences between the two regions of the gut were analysed using the Wilcoxon Signed Rank test. The effect of storage, for a particular group was also analysed using the Wilcoxon Signed Rank test as samples from the same horses were used in experiments on fresh and stored tissue.

5.1.3 Results

5.1.3.1 Duodenal preparations

In initial experiments, the responses of control tissue in terms of an increase in contractile rate and tone of the smooth muscle strips to different concentrations of physostigmine, established that there were final concentrations below which little response could be found (below $8.6x10^{-8}$M). Above $2.8x10^{-5}$M muscle strips showed increased tone, almost spasm and prolonged recovery time. In the intermediary range, tissues responded with measurable changes in both rate and tone. It was therefore decided to use a standard concentration of $8.6x10^{-6}$M and to compare responses between the four different groups.

Typical responses to physostigmine for duodenal muscle strips taken from a control horse and one affected by AGS are shown in Figure 5.1.1. In both traces there was an increase in the rate of contractions and an upward shift of baseline. The results for responses to physostigmine are shown in Table 5.1.1 and 5.1.2.

Following the addition of physostigmine ($8.6x10^{-6}$M) the rate of background contractions significantly increased in the control duodenal group ($P<0.0001$). Figure 5.1.2A shows the increase in the rate of contractions following physostigmine

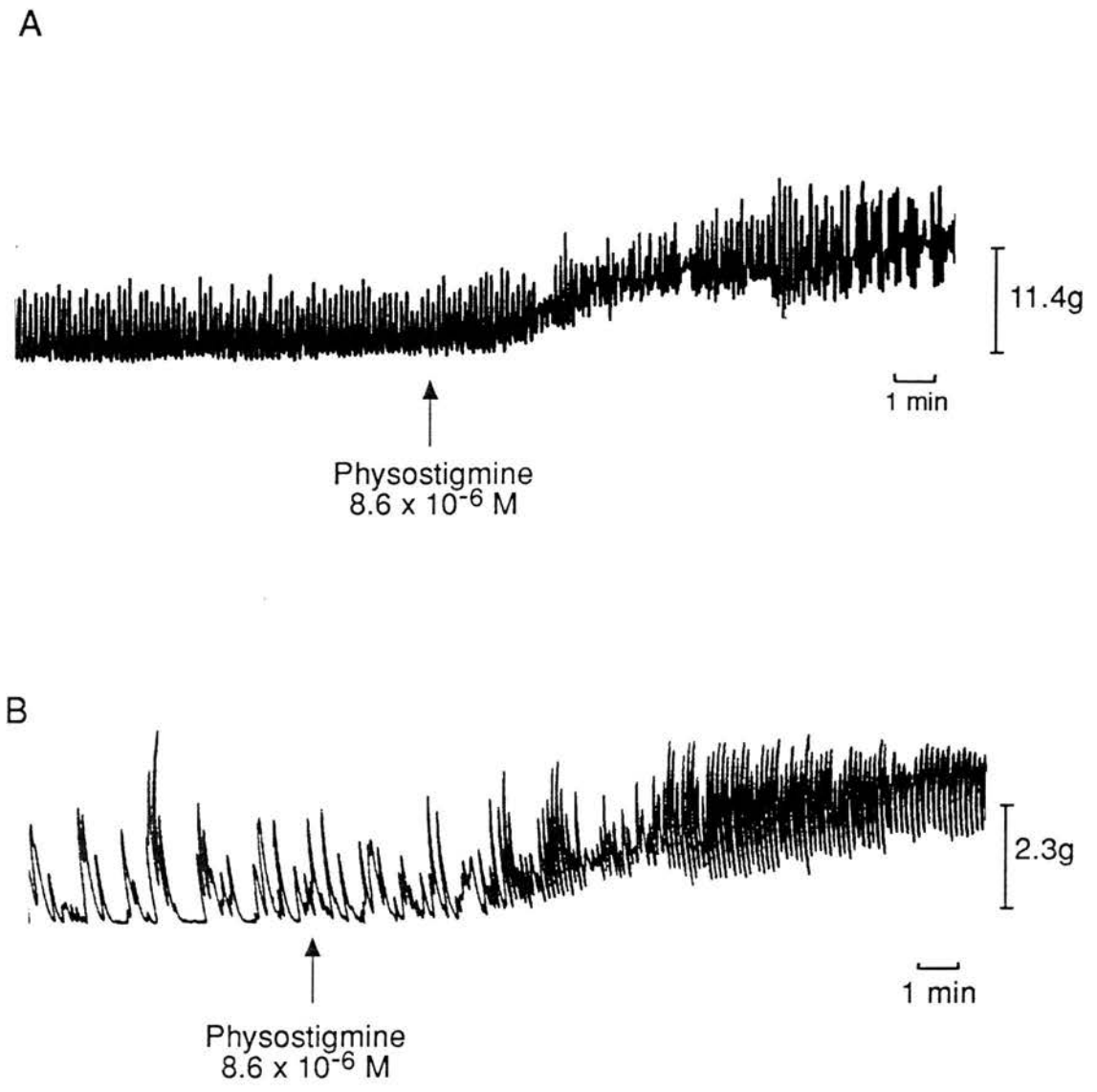


Figure 5.1.1. Effect of physostigmine ($8.6x10^{-6}M$) on an longitudinal muscle strip taken from A) the duodenum of a control horse, B) the duodenum of a horse suffering from acute grass sickness (note the different transducer calibrations).

	n	initial rate [contractions/min]	rate after physostigmine [contractions/min]	percentage increase [%]	latency [s]	tone increase [g]
Control	25	6.5 [ac] (3.4-11.5)	9.2[ad] (6.2-15.0)	43.7[e] (4.4-177.8)	90.0[f] (30.0-168.0)	5.1 (1.4-12.1)
Grass Sickness	30	3.8[bc] (0.8-8.6)	7.8[bd] (4.3-12.0)	129.5[e] (0.0-512.5)	160.0[f] (60.0-420.0)	3.3 (0.9-18.0)

Table 5.1.1. Effect of addition of physostigmine ($8.6x10^{-6}$M) on isolated duodenal muscle strips taken from control horses and those suffering from grass sickness. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test b,c,f = $P<0.0001$, a = $P<0.0005$, d,e = $P<0.005$.

	n	initial rate [contractions/min]	rate after physostigmine [contractions/min]	percentage increase [%]	latency [s]	tone increase [g]
Control	25	6.5 ^{abcd} (3.4-11.5)	9.2^{dh} (6.2-15.0)	43.7^{jk} (4.4-177.8)	90.0^{lmn} (30.0-168.0)	5.1^{p} (1.4-12.1)
AGS	10	3.8^{ae} (0.8-6.2)	7.8^{eh} (4.3-11.5)	129.5^{j} (17.7-512.5)	247.0^{l} (92.0-420.0)	1.8^{p} (0.9-5.0)
SAGS	8	3.2^{bf} (1.9-8.4)	8.4^{f} (5.2-10.6)	168.1^{k} (4.8-215.2)	190.0^{mo} (128.0-280.0)	3.1 (1.0-9.4)
CGS	12	4.3^{cg} (1.4-8.6)	7.4^{g} (5.2-12.0)	79.4 (0.0-360.0)	126.0^{no} (60.0-192.0)	5.0 (2.0-18.0)

Table 5.1.2 Effect of addition of physostigmine ($8.6x10^{-6}$M) on isolated duodenal muscle strips taken from control horses and those suffering from acute grass sickness (AGS), subacute grass sickness (SAGS), and chronic grass sickness (CGS). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other d = P<0.0001, e,g = P<0.01, f = P<0.05 when analysed using Wilcoxon Signed Rank test, m = P<0.0001, l = P<0.0005, a = P<0.001, b = P<0.005, j,n,o,p = P<0.01, c,h,k = P<0.05 when analysed using the Mann-Whitney U-test.

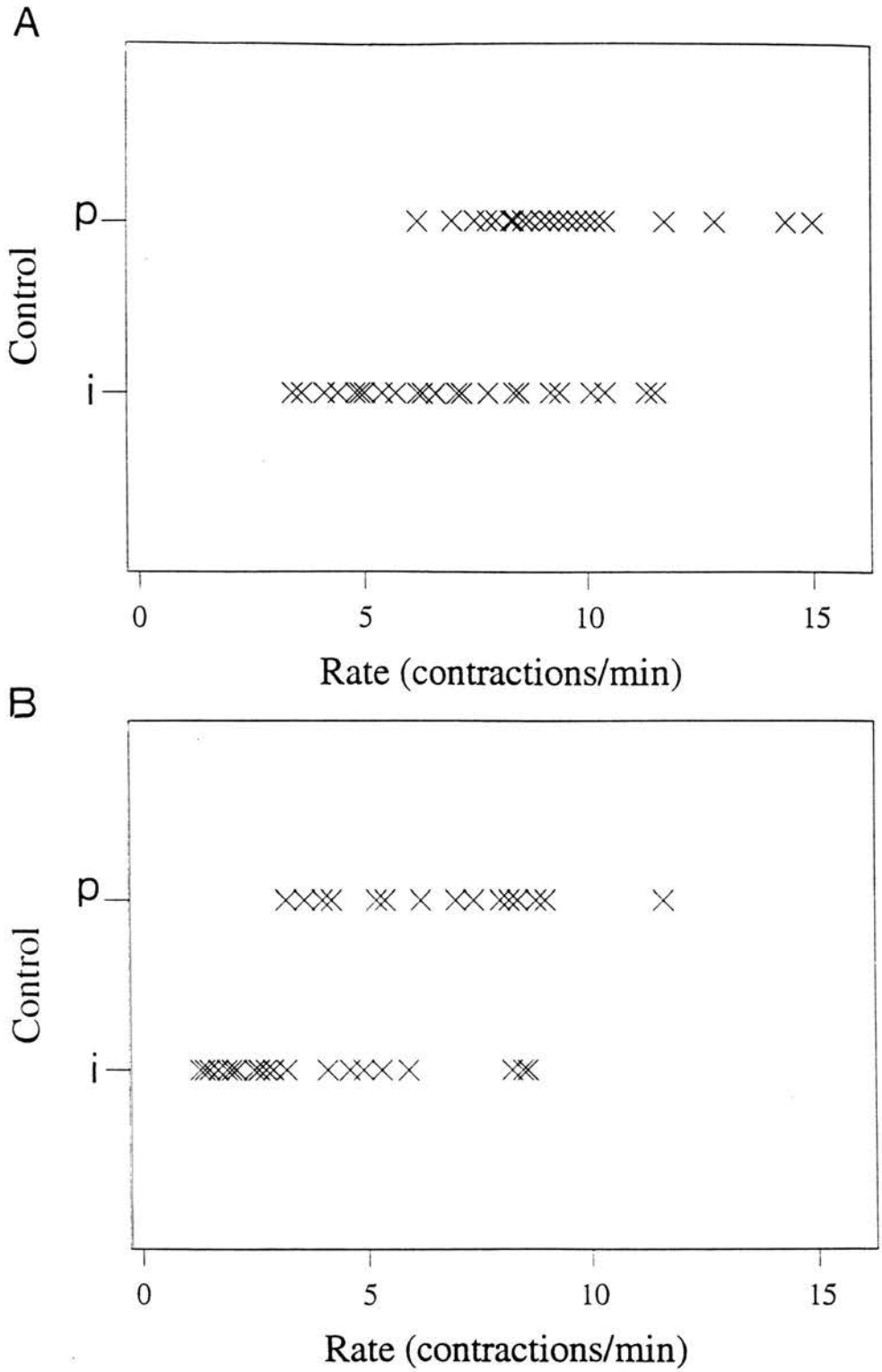


Figure 5.1.2. The effect of physostigmine ($8.6x10^{-6}$M) on the rate of contractions of A) duodenal smooth muscle strips taken from the control group, B) ileal smooth muscle strips taken from the control group. On the y-axis i corresponds to the initial rate of background contractions and p to the rate of contractions following treatment with physostigmine.

addition for the control duodenal group. The median rate increased from 6.5 to 9.2 contractions per minute for control duodenal strips (Table 5.1.1).

For all grass sickness affected duodenal tissue the initial rate of background contractions was significantly lower compared with duodenal control tissue (AGS $P<0.001$, SAGS $P<0.005$, CGS $P<0.05$) (Table 5.1.2). The addition of physostigmine ($8.6x10^{-6}M$) produced a significant increase in the rate of contractions in all three grass sickness groups compared with their initial rates (AGS and CGS $P<0.01$, SAGS $P<0.05$). However, these increased rates were still lower than the enhanced rate for the control group, significantly lower in the case of the AGS group ($P<0.01$) (Table 5.1.2).

The median percentage increase in rate was largest in the SAGS group (168%), followed by the AGS group (130%), with the lowest percentage increase for the control group (44%). For the AGS and SAGS grass sickness groups the percentage rate increase was significantly higher than the control group (AGS $P<0.01$, SAGS $P<0.05$).

Figure 5.1.3 illustrates the latency before a response to physostigmine for the duodenal grass sickness groups compared with the duodenal control group. The latency was significantly longer for all the grass sickness groups compared with the control group (SAGS $P<0.0001$, AGS $P<0.0005$ and CGS $P<0.01$).

The median increase in tone was very similar for the control and CGS groups. Only the AGS group was significantly lower than the control group ($P<0.01$) (Table 5.1.2).

5.1.3.2 Ileal preparations

Typical responses to physostigmine for ileal control tissue and that from a horse affected by AGS are shown in Figure 5.1.4. For both preparations the burst-like pattern of background contractions changed into more regular contractions following physostigmine addition, thus causing a increase in the rate of contractions. For the AGS muscle strip the latency before this increase in contractile rate was much longer than for the control strip (Figure 5.1.4). With the control ileal strip there was also an

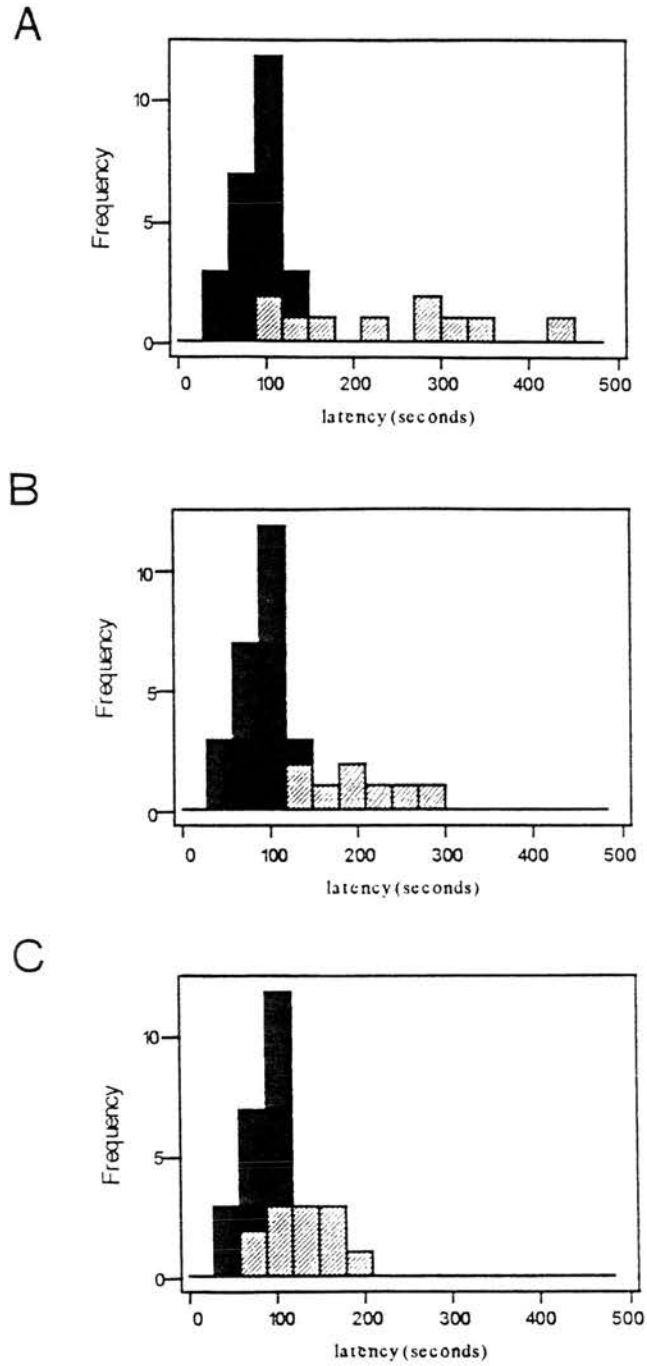


Figure 5.1.3. Distribution of the time period (latency) before a measurable response to physostigmine ($8.6x10^{-6}$M) addition occurred. The solid histogram represents the latency in seconds for the duodenal control group. The hatched histogram represents the latency histogram for the duodenal A) AGS group, B) SAGS group and C) CGS group.

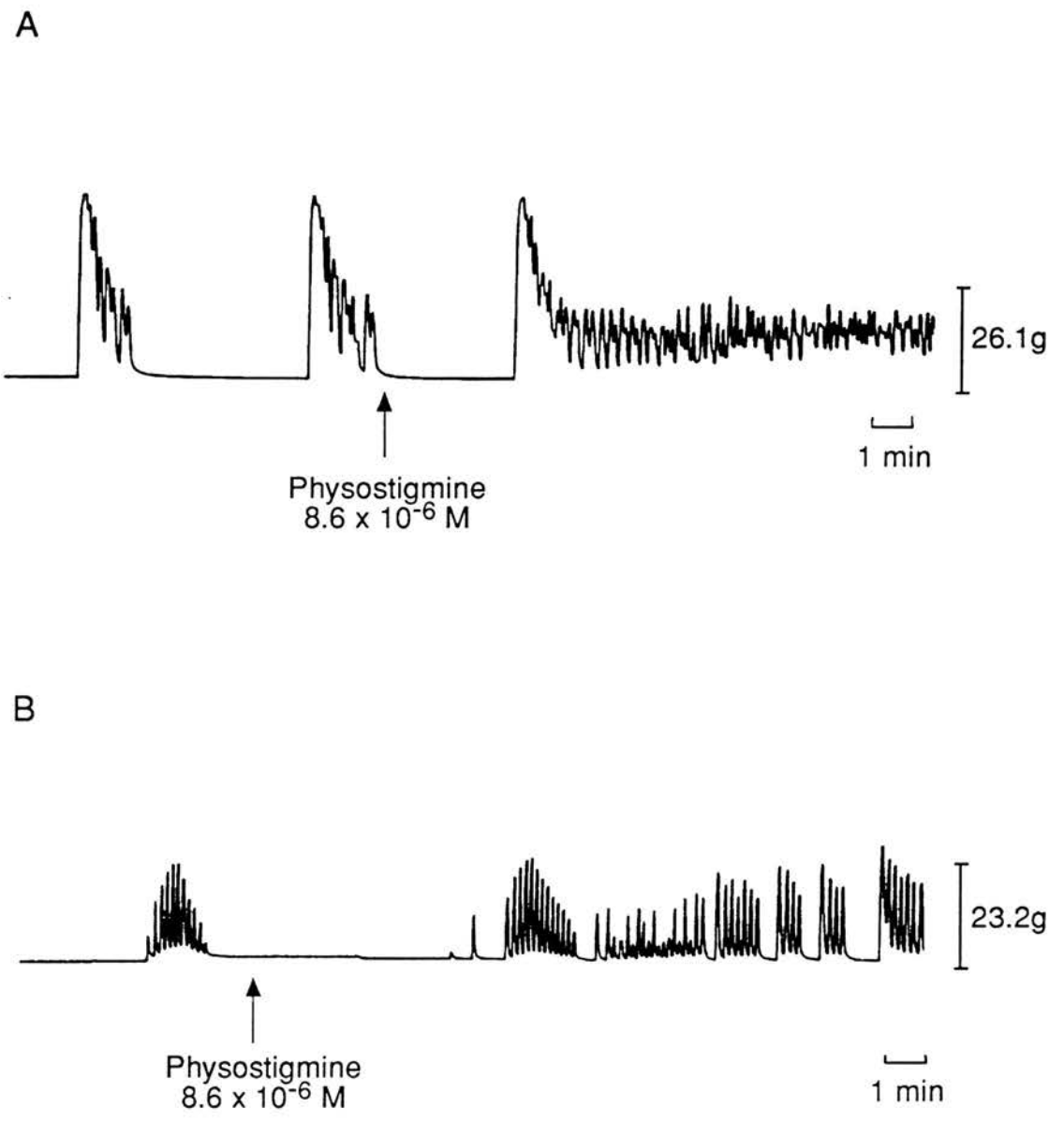


Figure 5.1.4. Effect of physostigmine ($8.6x10^{-6}M$) on an isolated longitudinal muscle strip taken from A) the ileum of a control horse, B) the ileum of a horse suffering from acute grass sickness.

increase in the tone of the preparation (upward shift of baseline), but with the AGS muscle strip this was not seen (Figure 5.1.4).

The median results for ileal preparations in response to physostigmine are shown in Tables 5.1.3 and 5.1.4. For control ileal preparations the rate of background contractions significantly increased following the addition of physostigmine ($P<0.0001$). Figure 5.1.2B shows the increase in the rate of contractions for the control ileal group.

All grass sickness affected ileal tissue had a lower, but non-significant, median initial rate of background contractions compared with the control ileal group (Table 5.1.4). Following addition of physostigmine, they all showed significant increases in their rate of contractions (AGS $P<0.005$, SAGS and CGS $P<0.05$). When compared with the enhanced rate following physostigmine for the control ileal group, the contractile rate for all three grass sickness ileal groups were significantly lower ($P<0.005$). Figure 5.1.5 shows the increase in rate of contractions following physostigmine for the duodenal and ileal AGS groups.

There was no significant difference between the percentage rate increase for any of the ileal groups. In the ileal region, it was the AGS group which had the lowest percentage rate increase following physostigmine addition, whereas in the duodenum it was the control group which showed the lowest increase.

The latency before a response could be seen followed a similar pattern as was found in the duodenum with AGS having the longest median latency of 228 seconds. Only the latency of the AGS and SAGS groups was significantly longer than the control ileal group ($P<0.05$) (Table 5.1.4).

In all AGS and SAGS muscle strips no increase in tone was ever observed, thus a value of zero was used. There were therefore significant differences between these two groups and the control and CGS groups ($P<0.001$) (Table 5.1.4). The range of values for increase in tone for the control and CGS ileal groups were very similar.

Table 5.1.4 shows any significant differences between the responses to physostigmine when the two regions were analysed using the Wilcoxon Signed Rank test.

	n	initial rate [contractions/min]	rate after physostigmine [contractions/min]	percentage increase [%]	latency [s]	tone increase [g]
Control	23	2.6^{ac} (0.0-8.6)	7.4^{ad} (3.2-16.4)	118.3 (2.3-363.2)	112.0^{e} (72.0-208.0)	1.2^{f} (0.0-21.0)
Grass Sickness	24	1.7^{bc} (0.6-6.2)	3.0^{bd} (1.2-8.4)	76.5 (3.2-300.0)	164.0^{e} (60.0-384.0)	0.0^{f} (0.0-20.8)

Table 5.1.3 Effect of addition of physostigmine ($8.6x10^{-6}$M) on isolated ileal muscle strips taken from control horses and those suffering from grass sickness. Values are medians with the range shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test. d = $P<0.0001$, a,b = $P<0.0005$, c = $P<0.01$, e,f = $P<0.05$.

	n	initial rate [contractions/min]	rate after physostigmine [contractions/min]	percentage increase [%]	latency [s]	tone increase [g]
Control	23	2.6^{a} * (0.0-8.6)	7.4^{acfg} Δ (3.2-16.4)	118.3 Δ (2.3-363.2)	112.0^{hi} (72.0-208.0)	1.2^{jk} (0.0-21.0)
AGS	10	1.8^{b} ♣ (0.8-4.2)	3.8^{bc} ♣ (1.2-8.4)	60.0 (15.1-245.4)	228.0^{h} (60.0-384.4)	0.0^{jl} ♦ (0.0-0.0)
SAGS	6	1.7^{c} ♣ (0.7-2.5)	2.9^{cf} (1.6-5.6)	137.6 (20.0-300.0)	222.0^{i} (84.0-324.0)	0.0^{km} ♣ (0.0-0.0)
CGS	8	1.5^{d} (0.6-6.2)	2.5^{dg} ♣ (1.8-6.6)	76.3 (3.2-200.0)	108.0^{lm} (60.0-304.0)	2.0^{lm} (0.0-20.8)

Table 5.1.4. Effect of addition of physostigmine ($8.6x10^{-6}$M) on isolated ileal muscle strips taken from control horses and those suffering from acute grass sickness (AGS), subacute grass sickness (SAGS), and chronic grass sickness (CGS). Values are medians with the range shown in brackets. Values with the same superscript are significantly different from each other a = P<0.0001, b = P<0.005 c,d = P<0.05 when analysed using Wilcoxon Signed Rank test, j,k,l,m = P<0.001, e,f,g = P<0.005, h,i = P<0.05 when analysed using the Mann-Whitney U-test. *, Δ, ♦, ♣ indicates there is a significant difference between the ileal and duodenal values (* P<0.0001, Δ P<0.005, ♦ P<0.01 and ♣ P<0.05).

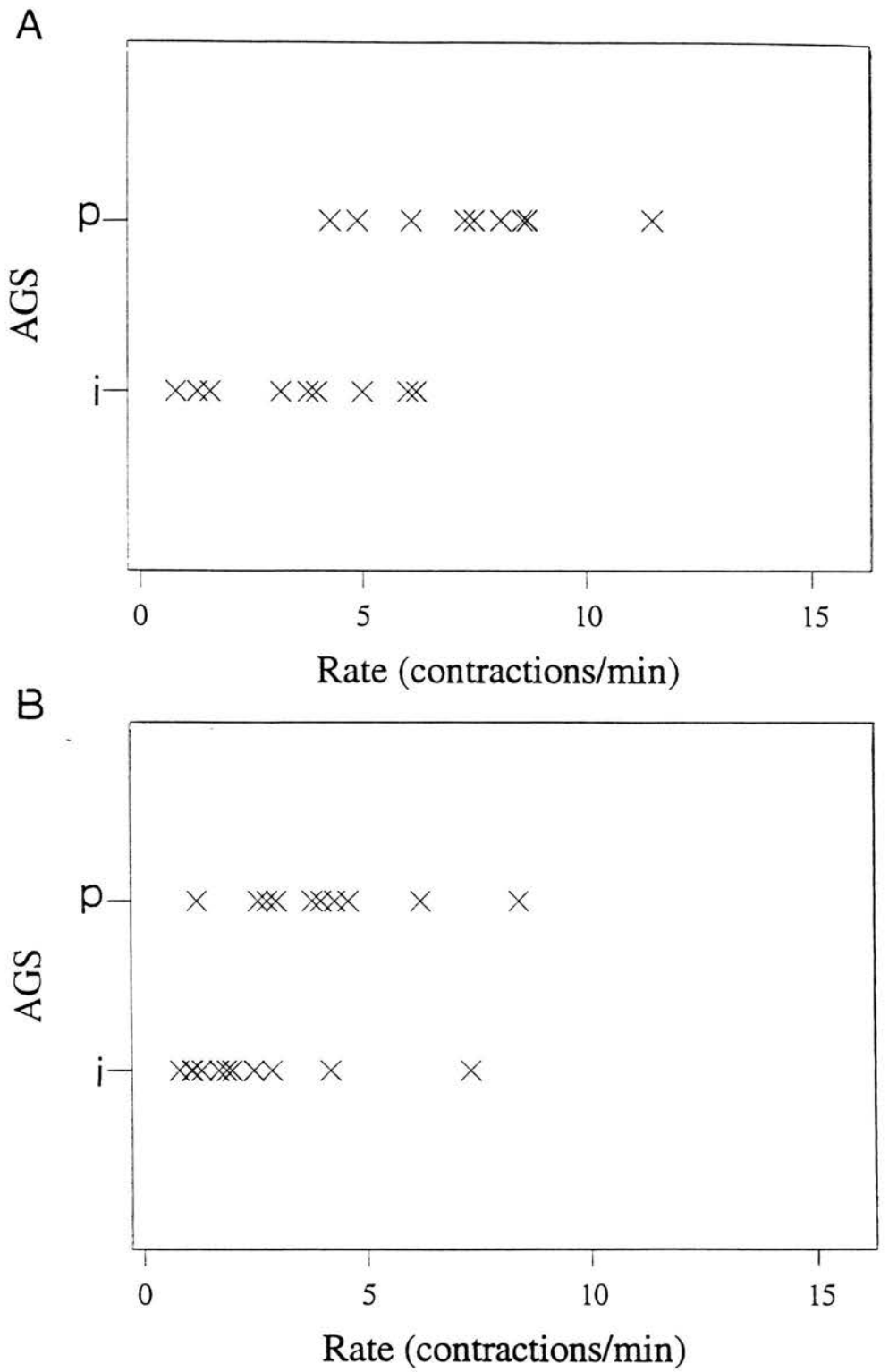


Figure 5.1.5. The effect of physostigmine ($8.6x10^{-6}$M) on the rate of contractions of A) duodenal smooth muscle strips taken from the AGS group, B) ileal smooth muscle strips taken from the AGS group. On the y-axis i corresponds to the initial rate of background contractions and p to the rate of contractions following treatment with physostigmine.

5.1.3.3 Effect of 24 hour storage on responses of duodenal preparations

The initial rate of background contractions for control duodenal tissue was significantly lower ($P<0.05$) for stored tissue compared with fresh tissue, with the median rate reducing from 6.5 to 4.6 contractions per minute (Tables 5.1.1 and 5.1.5). There was also a trend for the AGS and CGS groups to have lower initial contractile rates with stored tissue. Only the initial rate of background contractions for stored CGS tissue was significantly lower than the stored control group ($P<0.05$) (Table 5.1.5).

Following treatment with physostigmine ($8.6x10^{-6}M$) the rate of contractions for stored control tissue significantly increased ($P<0.0001$). This increased value was not significantly different from the increased rate of contractions following physostigmine for control fresh tissue. With both stored AGS and CGS tissue following storage and physostigmine addition there was a non-significant increase in the rate of contractions.

The CGS group had the largest percentage rate increase following physostigmine although there were no significant differences between any of the groups for this measurement (Table 5.1.6). Figure 5.1.6A shows the effect of physostigmine on the rate of contractions for control duodenal tissue following storage. The percentage increase for the duodenal control group following storage was significantly higher than for fresh tissue ($P<0.05$).

