THE ENTERIC NERVOUS SYSTEM AND INTERSTITIAL CELLS OF CAJAL OF THE HORSE

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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 are included in an appendix to the thesis.

NEIL P.H. HUDSON

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ABSTRACT

Gastrointestinal diseases, including obstructive and motility disorders such as grass sickness, are very common in the horse. Motility disorders may represent a dysfunction of the neural, muscular or pacemaker components (interstitial cells of Cajal, ICC) of bowel control. ICC are the c-Kit-immunoreactive cells responsible for the generation of pacemaker activity in gastrointestinal smooth muscle. Impairment of this ICC-mediated pacemaker action has been implicated in several motility disorders in humans and laboratory mammals. Equine dysautonomia (grass sickness) is a common, frequently fatal disease of horses characterised by dysfunction of the gastrointestinal tract. Neurons of the enteric nervous system (ENS) are primarily and most severely targeted by the putative neurotoxin causing the disease.
The aims of this study were:

- to characterise the morphology and neurochemical expression of the equine ENS using tissue culture and immunohistochemistry.

- to study the distribution of the ICC in the intestine in normal and grass sickness-affected horses.

- to perform a detailed *in vitro* investigation of the electrical properties and control of smooth muscle in both the healthy and diseased intestine.

- to test the hypothesis that impaired ICC-mediated control is responsible for intestinal dysfunction.

Fresh ileal samples were harvested from adult horses free from gastrointestinal disease euthanased on humane grounds. Small mammal (guinea pig and rat) systems were used for comparison and development of techniques. The tissues were microdissected to prepare wholemounts for immunohistochemistry and for either explant or dissociated culture systems of the ENS. Immunoreactivity was assessed using a standard indirect immunofluorescence technique. In the horse, explant culture systems were established using wholemounts of either the submucous plexus or the

muscularis externa (including the myenteric plexus). Dissociated cell cultures could only be obtained from the submucous plexus. This was in contrast to small mammal models where enteric neurons were grown in both the myenteric and submucous systems. Culture systems were maintained for up to 5 days in the horse and 8 days in the guinea pig. Immunoreactivity for a neuronal marker (*Pan*-N) and for glial cell markers (GFAP and S100) indicated the presence of both neurons and enteric glia in the tissue culture preparations. Further refinements to the techniques will be required before this *in vitro* model can be used for quantitative analysis.

To examine the ICC in the horse, samples were taken at multiple anatomically-defined sites from stomach to small colon. After tissue fixation in 10% phosphate-buffered formalin, 10 μm cryostat sections were processed using standard immunohistochemical techniques and the avidin-biotin-peroxidase method. The primary antiserum used was an affinity-purified polyclonal antibody raised against the c-Kit protein. Specific immunoreactivity for c-Kit was detected in all sites and three types of immunoreactive cell were identified. These were spindle-shaped cells in the region of the myenteric plexus with occasional cellular processes extending into the longitudinal muscle, stellate- or bipolar-shaped cells in the circular muscle layer and round cells in the submucosa. The round cells were shown to be mast cells with the use of toluidine blue staining whereas the other c-Kit-immunoreactive cells did not exhibit metachromasia and were therefore classified as ICC. In the duodenum, jejunum and ileum, ICC were found predominantly in the region of the myenteric plexus and to a lesser extent in the circular muscle. In contrast, in the large intestine (large colon, caecum, small colon) most cells were seen throughout the circular muscle and very few ICC were seen in the myenteric plexus area. A semi-quantitative grading system was used to examine any differences in ICC in grass sickness-affected ileum and pelvic flexure sections compared to normal animals. In horses with grass sickness, ICC were significantly decreased in both the myenteric plexus and circular muscle regions of both ileum and pelvic flexure compared to normal animals. It is possible therefore that the decline in ICC may be in some way responsible for the development of intestinal dysmotility in grass sickness.

Normal and diseased equine tissue from clinical cases and abattoir samples from pigs were collected and examined using *in vitro* microelectrode electrophysiological recordings from smooth muscle cells. Slow wave activity and spike potentials were recorded in normal equine and porcine ileal preparations. The slow wave activity was preserved in the presence of tetrodotoxin. A waxing and waning pattern of the slow wave activity was noted. Nifedipine abolished the spiking contractile activity of the smooth muscle but did not abolish the slow waves. The majority of ileal preparations from grass sickness-affected horses exhibited prominent slow wave activity with reduced slow wave frequency and increased duration suggesting that, although the neural elements are destroyed, the ICC-mediated pacemaker function remains intact.

This work will increase understanding of gastrointestinal dysmotility and information on the role of ICC will also offer benefits in potential developments in pharmacological therapy.