The latency before a response was observed was significantly longer for the stored control duodenal tissue than for fresh control tissue ($P<0.05$). The stored AGS group had a significantly ($P<0.05$) longer latency than the stored control group before a response to physostigmine could be seen (Table 5.1.6).

There were no significant differences between any of the groups for the increase in tone measurement. The AGS group had the lowest median value of 3.1g.

When the effects of storage on the AGS and CGS groups were compared with fresh AGS and CGS tissue there were no significant differences for any of the parameters measured, although it should be noted that the number of experiments used for stored tissue were low .

	n	initial rate [contractions/min]	rate after physostigmine [contractions/min]	percentage increase [%]	latency [s]	tone increase [g]
Control	18	4.6[a] (1.5-8.8)	8.3[ac] (5.2-12.3)	67.3 (23.9-376.2)	120.0[d] (52.0-224.0)	5.8 (1.2-20.0)
Grass Sickness	10	2.2[b] (1.0-6.8)	6.8[bc] (3.6-9.4)	176.5 (8.8-350.0)	236.0[d] (120.0-332.0)	3.8 (0.0-8.9)

Table 5.1.5. Effects of addition of physostigmine ($8.6x10^{-6}$M) on isolated duodenal muscle strips taken from control horses and those suffering from grass sickness following 24 hour storage at 4°C. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test a =$P<0.0001$, d =$P<0.001$, b = $P<0.005$, c =$P<0.05$.

	n	initial rate [contractions/min]	rate after physostigmine [contractions/min]	percentage increase [%]	latency [s]	tone increase [g]
Control	18	4.6[ab] ♠ (1.5-8.8)	8.3[b] (5.2-12.3)	67.3 ♠ (23.9-376.2)	120.0[c] ♠ (52.0-224.0)	5.8 (1.2-20.0)
AGS	5	2.9 (1.3-6.3)	5.6 (3.8-9.3)	204.0 (111.4-315.4)	270.0[c] (232.0-332.0)	3.1 (1.1-6.8)
CGS	5	2.2[a] (1.6-3.4)	7.7 (3.6-9.4)	224.6 (63.6-350.0)	132.0 (120.0-332.0)	7.3 (0.0-8.0)

Table 5.1.6 Effects of addition of physostigmine ($8.6x10^{-6}$M) on isolated duodenal muscle strips taken from control horses and those suffering from acute grass sickness (AGS) and chronic grass sickness (CGS) following 24 hour storage at 4°C. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other b = $P<0.0001$ when analysed using the Wilcoxon Signed Rank test, a,c = $P<0.05$ when analysed using the Mann-Whitney U-test. ♠ indicates there is a significant difference from the equivalent fresh value ($P<0.05$) (i.e. Table 5.1.1).

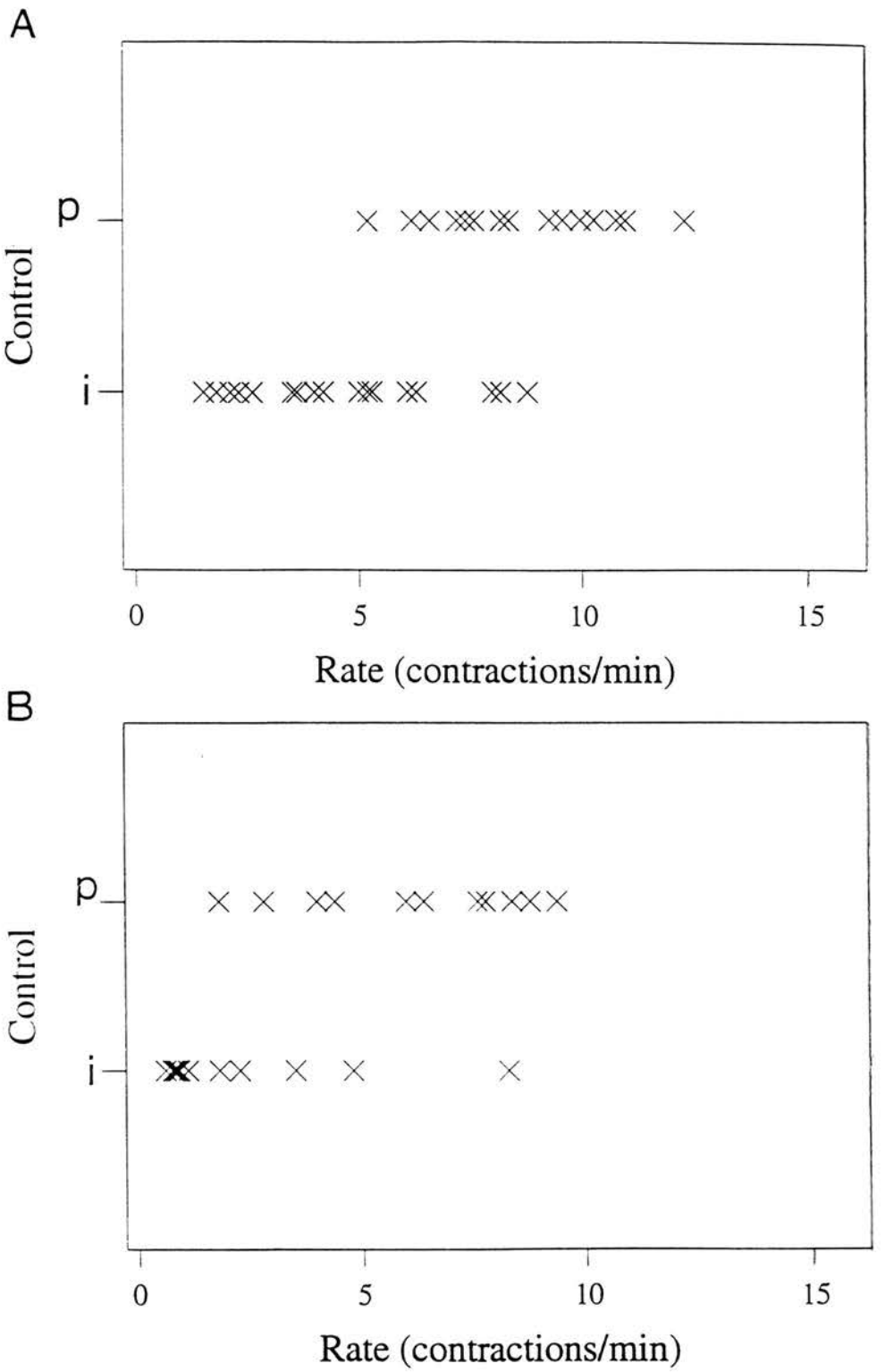


Figure 5.1.6. The effect of physostigmine ($8.6x10^{-6}$M) on the rate of contractions of A) duodenal smooth muscle strips taken from the control group following storage at 4°C for 24 hours, B) ileal smooth muscle strips taken from the control group following storage at 4°C for 24 hours. On the y-axis i corresponds to the initial rate of background contractions and p to the rate of contractions following treatment with physostigmine.

The stored control results were compared with fresh AGS, SAGS and CGS values using the Mann-Whitney U-test to see if the neuronal damage which occurred due to storage was similar to that known to occur in grass sickness cases. There were significant differences between the stored control latency value and that of fresh AGS and SAGS values ($P<0.05$). There was also a significant difference in the tone results for the fresh AGS group compared with the stored control duodenal group ($P<0.01$). There were no significant differences between the values for contractile rate either before or after physostigmine addition.

5.1.3.4 Effect of 24 hour storage on responses of ileal preparations

Although the median initial rate of contractions for stored control ileal tissue was lower than for fresh tissue, the difference was not significant (Table 5.1.3 and 5.1.8). There was also no significant difference between AGS and CGS stored and fresh values with respect to their initial contractile rate. Following treatment with physostigmine ($8.6x10^{-6}M$) there was a significant ($P<0.005$) increase in the rate of contractions for control stored ileal tissue. The addition of physostigmine produced a non-significant increase in the median rate of contractions for both grass sickness groups. The increased rate of contractions for the stored AGS group was significantly lower ($P<0.05$) than for the stored control group. Figure 5.1.6B shows the effect of physostigmine on the rate of contractions for control ileal tissue following storage.

As there was only a small increase in the rate of contractions, the medians of the percentage rate increase were low for both the grass sickness groups. The percentage increase for the stored control ileal group was significantly higher than for fresh tissue ($P<0.05$).

The stored ileal AGS group had a significantly ($P<0.005$) longer latency than the stored ileal control group.

As was found with fresh tissue there was no increase in tone following physostigmine with the AGS group. The increase in tone for the CGS group also had a median value of 0g (Table 5.1.8).

	n	initial rate [contractions/min]	rate after physostigmine [contractions/min]	percentage increase [%]	latency [s]	tone increase [g]
Control	11	1.1[a] (0.6-8.3)	6.4[ab] (1.8-9.4)	237.8[c] (1.2-611.1)	152.0[d] (104.0-360.0)	2.5[e] (0.0-22.5)
Grass Sickness	9	1.4 (0.5-4.4)	3.4[b] (1.0-6.2)	70.0[c] (10.0-233.3)	416.0[d] (160.0-640.0)	0.0[e] (0.0-1.5)

Table 5.1.7. Effect of addition of physostigmine ($8.6x10^{-6}$M) on isolated ileal muscle strips taken from control horses and those suffering from grass sickness following 24 hour storage at 4°C. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test a,d = $P<0.005$, b,c,e = $P<0.05$.

	n	initial rate [contractions/min]	rate after physostigmine [contractions/min]	percentage increase [%]	latency [s]	tone increase [g]
Control	11	1.1[a] ♣ (0.6-8.3)	6.4[ab] (1.8-9.4)	237.8 ♠ ♣ (1.2-611.1)	152.0[c] (104.0-360.0)	2.5 [d] (0.0-22.5)
AGS	5	1.0 (0.8-4.4)	2.2[b] (1.0-6.2)	40.9 (10.0-233.3)	416.0[c] (380.0-640.0)	0.0[de] (0.0-0.0)
CGS	4	2.0 (1.4-2.1)	3.4 (2.9-4.0)	90.5 (70.0-107.1)	372.0 (160.0-432.0)	0.0[e] (0.0-1.5)

Table 5.1.8. Effect of addition of physostigmine ($8.6x10^{-6}$M) on isolated ileal muscle strips taken from control horses and those suffering from acute grass sickness (AGS) and chronic grass sickness (CGS) following 24 hour storage at 4°C. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other, a = $P<0.005$ when analysed using the Wilcoxon Signed Rank test, c = $P<0.005$, d,e = $P<0.001$, b = $P<0.05$ when analysed using the Mann-Whitney U-test. ♠ indicates there is a significant difference from the equilvalent fresh value ($P<0.05$) (i.e. Table 5.1.2). ♣ indicates the value is significantly different from the duodenum stored results ($P<0.05$).

When the stored ileal control data was compared with stored duodenal control data there was a significant difference in the percentage increase following physostigmine ($P<0.05$). Also in the initial contractile rate of control tissue ($P<0.05$). There were no significant differences for the grass sickness groups although again the number of experiments with stored tissue was low (n=5 for AGS and n=3 for CGS).

When the stored ileal control group results were compared with fresh ileal AGS, SAGS and CGS results, there were no significant differences in the initial rate of contractions. There was a significant difference between fresh SAGS and stored control results for rate of contractions following physostigmine addition ($P<0.05$), with the stored control value been higher. For the percentage rate increase there were significant differences between control stored results and fresh AGS ($P<0.01$) and CGS groups ($P<0.05$). There were no significant differences between the latency results. The tone of the ileal AGS and SAGS groups were significantly different from the stored ileal control group ($P<0.01$).

5.1.4 Discussion

The responses to physostigmine were measured as increases in the rate of contractions and tone (baseline) of the smooth muscle strips. The traces produced were similar to those described in laboratory animals (Perry, 1968). The latency before an alteration in these parameters was also recorded.

Physostigmine enhanced the motility of all tissues studied from both fresh and stored tissue, taken from both control horses and those affected by the three clinical forms of grass sickness. The increase in contractile rate was significant in all the fresh muscle strips and in control horses following storage ($P<0.05$ or less). Although the median contractile rate increased in the AGS and CGS groups following physostigmine addition after storage, this increase was not significantly higher in either region of the small intestine. This may be because there was already substantial neuronal damage due to grass sickness (Doxey *et al.*, 1992; Scholes *et al.*, 1993a) and that following storage there

were insufficient numbers of healthy neurones to produce enough acetylcholine to alter motility significantly. There have been no reports of the type of neurones damaged in grass sickness. There have been some immunohistochemical studies which have reported a depletion of both peptide-containing cells and nerves in grass sickness cases (Sabate *et al.*, 1983; Bishop *et al.*, 1984; Vaillant, 1991; Pearson, G.T. and Woodman, M.P., personal communication).

As the grass sickness tissue had low initial contractile rates, following the addition of physostigmine the percentage increase in rate was significantly higher for both the fresh duodenal AGS and SAGS groups than the duodenal control group. For ileal tissue, the control group had the highest median percentage increase value. This pattern continued with stored tissue i.e. in the duodenal preparations the grass sickness groups had the highest median percentage increase in contractile rate whereas in the ileal regions it was the control group which had the highest value.

In the duodenal region only the AGS group had a significantly lower rate of contractions following physostigmine than the control group, whereas in the ileum all three grass sickness groups had a significantly lower enhanced rate compared with the ileal control group. This suggests that the ileal region was the worse affected in grass sickness.

Initially the absolute values for tone of the preparations were not known. As discussed in Chapter 3 all duodenal preparations had a uniform tension of 2g applied, and during the equilibration period these preparations relaxed and then maintained a constant baseline with regular contractions. For ileal tissue the situation was more complicated as the initial tension applied to the preparations varied depending on animal size and muscle thickness. However, as with duodenal strips, the tissue relaxed during the equilibration period to establish a constant baseline.

All duodenal muscle strips showed a positive increase in tone in response to physostigmine. Only duodenal AGS fresh tissue showed a significantly lower increase in tone compared with the duodenal control group

($P<0.01$). This was probably due to a smaller accumulation of acetylcholine in the AGS group. Indeed in the ileal region, no AGS or SAGS muscle strips showed any increase in tone following physostigmine addition (Table 5.1.2). This indicates that the number of surviving cholinergic neurones in grass sickness cases may be lower in the ileum than in the duodenum i.e. that grass sickness causes greatest damage in the ileum. This has indeed been suggested (Scholes *et al.*, 1993a; Doxey, D.L., Milne, E.M., Woodman, M.P., Gilmour, J.S. and Chisholm, H.K. submitted for publication). However, it may be that there are a lower number of cholinergic neurones in the ileal region of the horse initially and that damage due to grass sickness is in fact uniform throughout the small intestine. No references to the population of cholinergic neurones throughout the length of the gastrointestinal tract could be found in any species.

The significantly prolonged latency before a response to physostigmine with AGS and SAGS tissue provides further evidence for a functional deficit of cholinergic neurones in grass sickness. Fewer surviving cholinergic neurones would be expected to take longer to release enough acetylcholine to affect motility.

The effect of storage was investigated to see if stored control tissue would respond similarly to fresh grass sickness tissue and thus provide a model for grass sickness *in vitro* research. As there were some significant differences in the responses to physostigmine between control tissue following storage and fresh grass sickness tissue, particularly AGS tissue, it must be concluded that 24 hour storage at 4°C is not a suitable model for grass sickness.

Following 24 hour storage, grass sickness tissue showed no significant increase in the rate of contractions following treatment with physostigmine. This may be because there was substantial neuronal damage already and that following storage there were insufficient healthy neurones to produce sufficent acetylcholine to alter motility.

The enhanced rate of contractions and increased tone of equine intestinal tissue produced *in vitro* by physostigmine establish a pharmacological basis for the observed therapeutic usefulness of certain prokinetic drugs which may

activate or supplement the enteric cholinergic system in selected cases of grass sickness. Milne. E.M., Doxey, D.L., Woodman, M.P., Cuddeford, D. and Pearson, R.A. (in press) give details of the use of cisapride in the treatment of selected cases of CGS (see Chapter 5.3).

In conclusion, physostigmine has been found to be a useful pharmacological tool in showing differences between the control and grass sickness groups. It allowed the accumulation of endogenously released acetylcholine to be assessed. The effect of increased levels of acetylcholine is to bring the membrane potential of smooth muscle to the threshold required for the generation of spikes (action potentials) (Grundy, 1985). This causes an increase in the rate and amplitude of contractions. For grass sickness tissue the results suggest there was substantial cholinergic neuronal damage in the duodenum and ileum with the result that the accumulation of acetylcholine was reduced, therefore the increase in rate of background contractions and tone in response to physostigmine was limited. There was also a significantly longer latency before any responses could be observed.

5.2 RESPONSES TO BETHANECOL

5.2.1 Introduction

Acetylcholine has practically no therapeutic applications because of its diversity of action and its rapid hydrolysis by acetylcholinesterase and plasma butyrylcholinesterase (Taylor, 1990a). As a result, numerous derivatives have been synthesized in an attempt to obtain cholinergic drugs with more selective and prolonged actions. Of the many synthetic derivatives investigated only a few have had a clinical application, for example, carbachol, used in the treatment of impacted colic in the horse and bethanecol, used to stimulate gastrointestinal motility in post operative ileus (POI) and to stimulate urinary bladder contractions. Bethanecol has also been used to enhance gastric emptying and minimize gastro-oesophageal reflux in foals with gastric ulceration (Allen, Pingle, Smith, Conlon and Burgmann, 1993).

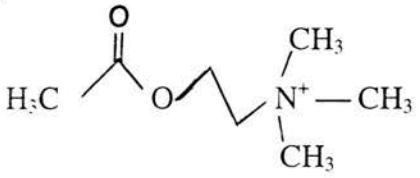
Bethanecol is a cholinergic agonist which largely has muscarinic actions. It also acts with some selectivity on the smooth muscle of the gastrointestinal tract and urinary bladder (Taylor, 1990a). It is an unsubstituted carbamyl ester which is totally resistant to hydrolysis by acetylcholinesterase or nonspecific cholinesterases. The muscarinic action of bethanecol is selectively blocked by atropine (Pugh, 1985).

Bethanecol is capable of producing increases in the amplitude of contractions and tone in the gut, thereby increasing peristaltic activity (Taylor, 1990a). It also enhances the secretory activity of the gastrointestinal tract. However, the results of studies using dogs, by Summers and Flatt (1988), indicate that bethanecol may cause smooth muscular contractions without resulting in coordinated propagating contractions that are necessary for the movement of ingesta. Gerring and Hunt (1986) used bethanecol in combination with yohimbine in their investigation to restore propulsive activity in an experimental model of POI in horses. They concluded that there was some increase in electromechanical activity with these drugs although metoclopramide had a more beneficial effect.

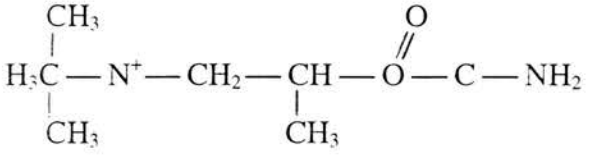
In human gastrointestinal disorders, bethanecol is of value in certain cases of postoperative abdominal distension and gastric atony or gastroparesis (Malagelada, Rees, Mazzotta and Go, 1980; Taylor, 1990a; Rang and Dale, 1991). It has also been

used in the treatment of oesphageal reflux (Saco, Orlando, Levinson, Buzymki, Jones and Frakes 1982).

Structure of acetylcholine



Structure of bethanecol



In skeletal muscle following denervation, hypersensitivity or supersensitivity of the muscle may occur (Janig, 1987). There are a few reports of denervation hypersensitivity in smooth muscle, for example parasympathetic and sympathetic hypersensitivity of the iris in ocular hypertension in humans (Clark and Mapstone, 1987; Clark, 1989). Experiments have also been carried out using laboratory animals, where denervation of regions of the gastrointestinal tract have been induced pharmacologically, to study the responsiveness of denervated tissue to selected pharmacological agents (Herman and Bass, 1990; Osinki and Bass, 1993;1994). Wright and Shepherd (1965) suggested that denervation hypersensitivity may occur in Hirschsprung's disease in humans.

In this Chapter, a series of experiments was conducted on equine intestine to investigate the sensitivity of the smooth muscle strips to bethanecol. In grass sickness, alteration of cholinergic sensitivity may be expected if equine gastrointestinal muscle behaves like other excitable tissue following denervation, with hypersensitivity occurring.

5.2.2 Materials and methods

The *in vitro* method was used (Chapter 3). Smooth muscle strips were taken from the duodenum and ileum from eight control horses, nine horses suffering from AGS, and nine horses suffering from CGS (see Appendix E for details). Molar concentrations of bethanecol chloride (Sigma) were added to the smooth muscle strips (the range of final concentrations was 3mM-0.0001mM). The individual data for each experiment was normalised as a percentage of its maximum response. For each individual experiment the objective was to find the concentration of bethanecol which gave 50% of the maximum contractile response evoked by bethanecol. There was not, therefore, a measurement for every concentration of bethanecol for every experiment. Dose response curves were then constructed for a) control data vs all the data for grass sickness tissue and b) control vs AGS and CGS tissue using the median value for each of the concentrations used. Responses at each concentration were analysed separately using the Mann-Whitney U-test to see if there were any significant differences between the groups. To assess the maximum contractile response the defletion in mms was converted into grams using the calibration curves for each transducer/pen recorder combination (see Chapter 3.2). The value was then divided by the wet weight of the muscle strip to try to reduce some of the variation between animal size and muscle thickness.

5.2.3 Results

5.2.3.1 Duodenal muscle strips

With *in vitro* preparations of equine small intestine, the response to bethanecol was to produce a rapid contraction (Figure 5.2.1). The addition of bethanecol produced dose dependent contractions in all duodenal smooth muscle strips from both control horses and those affected by grass sickness. When all the grass sickness data were combined for both the duodenal and ileal regions and compared with the duodenal and ileal control data respectively there were no significant differences in the results. The dose response curves for the log of the concentration of bethanecol versus the percentage maximum response for the control, AGS and CGS duodenal strips are shown in Figure 5.2.2. The points shown in Figure 5.2.2 are the median

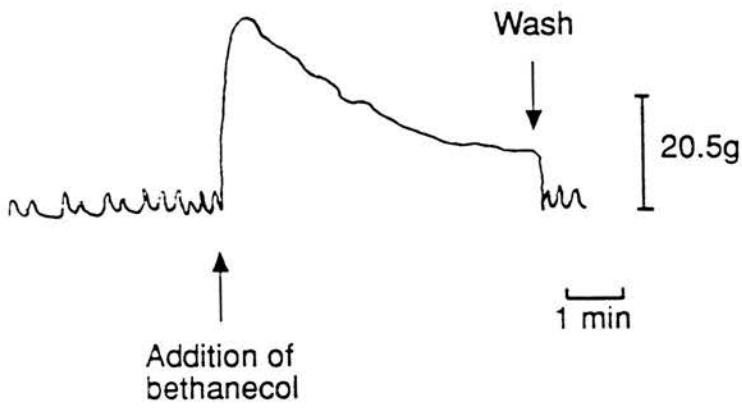


Figure 5.2.1. Response of duodenal muscle strip taken from a control horse to 0.1 mM bethanecol. The contractile response occurs within 10 seconds.

values. Details of the numbers involved and the range of values are given in Tables 5.2.1A, 5.2.1B and 5.2.1C. The maximum contraction obtained in each experiment was either at 3mM or 1mM bethanecol (equivalent to -2.52 or -3 on the log scale respectively). At 3mM bethanecol 62.5% of control duodenal strips had their maximum response compared with 50% for AGS duodenal strips and only 30% for CGS duodenal strips. The maximum responses for smooth muscle strips in grams per gram of wet tissue (median and range) were 39.4 (30.8-61.5) for the control group, 54.8 (32.7-82.2) for the AGS group and 54.9 (18.9-85.6) for the CGS group. However, there were no significant differences between any of the groups for this maximum contractile response.

The sensitivity of the three groups to bethanecol varied. The median values for the AGS group for the range of concentrations were consistently to the left of the control group, suggesting an increased sensitivity to bethanecol for AGS tissue, especially at lower bethanecol concentrations. When these data were analysed using the Mann-Whitney U-test there was only a significant difference ($P<0.05$) between the control and AGS groups at the 0.01mM concentration (-5 on the log scale). This was because at some concentrations there were no actual data for either groups for reasons explained in 5.2.2 and at other points the data were only a mean of two experiments.

The CGS group tended to be less sensitive than the control group at higher concentrations of bethanecol. However, at lower concentrations of bethanecol the tissue became more sensitive than the control tissue, with median values again lying to the left of the control values. Although, there were no significant differences between the control and CGS groups at any concentrations.

At 0.1mM and 0.001mM bethanecol (-4 and -6 on the log scale respectively) there was a significant difference betweeen the AGS and CGS duodenal groups ($P<0.05$).

A reference line was drawn at the 50% level so the ED_{50} values could be measured. These were calculated at 0.00094mM for the AGS group, 0.02mM for the control group and 0.071mM for the CGS group.

A)

Concentration mM	log of concentration	n	median (range) % of maximum response
3	-2.52	5	100.0 (100.0-100.0)
1	-3	8	92.5 (72.4-100.0)
0.6	-3.22	2	83.6 (82.1-85.0)
0.3	-3.52	2	69.6 (57.1-82.1)
0.1	-4	7	66.0 (50.0-71.7)
0.03	-4.52	7	55.3 (52.2-62.5)
0.01	-5	8	41.2 (28.6-50.0
0.001	-6	2	27.3 (26.4-28.2)

B)

Concentration mM	log of concentration	n	median (range) % of maximum response
3	-2.52	5	100.0 (100.0-100.0)
1	-3	9	95.0 (75.0-100.0)
0.1	-4	5	72.2 (58.3-78.4)
0.03	-4.52	3	57.1 (50.0-69.4)
0.01	-5	5	55.2 (41.7-67.6)
0.001	-6	5	51.4 (40.0-58.9)
0.0003	-6.52	3	36.1 (28.6-44.3)
0.0001	-7	2	25.1 (18.8-31.3)

C)

Concentration mM	log of concentration	n	median (range) % of maximum response
3	-2.52	3	100.0 (100.0-100.0)
1	-3	9	100.0 (69.6-100.0)
0.6	-3.22	2	80.1 (78.3-81.8)
0.3	-3.52	5	63.6 (56.5-73.1)
0.1	-4	7	50.0 (30.4-75.0)
0.06	-4.22	2	49.4 (45.7-53.1)
0.03	-4.52	2	46.8 (42.4-51.1)
0.01	-5	6	41.5 (33.3-63.6)
0.003	-5.52	3	46.7 (31.3-48.6)
0.001	-6	4	31.1 (29.7-37.8)

Table 5.2.1. The normalized contractile response of duodenal muscle strips to bethanecol. Values are medians of the maximum response, A) for the control group, B) for the AGS group and C) for the CGS group. The range of values are shown in brackets.

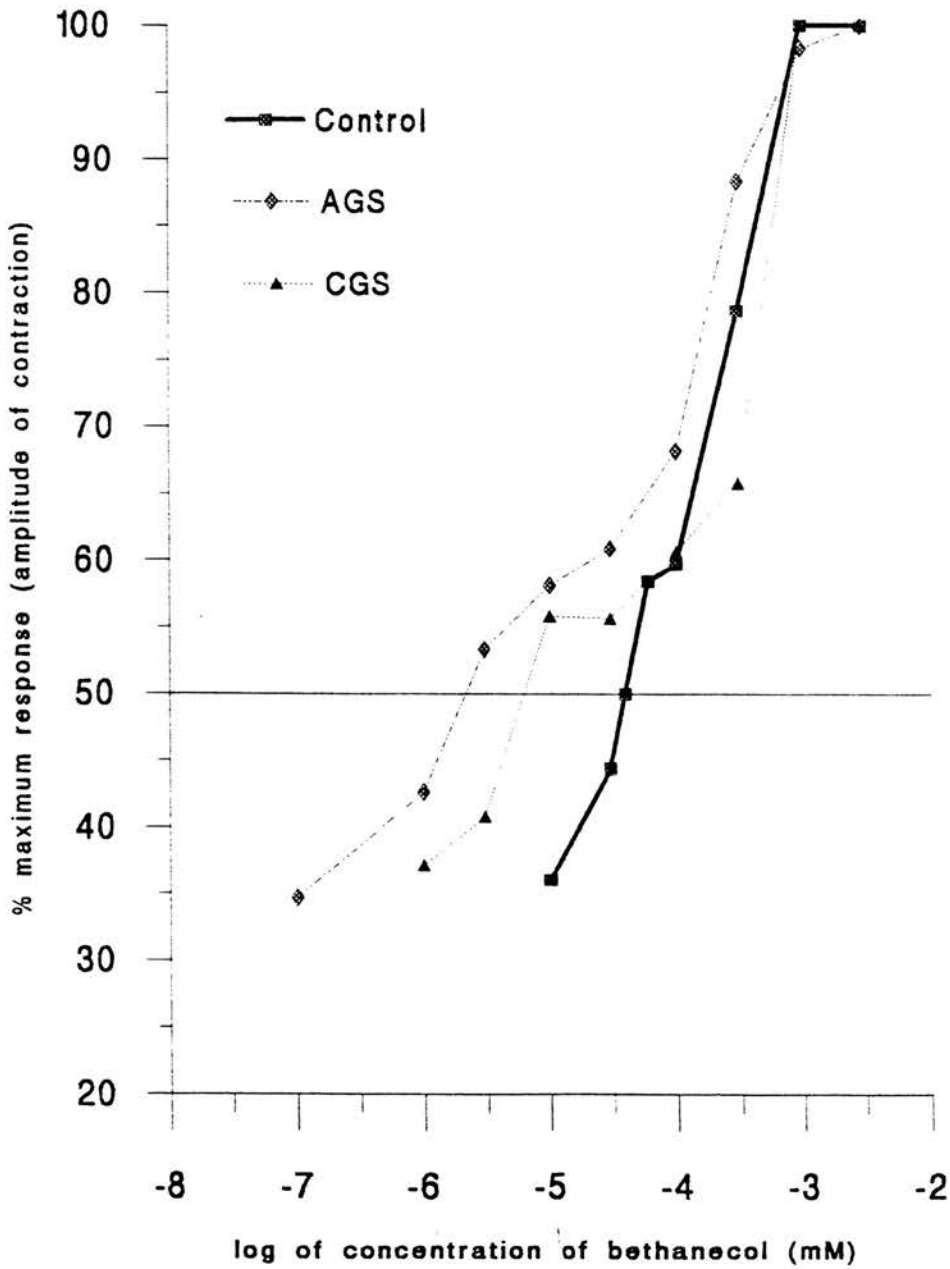


Figure 5.2.2. Dose response curves for bethanecol on strips of equine duodenum. The points shown are median values. 'Control' refers to horses euthanased owing to diseases unrelated to grass sickness. AGS and CGS refers to horses suffering from acute and chronic grass sickness respectively.

5.2.3.2 Ileal muscle strips

Figure 5.2.3 shows the dose response curves for bethanecol for ileal muscle strips divided into control, AGS and CGS groups. The median values at each concentration are plotted. The range and population size are shown in Tables 5.2.2A, 5.2.2B, and 5.2.2C. As was found with duodenal tissue, the maximum contractile response was observed at 1mM or 3mM for all ileal groups. At 3mM only 14.3% of control ileal strips had their maximum response compared with 42.9% for the ileal AGS group and 50% for the CGS group. The maximum responses of ileal smooth muscle strips in grams per gram of tissue (median and range) were 33.9 (29.3-49.6) for the control group, 61.0 (43.9-88.9) for the AGS group and 51.1 (25.9-108.8) for the CGS group. The maximum values found for the AGS ileal group was significantly higher than for the control ileal group ($P<0.05$).

Also, as was observed with the duodenal preparations, the median values for the ileal AGS dose response curve were located to the left side of the control group. There were significant differences between the AGS and control groups at 0.03mM and 0.01mM bethanecol (equivalent to -4.52 and -5 on the log scale) ($P<0.05$). The results for the ileal CGS group were situated to the right side of control values at high concentations of bethanecol but crossed over to the left side of control values at lower bethanecol concentrations. This 'cross over' occurred at a higher concentration of bethanecol than for the duodenal CGS group. As was observed with the duodenal region there were no significant differences between the CGS and control groups at any of the concentrations of bethanecol. There were also no significant differences between the AGS and CGS values at any point on the dose response curves.

The ED_{50} values for the ileal groups did not follow the same ranking as the duodenal groups. The values were 0.0022mM for the AGS group, 0.01mM for the CGS group and 0.04mM for the control group.

The statistical comparison of the responses to the same concentration of bethanecol between the two regions of the small intestine for each of the three groups was carried out using the Mann-Whitney U-test. Although some of the values for the two regions were from the same horses, there were insufficient numbers to use a

A)

Concentration mM	log of concentration	n	median (range) % of maximum response
3	-2.52	1	100.0 (100.0-100.0)
1	-3	7	100.0 (93.8-100.0)
0.3	-3.52	2	78.7 (76.4-81.0)
0.1	-4	6	59.7 (45.2-73.2)
0.06	-4.22	2	58.5 (56.3-60.7)
0.04	-4.4	2	50.0 (50.0-50.0)
0.03	-4.52	6	44.4 (36.0-58.5)
0.01	-5	6	36.1 (31.3-68.8)

B)

Concentration mM	log of concentration	n	median (range) % of maximum response
3	-2.52	3	100.0 (100.0-100.0)
1	-3	7	98.4 (79.3-100.0)
0.3	-3.52	2	88.4 (85.6-91.2)
0.1	-4	3	68.2 (61.8-93.3)
0.03	-4.52	4	60.9 (52.9-70.8)
0.01	-5	6	58.2 (49.5-74.5)
0.003	-5.52	2	53.3 (49.9-56.7)
0.001	-6	3	42.7 (40.0-43.3)
0.0001	-7	2	34.7 (28.9-40.5)

C)

Concentration mM	log of concentration	n	median (range) % of maximum response
3	-2.52	5	100.0 (100.0-100.0)
1	-3	9	100.0 (77.3-100.0)
0.3	-3.52	3	65.8 (62.2-74.1)
0.1	-4	3	60.7 (56.7-78.8)
0.03	-4.52	5	55.7 (35.5-69.2)
0.01	-5	7	55.8 (28.6-63.1)
0.003	-5.52	4	40.9 (38.5-60.6)
0.001	-6	4	37.2 (31.1-45.5)

Table 5.2.2. The normalized contractile response of ileal muscle strips to bethanecol. Values are medians of the maximum response, A) for the control group, B) for the AGS group and C) for the CGS group. The range of values are shown in brackets.

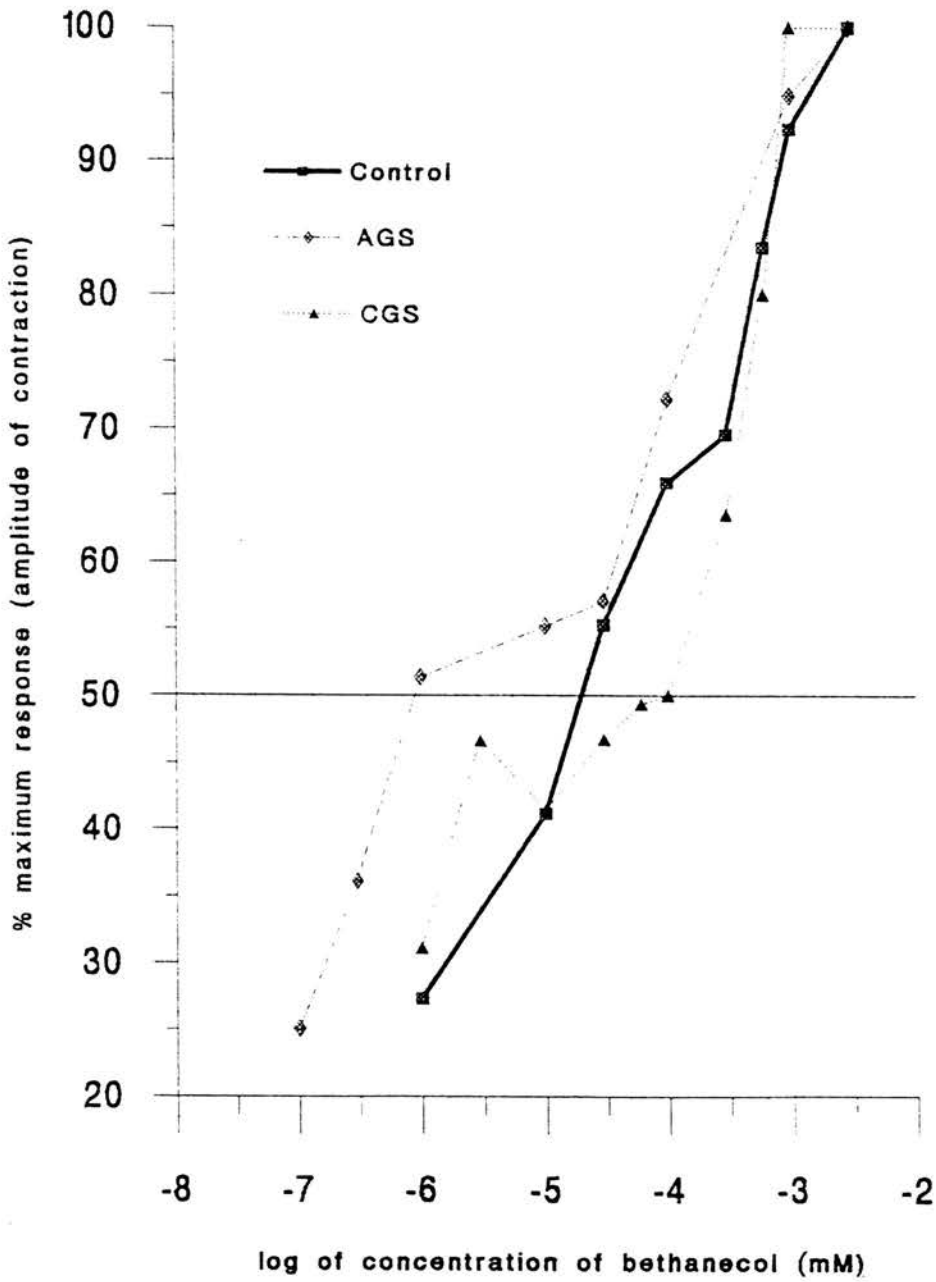


Figure 5.2.3. Dose response curves for bethanecol on strips of equine ileum. The points shown are median values. 'Control' refers to horses euthanased owing to diseases unrelated to grass sickness. AGS and CGS refers to horses suffering from acute and chronic grass sickness respectively.

paired statistical test. There were no significant differences between the two regions in terms of contractile reponse to different concentrations of bethanecol, for both the AGS and CGS groups. The control ileal tissue had a significantly lower response than duodenal tissue at 0.03mM bethanecol ($P<0.05$).

5.2.4 Discussion

As proposed in the introduction, hypersensitivity of muscarinic receptors in the gut may be expected to occur in the horse, following an attack of grass sickness.

A number of studies have investigated the effects of denervation with regard to tissue sensitivity and the mechanisms involved. Most work has concentrated on skeletal muscle, but some work has been carried out using smooth muscle in laboratory animals (Herman and Bass, 1990; Osinski and Bass, 1993; 1994).

In skeletal muscle when a nerve is cut and its terminals allowed to degenerate, the structure supplied by it becomes supersensitive to the transmitter substance released by the terminals. Several mechanisms are known to contribute to this denervation supersensitivity depending on the organ involved. For example, a change in the number or affinity of cellular receptors may occur. It has been reported that the number of nicotinic cholinergic receptors increases following surgical denervation. They also spread from the narrow locality of the end plate to cover the whole muscle membrane i.e. the receptors are no longer localized (McConnell and Simpson, 1976). Fambrough (1979) recorded the appearance of extrajunctional nicotinic receptors seemed to be a major factor in the development of supersensitivity in denervated skeletal muscle. Nilvebrant, Ekstrom and Malmberg (1986) reported that the density of muscarinic receptors was increased in denervated hypertrophied urinary bladders in rats. However, denervation supersensitivity of the iris of the cat has been reported without any changes in receptor density (Sachs, Kloog, Korczyn, Heron and Sokolovsky, 1979). Therefore, it must be concluded that in some cases, post junctional cells become supersensitive without a corresponding increase in the number of receptors (Rang and Dale, 1991). Herman and Bass (1990) measured isometric responses *in vitro* of naive and myenterically denervated rat jejunal circular muscle 15 and 30 days following denervation. Their results suggested that there were differences

in tissue sensitivity, but that within 30 days reinnervation had occurred to some degree. They thought this was most likely to be due to neurones located in the submucous plexus.

Loss of the mechanism for transmitter removal may also lead to supersensitivity. When sensitivity occurs as a result of the loss of uptake or degradation mechanisms for a specific neurotransmitter it is sometimes known as deviation supersensitivity. This develops within 24-48 hours and is specific for a given neurotransmitter and chemically related agonist (Trendelenburg, 1966; Osinski and Bass, 1994). There may be some alteration in ion channels. Powers and Colucci (1985), in their studies with rats treated with reserpine, suggested that voltage dependent calcium channel numbers may be modulated by the cell during supersensitivity.

There may also be some changes in the electrophysiological properties of the denervated tissue. Goto, Westfall and Fleming (1978) found that, while the resting potential of the smooth muscle cells of the guinea pig vas deferens is reduced by denervation, the threshold membrane potential for action potential generation is not changed.

The relative importance of these mechanisms is not known but there is evidence that several changes occur in the physiological status which render smooth muscle more sensitive to stimuli (Fleming, 1976; Westfall, 1981). Osinski and Bass (1994) describe how the mechanism responsible for the change in sensitivity depends on the particular animal species and tissues examined, as well as on the method of denervation used. Goto *et al.* (1978) suggested that in smooth muscle two mechanisms appeared to be particularly important; changes in electrical properties and cellular binding and/or movement of calcium.

Supersensitivity can also occur, but is less marked, when neural transmission is interrupted, for example by pharmacological agents. Long term blockade of post synaptic receptors causes receptors to proliferate leaving the post-synaptic cell supersensitive when the blocking agent is removed. Phenomena such as this are important in the central nervous system where such supersensitivity can cause rebound effects when drugs are given for some time and then stopped abruptly.

The characteristics of the population of muscarinic receptors in the gastrointestinal tract of the horse have not been reported to date. Osinski and Bass (1994) suggest that the smooth muscle cells of the longitudinal muscle layer of the rat ileum contain a mixture of muscarinic subtypes. Utilizing ligand-binding techniques Candell, Yun, Tran, and Ehlert (1990) reported that the majority (84%) of muscarinic receptors in the rat gut displayed properties of the M_2 subtype and the remaining (16%) appeared to be M_3 receptors. Bethanecol works on all these receptor subtypes.

In the present study measurement of the peak contractile amplitude produced by equimolar concentrations of bethanecol demonstrated significant differences in the sensitivity of tissue taken both from control horses and those affected by acute and chronic grass sickness. One problem encountered with ileal experiments was that the background amplitude of contractions were already large and therefore when low concentrations of bethanecol were applied it was difficult to distinguish between the contractile response to bethanecol and these background contractions.

The maximum response to bethanecol for all tissue occurred at either 3mM or 1mM. In the duodenum 63% of the control group had its maximum response at 3mM compared with only 14% in the ileum control group. This suggested that in the ileum there may be a lower number of muscarinic receptors. Therefore in most samples the maximum contractile response could be obtained at 1mM bethanecol in this ileal region. The results for the grass sickness groups are more difficult to interpret. In AGS about 50% of the preparations in both regions had their maximum response at 3mM, whereas with the CGS in the duodenum 30% had their maximum response at 3mM compared with 50% in the ileum. If the damage caused by grass sickness is not uniform throughout the length of the small intestine as has been suggested (Scholes *et al.*, 1993a; Doxey, D.L., Milne, E.M., Woodman, M.P., Gilmour, J.S. and Chisholm, H.K. submitted for publication), more damage to the ileum, which already appears to have a lower population of muscarinic receptors, would mean that a higher concentration of bethanecol would be needed to produce the maximum response. With duodenal preparations, there may be less damage to cholinergic neurones and less of an effect on the sensitivity of muscarinic receptors.

It was interesting that for the two regions the median values for the maximum contractile response for the three groups was very similar. The AGS group had the largest maximum contractile response to bethanecol, followed by the CGS group and finally the control group in both regions. Osinski and Bass (1994) reported that increased sensitivity of a tissue may result in an increase in the maximum response observed. The AGS ileal group maximum response was significantly higher than the control ileal group ($P<0.05$). Fleming and Westfall (1988) state that this increase in the maximum response to drugs is due to improved electrical coupling between the smooth muscle cells. The fact that the maximum response of both regions of the small intestine was not depressed in grass sickness cases suggests that grass sickness does not change the contractile mechanism of the smooth muscle (McPhillips, 1969).

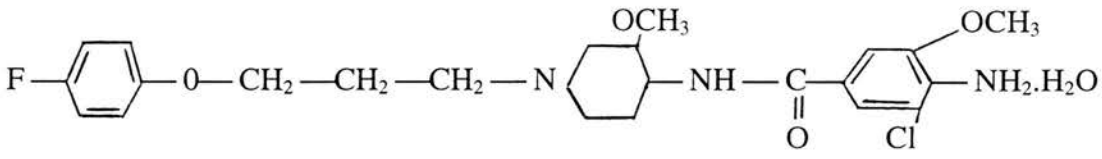
When a tissue becomes supersensitive it causes a leftward shift of the dose response curve and a lower ED_{50} value (Osinski and Bass, 1994). This certainly was the case for AGS tissue compared with control tissue in both regions of the small intestine. In the ileal region the CGS group also had a lower ED_{50} value than the control group, which suggested that the ileum may be damaged primarily in grass sickness.

In conclusion, these results provide evidence for altered muscarinic receptor sensitivity in grass sickness, but that the contractile mechanism remains unaltered.

5.3 RESPONSES TO CISAPRIDE

5.3.1 Introduction

Cisapride is a synthetic substituted benzamide with the formula:



It is a prokinetic drug which is used clinically as it enhances gastrointestinal motility along the entire length of the gastrointestinal tract. King and Gerring (1988) reported that cisapride restores electrical and mechanical activity, improves coordination between gastric and small intestinal activity cycles and decreases the stomach to anus transit time in clinically normal horses. This was also observed in ponies in which post operative ileus (POI) has been induced experimentally (Gerring and King, 1989). The successful use of cisapride in reducing the incidence of POI following colic surgery has also been reported (Gerring and King, 1989; De Geest, Vlaminck, Muylle, Deprez, Sustronck and Picavet, 1991; Van der Velden and Klein, 1993), particularly in the prevention of idiopathic POI (Gerring, King, Edwards, Pearson, Walmsley, Greet, 1991). However, its mechanism(s) of action is uncertain.

It is thought to act selectively on the gut, appearing to have no central nervous system effects (McCallum, Prakash, Campoli-Richards and Goa, 1988). In studies of guinea pig ileum *in vitro,* cisapride does not shift the dose response curve for acetylcholine and methacholine to the left as occurs with neostigmine. This finding tends to eliminate the possibility that cisapride acts by acetylcholinesterase inhibition or by sensitisation of muscarinic receptors in smooth muscle (Schuurkes, Van Nueten, Van Daele, Reynjens and Janssen, 1985).

The effects of cisapride are not abolished by ganglion-blocking concentrations of hexamethonium, therefore a postganglionic target for cisapride is implicated (Schuurkes *et al.*, 1985). It seems likely that cisapride acts principally by an indirect

cholinomimetic mechanism which facilitates acetylcholine release by the post ganglionic nerve endings in the myenteric plexus of the gut (Lee, Chey, You, Shah and Hamilton, 1984; Schuurkes *et al.*, 1985; Chen, Wiley and Owyang, 1986). However, contractions of colonic muscle strips caused by cisapride are insensitive to atropine and tetrodotoxin, suggesting that cisapride stimulates the smooth muscle directly in this region (Chen *et al.*, 1986; Van Nueten and Schuurkes, 1984).

What follows is an introductory study of responses to cisapride *in vitro* of equine small intestinal strips taken from horses with and without equine grass sickness. Tissue from surviving horses with CGS were not available.

5.3.2 Materials and Methods

Fresh tissue was used from five control horses, five horses suffering from AGS and five horses suffering from CGS to study the responses to cisapride (Janssen Pharmaceutica) [see Appendix F for details]. In early experiments the solubility of cisapride was found to be a problem, as in order to make up solutions with high concentrations, the solvents used (such as acetone or methanecol) had an effect on the background contractions of the preparations. At low concentrations ($2.8x10^{-7}M$) no response to cisapride could be detected during a 45 minute monitoring period. A standard final concentration was therefore used of $2.8x10^{-5}M$.

Following the addition of cisapride and a 15 minute incubation period the effects on the rate and amplitude of background contractions were measured over a seven minute period. The results are again expressed as medians with the range also shown. Initially the data from the control horses were compared with all the grass sickness data. Then the grass sickness data were divided into AGS and CGS groups. No horses suffering from SAGS were used in these experiments. The initial contractile rate and amplitude for each group compared with those following the addition of cisapride were analysed using the Wilcoxon Signed Rank Test. Between the three treatment groups (i.e. control, AGS, CGS) the analysis of results was carried out using the Mann-Whitney U-test. As the same horses were used to investigate the two regions of the small intestine, analysis within a group but between the two regions was also carried out using the Wilcoxon Signed Rank Test.

5.3.3 Results

5.3.3.1 Duodenal muscle strips

Figure 5.3.1 shows the response of two strips of duodenal smooth muscle to cisapride, one from a control horse and the other from a horse suffering from AGS. In the trace taken from a control horse there appeared to be an increase in the rate and amplitude of the contractions, whereas in the trace taken from the horse suffering from AGS only the rate of contractions appeared to increase following the addition of cisapride. In some control duodenal preparations there appearred to be a reduction in contractile amplitude. The responses of fresh duodenal tissue to cisapride are shown in Tables 5.3.1 and 5.3.2. There was a trend for an increase in rate of background contractions and a decrease in amplitude of contractions in both control and grass sickness groups. There was a significant increase in contractile rate following cisapride for the grass sickness group ($P<0.01$) (Table 5.3.1).

Between the three groups only the rate following cisapride for the control and AGS groups were significantly different from each other ($P<0.05$).

5.3.3.2 Ileal muscle strips

Figure 5.3.2 shows the response of ileal control and AGS preparations to cisapride. In the control tissue it appeared that cisapride disrupted the characteristic pattern of the background contractions. The responses for ileal tissue to cisapride are shown in Tables 5.3.3 and 5.3.4. As was found with duodenal strips there was a significant difference in the rate of background contractions following cisapride addition for the grass sickness group ($P<0.01$) (Table 5.3.3).

There was a significant difference in the rate of contractions following cisapride between the ileal control and AGS groups ($P<0.05$). When the results were compared for each group between the two regions there were no significant differences.

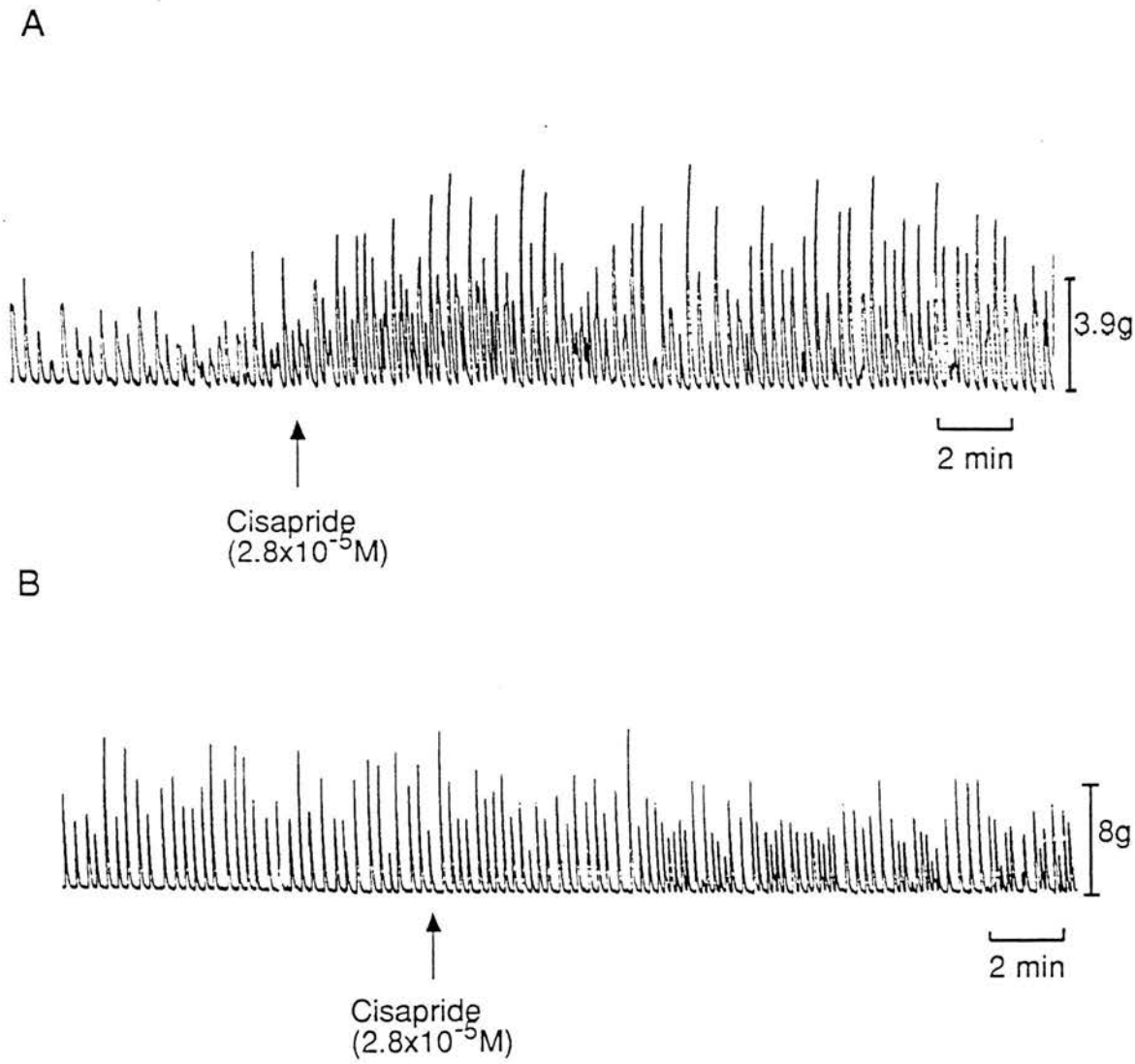


Figure 5.3.1. Responses of duodenal smooth muscle strips to cisapride $2.8x10^{-5}$M, A) taken from a control horse and B) taken from a horse suffering from acute grass sickness.

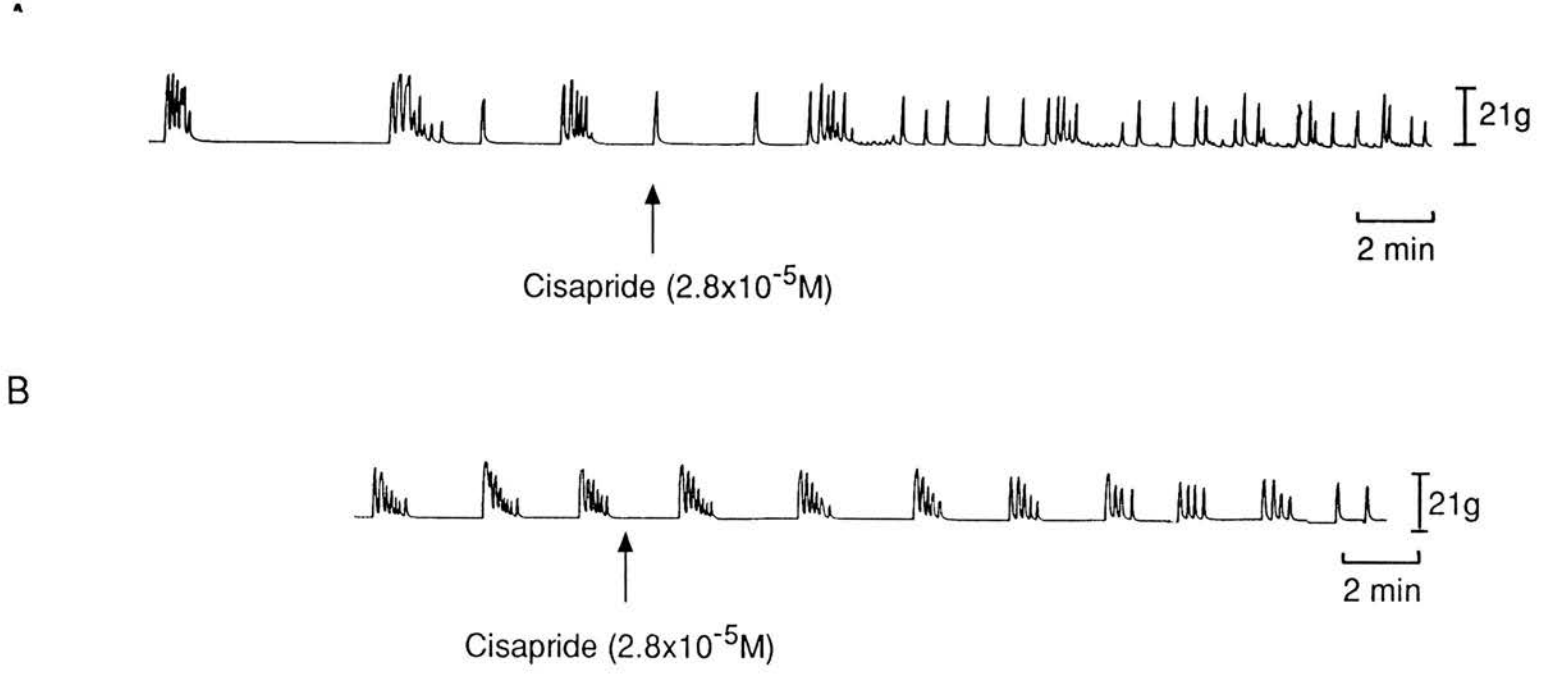


Figure 5.3.2. Responses of ileal smooth muscle strips to cisapride 2.8×10^{-5}M, A) taken from a control horse and B) taken from a horse suffering from acute grass sickness.

	n	initial rate of contractions [contractions/ min]	initial amplitude of contractions [g/g tissue]	contractile rate after cisapride [$2.8x10^{-5}$M] addition	contractile amplitude after cisapride addition
Control	5	5.3 (2.7-10.2)	12.4 (6.2-18.5)	6.3 (3.9-10.9)	11.9 (3.9-19.9)
Grass Sickness	10	2.9 [a] (1.1-5.9)	13.5 (4.4-26.7)	3.6 [a] (1.5-7.4)	11.3 (4.1-25.5)

Table 5.3.1. The effect of cisapride ($2.8x10^{-5}$M) on the rate and amplitude of background contractions of equine duodenal muscle strips. Values are medians with the range shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Wilcoxon Signed Rank test ($P<0.01$).

	n	initial rate of contractions [contractions/ min]	initial amplitude of contractions [g/g tissue]	contractile rate after cisapride [$2.8x10^{-5}$M] addition	contractile amplitude after cisapride addition
Control	5	5.3 (2.7-10.2)	12.4 (6.2-18.5)	6.3[a]♣ (3.9-10.9)	11.9 (3.9-19.9)
AGS	5	3.0 (1.1-5.0)	17.6 (4.4-26.7)	3.3[a] (1.5-5.7)	10.4 (4.1-25.5)
CGS	5	2.7 (2.1-5.9)	13.4 (10.1-24.0)	4.0 ♣ (3.1-7.4)	11.6 (9.8-12.0)

Table 5.3.2. The effect of cisapride ($2.8x10^{-5}$M) on the rate and amplitude of background contractions of equine duodenal muscle strips. Values are medians with the range shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test ($P<0.05$). When the data were analysed using the Wilcoxon Signed Rank test for the effect of cisapride within a particular group there was no statistical significance, however, values which have a ♣ are likely to indicate a biological significance as their P values were <0.06.

	n	initial rate of contractions [contractions/ min]	initial amplitude of contractions [g/g tissue]	contractile rate after cisapride [$2.8x10^{-5}$M] addition	contractile amplitude after cisapride addition
Control	5	2.2 (1.2-5.6)	12.7 (5.3-23.9)	2.9^{a}♣ (2.4-7.1)	7.8 (4.0-11.5)
Grass Sickness	10	1.8^{a} (0.7-4.5)	10.7 (2.9-27.5)	2.1^{a} (0.9-7.0)	8.1 (2.0-36.4)

Table 5.3.3 The effect of cisapride ($2.8x10^{-5}$M) on the rate and amplitude of background contractions of equine ileal muscle strips. Values are medians with the range shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Wilcoxon Signed Rank test ($P<0.01$).

	n	initial rate of contractions [contractions/ min]	initial amplitude of contractions [g/g tissue]	contractile rate after cisapride [$2.8x10^{-5}$M] addition	contractile amplitude after cisapride addition
Control	5	2.2 (1.2-5.6)	12.7 (5.3-23.9)	2.9^{a}♣ (2.4-7.1)	7.8 ♣ (4.0-11.5)
AGS	5	1.8 (0.7-2.0)	12.2 (2.9-27.5)	2.0^{a}♣ (0.9-2.1)	11.6 (2.0-31.3)
CGS	5	1.8 (1.5-4.5)	8.1 (3.0-21.4)	3.0 ♣ (1.6-7.0)	8.1 (2.0-36.4)

Table 5.3.4. The effect of cisapride ($2.8x10^{-5}$M) on the rate and amplitude of background contractions of equine ileal muscle strips. Values are medians with the range shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test ($P<0.05$). When the data were analysed using the Wilcoxon Signed Rank test for the effect of cisapride within a group there was no statistical significance between any of the values, however, values which have a ♣ are likely to indicate a biological significance as their P values were <0.06.