ABBREVIATIONS

AGS	Acute grass sickness
bFGF	Basic fibroblast growth factor
CGRP	Calcitonin gene-related peptide
CGS	Chronic grass sickness
c-Kit-I	C-Kit-immunoreactivity
CM	Circular muscle
CNS	Central nervous system
DAB	Diaminobenzidine
EDTA	Ethylenediaminetetraacetate
ENS	Enteric nervous system
ESP	External submucous plexus
FITC	Fluorescein 5-isothiocyanate
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
ICC	Interstitial cells of Cajal
ISP	Internal submucous plexus
LDH	Lactate dehydrogenase
LM	Longitudinal muscle
MAP	Microtubule-associated protein
MP	Myenteric plexus
NFM	Neurofilament M
NID	Neuronal intestinal dysplasia
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
Pan-N	*Pan*-neurofilaments
PBS	Phosphate-buffered saline
PGP 9.5	Protein gene product 9.5
PI3-kinase	Phosphatidylinositol 3′-kinase
POI	Postoperative ileus
R^2	$(\text{Correlation coefficient})^2$
RMP	Resting membrane potential
RPM	Revolutions per minute
RT-PCR	Reverse-transcription polymerase chain reaction
SCF	Stem cell factor
S.E.M	Standard error of the mean
SGS	Subacute grass sickness
SMP	Submucous plexus
TTX	Tetrodotoxin
VIP	Vasoactive intestinal polypeptide

CHAPTER 1: INTRODUCTION

1.1 The enteric nervous system: structure and function

The enteric nervous system (ENS), or neuronal system of the intestinal wall, has a central role in the control of most gastrointestinal functions (Furness and Costa, 1987). The ENS is considered to be the third component of the autonomic nervous system; the others being the sympathetic and parasympathetic nervous systems (Costa *et al.*, 1987). It is composed of intrinsic neurons (enteric neurons) and the processes of both afferent (sensory neurons) and efferent (sympathetic and parasympathetic) extrinsic neurons. The ENS consists of a complicated three-dimensional network of neurons organised into the submucous plexus (SMP) and the myenteric plexus (MP) (Figure 1). The SMP lies in the region between the mucosa and the two main muscle layers of the intestinal wall (*muscularis externa*). The MP lies between the circular and longitudinal intestinal muscle layers of the *muscularis externa*. The MP is concerned primarily with motility while the SMP is concerned with transepithelial ion transport, mucosal blood flow and secretomotor functions (Furness and Bornstein, 1995).

The ENS can continue to control many of the intestinal motor and secretory activities even if the extrinsic innervation to the gut is removed (Berne and Levy, 1993). As many as 25 neurotransmitter or neuromodulatory substances have been proposed to be active in the ENS (Wood, 1987) and the list is increasing. The MP contains motor neurons, sensory neurons and interneurons. In the majority of cases, enteric neurons contain more than one neurotransmitter or neuromodulator. As a broad generalisation, the main excitatory neurotransmitters are acetylcholine and substance P and the main inhibitory neurotransmitters are vasoactive intestinal polypeptide (VIP) and nitric oxide (NO), but in reality the functions of the various neurochemicals are more complex. In addition to the functions of the SMP listed above, some SMP neurons are interneurons projecting to other SMP neurons or to MP neurons (Berne and Levy, 1993).

Much of the understanding of the ENS comes from work on the guinea pig and rat but the equine ENS has been shown to consist of a MP and a two-layered SMP (internal and external SMPs) (Pearson, 1994) [Figure 2]. Further examination of the equine ENS involving neuronal characterisation and distribution has been described by Doxey *et al.* (1995b). Enteric glial cells, or the supporting cells of the ENS, have also been the subject of much research. Enteric glia are different cells to the non-myelinating Schwann cells of the peripheral nervous system but both are derived embryologically from the neural crest and only diverge relatively late in ontogeny (Gershon and Rothman, 1991). The interstitial cells of Cajal are the pacemakers and mediators of neurotransmission in the gastrointestinal tract (Sanders, 1996). As such, they are intimately associated with the ENS but they will be discussed in greater detail later in this thesis.

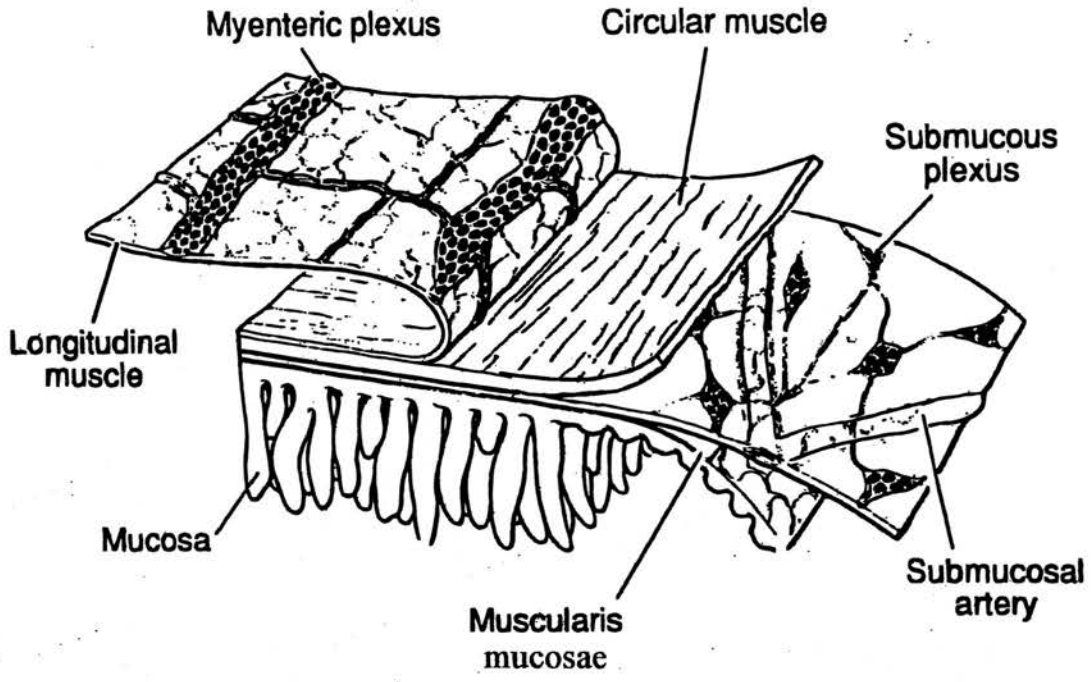

Figure 1. Schematic illustration of the architecture of the small intestine. (Adapted from Furness & Costa (1980) *Neuroscience* **5**, 1-20).