5.3.4 Discussion

It is thought that cisapride exerts its action by increasing acetylcholine release from enteric post ganglionic nerve endings. As some morphologically normal neurones can still be found in the myenteric plexus of the gut in certain cases of equine grass sickness (Doxey, *et al.*, 1992, Pogson *et al.*, 1992, Doxey, D.L., Milne, E.M., Woodman, M.P., Gilmour, J.S. and Chisholm, H.K. submitted for publication, also see Chapter 7) it was thought that cisapride might be of therapeutic benefit in the treatment of selected cases of CGS (Milne, E.M., Doxey, D.L., Woodman, M.P., Cuddeford, D. and Pearson, R.A. in press) who showed that cisapride therapy was associated with an increased rate of passage of ingesta and increased gut sounds in chronic grass sickness cases. Gerring and King (1989) found that cisapride seemed to be active in two presumed cases of grass sickness in their study, however, a precise diagnosis could not be confirmed in the case that recovered.

The results of these *in vitro* experiments do not provide any statistical support that cisapride has a prokinetic effect on equine intestinal motility from control horses. However, Tables 5.3.1 and 5.3.3 showed a significant increase in contractile rate for grass sickness tissue ($P<0.01$) following cisapride addition. Also in other cases the effect of cisapride approached a significant difference ($P<0.06$) and it may be the case that with a greater number of experiments more statistically significant differences would be found. Indeed many of the traces appeared to show changes in rate and/or amplitude but when analysed there was no statistical significance (Figures 5.3.1 and 5.3.2). In conclusion, there appeared to be more of an effect on the contractile rate than amplitude following cisapride addition in these experiments.

The 15 minutes allowed for incubation before the response to cisapride was measured, may not have been suitable. With experiments using guinea pig ileum *in vitro,* following the addition of cisapride the effect was measured for the initial 15 minutes (Schuurkes *et al.*, 1985). In their study they found that cisapride enhanced the contractile response to electrical stimulation over a range of cisapride concentrations. They used a range of concentrations 10^{-6} - 10^{-9}M compared with 2.8×10^{-5}M in these experiments.

More recent work in the guinea pig, investigating the mechanism of action of cisapride, has suggested that the effect of cisapride is mediated via serotonergic 5-hydroxytrytamine receptors, which results in facilitation of cholinergic transmission (Meulemans and Schuurkes, 1992). It would be interesting to carry out further experiments using equine intestine *in vitro* and cisapride to see if this mechanism of action was the same in the horse. De Ridder and Schuurkes (1993) suggested that serotonergic 5-hydroxytrytamine receptors were not involved in cisaprides action in their experiments using canine antrum.

CHAPTER 6

ADRENERGIC SYSTEM

6.1 Introduction

Greig (1928) first reported the similarity of some of the clinical signs of grass sickness with those of sympathicotonia i.e. patchy sweating, muscle tremors, rapid pulse rate and reduced gut activity. Histopathological lesions have been found in sympathetic regions of the peripheral and central nervous systems in grass sickness affected tissue (Barlow, 1969; Gilmour, 1973a; Hodson, Wright, Edwards, Mescall and Suswillo, 1984; Pogson *et al.*, 1992). There is also an increase in plasma catecholamine levels in horses suffering from grass sickness (Hodson, Causon and Edwards, 1984; Hodson, Wright and Hunt, 1986). It was therefore thought that there may be some differences in the responses of small intestinal tissue to selected adrenergic agents between control horses and those affected by grass sickness, which could be investigated using the *in vitro* technique.

In most laboratory species, catecholamines cause a relaxation of the smooth muscle of the small intestine. This inhibition of motility can be observed both *in vitro* and *in vivo.* However, there are two exceptions: isolated terminal ileum of the guinea pig (Munro, 1951; 1952) and the entire small intestine of the horse (Tanaka and Ohkubo, 1941; Brunaud and Labouche, 1947; Ruckebusch *et al.*, 1971). In both species adrenaline and noradrenaline added to isolated intestine in an organ bath cause a contraction. This observation was also thought to be worthy of further investigation.

The aims of this Chapter were to investigate the *in vitro* responses of equine small intestine to catecholamines and determine which receptors were involved, also to see whether there was any significant alteration in responses with tissue taken from horses suffering from grass sickness.

6.2 Materials and Methods

Strips of smooth muscle from the duodenum and ileum of control horses and those affected by grass sickness were set up in organ baths and the contractile activity measured isometrically as described in Chapter 3. The numbers of horses used in this series of experiments were 25 controls, 9 with AGS, 12 with SAGS and 12 with CGS.

Details of the horses used can be found in Appendix G. The drugs used were noradrenaline hydrochloride (Aldrich), adrenaline hydrogen tartrate (BDH), atropine sulphate (Sigma). These were superfused with the tissue and the response recorded. The adrenergic antagonists used were (S)-propranolol hydrochloride (Sigma), a β antagonist, phentolamine hydrochloride (Sigma), an α antagonist, prazosin hydrochloride (Sigma), a selective α1 antagonist, and yohimbine hydrochloride (Sigma), a selective α2 antagonist. These were added 15 minutes prior to the addition of the catecholamines. To facilitate comparison between horses and to correct for the variation in tissue thickness, which varied with the size of horses, the peak force in grams of the contractile response to noradrenaline was divided by the wet weight of the muscle strip. To measure the effect of noradrenaline on the rate of contractions, following the initial contractile response, a seven minute period was used and the number of contractions counted by hand.

6.2.1 Statistical analysis

The results are expressed in terms of medians with the range of values shown in brackets. For each set of results there is one Table which shows control tissue verses all the grass sickness data combined and a second Table where the grass sickness data is divided into AGS, SAGS and CGS groups. Muscle strips used to investigate responses to noradrenaline (both contractile response and effect on contractile rate) following treatment with antagonists, were from the same horses as those used to investigate the effect of noradrenaline alone, therefore differences in the responses for a particular group were analysed using the Wilcoxon Signed Rank test. Differences between the control and grass sickness groups were analysed using the Mann Whitney U-test. The effect on responses to adrenergic agents following storage of tissue for 24 hours at 4°C in unoxygenated modified Krebs was also investigated (see Chapter 3.4). Differences in the responses for a particular group between fresh and stored results were analysed using the Wilcoxon Signed Rank test.

6.3 Results

6.3.1 Contractile response to noradrenaline

In initial experiments it was found both regions of the small intestine contracted dose dependantly to noradrenaline and adrenaline. Noradrenaline was then used for further investigation of the adrenergic system. At low concentrations of noradrenaline (e.g. $7.1x10^{-8}$M) it was difficult to distinguish between this contractile response from that of the background contractions. It was therefore decided to use a standard final concentration of $7.1x10^{-6}$M as responses could clearly be seen in both control and grass sickness affected tissue and were very repeatable.

Figure 6.1A shows a typical response to noradrenaline ($7.1x10^{-6}$M) in a duodenal muscle strip taken from a control horse. There is an immediate contraction with enhanced tone (upward shift of baseline) which lasts two to three minutes after which the background contractions continue, but at a reduced rate. Figure 6.1B shows a typical response to noradrenaline of an ileal strip taken from a control horse; again there was an immediate contraction. After a few minutes the background contractile activity returns to its characteristic burst-like pattern (see Chapter 4.3.2.2). The pattern of response to noradrenaline of strips of grass sickness affected tissue was similar to that of control horses with an immediate contraction (Figure 6.2A), although with some ileal AGS and SAGS muscle strips no contractile response to noradrenaline was observed at all (Figure 6.2B). Also with about 50% of grass sickness affected tissue from both regions of the small intestine, after the initial contractile response to noradrenaline, the background activity was inhibited until the muscle strip was washed. The results of the peak contractile response to noradrenaline for both control and grass sickness affected horses are shown in Table 6.1.

For control horses the range of values for both regions for the response to noradrenaline was very similar, although the median value was larger in the ileum at 43g compared with 32g in the duodenum. The median and range of contractile values in response to noradrenaline for the grass sickness groups were also similar in both regions, with the exception of the ileal AGS group. With the ileal AGS muscle strips, three out of eight showed no contractile response to noradrenaline at all therefore this

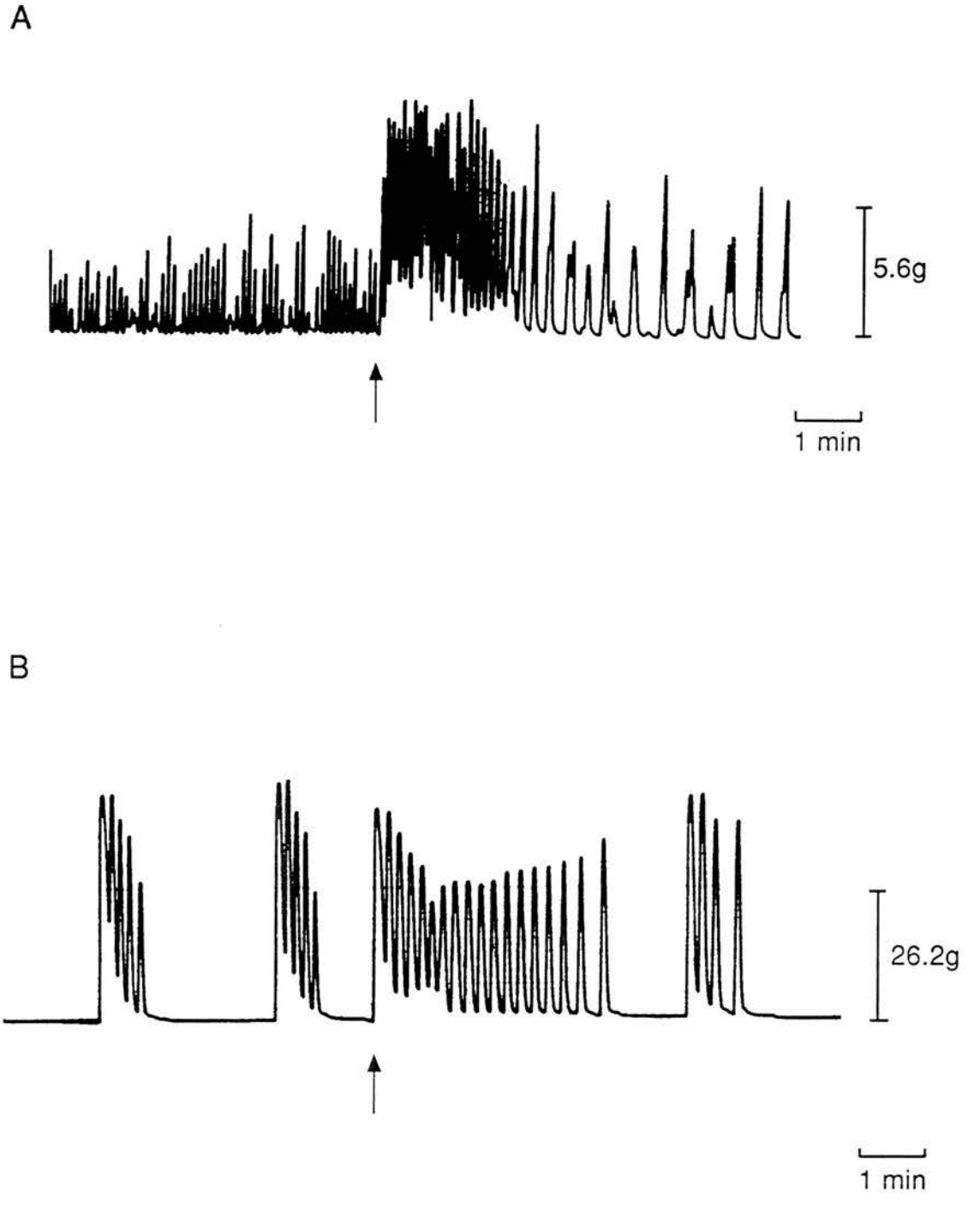


Figure 6.1. Smooth muscle strips taken from the small intestine of control horses; A) duodenum, B) ileum. Arrow indicates the addition of noradrenaline (7.1×10^{-6}M).

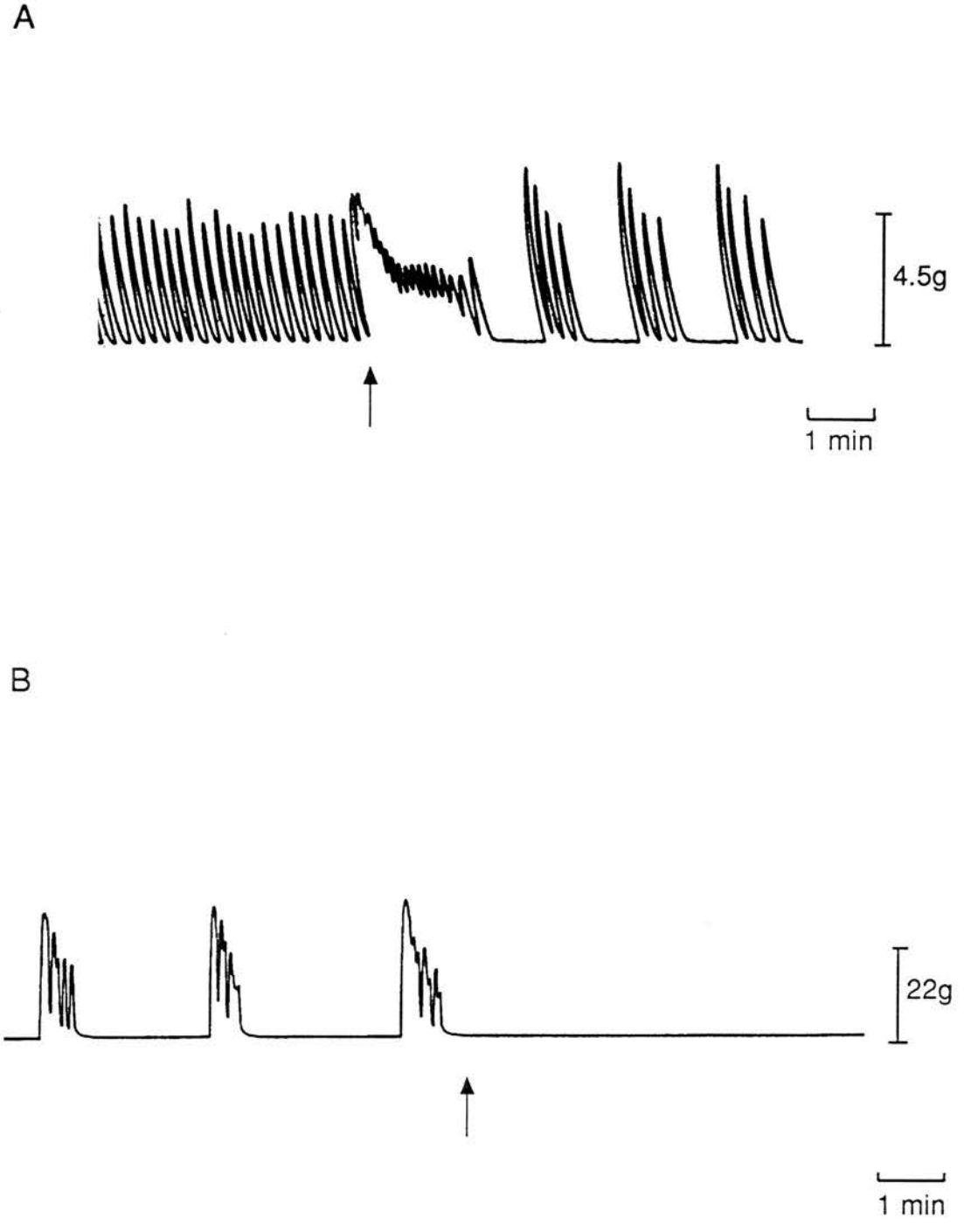


Figure 6.2. Smooth muscle strips taken from the small intestine of horses suffering from acute grass sickness; A) duodenum, B) ileum. Arrow indicates the addition of noradrenaline (7.1×10^{-6}M).

	n	contractile response to noradrenaline [g/g of tissue]	n	response to noradrenaline following pretreatment with propranolol [$1x10^{-4}$M]
Control	20	32.1 (8.4-115.3)	11	34.3 (8.4-93.8)[b]
Grass Sickness	30	29.8 (10.8-90.9)[a]	24	21.4 (0.0-63.2)[ab]

Table 6.1. Median peak contractile response to noradrenaline ($7.1x10^{-6}$M) in grams per gram of tissue, of muscle strips taken from the duodenum from control horses and those suffering from grass sickness. The range of values is shown in brackets. Values with the same superscript are significantly different from each when analysed using the Mann-Whitney U-test b = $P<0.005$ and a = $P<0.05$.

	n	contractile response to noradrenaline [g/g of tissue]	n	response to noradrenaline following pretreatment with propranolol [$1x10^{-4}$M]
Control	20	32.1 (8.4-115.3)	11	34.3 (8.4-93.8)[bc]
AGS	6	24.4 (16.0-31.2)	6	19.3 (12.3-26.4)[b]
SAGS	12	27.0 (16.2-52.3)	8	24.0 (8.1-63.2)
CGS	12	34.0 (10.8-90.9)[a]	10	24.8 (0.0-43.4)[ac]

Table 6.2 Median peak contractile response to noradrenaline ($7.1x10^{-6}$M) in grams per gram of tissue, of muscle strips taken from the duodenum from control horses and those suffering from grass sickness. AGS: acute grass sickness, SAGS: subacute grass sickness, CGS: chronic grass sickness. The range of values is shown in brackets. Values with the same superscript are significantly different from each other; a = $P<0.05$ when analysed using the Wilcoxon Signed Rank test. b = $P<0.01$ and c = $P<0.05$ when analysed using the Mann-Whitney U-test.

	n	contractile response to noradrenaline [g/g of tissue]	n	response to noradrenaline following pretreatment with propranolol [$1x10^{-4}M$]
Control	16	43.0 (13.8-118.3)[a]	9	25.3 (7.0-122.9)
Grass Sickness	31	24.7 (0.0-65.6)[a]	21	21.7 (0.0-58.7)

Table 6.3. Median peak contractile response to noradrenaline ($7.1x10^{-6}M$) in grams per gram of tissue, of muscle strips taken from the ileum from control horses and those suffering from grass sickness. The range of values is shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test a = $P<0.05$.

	n	contractile response to noradrenaline [g/g of tissue]	n	response to noradrenaline following pretreatment with propranolol [$1x10^{-4}M$]
Control	16	43.0 (13.8-118.3)[d]	9	25.3 (7.0-122.9)
AGS	7	3.2 (0.0-33.9)[def]	5	19.8 (5.9-36.2)
SAGS	12	27.6 (0.0-62.1)[e]	8	23.0 (0.0-58.7)
CGS	12	30.8 (4.5-65.6)[f]	8	23.6 (3.5-58.4)

Table 6.4. Median peak contractile response to noradrenaline ($7.1x10^{-6}M$) in grams per gram of tissue, of muscle strips taken from the ileum from control horses and those suffering from grass sickness. AGS: acute grass sickness, SAGS: subacute grass sickness, CGS: chronic grass sickness. The range of values is shown in brackets. Values with the same superscript are significantly different from each other d,f = $P<0.01$ and e = $P<0.05$ when analysed using the Mann-Whitney U-test.

group had a low median contractile value of 3.2g (Figure 6.2B). The contractile responses for the ileal control, SAGS and CGS groups were therefore all significantly higher than the ileal AGS group ($P<0.01$ for the control and CGS groups and $P<0.05$ for the SAGS group) (Table 6.4). For the duodenal strips there was no significant difference in contractile response to noradrenaline between the three grass sickness groups and the control group.

When the results were analysed between the two regions of the small intestine there were found to be no significant differences between the contractile response to noradrenaline for any of the four groups.

The contractile response to noradrenaline was not due to an indirect cholinergic effect as the response occurred in the presence of atropine ($8.6x10^{-6}M$) at a concentration capable of blocking muscarinic receptors (Malone, Brown, Trent and Turner, submitted for publication). Therefore, to investigate which adrenoceptors were involved in this response, propranolol, a beta antagonist ($1x10^{-4}M$ - $1x10^{-6}M$) and phentolamine, an alpha antagonist ($1x10^{-4}M$ -$1x10^{-6}M$), were used. Following pretreatment with propranolol a similar contractile response to noradrenaline and adrenaline was recorded (Figure 6.3).

There was no significant alteration in contractile response to noradrenaline ($7.1x10^{-6}M$) following pretreatment with propranolol ($1 x10^{-4}M$) in any of the groups for both regions of the small intestine, with the exception of the duodenal CGS group in which the contractile amplitude was significantly reduced following pretreatment with propranolol ($P<0.05$) (Table 6.2). With duodenal tissue, the response to noradrenaline following treatment with propranolol showed there were significant differences between the AGS and CGS groups and the control group ($P<0.01$ for the AGS group and $P<0.05$ for the CGS group) (Table 6.2).

There was no significant difference between any of the four groups for the contractile response of the ileum to noradrenaline following pretreatment with propranolol (Table 6.4). There was no significant difference between the duodenal and ileal results for any of the groups for response to noradrenaline following treatment with propranolol.

Figure 6.3. Duodenal muscle strip taken from a control horse. Arrow indicates the addition of noradrenaline (7.1×10^{-6}M) following pretreatment for 15 minutes with propranolol (1×10^{-4}M). After the initial contractile response, the rate and amplitude of the spontaneous activity was greater than after noradrenaline alone (compare with Figure 6.1A).

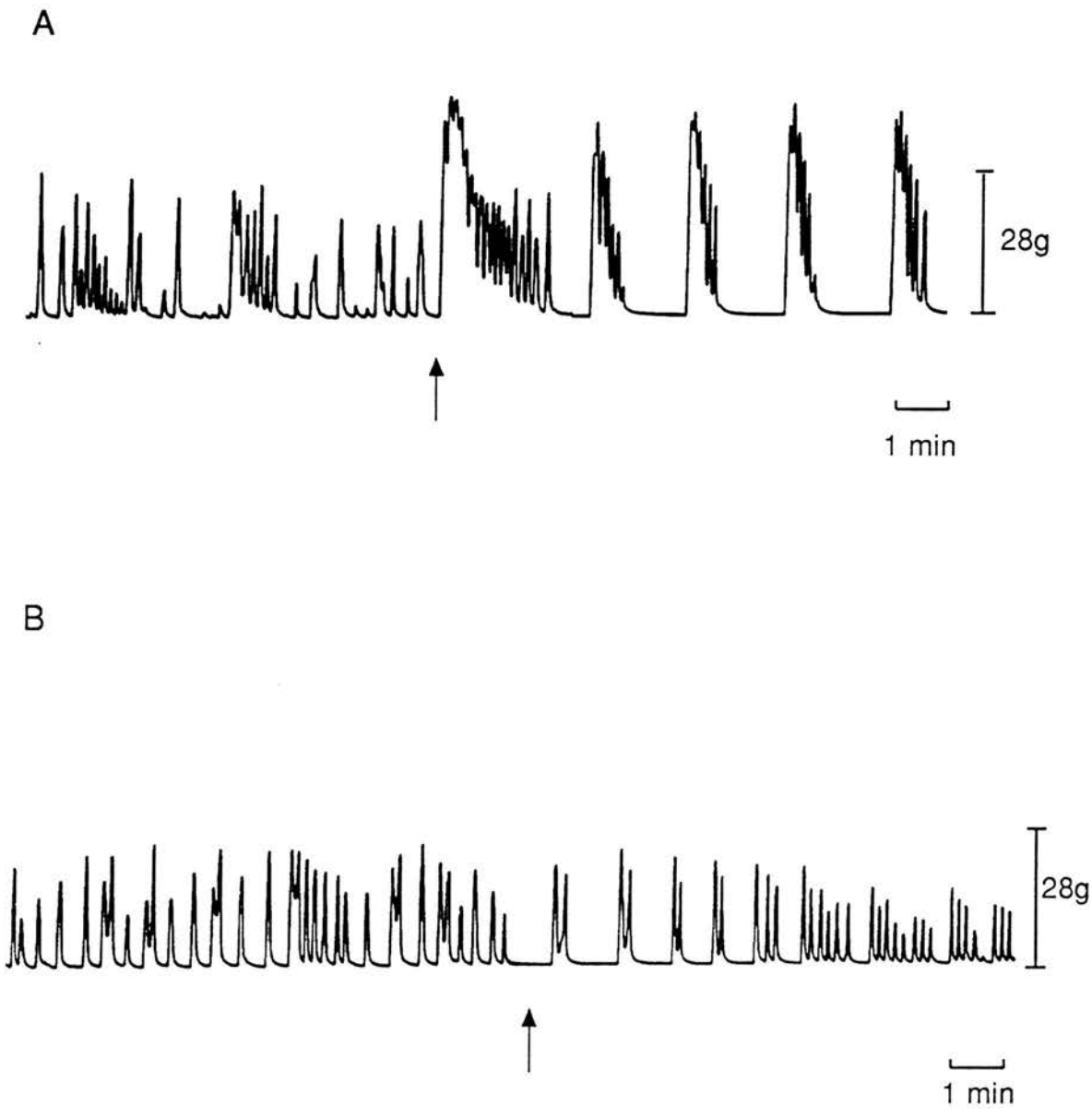


Figure 6.4. Smooth muscle strips taken from the duodenum of control horses; A) shows the response to noradrenaline ($7.1x10^{-6}$M) following pretreatment with phentolamine ($1x10^{-6}$M) for 15 minutes, B) shows the response to noradrenaline ($7.1x10^{-6}$M) following pretreatment with phentolamine ($1x10^{-4}$M) for 15 minutes.

Following pretreatment with phentolamine ($1x10^{-4}M$) for 15 minutes, there was no contractile response on the addition of noradrenaline with either control or grass sickness affected tissue. This was the case in both regions of the small intestine (Figure 6.4B). Following the addition of noradrenaline ($7.1x10^{-6}M$) background contractions either continued at a reduced rate and amplitude, or after a few minutes, there was complete inhibition of this activity until the preparation was washed. At lower concentrations of phentolamine ($1x10^{-6}M$) a contractile response could be obtained (Figure 6.4A).

In the presence of an alpha 1 antagonist, prazosin ($2.8x10^{-6}M$), the contractile response was observed (Figure 6.5). However, in the presence of yohimbine ($2.8x10^{-5}M$), an alpha 2 antagonist, the contractile response to noradrenaline was absent (Figure 6.6).

6.3.2 Effect of noradrenaline on rate of background contractions

As described in Chapter 4, the rate of background contractions in control duodenal preparations was significantly greater than for control ileal preparations. Also the median rates of background contractions in grass sickness affected tissue were lower than for the control groups in both regions of the small intestine.

Tables 6.5 and 6.6 show the initial rate of background contractions and the rates following noradrenaline alone (rate 1), noradrenaline after pretreatment with propranolol (rate 2) and noradrenaline after pretreatment with phentolamine (rate 3). For the control duodenal group rates 1, 2, and 3 were all highly significantly lower compared with the initial rate of contractions ($P<0.0001$, $P<0.01$, $P<0.001$, respectively) (Table 6.6). With duodenal strips from all three grass sickness affected groups only rate 1 was significantly lower than their initial rates ($P<0.01$). For duodenal muscle strips there was no significant difference for rate 1, rate 2, or rate 3 between the control and grass sickness groups.

For the control ileal group only rate 1 was significantly lower than the initial rate of contractions ($P<0.01$). For grass sickness ileal tissue only the SAGS and CGS tissue had a significantly lower contractile rate following the addition of noradrenaline than the initial contractile rate ($P<0.05$) (Table 6.6). There were no significant

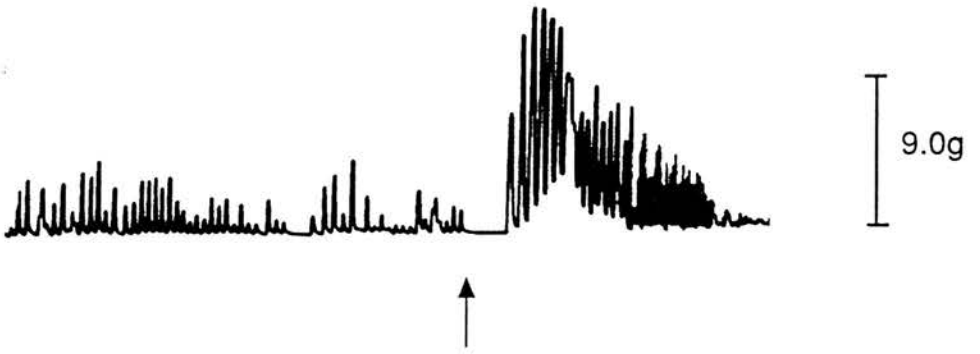


Figure 6.5. Duodenal muscle strip taken from a control horse. Arrow indicates the addition of noradrenaline ($7.1x10^{-6}M$) following pretreatment with prazosin ($2.8x10^{-6}M$), an alpha 1 antagonist, for 15 minutes.

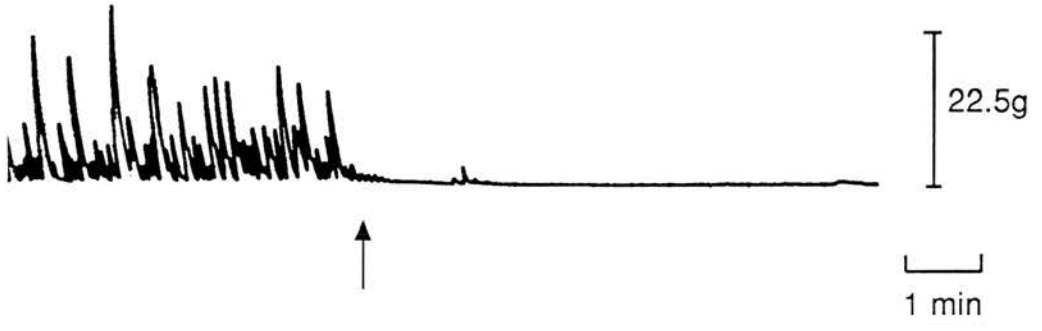


Figure 6.6. Duodenal muscle strip taken from a control horse. Arrow indicates the addition of noradrenaline ($7.1x10^{-6}M$) following pretreatment with yohimbine ($2.8x10^{-5}M$), an alpha 2 antagonist, for 15 minutes. No contractile response was observed. The background contractions were also inhibited until the preparation was washed.

A) Duodenum

	initial rate [contractions/min]	1) rate after noradrenaline	2) rate after propranolol and noradrenaline	3) rate after phentolamine and noradrenaline
Control	7.1 (2.6-11.2)[a]	0.6 (0.0-6.8)	2.0 (1.2-5.4)	1.6 (0.0-10.6)
Grass Sickness	3.4 (0.4-9.6)[a]	0.4 (0.0-2.4)	2.6 (1.0-10.4)	3.2 (0.0-9.6)

B) Ileum

	initial rate [contractions/min]	1) rate after noradrenaline	2) rate after propranolol and noradrenaline	3) rate after phentolamine and noradrenaline
Control	2.5 (0.0-10.0)	0.2 (0.0-2.3)	0.7 (0.0-1.8)[b]	1.2 (0.0-2.7)
CGS	1.7 (0.0-9.3)	0.0 (0.0-3.2)	2.0 (0.0-5.9)[b]	1.8 (0.0-7.6)

Table 6.5. Median rate of contractions per minute for muscle strips taken from A) the duodenum, B) the ileum, from control horses and those affected by grass sickness. The range of values is shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test a = $P<0.0001$ and b = $P<0.05$ (analysis was only carried out between groups).

A) Duodenum

	initial rate [contractions/min]	1) rate after noradrenaline	2) rate after propranolol and noradrenaline	3) rate after phentolamine and noradrenaline
Control	7.1 (2.6-11.2)^{abcde}{20}	0.6 (0.0-6.8)^{c}{20}	2.0 (1.2-5.4)^{d}{13}	1.6 (0.0-10.6)^{e}{20}
AGS	3.2 (1.3-5.2)^{af}{9}	0.6 (0.0-2.4)^{f}{9}	1.8 (1.2-4.4) {8}	2.4 (0.0-5.8) {9}
SAGS	4.1 (2.3-9.0)^{g}{12}	0.8 (0.0-2.4)^{g}{12}	3.7 (1.8-10.4) {8}	5.0 (0.0-8.0) {5}
CGS	3.2 (0.4-9.6)^{bh}{12}	0.0 (0.0-2.0)^{h}12}	2.6 (1.0-6.2) {8}	3.2 (1.0-9.6) {6}

B) Ileum

	initial rate [contractions/min]	1) rate after noradrenaline	2) rate after propranolol and noradrenaline	3) rate after phentolamine and noradrenaline
Control	2.5 (0.0-10.0)^{i}{20}	0.2 (0.0-2.3)^{i}{19}	0.7 (0.0-1.8)^{l}{12}	1.2 (0.0-2.7) {14}
AGS	1.1 (0.3-3.0) {9}	0.0 (0.0-3.2) {9}	1.3 (0.9-2.0)^{m}{4}	1.2 (0.6-6.6) {8}
SAGS	1.9 (0.0-3.0)^{j}{10}	0.0 (0.0-1.2)^{j}{10}	0.8 (0.0-2.4) {5}	1.8 (0.0-6.6) {7}
CGS	2.0 (0.0-9.3)^{k}{12}	0.4 (0.0-2.2)^{k}{12}	2.2 (1.9-5.9)^{lm}{6}	2.7 (0.0-7.6) {6}

Table 6.6. Median rate of contractions per minute for muscle strips taken from A) the duodenum, B) the ileum, from control horses and those affected by grass sickness, AGS: acute grass sickness, SAGS: subacute grass sickness, CGS: chronic grass sickness. The range of values is shown in brackets. { } is the number of muscle strips used. Values with the same superscript are significantly different from each other; c,e = $P<0.0001$, d,f,g,h,i = $P<0.01$ and j,k = $P<0.05$ when analysed using the Wilcoxon Signed Rank test. a,b,l = $P<0.001$ and m = $P<0.05$ when analysed using the Mann-Whitney U-test.

differences between the initial rate and rate 1 for the ileal AGS group (Table 6.6). Rate 2 was significantly higher in the ileal CGS group compared with control and AGS ileal groups ($P<0.001$ and $P<0.05$ respectively). There were no significant differences between any of the other rates between the control and grass sickness groups.

There was no significant difference between rate 1, rate 2 or rate 3 for each group between the duodenal and ileal samples, with the exception of rate 1 ($P<0.01$) and rate 2 ($P<0.05$) for the control group. It should be noted that the addition of propranolol or phentolamine themselves caused no significant change in the rate of background contractions in both control and grass sickness affected groups (see Chapter 4.3.3.3).

6.3.3 Effect of tissue storage

6.3.3.1 Effect of storage on contractile response

The contractile responses to noradrenaline ($7.1x10^{-6}M$) following storage for 24 hours at 4°C for control and grass sickness tissue are shown in Tables 6.7-6.10. The responses were very similar to those for fresh tissue. The responses of duodenal tissue from the AGS group to noradrenaline were significantly lower than in both the control and CGS groups ($P<0.05$). Following pretreatment with propranolol ($1x10^{-4}M$), as with fresh tissue, only the duodenal tissue from the CGS group showed a significant reduction in contractile response ($P<0.05$).

In two of the seven AGS ileal preparations no contraction in response to noradrenaline was obtained. Following pretreatment with propranolol, all the ileal AGS preparations showed a contractile response (Table 6.10). There was a significant difference ($P<0.05$) between the control group and the SAGS group in the ileal preparations in the response to noradrenaline following pretreatment with propranolol. There were no significant differences between the two regions of the small intestine for the contractile response to noradrenaline alone and noradrenaline following pretreatment with propranolol.

Following storage there was still no contractile response to noradrenaline following pretreatment with phentolamine ($1x10^{-4}M$) in any of the groups.

6.3.3.2 Effect of storage on background contractions

Following storage, for duodenal preparations, the rate of background contractions following noradrenaline (rate 1) was significantly lower than the initial rate of contractions for the control and SAGS groups ($P<0.05$) (Table 6.12). The rate of contractions of duodenal tissue following the addition of noradrenaline was significantly higher for duodenal tissue from the control group than all the grass sickness groups ($P<0.05$ for AGS and SAGS, $P<0.001$ for CGS). There was also a significant difference between the AGS and CGS groups for rate 2 ($P<0.05$).

With the ileal tissue used in this series of experiments the initial rate of contractions for the CGS group was significantly higher than the control, AGS and SAGS groups ($P<0.01$ for control, $P<0.05$ for AGS and SAGS). There was no significant alteration in contractile rate following the addition of noradrenaline for any of the ileal groups (Table 6.12). There were no significant differences between rate 1, rate 2, rate 3 between the two regions for each group with the exception of rate 1 for the control ($P<0.01$) and SAGS ($P<0.05$) groups.

Where results were available for responses to noradrenaline before and after storage for the same horses, the analysis was carried out using the Wilcoxon Signed Rank test. For amplitude of the contractile response to noradrenaline alone and noradrenaline following pretreatment with propranolol, there were no significant differences between fresh and stored tissue for both regions of the small intestine. For the rate of contraction the only significant difference was between the initial rate for the control groups from both regions ($P<0.01$).

	n	contractile response to noradrenaline [g/g of tissue]	n	response to noradrenaline following pretreatment with propranolol [$1x10^{-4}$M]
Control	12	39.0 (5.9-105.1)	9	35.5 (13.9-74.9)[a]
Grass Sickness	20	23.5 (4.9-76.8)	18	12.8 (4.4-63.1)[a]

Table 6.7. Median peak contractile response in grams/gram of tissue to noradrenaline ($7.1x10^{-6}$M) for duodenal muscle strips taken from control horses and those affected by grass sickness following storage for 24 hours at 4°C. The range of values are shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test a, =P<0.05.

	n	contractile response to noradrenaline [g/g of tissue]	n	response to noradrenaline following pretreatment with propranolol [$1x10^{-4}$M]
Control	12	39.0 (5.9-105.1)[a]	9	35.5 (13.9-74.9)[cd]
AGS	6	14.3 (4.9-28.2)[ab]	5	11.4 (4.4-30.5)[ce]
SAGS	8	19.8 (6.1-58.0)	7	10.6 (5.7-62.9)[df]
CGS	6	35.1 (15.2-76.8)[bg]	6	31.8 (14.3-63.1)[efg]

Table 6.8. Median peak contractile response in grams/gram of tissue to noradrenaline ($7.1x10^{-6}$M) for muscle strips taken from the duodenum from control horses and those affected by grass sickness following storage for 24 hours at 4°C. AGS: acute grass sickness, SAGS: subacute grass sickness, CGS: chronic grass sickness. The range of values are shown in brackets. Values with the same superscript are significantly different from each other; g = P<0.05 when analysed using the Wilcoxon Signed Rank test, a,b,c,d,e,f, = P<0.05 when analysed using the Mann-Whitney U-test.

	n	contractile response to noradrenaline [g/g of tissue]	n	response to noradrenaline following pretreatment with propranolol [$1x10^{-4}M$]
Control	14	37.1 (13.3-88.9)	5	59.6 (12.3-75.9)[b]
Grass Sickness	23	27.9 (0.0-85.9)	17	13.0 (3.0-45.1)[b]

Table 6.9. Median peak contractile response in grams/gram of tissue to noradrenaline ($7.1x10^{-6}M$) for ileal muscle strips taken from control horses and those affected by grass sickness following storage for 24 hours at 4°C. The range of values are shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test a,b =$P<0.05$.

	n	contractile response to noradrenaline [g/g of tissue]	n	response to noradrenaline following pretreatment with propranolol [$1x10^{-4}M$]
Control	14	37.1 (13.3-88.9)[h]	5	59.6 (12.3-75.9)[i]
AGS	7	33.6 (0.0-85.9)	4	17.1 (10.4-45.2)
SAGS	10	13.0 (0.0-57.4)[h]	9	9.2 (5.4-45.1)[i]
CGS	6	30.4 (13.3-49.0)	4	23.8 (8.5-30.7)

Table 6.10. Median peak contractile response in grams/gram of tissue to noradrenaline ($7.1x10^{-6}M$) for muscle strips taken from the ileum, from control horses and those affected by grass sickness following storage for 24 hours at 4°C. AGS: acute grass sickness, SAGS: subacute grass sickness, CGS: chronic grass sickness. The range of values are shown in brackets. Values with the same superscript are significantly different from each other h,i, = $P<0.05$ when analysed using the Mann-Whitney U-test.

A) Duodenum

	initial rate [contractions/min]	1) rate after noradrenaline	2) rate after propranolol and noradrenaline	3) rate after phentolamine and noradrenaline
Control	4.2 (1.3-8.9)[a]	1.8 (0.4-7.0)[b]	2.2 (0.7-7.8)	0.8 (0.0-7.7)
Grass Sickness	2.0 (1.0-9.8)[a]	1.0 (0.0-2.0)[b]	1.9 (0.6-3.2)	1.6 (0.4-6.8)

B) Ileum

	initial rate contractions/min]	1) rate after noradrenaline	2) rate after propranolol and noradrenaline	3) rate after phentolamine and noradrenaline
Control	0.0 (0.0-2.4)[c]	0.0 (0.0-4.8)	1.0 (0.0-2.0)	1.0 (0.0-2.3)
Grass Sickness	0.8 (0.0-7.4)[c]	0.2 (0.0-2.0)	3.2 (0.0-6.6)	1.5 (0.0-3.2)

Table 6.11. Median rate of contractions per minute for muscle strips A) from the duodenum and B) from the ileum, taken from control and grass sickness affected horses following 24 hours storage at 4°C. The range of values are shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test b =$P<0.005$, a,c = $P<0.05$ (analysis was only carried out between groups).

A) Duodenum

	initial rate [contractions/min]	1) rate after noradrenaline	2) rate after propranolol and noradrenaline	3) rate after phentolamine and noradrenaline
Control	4.2 (1.3-8.9)^{ab}{17}♥	1.8 (0.4-7.0)^{bdef}{15}	2.2 (0.7-7.8) {10}	0.8 (0.0-7.7) {9}
AGS	1.9 (1.1-9.8) {6}	1.0 (0.4-1.6)^{d}{5}	1.2 (0.6-2.0)^{g}{5}	1.5 (1.0-2.4) {4}
SAGS	2.2 (1.0-6.8)^{c}{9}	1.0 (0.6-2.0)^{cc}{8}	2.0 (1.2-3.2) {9}	1.6 (0.8-6.8) {7}
CGS	2.0 (1.5-3.4)^{a}{6}	0.6 (0.0-1.5)^{f}{5}	2.1 (1.6-3.0)^{g}{6}	1.3 (0.4-4.8) {4}

B) Ileum

	initial rate contractions/min]	1) rate after noradrenaline	2) rate after propranolol and noradrenaline	3) rate after phentolamine and noradrenaline
Control	0.0 (0.0-2.4)^{h}{18}♥	0.0 (0.0-4.8) {14}♣	1.0 (0.0-2.0)^{k}{7}	1.0 (0.0-2.3) {7}
AGS	0.7 (0.0-1.2)^{i}{6}	0.3 (0.0-1.0) {6}	0.3 (0.0-0.7) {3}	1.5 (0.1-2.0) {3}
SAGS	0.6 (0.0-2.8)^{j}{9}	0.0 (0.0-1.8) {9}♦	4.0 (0.2-6.6) {7}	2.0 (0.0-3.2) {7}
CGS	1.9 (0.8-7.4)^{hij}{6}	1.0 (0.0-2.0) {6}	3.4 (2.2-3.8)^{k}{3}	0.6 (0.0-2.4) {5}

Table 6.12. Median rate of contractions per minute for muscle strips A) from the duodenum and B) from the ileum, taken from control and grass sickness affected horses following 24 hours storage at 4°C. AGS: acute grass sickness, SAGS: subacute grass sickness, CGS: chronic grass sickness. { } is the number of muscle strips used. The range of values are shown in brackets. Values with the same superscript are significantly different from each other; b,c = $P<0.05$ when analysed using the Wilcoxon Signed Rank test, f,h = $P<0.01$ and a,d,e,g,i,j,k = $P<0.05$ when analysed using the Mann-Whitney U-test. ♥ indicates a significant difference from the equilvalent fresh value when the data were analysed for statisical differences using the Wilcoxon Signed Rank test ($P<0.01$). ♣ indicates there was a significant difference from the equivalent duodenal value ($P<0.01$). ♦ indicates there was a significant difference from the equivalent duodenal value ($P<0.05$).

6.4 Discussion

These results agree with the reports in the literature that *in vitro* equine small intestine contracts in response to catecholamine addition (Tanaka and Ohkubo, 1941; Brunaud and Labouche, 1947; Ruckebusch *et al.*, 1971; Malone, E.D., Brown, D.R., Trent, A.M. and Turner, T.A. [submitted for publication]). The isometric traces shown here of horse intestine *in vitro* in response to noradrenaline are similar to those presented by Ruckebusch *et al.* (1971). Ruckebusch used similar concentrations of noradrenaline (0.5-1x10^{-6}M) and adrenaline (1x10^{-6}M-1x10^{-7}) as these present studies. Unfortunately none of the other studies have quantified the amplitude of the contractile response, with the exception of Malone and co-workers who gave absolute amplitude values, with no account of tissue size, therefore their data could not be easily compared with the results from this study.

The results using adrenergic antagonists suggest that this contractile response to noradrenaline in equine small intestine was due to excitatory α2 receptors. Malone, E.D., Brown, D.R., Trent, A.M. and Turner, T.A. (submitted for publication) also report that the contractile response to noradrenaline in their study was selectively inhibited by pretreatment with yohimbine, an α2 antagonist. Ruckebusch reported that both propranolol and phentolamine caused a reduction in the amplitude of the contractile response. However, this observation was not quantified by Ruckebusch. Tanaka and Ohkubo (1941) and Brunaud and Labouche (1947) reported that ergotamine (an alpha antagonist) prevents the contractile response to adrenaline *in vitro* in the horse. Bailey (1968) found a biphasic response to adrenaline or noradrenaline using strips of smooth muscle from the stomach wall of the guinea pig, in which relaxation was followed by a contraction. On analysis with adrenergic antagonists he found the inhibitory component appeared to be mediated via β receptors and the excitatory component via α receptors.

In conclusion, it would appear that the contractile response in the horse was due to α2 excitatory receptors. Noradrenaline appeared to have two effects; when the excitatory one was blocked, the β response was to reduce the rate of background contractions. It may also cause a relaxation of tone, however, with the low

sensitivities chosen to measure the contractile response, no alteration was seen in these experiments.

Grass sickness affected tissue responded similarly to tissue taken from control horses in that, there were no significant differences for the contractile response to noradrenaline between any of the grass sickness groups and the control group for the duodenal region. For the ileal region only the AGS group had a significantly lower contractile response to noradrenaline from the other grass sickness groups and the control group (control and CGS $P<0.01$, SAGS $P<0.05$). However, there were no significant differences between the duodenal and ileal AGS groups.

In about 40% of ileal AGS cases, no contractile response to noradrenaline could be achieved until pretreatment with propranolol, thus causing this group to have a low median value of 3.2g/g of tissue. Other grass sickness muscle strips also showed no contractile response to noradrenaline (Tables 6.2 and 6.4). The reason for this is unclear. The increased levels of circulating catecholamines found in grass sickness (Hodson *et al.*, 1984; Hodson *et al.*, 1986) may have desensitized the adrenergic receptors in ileal tissue of AGS cases. Alternatively it may be an actual consequence of the disease that receptor number or availability is reduced. An alteration of receptor populations in certain diseases have been reported. For example, a decrease in cardiac beta-adrenoceptor function is a general phenomenon in all kinds of human heart failure (Brodde, Zertowski, Borst, Maier and Michel, 1989). Wright and Shepherd (1965) suggested there may be alteration in receptor sensitivity in Hirschsprung's disease in humans. After propranolol treatment a contractile response was evoked in nearly all muscle strips. Propranolol may have the property of unmasking or revealing α receptors responsible for the excitatory effect of noradrenaline. Gagnon, Deuroede and Belisle (1972) suggested this could be the explanation for the action of oxprenolol, a β antagonist, in their experiments using isolated strips of human colon.

As there was no significant difference between the contractile response to noradrenaline alone, or noradrenaline following pretreatment with propranolol between the two regions of the small intestine, it would suggest that the population of α receptors was relatively uniform throughout the length of the small intestine in horses (see Chapter 5.1.4).

The rate of contractions was significantly reduced in all four groups and both regions of the small intestine following the addition of noradrenaline with the exception of the ileal AGS group. In this ileal AGS group the initial rate of contractions was already low. Tanaka and Ohkubo (1941) and Ruckebusch *et al.* (1971) also reported that background activity was supressed following the contractile response to noradrenaline. The addition of noradrenaline following pretreatment with propranolol and phentolamine caused no significant effect on the rate of contractions compared with initial rates with the exception of the duodenal control group where both median rates were significantly lower than initially. If there was an inhibitory β effect of noradrenaline, following α blockade, a decrease in the rate of contractions would be expected. With several preparations following phentolamine and noradrenaline addition, background contractions were inhibited until the preparation was washed (Figure 6.6).

Following storage for 24 hours at 4°C there were no significant differences in the contractile response to noradrenaline alone and noradrenaline following pretreatment with propranolol for all four groups and both regions of the small intestine. This would suggest the contractile response is independent of nervous elements. Gagnon *et al.* (1972) also found this storage period had no effect on responses to adrenaline in their study using isolated preparations of human colon. Although within the ileal AGS group there were still some preparations which showed no contractile response to noradrenaline, the median value was now not significantly different from the other ileal groups. Following storage there was still no contractile response to noradrenaline in the presence of phentolamine for all four groups.

The only significant effect on contractile rates following storage was on the initial contraction rates of the control duodenal and ileal groups, which significantly reduced ($P<0.01$). There have been other reports of storage for 24 hours at 4°C having no significant effect on the contractile response to noradrenaline, following β blockade with oxprenolol, on strips of human sigmoid colon (Gagnon *et al.*, 1972).

With regards to the increased levels of circulating catecholamines observed in grass sickness cases. It is known that most of the plasma adrenaline in the horse comes from the adrenal medulla and that the increase in plasma adrenaline is most

likely to be caused by stress (Hodson *et al.*, 1984). However, Hodson, *et al.* (1986) showed that the levels of noradrenaline, adrenaline, cortisol and adrenocorticotrophic hormone were significantly higher in grass sickness cases than in control, stressed and colic cases ($P<0.05$ or less). Most plasma noradrenaline, in those species studied, is released from vasomotor nerves (Vane, 1969). Therefore, some of the increase in plasma noradrenaline in grass sickness cases may be caused by increased noradrenergic activity. Infusion of adrenaline in the horse has been reported to cause muscle tremors (Snow, 1977) and sweating (Evans, 1966). Snow (1977; 1979) reported that sweating is mediated by $\beta 2$ receptors in the horse, in contrast to α-mediation in the cow, sheep and goat (Findlay and Robertshaw, 1965; Robertshaw, 1968). Adrenaline has been given intramuscularly to resting horses without adverse long term effects (Anderson and Aitkon, 1977). There have been a number of studies on the effect of exercise on plasma catecholamines in the horse (Martinez, Godoy, Naretto and White, 1988; Snow, Harris, MacDonald, Forster and Marlin, 1992). From the findings of these studies it therefore seems likely that the increase in plasma catecholamines seen in grass sickness is a consequence rather than a cause of the disease.

In summary, it would appear that in equine small intestine *in vitro*, the addition of noradrenaline causes a contractile response due to excitatory $\alpha 2$ receptors, which are probably located within the smooth muscle itself. If these receptors are blocked, the rate of background contractions is reduced following the addition of noradrenaline due to the inhibitory effects of β adrenoceptors.

CHAPTER 7

HISTOLOGY OF THE ENTERIC NERVOUS SYSTEM

7.1 Introduction

It has been clear for many years that neuronal degeneration occurs in cases of equine grass sickness within the autonomic ganglia and enteric plexuses (Obel, 1955; Barlow, 1969; Gilmour, 1973a). However, many of these reports are conflicting in their interpretation of the relationship between neuronal damage and clinical illness. Obel (1955) reported degenerative changes of autonomic ganglia to be extensive in both acute and chronic cases of grass sickness in her study of 14 cases. Chromatolytic and vacuolated cells were predominantly found in the submucous ganglia, and only slight changes were seen in the myenteric plexus. Barlow (1969) observed that the severity of the degenerative changes in the ganglia was directly proportional to the duration of the clinical illness in his group of five cases. Gilmour (1973a) did not agree with this; he found that degenerative cells were more extensive and severe in acute and subacute cases than in chronic cases. Quantification of the extent of damage to the enteric nervous system has only recently been carried out (Doxey *et al.*, 1992; Pogson *et al.*, 1992; Doxey, D.L., Milne, E.M., Woodman, M.P., Gilmour, J.S. and Chisholm, H.K. submitted for publication). Pogson *et al.* (1992) examined the jejunum of grass sickness cases and found a reduction in the number of myenteric and submucous neuronal cell groups in AGS. Also, the proportion of damaged and dead cells was significantly greater in all three grass sickness groups compared with control animals ($P<0.01$-$P<0.001$). Scholes *et al.* (1993a) has also studied the enteric nervous system of control horses and those affected by grass sickness, reporting that the ileal region showed neuronal damage in all grass sickness cases.

This Chapter describes a light microscopical investigation, of the enteric nervous system, of samples of small intestine adjacent to those used in the pharmacological experiments described in the previous Chapters. The relationship between selected pharmacological results and the histological findings is also discussed.

7.2 Materials and methods

Sections of equine duodenum and ileum (4cm) adjacent to the sample used for the pharmacological experiments were fixed in Bouins solution (Culling, Allison and Barr, 1985) within 45 minutes of euthanasia, during which time they were continually aerated with 95% oxygen and 5% carbon dioxide and kept in chilled modified Krebs solution (see Chapter 3.2). After 16-24 hours fixation the samples were washed and then stored in 70% alcohol at room temperature until further use. The material examined was taken from five control horses, five horses suffering from AGS and five horses suffering from CGS. Details of the horses used are shown in Appendix I.

The samples were trimmed, dehydrated and embedded in paraffin wax. Transverse 5μm sections were then cut using a rotary microtome (American Optical, Milton Keynes, UK). Every tenth section was examined allowing 50μm intervals between the sequential sections. This technique was used to avoid observing the same neurones twice. Pearson (1994) reported the mean diameter of neurones in the myenteric plexus of equine small intestine was 41μm. Five sections per horse were examined. The circumference of some samples of gut made it necessary for the sample to be cut in two, thus requiring ten half sections to be examined per horse. The tissue was then stained using Ehrlich's haematoxylin and eosin (Culling *et al.*, 1985).

Sections were examined using a light microscope (Vickers M15C binocular, York, UK). The entire section was examined and a subjective scoring system was applied to each section using the following parameters: 1) number of all neurones, 2) size of all normal neurones, and 3) proportion of abnormal neurones. Slides were coded and examined 'blind' to prevent bias. The range of scores used was zero to five, including half marks.

7.2.1 Scoring system

See Appendix K for further details of the scoring system used.

1) Number of neurones

Normal enteric ganglia contained nerve cell bodies full of Nissl substance. On staining they were basophilic and had a stippled appearance. Figure 7.1 shows a

ganglion within the duodenal myenteric plexus of a control horse containing clear nerve cell bodies. Figure 7.2 shows a ganglion within the duodenal submucous plexus of a control horse. The presence of normal neurones lead to a score in the range of 2-5 depending on the number of ganglia and nerve cell bodies within the plexus; the lower the score, the more severe the damage. Where it was evident that neurones had been destroyed leading to low numbers of neurones remaining, scores as low as 0.5 were given. Figures 7.3 and 7.4 shows a duodenal myenteric ganglion and an ileal submucous ganglion taken from a horse suffering from AGS. Note the reduced number of nerve cell bodies.

2) Size of neurones

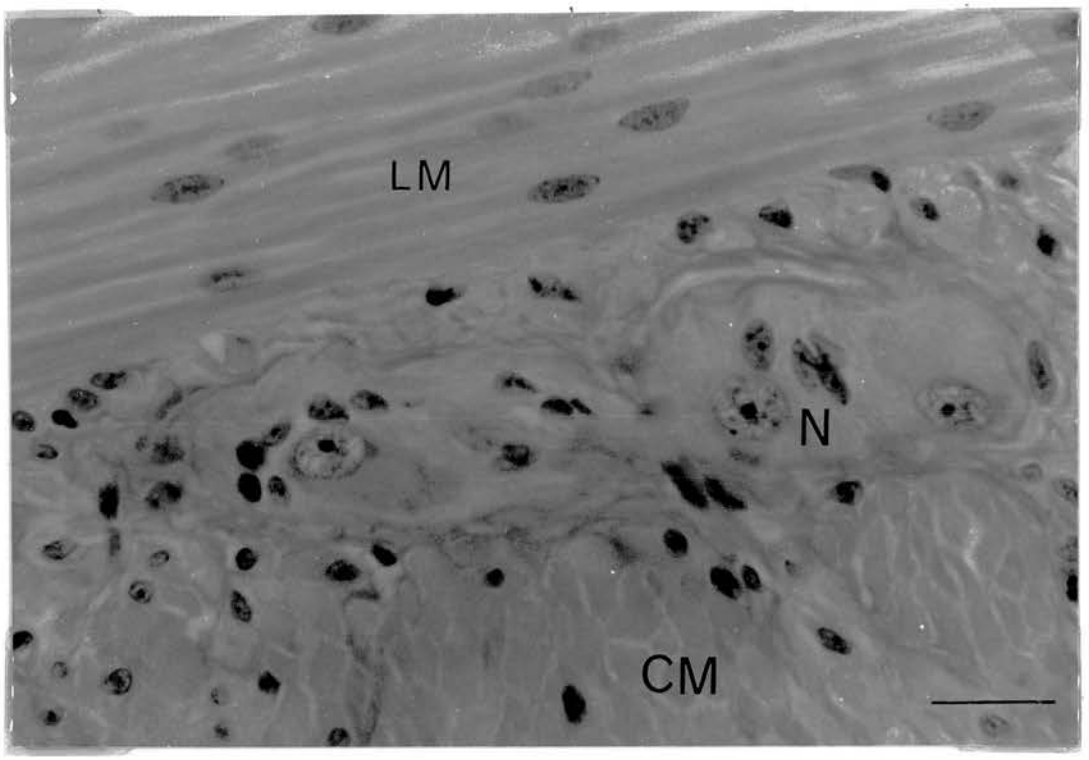
In grass sickness, neuronal degeneration occurs, nerve cells lose their nuclei and Nissl substance and cell bodies often appeared swollen. Only the neurones with normal morphology were considered when scoring for the size of neurones to see if a certain neuronal size (i.e. small or large) was preferentially damaged in grass sickness. There was little variation of size of normal neurones between the three groups therefore the scores given were between 1.5 and 4.