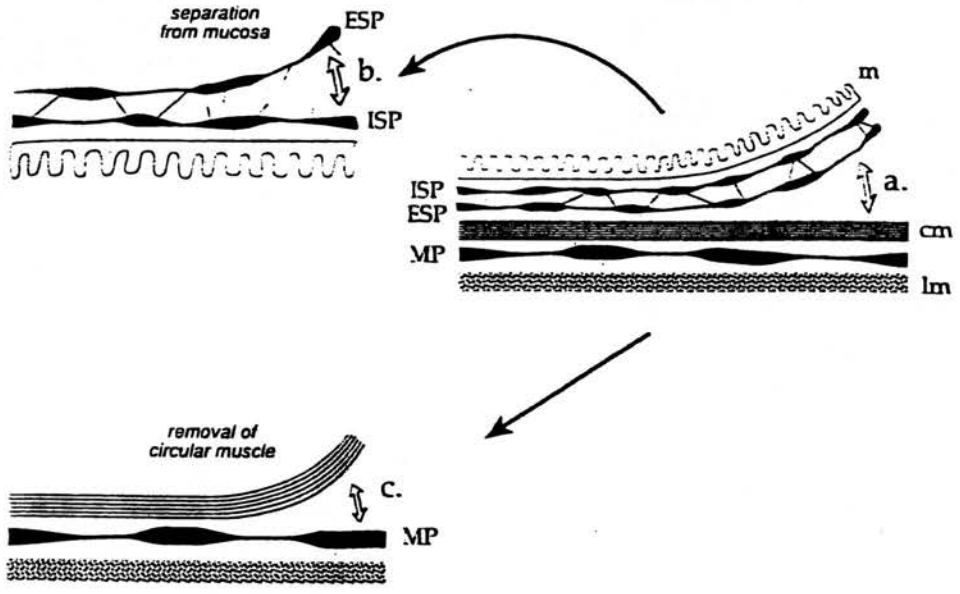

Figure 2a-c. Schematic representation of the microdissection of the equine small intestine. **a.** Separation of the mucosa and submucosa from the *muscularis externa*. **b.** Separation of the external submucous plexus (ESP) from the internal submucous plexus (ISP) and mucosa. **c.** Removal of the circular smooth muscle layer (cm) from the underlying myenteric plexus (MP) and longitudinal smooth muscle layer (lm). (Adapted from Pearson (1994) *Cell & Tissue Research* **276**, 523-534).

1.2 Dysfunction of the enteric nervous system and gastrointestinal tract

Gastrointestinal diseases, including motility and obstructive disorders, are common in both veterinary and human medicine. Disorders of the ENS can cause profound gastrointestinal dysfunction in both animals (Guilford, 1990) and humans (Heaton *et al.,* 1988; Goyal and Hirano, 1996; De Giorgio *et al.*, 2000b).

1.2.1 Equine dysautonomia (grass sickness) and dysautonomias in other species

Grass sickness (equine dysautonomia) is a common and often fatal disease of equids of unknown aetiology characterised by dysfunction of the alimentary tract (Milne, 1997a; Cottrell *et al.*, 1999) (Figures 3 and 4). It is a predominantly northern European disease especially seen in the north-east of the United Kingdom and was first recorded around 1909 in Scotland (Pinsent, 1989). There is an equine disease called "mal seco" in South America that has similar clinical and pathological characteristics (Uzal *et al.*, 1992). Grass sickness can be classified according to the duration and severity of the clinical signs. Acute grass sickness (AGS) usually results in death or euthanasia in less than 2 days, subacute grass sickness (SGS) in 2-7 days, and in chronic grass sickness (CGS) the duration of the disease is over 7 days (Doxey *et al.*, 1991). The prognosis for AGS and SGS is hopeless (Gilmour, 1988). Despite some authors' views that the prognosis for CGS is also hopeless (Gilmour, 1988; Pinsent, 1989), recent reports suggest that, in certain cases, recovery is possible with intensive nursing (Milne and Wallis, 1994; Doxey *et al.*, 1995a; Milne, 1997b; Doxey *et al.*, 1998; Doxey *et al.*, 1999). If there is the presence of a degree of intestinal motility, the ability to swallow and the absence of signs of severe colic (abdominal pain), a decision can be made to treat cases of CGS with an intensive nursing schedule (Milne and Wallis, 1994).

Grass sickness affects the autonomic nervous system and the neuronal pathology has been well documented in the autonomic ganglia (Figure 5) and the myenteric and submucous plexuses of the gut.