3) Proportion of abnormal neurones

Sections taken from horses suffering from grass sickness showed a number of abnormal nerve cells. Abnormalities varied from vacuolation of the nerve cell body to complete loss of neurones. Morphological changes observed in grass sickness affected tissue included pyknosis, cytoplasmic vacuolation and loss of Nissl substance. If greater than 75% of the remaining neurones were abnormal the score of 4 was given. Several abnormal neurones lead to a score in the range of 1.5-2.5. If only one or two abnormal cells or no abnormal cells were observed throughout the whole section, a score of 0.5 or zero was given respectively. Figure 7.4 shows abnormal nerve cells within a ganglion in the ileal submucous plexus of a horse suffering from AGS.

Figure 7.1. Myenteric plexus from the duodenal region of the small intestine of a control horse. Can see three normal neurones characterized by clear nuclei and Nissl sustance; LM = longitudinal muscle layer, N= neurone, CM = circular muscle layer. Haematoxylin and eosin, bar = 20μm

Figure 7.2. The submucous plexus from the duodenal region of the small intestine of a control horse. The neurones are located around the edge of the ganglia; S= submucosa, N= neurone. Haematoxylin and eosin, bar = 20μm


LM
N
CM

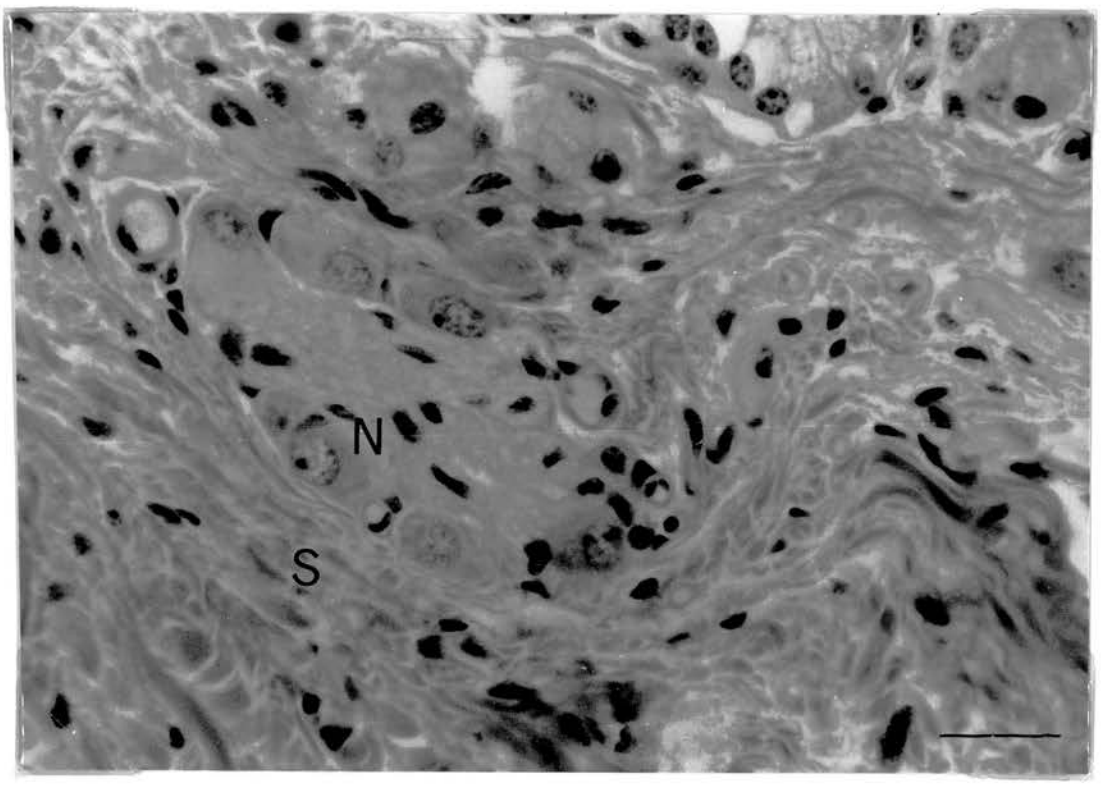
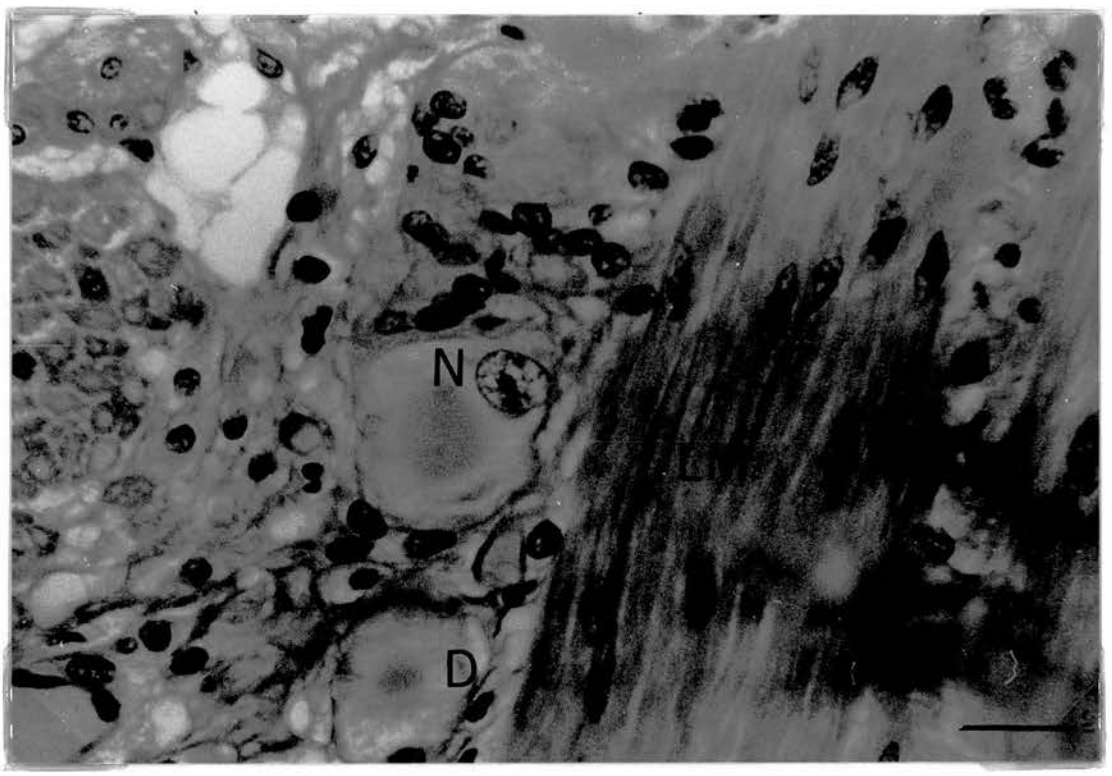
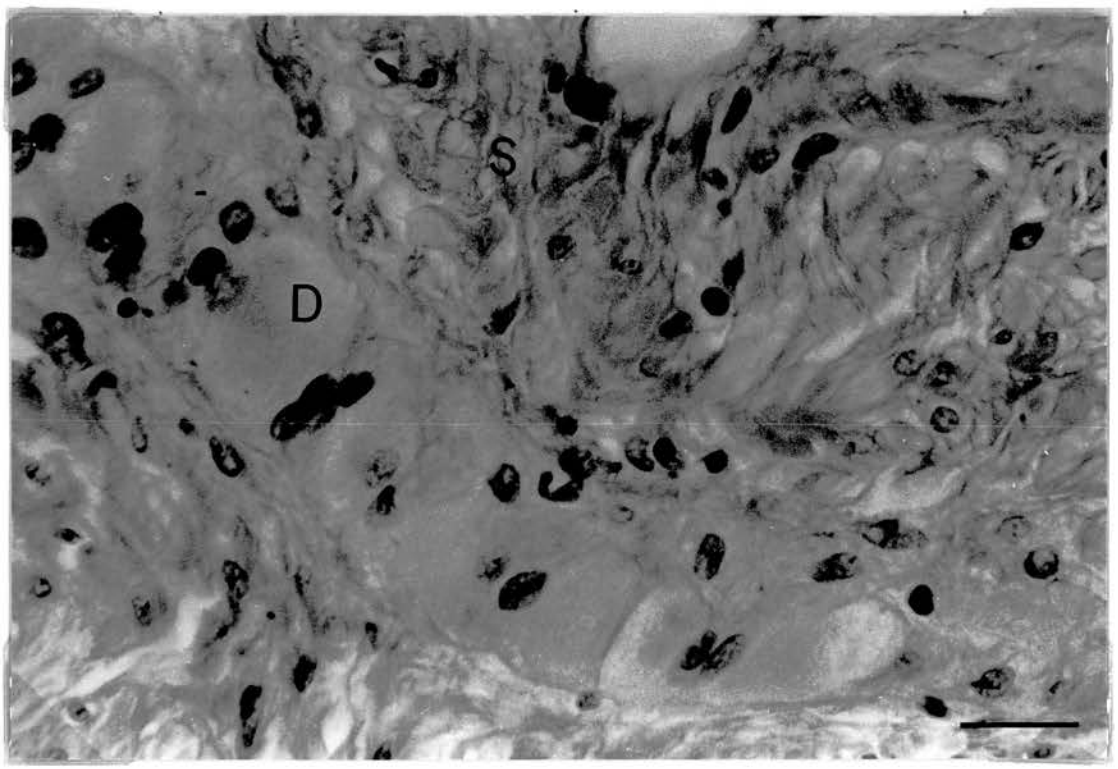

N
S

Figure 7.3. Group of neurones in the myenteric plexus from the duodenal region of the small intestine from a horse suffering from acute grass sickness. There is marked depletion of nerve cells, although the one remaining neurone appears normal: LM= longitudinal muscle, N= neurone, D = position where would have expected to see neurone. Haematoxylin and eosin, bar = 20μm

Figure 7.4. Group of neurones in the submucous plexus from the ileal region of the small intestine from a horse suffering from acute grass sickness. There is extensive chromatolysis (swelling of cells, loss of Nissl, eccentricity and pyknosis of nucleus). D= position would have expected to see neurone, S= submucosa. Haematoxylin and eosin, bar = 20μm


N
D


S
D

7.2.2 Statistical analysis

For each group in each region of the small intestine (duodenum and ileum) and each plexus (myenteric and submucous) data was available from five horses in the form of five or ten sections per horse. The median score for each horse was used in the Mann-Whitney U-test analysis for differences between the three groups (control, AGS and CGS) and between the two regions of the gut. For differences between the two plexuses within the same region of the gut the Wilcoxon Signed Rank test was used, also using the median score for each horse within a group.

For each of the three parameters subjectively scored, the Kruskal Wallis test was used to determine whether there were significant differences between the five sections used from each horse i.e. the within group variation.

7.2.3 Repeatability of scoring system

Initially 30 slides were scored 'blind'. This was repeated a few days later. Twenty six of the 30 were given second scores the same or 0.5 different from their initial scores. In the remaining four slides the difference in scores was 1 score.

7.3 Results

7.3.1 General observations

Several submucous and myenteric ganglia were observed in every transverse section. The structure of both ganglia was similar in both regions of the small intestine. Large ganglia were recognisable as elongated clusters of nerve cell bodies. Smaller ganglia merged with the surrounding connective tissue. The submucous plexus appeared to contain a higher proportion of large ganglia containing more nerve cell bodies than the myenteric plexus (Figure 7.1 and 7.2). These larger submucous ganglia were situated near the circular muscle, whereas small submucous ganglia lay close to the muscularis mucosae. In the submucous ganglia the neurones appeared to have a more peripheral position than in the myenteric plexus (Figures 7.1 and 7.2). Myenteric ganglia were located between the circular and longitudinal muscle layers, although they were also observed within the innermost longitudinal muscle. With horses suffering from grass sickness, degeneration and loss of enteric neurones to

some degree were observed in all sections (Figures 7.3 and 7.4). However, even in control horses some abnormal cells were seen.

The initial Kruskal Wallis tests showed that there were significant differences between the three groups and between the 15 horses used for all three parameters which were scored. There was no significant difference between the five (or ten) sections scored for each horse. To find out between which sets of data the significance lay, the Mann-Whitney U-test and Wilcoxon Signed Rank test were used. The results are shown in Tables 7.1, 7.2 and 7.3. Values with the same superscript are significantly different from each other.

7.3.2 Variation between horses within a group

The actual probability values, for the within group variation and the between group variation, for the three parameters which were scored, for the control and grass sickness groups are shown in Tables 7.4, 7.5 and 7.6. The AGS group showed the greatest within group variation. For neuronal numbers, the ileal myenteric plexus in the control horses had greater within group variation than between group variation. The ileal submucous plexus in the control horses also had significant within group variation but this was lower than the between group variation. This was found to be caused by the results for one particular horse which was 18 years old and suffering from Cushing's disease (hyperadrenocorticalism) (see Appendix E). For the AGS group, both regions of the gut and both plexuses showed significant within group variation, this was because the range of scores for the AGS horses was wide. For the CGS group there was no significant within group variation for neuronal number for either plexus or region.

For neuronal size there was no significant within group variation for the control and CGS groups. For the AGS group the duodenal myenteric plexus and the ileal submucous plexus showed significant within group variation.

For the proportion of abnormal cells, there was a significant difference between the horses used in the control group. With the AGS and CGS groups there was a significant difference between all the horses used with the exception of the ileal

1) Number of neurones

Duodenum/ Myenteric plexus		Ileum/ Myenteric plexus	
Control	3.8 (2.5-5.0)[abc]	Control	3.0 (1.5-4.0)[cde]
AGS	1.5 (0.5-3.0)[a]	AGS	1.5 (0.5-3.0)[d]
CGS	1.8 (1.0-2.5)[b]	CGS	1.5 (1.0-2.0)[e]

Duodenum/ Submucous plexus		Ileum/ Submucous plexus	
Control	3.0 (2.0-4.0)[fg]	Control	3.0 (1.8-4.0)[hj]
AGS	1.5 (0.8-2.0)[f]	AGS	1.8 (0.8-2.5)[hi]
CGS	2.0 (1.0-2.8)[g]	CGS	1.5 (0.8-2.3)[ji]

Table 7.1. The number of neurones present in the myenteric plexus and submucous plexus of 5μm transverse sections of equine duodenum and ileum. Values are the median scores with the range of values shown in brackets (The score presented in the table is the median value of the median scores for the five horses used for each group, the range presented in the table is the entire range of scores for five or ten sections for each of five horses). Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test ($P<0.05$).
c = $P<0.06$ when analysed using the Wilcoxon Signed Rank test.

2) Size of normal neurones

Duodenum/ Myenteric plexus		Ileum/ Myenteric plexus	
Control	3.0 (2.5-4.0)[a]	Control	3.0 (2.0-4.0)[c]
AGS	2.5 (1.5-3.0)	AGS	2.5 (1.8-3.0)
CGS	2.5 (1.8-3.5)[ab]	CGS	2.0 (1.5-2.5)[bc]

Duodenum/ Submucous plexus		Ileum/ Submucous plexus	
Control	2.5 (2.0-3.0)[ed]	Control	2.5 (1.5-3.5)[g]
AGS	2.0 (1.0-2.0)[d]	AGS	2.0 (1.5-3.0)[h]
CGS	2.0 (1.5-3.0)[ef]	CGS	1.5 (1.3-2.3)[fgh]

Table 7.2. The sizes of neurones in the myenteric and submucous plexuses of transverse sections of equine duodenum and ileum. Values are the median scores with the range of values shown in brackets (The score presented in the table is the median value of the median scores for the five horses used for each group, the range presented in the table is the entire range of scores for five or ten sections for each of five horses). Values with the same superscript are significantly different from each other e,g = $P<0.01$, a,c,d,h = $P<0.05$ when analysed using the Mann-Whitney U-test, b,f = $P<0.06$ when analysed using the Wilcoxon Signed Rank test.

3) Proportion of abnormal neurones

Duodenum/ Myenteric plexus		Ileum/ Myenteric plexus	
Control	0.5 (0.0-1.5)[ab]	Control	0.5 (0.0-1.5)[d]
AGS	2.0 (1.0-4.0)[a]	AGS	1.8 (0.5-2.5)[de]
CGS	3.0 (1.5-4.0)[bc]	CGS	1.3 (0.5-1.5)[ce]

Duodenum/ Submucous plexus		Ileum/ Submucous plexus	
Control	0.5 (0.0-1.0)[fg]	Control	0.5 (0.3-1.5)[i]
AGS	2.3 (1.0-3.5)[f]	AGS	1.5 (0.5-2.5)[ij]
CGS	3.0 (2.0-4.0)[gh]	CGS	0.5 (0.3-1.5)[hj]

Table 7.3. The number of abnormal neurones in the myenteric and submucous plexuses of 5μm transverse sections of equine duodenum and ileum. Values are the median scores with the range of values shown in brackets. (The score presented in the table is the median value of the median scores for the five horses used for each group, the range presented in the table is the entire range of scores for five or ten sections for each of five horses). Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test ($P<0.05$), c,h = $P<0.06$ when analysed using the Wilcoxon Signed Rank test.

1) neuronal number

region/ plexus	within group variation	between group variation	
		AGS	CGS
Duo/mp	NS	P=0.0122	P<0.0122
Ile/mp	P=0.007	P=0.0163	P=0.0122
Duo/sp	NS	P=0.0122	P=0.0122
Ile/sp	P=0.048	P=0.0122	P=0.0122

2) neuronal size

region/ plexus	within group variation	between group variation	
		AGS	CGS
Duo/mp	NS	NS	P=0.0472
Ile/mp	NS	NS	P=0.0367
Duo/sp	NS	P=0.0283	NS
Ile/sp	NS	NS	P=0.0196

3) Proportion of abnormal neurones

region/ plexus	within group variation	between group variation	
		AGS	CGS
Duo/mp	P=0.012	P=0.0122	P=0.0122
Ile/mp	P=0.008	P=0.0122	NS
Duo/sp	P=0.018	P=0.0122	P=0.0122
Ile/sp	P=0.002	P=0.0472	NS

Table 7.4. The probabilities for the within and between group variation for the control group for the subjective scoring carried out on histological sections of equine small intestine taken from horses with and without grass sickness. Key; Duo= duodenum, Ile= ileum, mp= myenteric plexus, sp= submucous plexus, AGS= acute grass sickness, CGS= chronic grass sickness, NS= not significant, P= probability, P<0.05 is taken to be of statistical significance.

1) neuronal number

region/ plexus	within group variation	between group variation	
		Control	CGS
Duo/mp	P=0.015	P=0.0122	NS
Ile/mp	P=0.006	P=0.0163	NS
Duo/sp	P=0.001	P=0.0122	NS
Ile/sp	P=0.020	P=0.0122	P=0.0472

2) neuronal size

region/ plexus	within group variation	between group variation	
		Control	CGS
Duo/mp	P=0.039	NS	NS
Ile/mp	NS	NS	NS
Duo/sp	NS	P=0.0283	NS
Ile/sp	P=0.043	NS	P=0.0122

3) proportion of abnormal neurones

region/ plexus	within group variation	between group variation	
		Control	CGS
Duo/mp	P=0.001	P=0.0122	NS
Ile/mp	NS	P=0.0122	P=0.0216
Duo/sp	P=0.010	P=0.0122	NS
Ile/sp	P=0.052	P=0.0472	P=0.0122

Table 7.5. The probabilities for the within and between group variation for the AGS group for the subjective scoring carried out on histological sections of equine small intestine taken from horses with and without grass sickness. Key; Duo= duodenum, Ile= ileum, mp= myenteric plexus, sp= submucous plexus, CGS= chronic grass sickness, Control= control horses, NS= not significant. P= probability, $P<0.05$ is taken to be of statistical significance.

1) neuronal number

region/ plexus	within group variation	between group variation	
		Control	AGS
Duo/mp	NS	P=0.0122	NS
Ile/mp	NS	P=0.0122	NS
Duo/sp	NS	P=0.0122	NS
Ile/sp	NS	P=0.0122	P=0.0472

2) neuronal size

region/ plexus	within group variation	between group variation	
		Control	AGS
Duo/mp	NS	P=0.0472	NS
Ile/mp	NS	P=0.0367	NS
Duo/sp	NS	NS	NS
Ile/sp	NS	P=0.0196	P=0.0122

3) proportion of abnormal neurones

region/ plexus	within group variation	between group variation	
		Control	AGS
Duo/mp	P=0.020	P=0.0122	NS
Ile/mp	P=0.026	NS	P=0.0216
Duo/sp	P=0.020	P=0.0122	NS
Ile/sp	NS	NS	P=0.0122

Table 7.6. The probabilities for the within and between group variation for the CGS group for the subjective scoring carried out on histological sections of equine small intestine taken from horses with and without grass sickness. Key; Duo= duodenum, Ile= ileum, mp= myenteric plexus, sp= submucous plexus, AGS= acute grass sickness, Control= control horses, NS= not significant, P= probability, $P<0.05$ is taken to be of statistical significance.

myenteric plexus for the AGS group and the ileal submucous plexus for the CGS group.

7.3.3 Variation between the groups

1) Number of neurones

The score for the number of neurones in the control group was significantly higher ($P<0.05$), in both plexuses and both regions of the small intestine, compared with the grass sickness groups (Table 7.1). The largest median score for the number of neurones for control tissue was given to the duodenal myenteric plexus. For grass sickness affected tissue, the largest median score was given to the duodenal submucous plexus in the CGS group.

There were no significant differences between the scores given to the two grass sickness groups in the myenteric plexus. For the submucous plexus there was a significant difference between the AGS and CGS groups in the ileal region ($P<0.05$). The duodenal myenteric plexus from the control group had a significantly higher score, for number of neurones, than the ileal myenteric plexus in the same horses ($P<0.05$). When comparing the two plexuses within the same region of the gut, there were no significant differences in the number of neurones for any of the groups.

2) Neuronal size

Only the neurones which looked morphologically normal were taken into consideration when scoring for neuronal size. Often damaged cells became swollen and therefore would have complicated the interpretation of the results. The variation in scores for neuronal size between the three groups was not as large as the variation of scores for neuronal number (Table 7.2). In the myenteric plexus, the size of the neurones for the CGS group, for both duodenal and ileal regions, was significantly smaller than the control group ($P<0.05$). There was no significant difference between the control and AGS groups for this region with regard to neuronal size. In the duodenal submucous plexus both the AGS and CGS groups scores were significantly lower than the control group ($P<0.05$). In the ileal submucous plexus only the CGS group had a significantly lower score than the control group ($P<0.01$). This was also

significantly lower than the AGS ileal submucous score ($P<0.05$). The size of neurones in the ileal myenteric plexus of the CGS group and ileal submucous plexus was significantly smaller than the duodenal myenteric plexus and duodenal submucous plexus, respectively ($P<0.05$). When comparing the two plexuses within the same region of the gut, there were no significant differences in size of neurones for either the control or grass sickness groups.

3) Proportion of abnormal neurones

The control group had a low median score for the proportion of abnormal neurones; only occasional abnormal cells were observed in this group (Table 7.3). Therefore, for the duodenal sections, the scores for the control group were significantly lower than the two grass sickness groups ($P<0.05$). In the ileal region the AGS group had a significantly higher score than both the control and CGS groups for both plexuses ($P<0.05$). There was a significant difference between the CGS duodenal and ileal values for both the myenteric and submucous plexus ($P<0.05$). In the duodenum there was no significant difference between the scores for the myenteric and submucous plexuses for the control and both grass sickness groups. When comparing the two plexuses, within the same region of the gut, there were no significant differences in the proportion of abnormal neurones for the control and both grass sickness groups.

7.3.4 Comparison of histological results with pharmacological findings

It was decided that the physostigmine results would be the most suitable to compare with the histological data as it was thought that these results showed a correlation with the number of active cholinergic neurones present in the *in vitro* preparations. Bethanecol and noradrenaline cause their contractile responses by their action on muscarinic and adrenergic receptors respectively and are therefore not as good an indicator of neuronal activity.

The results for responses to physostigmine ($8.6x10^{-6}M$) for the horses used in the histological study are shown in Table 7.7 and 7.8.

	n	initial rate [con/min]	rate after physos. [con/min]	percentage increase [%]	latency [s]	increase in tone [g]
Control	5	6.3[a]	9.3[ab]	44.8	94.0	6.0
		(4.4-7.2)	(7.8-10.2)	(33.3-90.91)	(68.0-96.0)	(3.3-12.1)
AGS	5	4.1	8.1	132.2	198.0	1.8
		(1.6-6.0)	(4.3-11.5)	(43.3-168.8)	(92.0-336.0)	(1.1-5.0)
CGS	5	4.4	7.3[b]	83.0	144.0	3.6
		(2.0-7.8)	(5.2-8.6)	(0.0-245.4)	(86.3-192.0)	(3.0-18.0)

Table 7.7. Responses to physostigmine ($8.6x10^{-6}$M) of isolated equine duodenal smooth muscle strips. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other; a = $P<0.05$ (Wilcoxon Signed Rank test) and b = $P<0.05$ (Mann-Whitney U-test). con/min = number of contractions per minute, physos. = physostigmine.

	n	initial rate [con/min]	rate after physos. [con/min]	percentage increase [%]	latency [s]	increase in tone [g]
Control	5	3.3^{c}	7.1^{cef}	75.4	101.0	1.6^{g}
		(1.4-8.3)	(3.2-8.8)	(6.7-271.4)	(84.0-208.0)	(0.0-17.0)
AGS	5	1.6^{d}	2.9^{de}	82.7	208.0	0.0^{gh}
		(0.8-2.9)	(1.2-4.6)	(30.0-180.4)	(112.0-384.0)	(0.0-0.0)
CGS	5	1.3	2.2^{f}	76.2	182.0	8.8^{h}
		(1.0-4.7)	(2.2-6.0)	(27.7-120.0)	(60.0-304.0)	(0.0-20.8)

Table 7.8. Responses to physostigmine ($8.6x10^{-6}$M) of isolated equine ileal smooth muscle strips. Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other;
c,d = $P<0.05$ (Wilcoxon Signed Rank test) and e,f,g,h = $P<0.05$ (Mann-Whitney U-test). con/min = number of contractions per minute, physos. = physostigmine.

Comparing Tables 7.7 and 7.8 with Tables 5.1.1 and 5.1.2 in Chapter 5.1 suggest that the horses chosen for the histological study were a representative sample of the control, AGS and CGS groups. The initial rate of background contractions, for which the maximum rate was found in the control tissue, corresponds to the group which was given the largest score for number of neurones in the enteric plexuses.

There was a significantly ($P<0.01$) lower score, for the number of neurones, in both plexuses, for control ileal compared with the control duodenal tissue in the histological studies. In the pharmacological experiments the background rate of contractions for ileal tissue was lower than for duodenal tissue.

In the myenteric plexus the AGS group had a significantly higher score for proportion of damaged cells than the control and CGS groups, this would therefore support the pharmacological results which suggested greatest damage in cases of AGS as they took longer to respond to the addition of physostigmine.

7.4 Discussion

The observations that enteric ganglia were numerous and consistently present in the myenteric and submucous plexuses of the small intestine in the control horses are in agreement with previous reports on the equine enteric nervous system (Hultgren, 1982; Vonderfecht *et al.*, 1983; Scholes *et al.*, 1993a).

There are varied reports on the number of cells per ganglion. Scholes *et al.* (1993a) reported that the structure of both the submucous and myenteric plexus was broadly similar throughout the gastrointestinal tract. They found 1-20 nerve cell bodies in the submucous ganglia and 1-10 nerve cell bodies in the myenteric ganglia of control horses. Pearson (1994) reported a mean of 20 cells per ganglion in the jejunal submucous plexus of young control horses (< eight weeks old). Pogson *et al.* (1992) reported the mean number of cells per ganglion to be five for the submucous plexus and 3.5 for the myenteric plexus in the equine jejunum. Doxey *et al.* (1992) and Pogson *et al.* (1992) found a higher number of ganglia per section in the submucous plexus compared with the myenteric plexus in equine jejunum. In this present study the variation in scored values for neuronal number between the plexuses, of a particular region, was not significantly different for both the control and grass

sickness groups. In grass sickness cases, the number of neurones was significantly reduced compared with control values ($P<0.05$) (Table 7.1). This is in agreement with other reports (Doxey *et al.*, 1992, Pogson *et al.*, 1992, Scholes *et al.*, 1993a).

In a report on the variation in neuronal size between the enteric plexuses in the horse, Pearson (1994) recorded that the mean diameter for neurones in the external submucous plexus (ESP) and internal submucous plexus (ISP) were 25+/-5μm and 26+/-6μm respectively. In the myenteric plexus they were found to be larger at 41+/-7μm. The results reported here are in agreement with those findings. In both the control and grass sickness groups, a larger score for the size of normal neurones was given to the myenteric plexus, compared with the submucous plexus (Table 7.2). The size of neurones was scored to determine whether larger or smaller neurones were differentially affected in cases of grass sickness. As the median scores for neuronal size for each plexus and region are significantly smaller ($P<0.05$) in the CGS group, than the control groups, it would suggest that it is the larger neurones that are affected in CGS. With AGS cases there was only a significant difference from the control group for the duodenal submucous plexus where larger neurones also seemed to be preferentially damaged.

As CGS cases had survived for a minimum of eight days before euthanasia, it is possible that all significant neuronal damage or degeneration has taken place by this stage. These results therefore suggest that it is the smaller neurones that survive an attack of grass sickness. There have been no previous reports of the size of neurones that are affected by grass sickness.

Pogson *et al.* (1992), in a study of autonomic and enteric ganglia found a relationship between the size of a ganglion and the size of the horse. In this present study no account of the size of the animals was taken. This relationship may have had an effect on the score for the size of neurone in the CGS group, as four out of the five horses were relatively small (see Appendix E).

Some abnormal neurones were present in the myenteric plexus of control horses. Degenerative changes of the myenteric plexus in the horse have been reported in other diseases (Brownlee, 1959; Gilmour, 1973a; Murray *et al.*, 1988; Burns,

Karcher and Cummings, 1990). Pogson *et al.* (1992) reported that 97% of cells appeared normal in their control group.

Scholes *et al.* (1993a) found with grass sickness affected horses, that some cells showed no morphological changes, whereas other preparations showed occasional vacuolation of nerve cell bodies and others had a complete loss of neurones. The most severe neuropathy was recorded in AGS ileal samples. Those authors also reported that in some acute cases no neurones could be recognised.

In these results, in the duodenum, the CGS group showed the greatest proportion of damaged cells although there was no significant difference between the CGS and AGS groups. In the ileum, the AGS group had the largest proportion of abnormal cells, which was significantly higher than the control and CGS groups ($P<0.05$). It may be that the ileum is the first site to be affected in most severe cases of AGS. With cases of CGS, which have a longer clinical duration, the damage may have spread to the duodenum thus giving this group the greatest proportion of abnormal cells in this region. With AGS, where euthanasia occurs within 48 hours, cell degeneration may still be in progress at the time of death and result in a lower score than in CGS for proportion of abnormal cells, in the duodenum regions. Doxey *et al.* (1992) found the greatest percentage of abnormal cells (74%) in the AGS group in their study, but their investigation was confined to the jejunum.

Pogson *et al.* (1992) found the degree of neuronal damage in grass sickness is related to the type/severity of clinical dysautonomia and concluded that both the rate of neuronal loss and the extent of neuronal damage, may influence the clinical manifestations of grass sickness.

As only a small number of horses were studied in each group there were sometimes significant differences in scores awarded between horses within a group. Tables 7.4-7.6 compared the within group variation with the between group variation. There are some cases where the within group variation was highly significant. A natural reduction in neuronal number with advancing age has been reported in laboratory species (Gabella, 1989). Should such a phenomenon occur in the horse, this may account for the variation in neuronal number in the ileal myenteric plexus of the control group, where one horse was 18 years old.

The proportion of abnormal neurones showed significant differences within the control group. Some horses had no abnormal cells, whereas in others several abnormal cells were seen. These may have been secondary to the conditions for which they were euthanased (Brownlee, 1959).

The histological results supported pharmacological studies with physostigmine, which indicated that the most severe neuronal damage occurred in cases of AGS. The percentage increase in the rate of contractions following physostigmine was greatest for the AGS group for both the duodenal and ileal region although there were no significant differences between the groups. The latency before a response was detected was also longest for the AGS group (although not significantly so), suggesting that the AGS group had the least number of active cholinergic neurones out of the three groups (see Chapter 5.1.4). The histological studies also suggest that the AGS group had the lowest number of neurones, with the exception of the ileal submucous plexus where the CGS group had a slightly lower median score for the number of neurones. The proportion of abnormal neurones in the AGS group had a similar median score in both plexuses and both regions, whereas the values for the CGS group was much higher in the duodenal region than the ileal region.

In conclusion, differences in the number of neurones, size of neurones, and the proportion of abnormal cells were evident using a simple scoring system on histological sections. Although there were some significant differences in the values awarded for horses within a particular group, particularly the AGS group, these results showed interesting differences between the control and grass sickness groups. These results confirm that extensive damage occurs to the enteric neurones in cases of grass sickness and there was some evidence that the smaller neurones may be least affected. Interestingly, there was good correlation between functional cholinergic responses and neuronal morphology.

CHAPTER 8

GENERAL CONCLUSIONS

The overall aim of this thesis was to study equine intestinal motility using an *in vitro* technique. It must be remembered however, that isometric recording of intestinal contractile activity, while it represents the final physiological output of the intestinal smooth muscle, is only an indirect measure of earlier events, such as an increase in membrane conductance, depolarization or hyperpolarization and changes in spiking activity (Paton, 1975). It also cannot be said with certainty that *in vitro* observations exactly reflect *in vivo* activity.

The function of the smooth muscle of the small intestine is to contract and relax in such a way as to provide propulsion and mixing of the contents (Bolten, 1989). This is achieved by the activity of the enteric nervous system, the extrinsic autonomic nervous system and the endocrine system (Kutchai, 1993).

In the disease grass sickness in horses there is a dysfunction of the autonomic nervous system, primarily affecting the alimentary tract (Gilmour, 1989). Degenerative changes are found within the autonomic ganglia, including those found within the myenteric and submucous plexuses in the intestinal wall (Obel, 1955; Gilmour, 1973a). Acute cases of grass sickness usually have dysphagia and intestinal stasis (Milne, 1991). Animals die, or require euthanasia, within 48 hours of the onset of clinical signs. In subacute cases the clinical signs are less intense but rapid loss of condition including weight loss are marked features. Most horses with SAGS die, or require euthanasia, within two to seven days of the onset of the disease. A few cases progress to the chronic form of the disease, classified as lasting eight days duration or more.

The aetiology of grass sickness is unknown, one theory is a neurotoxin is present (Gilmour, 1973b). If this is the case, does the neurotoxin have a selective action on certain types of neurones. As the disease causes such disruption to the function of intestinal smooth muscle it was thought that *in vitro* studies of responses to selected pharmacological agents would provide useful information on the extent of damage occurring to the enteric nervous system.

Like intestinal smooth muscle in other species, strips of equine small intestine showed regular background contractile activity. It was found that ileal muscle strips showed a burst-like pattern of background contractions whereas in duodenal tissue the contractions were more regular with a significantly higher contractile rate (Murray *et al.*, 1994). A higher contractile rate would be expected in the more proximal regions of the gastrointestinal tract (Davies and Gerring, 1983).

There was no significant difference in the amplitude of contractions between the two regions of the small intestine in control horses. With grass sickness tissue there was a trend for a lower rate of contractions and a higher amplitude of contractions, although the differences were not always statistically different. The addition of tetrodotoxin, atropine, propranolol, phentolamine, prozosin and yohimbine had no significant effects on the characteristics of the background contractions with both control and grass sickness tissue.

The addition of physostigmine demonstrated that the ileal region was most affected in cases of grass sickness. In the duodenal region only the AGS group had a significantly lower rate of contractions than the control group following the addition of physostigmine, whereas in the ileum all three grass sickness groups had significantly lower enhanced rates compared with the ileal control group ($P<0.05$). The duodenal AGS group showed a significantly lower increase in tone compared with the duodenal control group ($P<0.01$). In the ileal region no AGS or SAGS muscle strips had any increase in tone following the addition of physostigmine. There was a significantly prolonged latency with AGS and SAGS tissue before a response could be seen.

The effect of storage for 24 hours at 4°C was investigated to see if stored control tissue would respond similarly to fresh grass sickness tissue and thus provide a model for grass sickness *in vitro* research, however, there were found to be significant differences in some responses so it was concluded this would be an unsuitable model.

Dose response curves in response to bethanecol were constructed for the control, AGS and CGS groups. They showed that AGS tissue was supersensitive to this cholinergic agonist, indicated by a leftward shift of the dose response curve and a

lower ED_{50} value. In the ileal region the CGS group also had a lower ED_{50} value than the ileal control group. There have been a number of reports in the literature of denervation hypersensitivity in both skeletal and smooth muscle (Clark and Mapstone, 1987; Janig, 1989; Osinski and Bass, 1994).

Cisapride is a prokinetic drug which has been reported to enhance gastrointestinal motility along the entire gastrointestinal tract (King and Gerring, 1988). The results with cisapride in this study showed a trend to an increase in contractile rate ($P<0.06$) with both control and grass sickness affected tissue, although it must be concluded that more experiments to study the mechanism of action should be carried out.

In vitro equine small intestine contracts in response to catecholamine addition. It was concluded this was due to $\alpha 2$ adrenergic receptors and independent of nervous elements. There were no significant differences in the responses of grass sickness affected tissue to noradrenaline in these experiments, with the exception of the ileal AGS group, which had a significantly lower contractile response from the other grass sickness groups and the control group ($P<0.05$ or less). It may be an effect of the disease that receptor number or availability is reduced, as has been reported in other diseases (Wright and Shepherd, 1965; Brodde, Zerkowski, Borst, Maier and Michel, 1989).

There have been no previous attempts to correlate enteric neuronal damage with any functional responses. In these experiments a subjective histological scoring system established significant differences between the two plexuses and the two regions examined with regard to neuronal number, size of normal neurones and proportion of abnormal cells. The control, AGS and CGS groups were examined. Both grass sickness groups had a significantly lower score for neuronal number than the control group ($P<0.05$). There was also some evidence that it was the larger neurones that were effected by grass sickness. In the ileum the AGS group had a significantly higher proportion of abnormal cells than both the control and CGS groups. In the duodenum there was no significant difference between the AGS and CGS groups although they both had a significantly higher proportion of abnormal cells compared with the control group ($P<0.05$).

The histological results supported the pharmacological studies with physostigmine, which suggested that the most severe neuronal damage occurred in cases of AGS.

In conclusion, *in vitro* investigations of small intestinal motility can provide some useful information on the functional deficits which occur in horses suffering from grass sickness. The damage to the excitatory cholinergic enteric nervous system appears to be so great in cases of AGS that any therapeutic treatment would be of limited benefit. In CGS cases, treatment with prokinetic drugs such as cisapride have been shown to be of benefit in selected cases (Milne, E.M., Doxey, D.L., Woodman, M.P., Cuddeford, D. and Pearson, R.A., in press). However, it must be remembered that other non-intestinal factors will affect the horses chances of survival such as its ability to swallow, level of depression and degree of rhinitis.

Appendix A.

Within animal repeatability of organ bath experiments.

Chapter 3 describes the experimental protocol for the *in vitro* organ bath experiments. A limitation of this work was there was no investigation of the within animal repeatability with regard to responses to pharmacological agents. A constraint of clinical research, especially involving horses, is that there is a limited availability of suitable material. Experiments needed to be carried out on fresh tissue and therefore for each horse only one smooth muscle strip from a specific area of the small intestine was used to investigate the responses to a particular pharmacological agent. In the tables of results the total number of horses used to determine the response to a pharmacological agent are shown.

It was thought reasonable to expect that responses from adjacent strips of gastrointestinal tract would be similar. In the literature work in laboratory species on morphology, histology and immunohistology has found uniformity within a particular area of gut (Schultzberg, 1986; Furness and Costa, 1987). Gabella (1990) stated how the structure of the ganglionic network, size and shape of the meshes were strikingly regular and consistent in a particular segment of the gut of the guinea pig. In the jejunum-ileum the pattern of the myenteric plexus was uniform both around the circumference and along the length, showing no obvious gradient in any structural parameter. She found differences in the extent of the myenteric plexus in different species. If the structure and distribution of enteric nerves was the same in the horse within a particular region it would be likely that the *in vitro* response to pharmacological agents would be the same.

The two sites used for sampling were well defined :- the caudal flexure of the duodenum and the terminal ileum 10cm proximal to the ileocaecal fold. This would

reduce the possible variation between horses attributable to variations in sampling sites. It should also be noted that the order drugs were added to the smooth muscle preparations was randomised so that responses were not affected by the timing of the drug addition (Perry, 1968).

In summary within animal repeatability could have been investigated more thoroughly, however, the sample sites were accurately defined and there was no evidence that the responses of an adjacent strip of smooth muscle would have been any different. The number of horses used to investigate the responses to pharmacological agents were sufficient to show statistically significant differences between the control and grass sickness groups.

Appendix B. Horses used to investigate characteristics of background contractions (Chapter 4).

BREED	AGE	SEX	REASON FOR EUTHANASIA	DRUG TREATMENT
pony	16 yrs	gelding	ragwort poisoning	none
hunter	9 yrs	mare	laminitis	PBZ 3days previously
Welsh pony	4 yrs	stallion	ventricular septal defect	none
Clydesdale	10 yrs	mare	gut torsion	PBZ few hours before
pony	9 yrs	mare	liver disease	none
TB x	14 yrs	gelding	large colon displacement	buscopan day before, PBZ day died
Welsh pony	10 yrs	mare	liver disease/ eosinophilic gastroenteritis	none
Welsh x	16yrs	mare	volvulus of small intestine	xylazine, PBZ
Welsh pony	15 yrs	mare	orthopaedic	none
Shetland	9 mths	*	orthopaedic	none
Hanovarian	6 yrs	gelding	mitral value incompetence	none
TB	12 yrs	mare	pyelonephritis	flunixin, penicillin
pony type	*	gelding	experimental pony	none
TB x Welsh	4yrs	mare	AGS	buscopan, butorphanol, finadyne day before
Shetland	3yrs	stallion	AGS	none
Highland	6mths	colt	AGS	flunixin
Highland	4 yrs	mare	AGS	none
Highland	5yrs	filly	AGS	none
TB x	7yrs	mare	AGS	finadyne day euthanasia
TB	12yrs	stallion	AGS	none
pony	4yrs	mare	AGS	flunixin on day of euthanasia
Highland x	4yrs	gelding	AGS	none
Welsh x C/mara	10yrs	gelding	AGS	flunixin on day of euthanasia
Arab x Welsh	10yrs	gelding	AGS	buscopan on day of euthanasia
Welsh	4yrs	gelding	AGS	none
Clydesdale	3yrs	gelding	AGS	none

Appendix B continued.....

TB x	5yrs	*	SAGS	none
Highland x	7yrs	mare	SAGS	none
TB x	5yrs	mare	SAGS	equipalazone day before
TB x	6yrs	mare	SAGS	buscopan 3 days before, penicillin, dexamethazone day before
polo pony	7yrs	mare	SAGS	none
Highland x	10mths	stallion	SAGS	none
pony type	6yrs	gelding	SAGS	finadyne
Clydesdale	2yrs	filly	SAGS	none
Highland	*	gelding	SAGS	none
TB	9yrs	gelding	SAGS	finadyne 2 days before
TB x	7yrs	gelding	SAGS	finadyne on day euthanasia
TB x	10yrs	mare	SAGS	none
Welsh	12yrs	gelding	SAGS	finadyne until day before euthanasia
Norwegian Fjord	7yrs	mare	CGS	cisapride stopped 3 days before euthanasia
pony type	8yrs	mare	CGS	none
3/4 TB	13yrs	gelding	CGS	none
Clydesdale	2yrs	filly	CGS	depocillin upto euthanasia
Warmblood	2yrs	filly	CGS	cisapride upto day of euthanasia
TB x	2yrs	stallion	CGS	cisapride until day before euthanasia
Shetland	4yrs	filly	CGS	synulox day before euthanasia
Arab x	2yrs	gelding	CGS	none
Shetland	10yrs	mare	CGS	none
TB	yearling	mare	CGS	cisapride, finadyne day before euthanasia
Highland	3yrs	gelding	CGS	none
pony	6yrs	mare	CGS	cisapride until day of euthanasia
pony	18yrs	mare	CGS	equipalazone, depocillin, crystapen until day of euthanasia

Key: TB = Thoroughbred, x = crossbreed, C/mara = Connemara, yrs = years old, mths = months old, AGS = acute grass sickness, SAGS = subacute grass sickness, CGS = chronic grass sickness, PBZ = phenylbutazone, * = unknown data.

Appendix C. Horses used in physostigmine experiments (Chapter 5.1).

BREED	AGE	SEX	REASON FOR EUTHANASIA	DRUG TREATMENT
pony	16yrs	gelding	ragwort poisoning	none
Hunter type	9yrs	mare	laminitis	PBZ 3 days previously
Welsh pony	4yrs	stallion	ventricular septal defect	none
Gelderlander	12yrs	gelding	heart disease	frusemide for 11 days
TB x	7yrs	gelding	lung abscess	none
Clydesdale	2mths	filly	immuno-incompetence	gentamicin, blood transfusion, corticosteriods, penicillin
Dutch WB	17mths	gelding	orthopaedic	2g PBZ daily
TB x	11yrs	mare	gastric rupture	halothane, ketamine anaesthesia
TB	12yrs	mare	pyelonephritis	flunixin, penicillin
pony	18yrs	gelding	cushings disease	none
pony	*	gelding	experimental pony	none
pony	*	gelding	experimental pony	none
pony	*	gelding	experimental pony	none
welsh x Arab	20mths	*	liver disease	*
Icelandic	14yrs	mare	ruptured prepubic tendon of abdominal cavity	none
TBx	17yrs	gelding	colic- stangulation by lipoma	buscopan, flunixin, butorphanol, PBZ
Dartmoor x	24yrs	mare	stenosis of small intestine	buscopan
TBxDartmoor	3yrs	mare	bilateral laryngeal chondritis	penicillin
TB x	3yrs	mare	pharyngeal paralysis	none
TB	6yrs	mare	orthopaedic	flunixin, PBZ, penicillin
Welsh	15yrs	mare	orthopaedic	none
Hanovarian	6yrs	gelding	mitral value incompetence	none
Shetland	9mths	*	orthopaedic	*
TB	3yrs	gelding	wobbler	none
Shetland	*	gelding	experimental pony	none
Clydesdale	3yrs	gelding	AGS	none
Welsh pony	4yrs	gelding	AGS	none
TB	12yrs	stallion	AGS	none
Highland	2yrs	filly	AGS	finadyne day before
Highland	5yrs	filly	AGS	none
Highland	6mths	stallion	AGS	flunixin

Appendix C continued.....

pony	4yrs	gelding	AGS	none
Arab x Welsh Mountain	10yrs	gelding	AGS	buscopan few hours before euthanasia
WelshxC/mara	10yrs	gelding	AGS	flunixin on day of euthanasia
pony	4yrs	mare	AGS	flunixin on day of euthanasia
TB x	7yrs	gelding	SAGS	finadyne on day of euthanasia
pony	7yrs	mare	SAGS	none
Welsh	12yrs	gelding	SAGS	finadyne until day before euthanasia
TB x	6yrs	mare	SAGS	buscopan 3 days before, penicillin and dexamethazone day before euthanasia
Highland x	10mths	stallion	SAGS	none
TB	9yrs	gelding	SAGS	finadyne 2 days before euthanasia
Clydesdale	2yrs	filly	SAGS	none
Highland x	7yrs	mare	SAGS	none
Warmblood	2yrs	filly	CGS	cisapride upto euthanasia
Shire x	6yrs	mare	CGS	none
Shetland	4yrs	filly	CGS	ampocillin/clavulanic acid
TB	yearling	mare	CGS	cisapride and finadyne day before euthanasia
Norwegian Fjord	7yrs	mare	CGS	cisapride stopped 3 days before euthanasia
Clydesdale	5yrs	mare	CGS	cisapride until day before euthanasia
TB x	2yrs	stallion	CGS	cisapride until day before euthanasia
Halflinger	5yrs	gelding	CGS	cisapride until 5 days before euthanasia
pony	18yrs	mare	CGS	equiphazone, depocillin and crystapen until euthanasia
Arab	2yrs	gelding	CGS	none
Highland	3yrs	gelding	CGS	none
pony	6yrs	mare	CGS	cisapride until euthanasia

Key: TB = Thoroughbred, x = crossbreed, WB = Warmblood, yrs = years old, mths = months old, AGS = acute grass sickness, SAGS = subacute grass sickness, CGS = chronic grass sickness, PBZ = phenylbutazone, * = unknown data.

Appendix D. Analysis of physostigmine data when the grass sickness data are divided into different groups. There are now only two grass sickness groups. When the results were plotted there appearred to be a division in relation to latency before a response to physostigmine was observed, therefore a latency of 250 seconds was used as a dividing line. Horses with a longer latency period were put into the AGS group with those less than 250 seconds into the CGS group.

	n	initial rate [contractions /min]	rate after physostigmine [contractions/ min]	percentage increase [%]	latency [s]	tone increase [g]
Con	25	6.5^{ab} (0.6-8.3)	9.2^{de} (6.2-15.0)	43.7^{fg} (4.4-177.8)	90.0^{jk} (30.0-168.0)	5.1^{m} (1.4-12.1)
AGS	7	3.2^{ac} (0.8-3.8)	7.5^{d} (4.3-9.0)	175.0^{fh} (113.2-512.5)	280.0^{jl} (264.0-420.0)	2.0^{m} (1.0-5.0)
CGS	23	4.1^{bc} (1.4-8.6)	8.0^{e} (5.2-12.0)	100.0^{gh} (0.0-360.0)	140.0^{kl} (60.0-224.0)	3.5 (0.9-18.0)

Effect of addition of physostigmine ($8.6x10^{-6}$M) on isolated duodenal muscle strips taken from control horses (Con) and those suffering from acute grass sickness (AGS) and chronic grass sickness (CGS). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other, a, f, k, j, l = $P<0.001$, b, m = $P<0.01$, c, d, e, g, h = $P<0.05$ when analysed using the Mann-Whitney U-test.

Appendix D continued....

	n	initial rate [contractions /min]	rate after physostigmine [contractions/ min]	percentage increase [%]	latency [s]	tone increase [g]
Con	23	2.6[ab] (0.0-8.6)	7.4[cd] (3.2-16.4)	118.3 (2.3-363.2)	112.0[f] (72.0-208.0)	1.2 (0.0-21.0)
AGS	8	1.7[a] (0.7-2.3)	2.9[c] (1.6-5.6)	122.0 (20.0-245.4)	342.0[fg] (268.0-384.0)	0.0 (0.0-2.0)
CGS	14	1.7[b] (0.6-4.7)	3.4[d] (1.2-8.4)	58.7 (3.2-300.0)	140.0[g] (60.0-248.0)	0.0 (0.0-20.8)

Effect of addition of physostigmine ($8.6x10^{-6}$M) on isolated ileal muscle strips taken from control horses (Con) and those suffering from acute grass sickness (AGS) and chronic grass sickness (CGS). Values are medians with the range of values shown in brackets. Values with the same superscript are significantly different from each other, c, f, g = $P<0.001$, d = $P<0.01$, a, b = $P<0.05$ when analysed using the Mann-Whitney U-test.

Appendix E. Horses used in bethanecol experiments (Chapter 5.2).

BREED	AGE	SEX	REASON FOR EUTHANASIA	DRUG TREATMENT
Welsh pony	15yrs	mare	orthopaedic	none
pony	20yrs	mare	thyroid adenoma	none
Shetland	9mths	*	orthopaedic	*
pony	18yrs	gelding	cushings disease	none
Hanovarian	6yrs	gelding	heart disease	none
Dartmoor x	24yrs	mare	colic	buscopan
Shetland	*	gelding	experimental	none
Welsh x Arab	20mths	*	liver disease	*
Arab x Welsh	10yrs	gelding	AGS	buscopan day euthanasia
WelshxC/mara	10yrs	gelding	AGS	flunixin day euthanasia
TB	12yrs	stallion	AGS	none
Highland	5yrs	filly	AGS	none
Shetland	3yrs	stallion	AGS	none
pony	4yrs	gelding	AGS	none
Highland x	4yrs	gelding	AGS	none
pony	4yrs	mare	AGS	flunixin day euthanasia
Highland	6mths	colt	AGS	flunixin
Welsh M/tain	4yrs	gelding	CGS	none
3/4 TB	13yrs	gelding	CGS	none
Clydesdale	2yrs	gelding	CGS	depocillin
Shire x	6yrs	filly	CGS	none
Arab x	2yrs	mare	CGS	none
Shetland	4yrs	gelding	CGS	synulox day before
TB x	2yrs	gelding	CGS	PBZ day before
Shetland	10yrs	mare	CGS	none
Warmblood	2yrs	filly	CGS	cisapride upto euthanasia

Key: TB = Thoroughbred, x = crossbreed, C/mara = Connemara, M/tain, yrs = years old, mths = months old, AGS = acute grass sickness, CGS = chronic grass sickness, PBZ = phenylbutazone, * = unknown data.

Appendix F. Horses used in cisapride experiments (Chapter 5.3).

BREED	AGE	SEX	REASON FOR EUTHANASIA	DRUG TREATMENT
Dartmoor x TB	3yrs	mare	bilateral laryngeal chondritis	penicillin
TB x	3yrs	mare	respiratory disease	none
TB	6yrs	mare	orthopeadic	flunixin, penicillin, PBZ
Dutch WB	17mths	gelding	orthopaedic	2g PBZ daily
TB x	7yrs	gelding	lung abcess	none
Fell	4yrs	filly	AGS	none
Highland	4yrs	mare	AGS	none
pony x	8yrs	mare	AGS	none
TB x	5yrs	mare	AGS	none
TB x	7yrs	mare	AGS	finadyne
Shire x ID	6yrs	mare	CGS	none
TB x	2yrs	gelding	CGS	PBZ day before euthanasia
Halflinger x	5yrs	gelding	CGS	cisapride until 5 days before euthanasia
TB x	5yrs	stallion	CGS	none
3/4 TB	13yrs	gelding	CGS	none

Key: TB = Thoroughbred, x = crossbreed, yrs = years old, mths = months old, ID = Irish Draught, WB = Warmblood, AGS = acute grass sickness, CGS = chronic grass sickness, PBZ = phenylbutazone, * = unknown data.

Appendix G. Horses used in adrenergic experiments (Chapter 6).

BREED	AGE	SEX	REASON FOR EUTHANASIA	DRUG TREATMENT
Welsh pony	10yrs	mare	eosinophilic gastroenteritis/ liver disease	none
TB	3yrs	gelding	wobbler	none
TB x	7yrs	gelding	lung abcess	none
Clydesdale	2mths	filly	immuno-incompetence	corticosteriods, penicillin, gentamicin, blood transfusion
Dutch WB	17mths	gelding	orthopaedic	2g PBZ daily
TB x	11yrs	mare	gastric rupture	halothane, ketamine anaesthesia
TB	12yrs	mare	pyelonephritis	flunixin, penicillin
pony	18yrs	gelding	cushings disease	none
pony	*	gelding	experimental pony	none
pony	*	gelding	experimental pony	none
pony	*	gelding	experimental pony	none
Welsh x Arab	20mths	*	liver disease/thrombosis	*
pony	23yrs	mare	tapeworm cyst of liver	PBZ same day as euthanasia
Icelandic	14yrs	mare	ruptured prepubic tendon of abdominal cavity	*
TB x	17yrs	gelding	colic-strangulation by lipoma	buscopan, flunixin, butorphanol, PBZ
Welsh x	16yrs	mare	colic-volvulus of SI	xylazine, PBZ
TB x	14yrs	gelding	large colon displacement	buscopan day before, PBZ day of euthanasia
pony	9yrs	mare	liver disease	none
pony	20yrs	mare	thyroid adenoma	none
Gelderlander	12yrs	gelding	heart disease	frusemide for 11 days
TB x	7yrs	mare	AGS	finadyne
TB x	10yrs	mare	AGS	equipalazone
Highland	2yrs	filly	AGS	finadyne day before
pony x	8yrs	mare	AGS	none
Highland	6mths	colt	AGS	flunixin
pony	4yrs	gelding	AGS	none
TB x Welsh	4yrs	mare	AGS	buscopan, torbugesic, finadyne
TB	9yrs	gelding	AGS	none

Appendix G continued.....

Highland	4yrs	mare	AGS	flunixin on day of euthanasia
pony	2yrs	filly	SAGS	finadyne 2 days before euthanasia
Highland	*	mare	SAGS	finadyne
pony	4yrs	mare	SAGS	none
pony type	6yrs	*	SAGS	none
TB x	7yrs	gelding	SAGS	finadyne on day of euthanasia
pony	7yrs	mare	SAGS	none
Welsh	12yrs	gelding	SAGS	finadyne until day before euthanasia
TB x	6yrs	mare	SAGS	buscopan 3 days before, penicillin and dexamethazone day before euthanasia
Highland x	10mths	stallion	SAGS	none
TB	9yrs	gelding	SAGS	finadyne 2 days before euthanasia
Clydesdale	2yrs	filly	SAGS	none
Highland x	7yrs	mare	SAGS	none
TB x	5yrs	*	CGS	none
Clydesdale	2yrs	filly	CGS	depocillin until euthanasia
Welsh M/tain	4yrs	gelding	CGS	none
TB	yearling	mare	CGS	cisapride and finadyne day before euthanasia
TB x	2yrs	gelding	CGS	PBZ 1 day before euthanasia
Clydesdale	5yrs	mare	CGS	cisapride until day before euthanasia
TB x	2yrs	stallion	CGS	cisapride until day before euthanasia
Halflinger	5yrs	gelding	CGS	cisapride until 5 days before euthanasia
pony	18yrs	mare	CGS	equiphazone, depocillin and crystapen until euthanasia
Shetland	10yrs	mare	CGS	none
Highland	3yrs	gelding	CGS	none
pony	6yrs	mare	CGS	cisapride until euthanasia

Key: TB = Thoroughbred, WB = warmblood, x = crossbreed, M/tain = mountain, yrs = years old, mths = months old, SI = small intestine, AGS = acute grass sickness, SAGS = subacute grass sickness, CGS = chronic grass sickness, PBZ = phenylbutazone, * = unknown data.

Appendix H. Analysis of noradrenaline data when the grass sickness data are divided into different groups. There are now only two grass sickness groups. When the results were plotted there was no clear dividing line therefore the grass sickness groups were separated on duration of illness.

A) Duodenum

	initial contractile response to noradrenaline [g/g of tissue]	response to noradrenaline following pretreatment with propranolol [g/g of tissue]	contractile rate following noradrenaline addition [contractions/ min]	contractile rate after propranolol and noradrenaline [contractions/min]	contractile rate after phentolamine and noradrenaline [contractions/ min]
Con	32.1[a]	34.3[cd]	0.6	2.0	1.6
	(8.4-115.3)	(8.4-93.8)	(0.0-6.8)	(1.2-5.4)	(0.0-10.6)
AGS	20.1[ab]	16.6[c]	0.9	2.0	2.8
	(16.0-31.2)	(10.3-26.4)	(0.0-2.4)	(1.2-4.4)	(0.0-7.8)
CGS	37.7[b]	24.6[d]	0.2	2.6	4.6
	(10.8-90.9)	(0.0-63.2)	(0.0-2.0)	(1.0-6.2)	(1.0-9.6)

B) Ileum

	initial contractile response to noradrenaline [g/g of tissue]	response to noradrenaline following pretreatment with propranolol [g/g of tissue]	contractile rate following noradrenaline addition [contractions/ min]	contractile rate after propranolol and noradrenaline [contractions/ min]	contractile rate after phentolamine and noradrenaline [contractions/ min]
Con	43.0[e]	15.8	0.2	0.7[h]	1.2
	(13.8-118.3)	(0.0-72.2)	(0.0-2.3)	(0.0-1.8)	(0.0-2.7)
AGS	9.8[ef]	19.0	0.0[g]	0.9[i]	1.0
	(0.0-34.6)	(0.0-36.2)	(0.0-3.2)	(0.0-2.0)	(0.0-6.6)
CGS	44.7[f]	26.9	0.6[g]	2.2[hi]	2.7
	(4.5-65.6)	(3.5-58.7)	(0.0-2.2)	(1.9-5.9)	(0.0-7.6)

Median peak contractile response in grams/gram of tissue and rate of contractions per minute in response to noradrenaline addition (7.1×10^{-6} M) for muscle strips taken from A) the duodenum and B) the ileum, from control horses (Con) and those affected by AGS (duration of illness 3 days or less) and CGS (duration of illness 4 days or more). The range of values are shown in brackets. Values with the same superscript are significantly different from each other when analysed using the Mann-Whitney U-test e, f, h = $P<0.001$, b, c, i = $P<0.01$, a, d, g = $P<0.05$.