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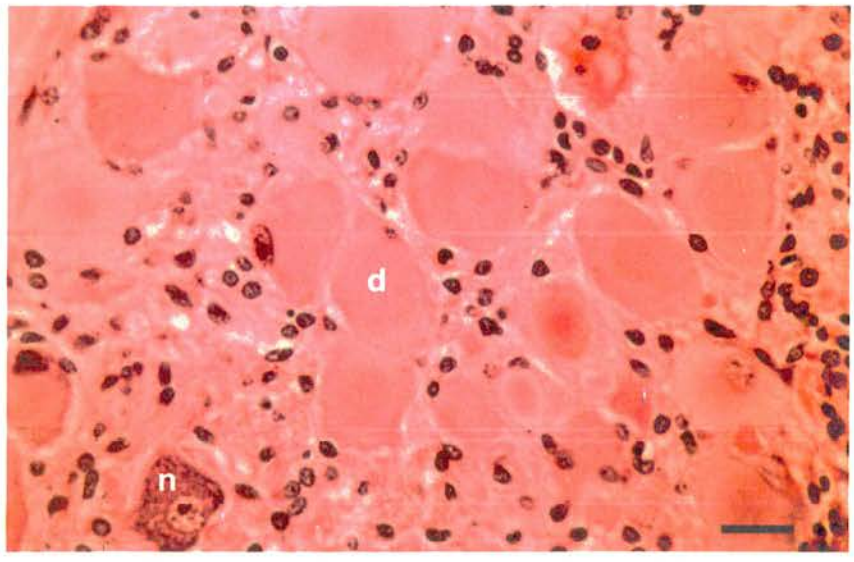


Figure 3. A horse with chronic grass sickness.
Figure 4. Colonic impaction of feed material from a case of subacute grass sickness.
Figure 5. Histological section of a stellate ganglion from a case of acute grass sickness
Note the degenerative neurons (d) and occasional normal neuron (n).
Bar = 30µm. Stain H & E

There is extensive chromatolysis with loss of Nissl substance, eccentricity or pyknosis of the nuclei, swelling and vacuolation (Obel, 1955). The pathology of the ganglia has been well described (Brownlee, 1959; Gilmour, 1975) and the disease has been shown to have more widespread effects in the central nervous system (Barlow, 1969). Neurons of the ENS are primarily and most severely targeted by the putative neurotoxin causing the disease. Doxey *et al.* (1992) reported a decrease in the number of neurons in both the MP and SMP of the intestine of grass sickness cases. The neuropeptide-containing neurons of the ENS are directly affected in the disease. The numbers of peptide-containing nerves with positive immunoreactivity for vasoactive intestinal polypeptide (VIP), substance P, enkephalin, bombesin, somatostatin and enteroglucagon are decreased dramatically (Hodson *et al.,* 1982; Sabate *et al.,* 1983; Bishop *et al.,* 1984). There has been only one report that describes an increase in the numbers of the supporting cells in the enteric ganglia (Scholes *et al.,* 1993a).

The aetiology of grass sickness is still unknown but currently two hypotheses are being investigated. Tocher *et al.* (1923) provided evidence suggesting that grass sickness may be caused by the bacterium *Clostridium botulinum*. Recently, Hunter *et al.* (1999) have re-investigated this hypothesis and have shown an association between *Clostridium botulinum* type C and grass sickness. Another avenue of research is examining the hypothesis that grass sickness is a consequence of oxidative stress and its potential neurotoxic effects (McGorum *et al.*, 1998).

The diagnosis of grass sickness *ante mortem* is based on clinical evaluation. Oesophageal contrast studies (Greet and Whitwell, 1986) and laboratory tests such as peritoneal fluid analysis (Milne *et al.*, 1990) are useful as adjuncts without providing definitive proof. Diagnosis can only be confirmed by demonstration of characteristic histopathology in the ENS or autonomic ganglia examined at *post mortem*. Histopathology of ileal biopsies taken at laparotomy are diagnostic (Scholes *et al.,* 1993b). This may be useful in cases which are showing similarities to surgical colic lesions but should be undertaken cautiously as laparotomy adversely affects the outcome in chronic cases where treatment is being considered (Milne *et al.*, 1994; Murphy and Love, 1996). The prognosis for survival of treated CGS cases has

improved dramatically in recent years to between 40-50% for all cases and up to 70% of specially selected cases, but it depends on careful selection of viable cases (Milne, 1997b; Milne and Wallis, 1994). The degrees of dysphagia, inappetance, colic and reduction in gut sounds are significantly less marked in survivors than non-survivors. The presence of severe rhinitis sicca is also a sign of poor prognosis (Milne *et al.*, 1994).

Dysautonomias do occur in other species such as the cat, dog and hare (Pollin and Griffiths, 1992; Griffiths and Whitwell, 1993; Longshore *et al.*, 1996; Schulze *et al.*, 1997), rabbit (rabbit mucoid enteropathy; Whitwell and Needham, 1996), and in man (Heaton *et al.*, 1988). In feline dysautonomia, or Key-Gaskell Syndrome, the number of enteric neurons containing VIP, substance P and met-enkephalin is reduced (Vaillant and Sharp, 1984).

1.2.2 Disorders of the equine gastrointestinal tract

Disorders of the gastrointestinal tract are amongst the most common problems in equine veterinary medicine. An understanding of the relationship between gastrointestinal motility and disease is crucial for the proper treatment of large animal patients (Navarre and Roussel, 1996). There are various gastrointestinal diseases in the horse that may have some relevance to any research into grass sickness especially with regard to motility problems. Burns *et al.* (1990) reported a case of equine myenteric ganglionitis that was classified as an equine form of chronic idiopathic intestinal pseudo-obstruction. Gastroenterologists describe pseudo-obstruction as a syndrome in which ineffective intestinal propulsion causes signs of obstructive bowel disease without evidence of a mechanical obstruction (Coulie and Camilleri, 1999). Ileocolonic aganglionosis (lethal white foal syndrome), involving the absence of MP ganglia in the white progeny of overo spotted horses, has been well described (Hultgren, 1982). This disease is an equine version of the human Hirschsprung's disease and is associated with a mutation of the *endothelin-B receptor* gene (Metallinos *et al.*, 1998; Yang *et al.*, 1998). Megacolon with myenteric hypoganglionosis has also been reported in a Clydesdale foal which, unlike the lethal

white foal syndrome described above, was not a pigment-related disorder (Murray *et al.*, 1988).

Horses with acute and chronic obstructive disorders of the gastrointestinal tract have been shown to have significantly reduced MP and neuron densities in the large colon and increased numbers of enteric glial cells when compared to normal horses (Schusser and White, 1997). This was part of a follow up on work done on MP and neuron densities in normal foals in which numerous enteric glial cells with foamy nuclei were described as surrounding each neuron (Schusser and White, 1994).

All horses undergoing a laparotomy for an acute abdominal crisis are at risk of developing postoperative ileus (POI), the pathophysiology of which remains obscure (Dart and Hodgson, 1998). Equine POI is often fatal and in a retrospective study of 259 postoperative abdominal crisis patients, POI accounted for 42.9% of postoperative fatalities (Hunt *et al.*, 1986). This means that the horse potentially could be an important model for human POI, not least because of the access to tissues from frequent fatalities in the clinical setting.

1.2.3 Disorders of the gastrointestinal tract of other animal species

Similar to the lethal white foal syndrome and Hirschsprung's disease, there is a form of intestinal aganglionosis seen in the lethal spotted mutant mouse (ls/ls). Indeed, this may be an appropiate model for the human disease (Chakder *et al.*, 1997). In the aganglionic segments of the intestine of these mice, there is a striking overgrowth of the *muscularis mucosae* (Tennyson *et al.,* 1986). The authors describe the supporting cells of the myelinated and unmyelinated nerves in the aganglionic segment as having voluminous perineural cytoplasm typical of immature Schwann cells. These cells also exhibit cytoplasmic intermediate filaments but otherwise have the typical morphology of peripheral Schwann cells, rather than enteric glia. The authors hypothesise that the extracellular matrix and/or cells of mesenchymal origin of the terminal bowel of the ls/ls mouse may prevent the in-growth of normal glial precursors as well as neurons of the enteric nervous system, but may allow or even encourage the in-growth of abnormal numbers of extrinsic axons.

A very interesting finding was noted by Bush *et al.* (1998) that mice in which enteric glia have been genetically-ablated, develop a fulminant jejuno-ileitis. The authors showed therefore that the enteric glia perform an essential role in maintaining the integrity of the intestine and suggest that the loss or dysfunction of these cells may contribute to the cellular mechanisms of inflammatory bowel disease.

In rats with experimentally-induced diabetes (to investigate diabetic autonomic neuropathy), the enteric neurons showed an increase in VIP-immunoreactivity but not substance P (Belai *et al.*, 1985). In another study, rats with experimentally-destroyed MPs have altered mucosal morphology and increased thickness of the smooth muscle layers (Hadzijahic *et al.*, 1993).

Feline constipation is a common problem in small animal veterinary practice (Yam, 1997). Acquired idiopathic megacolon is an important syndrome in middle aged to older cats (Washabau and Holt, 1999). The pathogenesis of this syndrome is unclear but *in vitro* isometric mechanical work has suggested the presence of an impairment of colonic smooth muscle function (Washabau and Stalis, 1996). The megacolon syndrome in the cat bears many similarities to the one seen in humans (Guilford 1996; Phillips and Pemberton, 1998).

In dogs, dysmotility syndromes are rarer but have been reported. Lamb and France (1994) described a case of chronic intestinal pseudo-obstruction in a dog that showed histopathological evidence of smooth muscle atrophy and fibrosis and MP neuronal vacuolation. Functional intestinal hypomotility has been reported in a dog in association with MP neuronal degeneration and necrosis (Aroch *et al.*, 1997).

Intestinal pseudo-obstruction has also been reported in a cow that presented with *postpartum* atony of the small and large intestine. Histopathological examination revealed ganglionitis of the cranial mesenteric ganglion and MP (Baker *et al.*, 1985).