Appendix I. Horses used for the histological study of enteric nervous system (Chapter 7).

BREED	AGE	SEX	REASON FOR EUTHANASIA	DRUG TREATMENT
TB	12yrs	mare	pyelonephritis	flunixin, penicillin
pony	18yrs	gelding	cushings disease	none
pony	*	gelding	experimental pony	none
Welsh x Arab	20mths	*	liver disease	*
pony	23yrs	mare	tapeworm cyst of liver	PBZ
Highland	5yrs	filly	AGS	none
Highland	6mths	colt	AGS	flunixin
Shetland	3yrs	stallion	AGS	none
pony	4yrs	gelding	AGS	none
TB x	10yrs	mare	AGS	equipalazone
Shetland	4yrs	filly	CGS	amoxycillin/clavulanic acid day before euthanasia
Arab x	2yrs	gelding	CGS	none
Clydesdale	5yrs	mare	CGS	cisapride until day before euthanasia
Halflinger	5yrs	gelding	CGS	cisapride until 5 days before euthanasia
pony	18yrs	mare	CGS	equipalazone, depocillin, crystapen

Key: TB = Thoroughbred, x = crossbreed, yrs = years old, mths = months old, PBZ = phenylbutazone, * = unknown data.

Appendix J. Possible effects of previous drugs horses may have received on gastrointestinal motility.

It should be noted that as discussed in Chapter 3.2.3 initial experiments were carried out to see whether the barbiturates used for euthanasia could be washed off the smooth muscle preparation. It was found that they could be washed off after several minutes of washing with fresh modified Krebs solution. It was therefore likely that any previous drug treatment that the horses may have received pre-euthanasia would also have been removed during the one hour eqilibration period. Horses received any drug treatment several hours before euthanasia, also the half-lives of the drugs used were relatively short. Possible actions on the gastrointestinal tract of the drugs used to treat any of the horses used in the *in vitro* experiments are discussed below.

1. Non steriodal anti-inflammatory drugs (NSAIDS)

Phenylbutazone (*Equipalozone*)

Phenylbutazone has analgesic, anti-inflammatory, antipyretic and mild uricosuric properties. The proposed mechanism of action is by inhibition of cyclo-oxgenase thereby decreasing prostaglandin synthesis (Rang and Dale, 1991). The serum half-life in horses is 3.5-6 hours and is dose dependant. Contraindications that are of relevance to the gastrointestinal tract, suggests that in foals and ponies there may be increased incidence of gastrointestinal ulceration (Plub, 1995). Buscopan is a NSAID related to phenylbutazone.

Flunixin

Flunixin has analgesic, anti-inflammatory and antipyretic properties. Flunixin does not appreciably alter gastrointestinal motility in the horse. Its serum half-life is about 1.6 hours in the horse. When used to treat colic flunixin may mask the behavioural and cardiopulmonary signs associated with endotoxemia or intestinal devitalization (Plub, 1995). In dogs gastrointestinal distress is the most likely adverse reaction to flunixin. It appears to be a relatively safe agent for use in the horse. Flunixin is also known as Finadyne.

2. Antibiotics

Penicillins

Penicillins act by inhibiting mucopeptide synthesis in the cell wall resulting in a defective barrier and an osmotically unstable spheroplast. They are extremely effective antibiotics and are widely used. A side effect of penicillins particulary the broad spectrum type given orally is the alteration of the bacterial flora in the gastrointestinal tract. This may be associated with gastrointestinal disturbances (Brander, Pugh, Bywater and Jenkins, 1991).

Gentamicin

Gentamicin belongs to the aminoglycoside antibiotic group. Its elimination half-life is 1.8-3.2 hours in the horse (Pugh, 1995).

3. Anaesthetic agents

Halothane

While the precise mechanism by which inhalent anaesthetics exert their general anaesthetic effect is not known some key pharmacological effects noted with halothane include; CNS depression, depression of body temperature regulating centres, increased cerebral blood flow, respiratory depression, hypertension, vasodilation and myocardial depression. The contraindications do not list any effects on the gastrointestinal tract (Plub, 1995; Data sheet, 1995/1996).

Ketamine

Ketamine is also a general anaestheic agent which has significant analgesic activity and a lack of cardiopulmonary effects. No effects on the gastrointestinal tract have been reported.

Xylazine

Xylazine is an alpha 2 adrenergic agonist, classified as a sedative/analgesic with muscle relaxant properties.

Butorphanol (*Torbugesic*)

Butorphanol is a synthetic opiate partial agonist, it is related structurally to morphine but exhibits pharmacologic actions similar to other partial agonists such as pentazocine and nalbuphine. It is considered to be a more potent an analgesic than morphine. It also pocesses significant antitussive activity. The duration of action is upto 4 hours in the horse. Adverse effects in horses may include transient ataxia and sedation. It also has the potential to decrease gastrointestinal motility especially at very high doses, although these effects are considered tranitory in nature (Barragry, 1994).

4. Cisapride

Cisapride is a prokinetic agent which increases gastrointestinal activity. Its mechanisms of action are discussed in Chapter 5.3.1 (Schuurkes *et al.*, 1985). Its elimination half-life is about 8-10 hours in the horse. Any grass sickness cases which had been treated with cisapride received their last dose of cisapride at least 12 hours before euthanasia.

5. Dexamethazone

Dexamethazone is a synthetic glucocorticoid. In the literature there are no reports of corticosteriods having an effect on gastrointestinal motility (Barragry, 1994).

6 Furosemide

Furosemide is used for its diuretic activity. It may induce fluid and electrolyte abnormalities. Other potential adverse effects include ortotoxicity, gastrointestinal abnormalities, haematological effects , weakness and restlessness.

Appendix K. Histological scoring system.

1) Number of neurones

Score	Description
1	very few neurones observed throughout entire section
2	some ganglia contained neurones which had normal appearance
3	at least one neurone in every ganglia
4	several ganglia containing neurones seen in both plexuses
5	numerous neurones throughout enteric plexuses

2) Size of normal neurones

The score awarded for size was very subjective, only the scores 1.5 to 4 were awarded. The animals size or age was not taken into account. The scoring system was however repeatable as described in Chapter 7.2.3

3) Proportion of abnormal neurones

Score	Description
0	no abnormal cells seen
1	only 1-3 abnormal cells observed throughout the whole section
2	25% neurones abnormal
3	50% nerones abnormal
4	75% or greater neurones abnormal, extensive chromatolysis
5	only the occasional neurone could be seen

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Room A
Afternoon Session

The Post Mortem Findings In 213 Equines With Special Reference To Lesions Of The Gastrointestinal Tract.

2.00 2A14 — 2.20 2A15

F Howie[*1], H Pirie[2], H Thompson[2], I McCandlish[2], P McNeil[2], & S Callanan.[2]
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Equine gastrointestinal disease is well recognised, however, few studies have been made into its significance as a cause of death or of subclinical lesions. To this end the lesions present at necropsy of all equines examined by the Glasgow University Veterinary School, Department of Veterinary Pathology over the period 1987-1991 were reviewed. This study revealed that the gastro-intestinal tract was by far the most frequently affected anatomical system in terms of both significant lesions, ie, those considered to have been the cause of death, and incidental lesions. The findings will be presented and those concerning the gastrointestinal tract discussed in detail.

Histopathological Changes In Brain Stem Nuclei Of Horses With 'Mal Seco' And With Grass Sickness.

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'Mal seco' is a disease of unknown aetiology affecting horses in Argentina. A histopathological study of the brain stem nuclei of 3 horses with 'mal seco' and of 4 horses with grass sickness was performed. Another 3 horses with a clinico-pathological diagnosis other than 'mal seco' or grass sickness were used as controls. At least one peripheral autonomic ganglion from each horse was also investigated. Degenerative histological changes were found in several brain stem nuclei and in the peripheral autonomic ganglia of all the 'mal seco' and grass sickness horses. No such changes were found in the brain stem nuclei and peripheral ganglia of the controls. The changes found in the brain stem nuclei and in the ganglia of both 'mal seco' and grass sickness cases were similar and consisted of chromatolysis, cytoplasmic vacuolation, interceilular and intracytoplasmatic eosinophilic bodies, pyknotic and eccentric nuclei and neuronophagia. These results provide further evidence to the hypothesis that 'mal seco' and grass sickness may be the same disease. This work was funded by the EEC (Grant CII-CT92-00061).

Damage To The Enteric Nervous System In Equine Dysautonomia (Grass Sickness).

2.40 2A16 — 3.00 2A17

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Histological examination of the myenteric and submucosal plexuses of the jejunum, ileum and small colon of normal horses and those suffering from dysautonomia was undertaken. Quantitative assessments were made of neurone numbers and the type of damage they had sustained. Both cell loss and damage occurred. It was particularly severe in the ileum and some animals had less than 5% of their normal neurone population left.

An *In Vitro* Study Of Small Intestinal Motility In Horses With And Without Grass Sickness.

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Equine grass sickness is a painful and usually fatal disease characterized by dysfunction of the autonomic nervous system, primarily of the alimentary tract. Degeneration of enteric neurones in the gastrointestinal tract have been demonstrated in grass sickness and to investigate differences in intestinal motility an *in vitro* technique was used. Responses to pharmacological agents are compared for normal and grass sickness affected tissue. To test the viability and capacity of enteric cholinergic neurones to produce acetycholine, an anticholinesterase agent and a muscarinic agonist were used. The phenomenon that noradrenaline when added to isolated small intestine of the horse causes contraction[(1)] was also investigated.
[(1)]Ruckebusch M., Grivel M.L. and Fargeas MJ. (1971). Sur l'Action Paradoxale de l'Adrenaline au Niveau de l'intestin Grêle chez le Cheval. Arch.int.Pharmacodyn. **194**, 387-402.
Acknowledgements Alison Murray is funded by the ADRA Equine Research & Grass Sickness Fund.

Taken from the Proceedings of the Association of Veterinary Teachers and Research Workers. Scarborough, U.K. 1994

Veterinary Research Communications, 18 (1994) 199–207

CHOLINERGIC ACTIVITY OF INTESTINAL MUSCLE *IN VITRO* TAKEN FROM HORSES WITH AND WITHOUT EQUINE GRASS SICKNESS

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ABSTRACT

Murray, A., Cottrell, D.F. and Woodman, M.P., 1994. Cholinergic activity of intestinal muscle *in vitro* taken from horses with and without equine grass sickness. *Veterinary Research Communications*, **18 (3)**, 199–207

Equine grass sickness (EGS) is a pan-dysautonomia of horses that involves central and peripheral neuronal degeneration and ultimately depletion. This is the first reported functional study on the motility of equine intestine taken immediately *post mortem* from horses with EGS. Strips of smooth muscle from the small intestine of healthy and EGS-affected horses were suspended in an organ bath and their motility was measured isometrically. The activity of the cholinergic system was studied. Physostigmine enhanced the motility of all muscle strips. Tissues taken from horses suffering from acute grass sickness (AGS) had the longest latency before a measurable response could be obtained ($p < 0.05$). The ileum appeared to be damaged by EGS to a greater extent than the duodenum. For the duodenal strips the enhanced rate of spontaneous contractions was significant ($p < 0.05$) for both normal tissue and that affected by grass sickness but this was not the case for the ileal strips. Muscarinic receptor sensitivity investigation using bethanecol suggested a hypersensitivity of receptors with AGS material,

Keywords: cholinergic, dysautonomia, grass sickness, horse, intestinal motility, *in vitro*

Abbreviations: AGS, acute grass sickness; CGS, chronic grass sickness; ED_{50}, median effective dose; EGS, equine grass sickness; VIP, vasoactive intestinal peptide

INTRODUCTION

Equine grass sickness (EGS) is a serious clinical disease in which horses suffer widespread dysautonomia. Although the cause of EGS is unknown, it is characterized by dysfunction of the autonomic nervous system, primarily affecting the alimentary tract (Greig, 1928). Histological examination of autonomic ganglia shows evidence of neuronal degeneration and ultimately depletion (Obel, 1955; Gilmour, 1973), and it is thought that a neurotoxin may be involved in the aetiology (Pollin and Griffiths, 1992). Neuronal damage is also observed in the enteric nervous system (Sabate *et al.*, 1983) with the greatest damage to neurons in the ileum (Scholes *et al.*, 1993). Motility of equine small intestine from clinically normal tissues has previously been studied by Ruckebusch and colleagues (1971). We have extended this technique to study tissue taken from horses in various clinical categories of EGS. In particular we tested the viability of the enteric cholinergic neurons and their capacity to release endogenous acetylcholine, as well as the sensitivity of the cholinergic receptors.

MATERIALS AND METHODS

Horses and ponies (100–450 kg) of each sex were killed by overdosage with barbiturate and then bled out. Samples of smooth muscle were taken from 20 normal and 12 grass sickness-affected horses. Horses were classified as 'normal' when euthanasia was due to reasons unrelated to EGS (orthopaedic (4), ragwort poisoning (1), laminitis (3), cardiovascular conditions (2), respiratory disease (2), immunoincompetence (1), experimental pony (3), urinary tract infection (1), trauma (1), liver disease (2)); as 'acute' when they had nasogastric reflux, intestinal stasis and were euthanized within 48 h of the initial diagnosis of equine grass sickness (AGS); and as 'chronic' when they had less severe clinical signs and had survived for 7 days or more (CGS). Diagnosis of EGS was confirmed by *post-mortem* histological identification of characteristic lesions in the caeliacomesenteric ganglia.

Samples of intestine were removed within 15 min of death and placed in modified Krebs solution (sodium chloride 119.8 mmol/L, potassium chloride 3.4 mmol/L, calcium chloride 3.0 mmol/L, magnesium chloride 0.6 mmol/L, sodium hydrogencarbonate 26.2 mmol/L, sodium hydrogenphosphate 1.3 mmol/L, glucose 6.7 mmol/L). The Krebs solution was maintained at 37°C and aerated continuously with a mixture of 95% oxygen and 5% carbon dioxide. The two sampling sites were the caudal flexure of the duodenum and the terminal ileum proximal to the ileocaecal fold. Strips of mucosa-free smooth muscle (3×0.5 cm) were cut parallel to the longitudinal muscle layer and suspended vertically in an organ bath (35 ml) that contained modified Krebs. Duodenal strips were loaded with a force of 2 g. The force used for the ileal strips was more variable as these were more muscular and had a tendency to coil tightly; for large horses the force needed was as high as 35 g. Tissue taken from horses affected with EGS required a lower tension, the maximum being 10 g.

Motility was measured isometrically (Ruckebusch *et al.*, 1971) using a Washington Type D strain gauge transducer and recorder (Washington 400 MD2 ink writing oscillograph). The parameters measured were rate of contraction (contraction peaks over a 10-min period, amplitude (isometric force in grams of an individual contraction that returned to the baseline), and tone (change in baseline). Latency was the time before these parameters altered following the application of a drug. A 1-h equilibration period was allowed. The drugs used were physostigmine sulphate (BDH Chemicals) and bethanecol chloride (Sigma); the concentrations given in the text are final concentrations. The data were analysed for statistical significance using the Mann–Whitney *U*-test and analysis of variance.

Samples of gut adjacent to the test strips were also fixed for histology, but the results from this work will be presented in a further paper comparing the histological and physiological results.

RESULTS

When samples of small intestine were collected *post-mortem* the following observations were made. 'Normal' intestine contained digesta, and spontaneous contractile activity with segmentation was apparent. AGS-affected small intestine was distended with fluid and there was very little visible movement of the gut.

CGS-affected gut was flaccid with very little digesta present, although there often appeared to be an increase in intestinal secretion.

Intestinal muscle strips were set up in organ baths and spontaneous contractions developed within the 1-h equilibration period. The pattern of motility in the duodenal strips was continuous spontaneous contractions (Table I), whereas the spontaneous contractions of the ileal strips occurred in bursts alternating with periods of quiescence (Figure 1c). The pattern of spontaneous activity was the same for normal and EGS-affected tissue.

TABLE I
Spontaneous activity (mean ± SEM) of intestinal smooth muscle strips *in vitro*

Muscle	Category of horse	Mean rate of spontaneous contractions (per min)	Mean amplitude of spontaneous contractions (g)
Duodenum	Normal	6.79 ± 0.53	7.35 ± 2.95
	AGS	3.12 ± 0.68	12.25 ± 4.95
	CGS	4.68 ± 1.02	14.35 ± 4.40
Ileum	Normal	3.66 ± 0.53	12.73 ± 3.57
	AGS	3.96 ± 0.76	15.48 ± 1.72
	CGS	2.90 ± 1.08	8.95 ± 2.45

Physostigmine

There was little response by normal tissue to concentrations of physostigmine below 8.6×10^{-8} mol/L. Above 2.8×10^{-5} mol/L, the tissues went into atony, with sustained contraction and a prolonged recovery time. In the intermediate range, tissue responded with measurable changes in both rate and tone (Figure 1a). It was therefore decided to use a concentration of 8.6×10^{-6} mol/L to compare the responses of the various tissues.

On addition of physostigmine, the rate of spontaneous contractions was enhanced (Table II). Physostigmine caused a significantly greater proportional increase in the rate of contraction of tissues from horses with AGS compared to preparations from normal horses ($p < 0.05$).

The rates of contractions after the addition of physostigmine to AGS or CGS preparations of the ileum were not significantly different from their initial rates, whereas with preparations from the duodenum the rate of contractions increased significantly in all three groups ($p < 0.05$) after physostigmine was added.

For the duodenal strips, the latency before a response increased with the severity of EGS. With the ileal strips, the shortest latency was given by CGS and the longest by

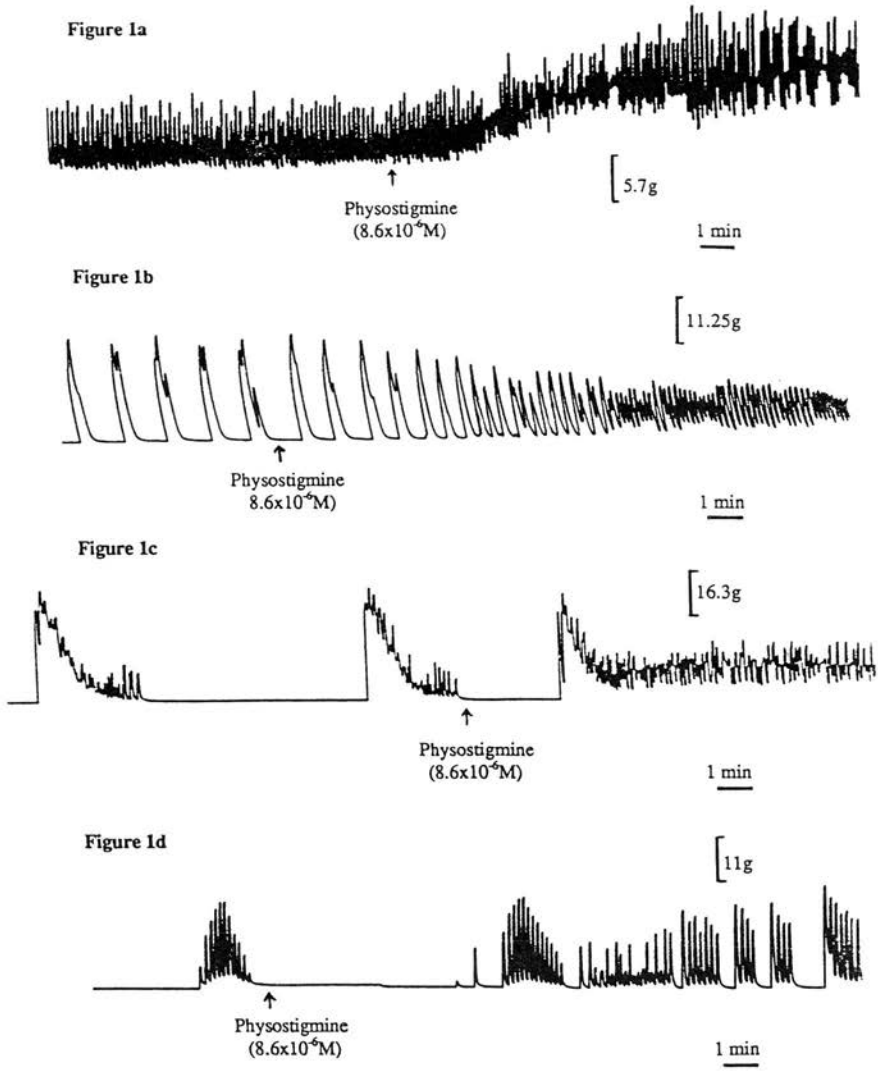

Figure 1. (a) Isometric trace from a duodenal muscle strip taken from a normal horse, showing regular spontaneous activity. On addition of physostigmine (8.6×10^{-6} mol/L), there is a short latency followed by an increase in both rate of contractions and tone (baseline). (b) Isometric trace from a duodenal muscle strip taken from a horse suffering from AGS; the initial rate of spontaneous contractions is much lower than that for normal horses. On addition of physostigmine (8.6×10^{-6} mol/L), the latency before a response is observed is much longer and, although there is a rate increase, there is little increase in tone. (c) Isometric trace from an ileal muscle strip taken from a normal horse, showing two bursts of spontaneous contractions. After the addition of physostigmine (8.6×10^{-6} mol/L), the interburst interval is shortened and the spontaneous activity becomes more regular. There is also a small increase in tone. (d) Isometric trace from an ileal muscle strip taken from a horse suffering from AGS. A similar response as above (c) is observed but there is no increase in tone on the addition of physostigmine (8.6×10^{-6} mol/L)

TABLE II
The effects of physostigmine (8.6×10^{-6} mol/L) on isolated strips of horse small intestine. All values are means ± SD SEM

Category of horse*	*n*	Initial contraction rate (per min)	Contraction rate (per min) after physostigmine	Mean percentage increase in rate (per min)	Mean response time (s)	Mean increase in tone (g)
Duodenum						
Normal	20	$6.79 \pm 0.53^{a,d}$	$9.87 \pm 0.41^{a,e}$	55.27 ± 9.83^{f}	80.95 ± 4.76^{g}	$6.05 \pm 0.67^{i,l}$
AGS	6	$3.12 \pm 0.68^{b,d}$	$7.80 \pm 0.93^{b,e}$	221.10 ± 68.80^{f}	$245.0 \pm 45.6^{g,h}$	$1.77 \pm 0.40^{i,k}$
CGS	5	4.68 ± 1.02^{c}	8.20 ± 0.77^{c}	140.0 ± 87.0	98.10 ± 12.0^{h}	$12.43 \pm 2.62^{k,l}$
Ileum						
Normal	19	3.66 ± 0.53^{m}	$7.63 \pm 0.73^{m,n}$	147.9 ± 24.6^{o}	117.2 ± 12.0^{p}	5.99 ± 1.97^{r}
AGS	6	3.96 ± 0.76	5.78 ± 0.73	54.4 ± 19.8^{o}	$203.0 \pm 39.7^{p,q}$	$0.00 \pm 0.00^{r,s}$
CGS	5	2.90 ± 1.08	3.84 ± 0.98^{n}	73.0 ± 34.0	80.6 ± 13.8^{q}	12.43 ± 5.92^{s}

* 'Normal' refers to horses that were euthanized owing to diseases unrelated to grass sickness. 'AGS' and 'CGS' refer to horses that were suffering from acute and chronic grass sickness, respectively. For definitions, see Materials and Methods
Values with the same superscript are significantly different from each other when analysed by the Mann–Whitney *U*-test ($p < 0.05$)

AGS. An increase in tone in the intestinal strips was usually observed in response to physostigmine, with the exception of ileal AGS preparations. In both duodenal and ileal strips, the largest increase in tone was observed with tissue from horses suffering from CGS ($p<0.05$).

Bethanecol

Bethanecol caused dose-dependent contractions with all smooth-muscle strips (Figure 2). However, the sensitivity of the tissues varied. Figure 3 shows the dose–response curves with bethanecol. To calculate the ED_{50} values, the individual data for each experiment were normalized as a percentage of its maximal response. The maximum response of the tissues as contraction amplitude in grams per gram of tissue (mean ± SEM) were normal 38.6 ± 5.1, AGS 45.2 ± 9.1 and CGS 29.9 ± 4.8. AGS tissue was also more sensitive to lower concentrations of bethanecol than normal or CGS tissue. At 1×10^{-5} mol/L bethanecol (equivalent to −5 on the log scale, Figure 3), the effects for the three different tissues were significantly different ($p<0.05$). At other concentrations the differences between the tissues were not significant, possibly because of the small sample sizes. The ranking of the response at each concentration of bethanecol showed the same trend: AGS, followed by normal, then CGS. AGS material contracted to 50% of its maximum response at a much lower concentration of bethanecol (8.7×10^{-7} mol/L) than normal (1.5×10^{-5} mol/L) or CGS (5.6×10^{-5} mol/L) material. The duration of the response to bethanecol was more prolonged with material from AGS cases.

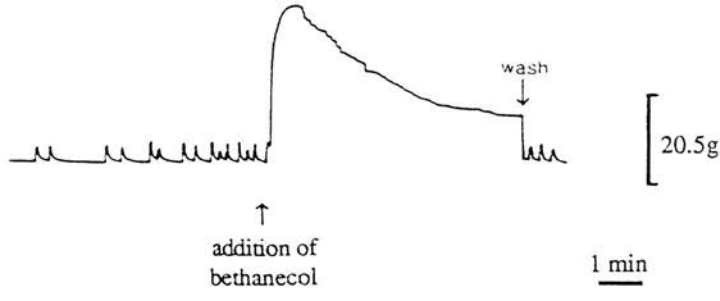

Figure 2. The response of a duodenal muscle strip taken from a normal horse to 0.1 mmol/L bethanecol

DISCUSSION

This *in vitro* preparation of equine small intestine demonstrated the presence of active cholinergic mechanisms that were impaired in EGS. There were differences in the sensitivity between tissues taken from normal horses and those with acute or chronic EGS with both an anticholinesterase drug (physostigmine) and a muscarinic agonist (bethanecol). It therefore appears that these results provide functional correlates with the enteric neuronal cell degeneration known to occur in EGS (Sabate *et al.*, 1983).

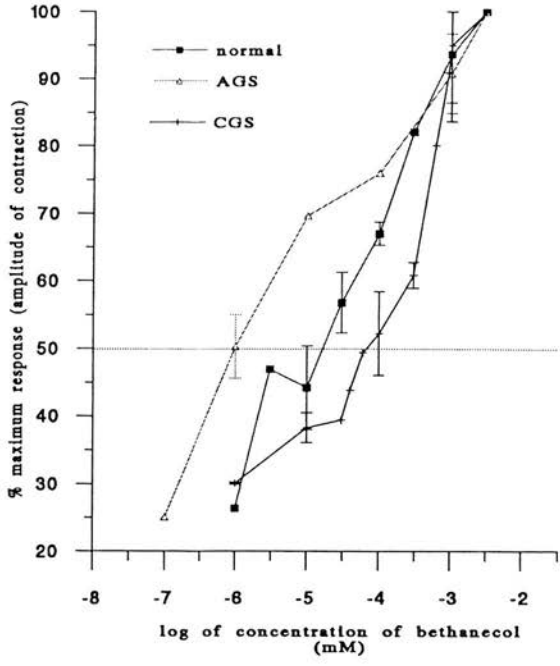

Figure 3. Dose–response curves with bethanecol for strips of equine duodenum. 'Normal' refers to horses euthanized owing to diseases unrelated to equine grass sickness (n = 5). 'AGS' and 'CGS' refer to horses suffering from acute and chronic equine grass sickness (n = 3 and n = 7, respectively)

Physostigmine

Physostigmine enhanced the motility of all the tissues studied, demonstrating the viability of active cholinergic enteric neurons. However, in no case did the enhanced rate of contractions in tissue from EGS cases approach the maximum found with normal tissue, suggesting a reduced release of endogenous acetylcholine by tissues taken from acute or chronic cases of EGS.

The prolonged latency with AGS tissue provides further evidence of a functional deficit resulting from the nerve damage seen histologically in AGS. Fewer surviving cholinergic neurons would be expected to take longer to release enough acetylcholine to affect motility.

It appeared that the two regions of the gut were affected by EGS to different degrees, with the ileum experiencing the greatest damage. Histological results have also shown the greatest neuronal damage in the ileum (Scholes *et al.*, 1993). Functional identification of the damaged neurons has yet to be made.

The increase in tone produced by physostigmine was significantly greater in tissue taken from CGS cases than in that from normal or AGS cases. This was a relative change because the CGS duodenum was observed to be flaccid at collection and absolute values for the tone are not known.

The enhanced rate of contraction and increased tone of equine intestinal tissue

produced *in vitro* by physostigmine establishes a pharmacological basis for the observed therapeutic usefulness of prokinetic drugs that may activate or supplement the enteric cholinergic system in certain cases of grass sickness (Milne *et al.*, 1994).

Bethanecol

Measurement of the peak tone produced by equimolar concentrations of bethanecol demonstrated differences in the sensitivity of tissue taken from normal animals and those with EGS. As shown in Figure 3, to reach 50% of its maximum response, normal tissue requires 17 times as much bethanecol as tissue from an animal with AGS. A number of mechanisms may be involved in this altered sensitivity to a muscarinic agonist. There might be denervation hypersensitivity analogous to that found in skeletal muscle (Janig, 1989) in which, following denervation, extrajunctional muscarinic receptors of the fetal type are expressed on the muscle membrane, thus increasing the sensitivity of the muscle (Ganong, 1991). Secondly, it is known that pharmacologically active substances are released into gut tissue in EGS; these may sensitize the muscle, and make it more excitable. Hyperactivity and subsequent inactivation of neurons containing both VIP and substance P during EGS has been proposed (Bishop *et al.*, 1984; Hodson and Wright, 1987) and these mechanisms may play a role in the changes in the excitability of muscle to cholinergic agents, as reported here.

These results indicate that the loss of enteric neurons in EGS (Scholes *et al.*, 1993) leads to a reduction in the release of acetylcholine by cholinergic neurons that accordingly reduces intestinal motility, the greatest detrimental effect occurring in AGS and the ileum being affected to a greater extent than the duodenum. In addition, sensitivity of the muscarinic receptors is altered, being enhanced in AGS but reduced in CGS.

ACKNOWLEDGEMENTS

Alison Murray is funded by the A.D.R.A. and Grass Sickness Fund. The authors wish to thank the staff of the Department of Veterinary Clinical Studies for supplying clinical material and case notes.

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(Accepted: 5 May 1994)