1.2.4 Disorders of the human gastrointestinal tract

In humans, there are numerous diseases which affect the ENS that are useful to compare to diseases in the horse, including Chagas' disease (American Trypanosomiasis), diabetic autonomic neuropathy, familial dysautonomia, intestinal aganglionosis (Hirschsprung's disease) and chronic idiopathic intestinal pseudo-obstruction (Heaton *et al.,* 1988). In Hirschsprung's disease, an absence of ganglion cells and their intrinsic nerve fibres, and proliferation of extrinsic nerve fibres are cardinal features. One study of Hirschsprung's disease showed that nitric oxide synthase (NOS) was selectively absent in the plexus areas and in the musculature of the aganglionic segments suggesting that this may contribute to the absence of smooth muscle relaxation in these segments (Vanderwinden *et al.*, 1993). Other relevant diseases include neuronal intestinal dysplasia (NID) (Krammer *et al.,* 1993b), and idiopathic chronic constipation where the total number of MP neurons in the colon is decreased, VIP-positive neurons are decreased and NOS-positive neurons are increased. VIP and nitric oxide (NO) are nonadrenergic, noncholinergic inhibitory neurotransmitters. The hypothesis put forward is that the excessive production of nitric oxide may cause the persistent inhibition of contractions (Cortesini *et al.,* 1995). In a post-measles encephalitis syndrome, the loss of myenteric ganglion cells, Schwann cell proliferation, and intranuclear inclusions in neurons and glial cells of the MP, spinal cord, dorsal root, and coeliac plexus can sometimes result in hyperactive, uncoordinated gastrointestinal muscle activity (Heaton *et al.,* 1988).

Motor disorders of the gastrointestinal tract are very common (Huizinga *et al.,* 1997). Comparative intestinal motility disorders, such as postoperative ileus and intestinal pseudo-obstruction, offer a direct pathological perspective on disruption to normal intestinal physiology. Ileus is the syndrome of functional inhibition of propulsive bowel motility (Livingston and Passaro, 1990). The most common setting for ileus is after operations in the peritoneal cavity (Turnage and Bergen, 1998). Postoperative ileus (POI) is thought to be the result of loss of normal co-ordination of intestinal contraction by the intrinsic electrical activity of the gut (Turnage and Bergen, 1998). Recently, it has been suggested that POI is mediated through a leukocytic

inflammatory response in the *muscularis externa* (Kalff *et al.*, 1999). Leukocyte-derived inducible nitric oxide (NO) has been shown in experimental rats to modulate postoperative motility changes and participate in the development of suppressed muscle contractility (Kalff *et al.*, 2000). POI remains a poorly understood common clinical problem and the lack of a specific therapy means that it has a huge economic impact because of sequelae such as prolonged hospitalisation (Livingston and Passaro, 1990; Kalff *et al.*, 1999; Prasad and Matthews, 1999). Pseudo-obstruction syndromes (where ineffective intestinal propulsion causes signs of obstructive bowel disease without a physical obstruction) are increasingly being recognised in medical gastroenterology and are thought to represent an impairment of the intrinsic neuromuscular or extrinsic control of gut motility (Coulie and Camilleri, 1999). Pseudo-obstruction is considered to be a severe gastrointestinal motor disorder (Di Lorenzo, 1999). Primary and secondary disorders of the enteric nervous system or smooth muscle, or extrinsic innervation, can result in acute or chronic gut dilatations (Kamm, 1996) such as megacolon (Phillips and Pemberton, 1998). Constipation is one of the most common digestive disorders in humans (Sonnenberg *et al.*, 1994) and therefore animal examples such as feline constipation may be useful for comparative studies. In human slow transit constipation, excitatory nerve fibres have been shown to be present in the circular muscle but those showing immunoreactivity for tachykinins and enkephalin are reduced (Porter *et al.*, 1998). In idiopathic megarectum, there is an increase in VIP- and NO-containing nerve fibres in the *muscularis mucosae* and *lamina propria* and a decrease in the longitudinal muscle layer in rectal tissue (Gattuso *et al.*, 1996). A case of idiopathic myenteric ganglionitis presenting for intractable vomiting has been reported (De Giorgio *et al.*, 2000a). In this young adult there was a marked decrease in substance P-immunoreactivity in nerve fibres and myenteric neurons in tissue biopsies of the stomach.

1.3 Immunohistochemistry of the enteric nervous system

Immunohistochemistry is an extremely useful research tool for investigating the anatomy and function of the ENS. Much of the knowledge of the

immunohistochemistry of the intestine has been gathered from whole-mount studies in the guinea pig (Llewellyn-Smith *et al.,* 1985), but there is an increasing amount of work being done in other species. There have been immunohistochemical studies in animals such as the pig (Scheuermann *et al.*, 1989; Krammer and Kühnel, 1992) and also in humans (Krammer *et al.*, 1993a). Extrapolation of findings from other animals to the horse may not be justifiable because differences in structure and organisation of the ENS exist (Pearson, 1994).

Whole-mount preparations are more suitable than tissue sections for evaluation in diseases such as Hirschsprung's disease and NID because the three-dimensional morphology can be more easily appreciated (Krammer *et al.*, 1993a). These authors highlight the disadvantages of working with adult human gut (which is quite thick) as being the difficulty in the preparation of the whole-mounts and the limited penetration of antibodies. Immunohistochemistry can be useful in revealing pathology and neuronal counts in diseases such as NID using antibodies to PGP 9.5 (protein gene product 9.5) (Krammer *et al.,* 1993b). In Hirschsprung's disease, it has been shown by Kato *et al.* (1990) that the supporting cells of the enteric neurons (the enteric glia) express glial fibrillary acidic protein (GFAP), S100, and glutamate synthetase (a key enzyme for ammonia detoxification and glutamate metabolism in the central nervous system), whereas the supporting cells of the extrinsic components which accompany peripheral nervous system axons, are negative or very weakly positive for glutamate synthetase. The authors conclude that glutamate synthetase immunocytochemistry may delineate intrinsic and extrinsic neural components in the ENS, and may provide an important clue for the differential diagnosis of Hirschsprung's disease.

There have been a moderate number of immunohistochemical studies on sections from the equine intestine (Bishop *et al.*, 1984; Cummings *et al.,* 1984; Kotze and Van Aswegen, 1990; Burns and Cummings, 1993) but only two using whole-mount preparations (Burns and Cummings, 1991; Pearson, 1994). The latter report describes the stucture of the equine ENS and reports the presence of neurons showing positive immunoreactivity for galanin, VIP, neuropeptide Y and substance P. The problems of whole-mount preparation and antibody penetration become evident when one

begins to work on adult equine intestine which is very thick. The two equine studies using wholemounts overcame such problems by using either foal tissue (Pearson, 1994) or enzymatic digestion of smooth muscle (Burns and Cummings, 1991). Sections may therefore be preferable when examining adult equine tissue, despite the loss of a degree of three-dimensional morphology.

1.4 Tissue culture of the enteric nervous system

The development of a technique for culturing explants of the MP and SMP from the guinea pig ENS by microdissection and enzymatic methods has led to a greater understanding of ENS physiology (Jessen *et al.,* 1978; Jessen *et al.*, 1983). Three types of culture are possible: organotypic (the whole intestine is cultured), explant (intact plexuses) and dissociated cell cultures (Willard and Nishi, 1989). Organotypic cultures have the advantage over dissociated cultures, and to a lesser extent explant cultures, in that the ultrastructural features of the ENS are kept intact (Song *et al.*, 1995). However, dissociated cultures are potentially valuable because the neurons continue to express many of their differentiated properties (Nishi and Willard, 1985). Dissociated cell culture techniques have been markedly refined (Saffrey *et al.,* 1991; Jaeger, 1995; Schäfer *et al.,* 1995). Refinements to ENS tissue culture methodology in the rat have increased the amounts of myenteric plexus that can be obtained (Schäfer et al., 1997). Jessen *et al.* (1983) described three types of cell in their culture system: neurons, glial cells and fibroblasts. Fibroblasts have larger, flatter nuclei and dark cytoplasmic granules whereas glia are often flat on one side with a long process on the other and have a greater migratory tendency (Willard and Nishi, 1989). Immunohistochemistry can be used in conjunction with cultures to examine the cellular characteristics of the systems being studied (Saffrey *et al.,* 1992b; Schäfer *et al.,* 1995).

Cultures are useful both for correlating the morphological, biophysical, pharmacological and synaptic properties of neurons and for testing the ability of altered environmental conditions to change these properties (Nishi and Willard, 1985). Indeed, recent research has been examining the effects of various trophic

factors on ENS cultures, with the aim of increasing our understanding of the development of the ENS (Schäfer *et al.*, 1998; Schäfer and Mestres, 1999). Furthermore, the SMP culture system has been shown to be a suitable one for characterising neurotransmitter release from enteric neurons (Hanani, 1993).

Therefore, culture systems offer considerable potential for the development of *in vitro* models for diseases of the ENS. Hanani (1993) and Schäfer and Mestres (1997) have successfully cultured myenteric neurons from the human large intestine but there appear to be no previous reports of equine enteric neurons being grown in tissue culture.

1.5 The interstitial cells of Cajal (ICC)

The interstitial cells of Cajal (ICC) are a group of cells in the gastrointestinal tract that were first described by Santiago Ramón y Cajal (Cajal, 1893; Cajal, 1911). The ICC are currently attracting much interest because of their role in the control of intestinal motility (Hagger *et al.*, 1997). These cells form a network in close association with the smooth muscle and nerves of the intestine (Vanderwinden *et al.*, 1996a).

1.5.1 The function of the ICC

The ICC are considered to be the pacemakers and mediators of neurotransmission in the gastrointestinal tract (Langton *et al.*, 1989; Torihashi *et al.*, 1995; Sanders, 1996). Furthermore, it has been confirmed recently that ICC generate a rhythmic pacemaker current and are the initiators of slow waves in the gastrointestinal tract (Ward *et al.*, 1997; Koh *et al.*, 1998; Thomsen *et al.*, 1998; Lee *et al.*, 1999). Slow waves are rhythmical oscillations of the smooth muscle membrane potential (Weisbrodt, 1974). Contractile activity occurs when spike bursts are superimposed on slow waves. Slow wave activity is the rate-limiting step for peristaltic activity (Hagger *et al.*, 1997). There is considerable variation in the distribution of ICC in man and in other mammals (Christensen *et al.*, 1992, Hagger *et al.*, 1997). ICC have been described as

being interposed between nerves and muscle cells where they form gap junctions with each other and nearby smooth muscle cells (Langton *et al.,* 1989). They are innervated by enteric neurons and can send extensions into both smooth muscle layers, between muscle cells and into connective tissue septa (Rumessen and Thuneberg, 1991).

A classification scheme for the different types of ICC that may be seen has been published (Sanders, 1996). These cell types are: ICC in the myenteric region (stomach, small bowel, colon), ICC at the submucosal surface of the circular muscle of the colon, ICC of the deep muscularis plexus of the small intestine, and intramuscular ICC in the circular and longitudinal muscle layers. The pacemaking activity of the stomach and small intestine originates from the ICC in the region of the MP whereas in the large intestine it resides in the inner circular muscle layer and, to a lesser extent in the region of the MP. The intramuscular ICC have a role in mediation of neurotransmission and propagation of electrical events (Sanders, 1996). Indeed recently, Ward *et al.* (2000a) have shown synaptic-like structures between excitatory nerve terminals and intramuscular ICC in the murine stomach. This study also provided functional data supporting the hypothesis that excitatory neurotransmission requires this close contact between neurons and ICC in order to achieve a response in the smooth muscle.

ICC have been shown to express the proto-oncogene *c-kit*, which encodes a receptor tyrosine kinase (Huizinga *et al.*, 1995). The natural ligand of the c-Kit protein is stem cell factor (Zsebo *et al.,* 1990). In addition to the functional importance of c-Kit, antibodies raised against c-Kit are now available for the immunohistochemical labelling of ICC (Ward *et al.*, 1994).

1.5.2 The ICC in animals and man in health

The distribution of ICC has been examined in humans and some animals including the guinea pig, mouse, rat, cat, dog, rabbit, ferret, opossum and pig, and there does appear to be some interspecies variation (Christensen *et al.,* 1992; Burns *et al.,* 1997; Hagger *et al.*, 1997; Henry *et al.,* 1998; Rømert and Mikkelson, 1998).

Immunohistochemical studies of ICC using antibodies against c-Kit have been extremely useful in examining the human gastrointestinal tract in health (Hagger *et al.* 1998a; Hagger *et al.,* 1998b; Rømert and Mikkelson, 1998; Kenny *et al.*, 1999; Torihashi *et al.,* 1999a; Wester *et al.,* 1999). These studies stand as useful comparisons with pathological conditions. To date, there have been no previous studies of the ICC in the horse.

1.5.3 The ICC in gastrointestinal dysfunction

Impairment of ICC-mediated control of electrical events in smooth muscle has been implicated in several motility disorders in laboratory mammals and in humans. The development of ICC and generation of electrical intestinal rhythmicity are blocked in W/W^v mice that have a mutation of the *c-kit* gene (Ward *et al.*, 1994). Furthermore, Ws/Ws mutant rats lacking a functional *c-kit* gene have disturbed intestinal movement that is attributed to a lack of ICC (Isozaki *et al.*, 1995). The natural ligand of the c-Kit protein is stem cell factor (SCF) (Zsebo *et al.*, 1990). Sl/Sl^d mutant mice with a mutation of the SCF gene have disrupted development of ICC and disturbed electrical rhythmicity (Ward *et al.,* 1995). Interestingly, glial-derived neurotrophic factor knockout mice that lack enteric nerves (a lethal knockout) have normal ICC distribution and slow wave activity at birth (Ward *et al.*, 1999b). This suggests that enteric neurons are not required for the development of functional ICC. This is an interesting comparison for grass sickness which, crudely-speaking, could be considered a large animal disease where the enteric neurons have been "knocked out" by the putative toxic agent.

Abnormalities of the ICC have been implicated in disorders of the human gastrointestinal system such as Hirschsprung's disease (Yamataka *et al.,* 1995; Vanderwinden *et al.*, 1996b), infantile hypertrophic pyloric stenosis (Langer *et al.*, 1995; Vanderwinden *et al.*, 1996a), ulcerative colitis (Rumessen, 1996), chronic idiopathic intestinal pseudo-obstruction (Isozaki *et al.*, 1997; Yamataka *et al.*, 1998), transient neonatal intestinal pseudo-obstruction (Kenny *et al.*, 1998b), constipation associated with piebaldism (Giebel and Spritz, 1991), colonic hypomotility in

patients with anorectal malformations (Kenny *et al.*, 1998a), megacolon (Faussone-Pellegrini *et al.*, 1999), slow transit constipation (He *et al.*, 2000) and Chagas' disease (Hagger *et al.*, 2000). Immunohistochemical evaluation of the ICC using antibodies to c-Kit has been useful in several recent studies of human gastrointestinal diseases (Yamataka *et al.*, 1995; Vanderwinden *et al.*, 1996a; Vanderwinden *et al.*, 1996b; Isozaki *et al.*, 1997; Horisawa *et al.*, 1998; Kenny *et al.*, 1998a; Kenny *et al.*, 1998b; Yamataka *et al.*, 1998; Faussone-Pellegrini *et al.*, 1999; He *et al.*, 2000; Hagger *et al.*, 2000).

To date, there have been no studies of ICC in any of the numerous veterinary gastrointestinal diseases. Indeed, because ICC are important motility regulating cells, it is more than likely that they are involved, either primarily or secondarily, in the pathogenesis of some gastrointestinal disease processes in which there is a prominent element of motility disturbance (Rumessen *et al.*, 1993).

1.6 Electrophysiology of gastrointestinal smooth muscle

Spontaneous electrical activity of the intestine such as slow waves can be recorded *in vitro* using intracellular microelectrodes to impale individual smooth muscle cells. Slow waves are rhythmical oscillations of the smooth muscle cell membrane potential and are the rate-limiting step for peristaltic activity. The generation of slow waves is an exclusive feature of the ICC but not smooth muscle cells which lack the basic ionic mechanisms needed to generate or regenerate slow waves (Horowitz *et al.*, 1999). Calcium currents are integral to the generation and spread of pacemaker activity. Spontaneous activation of low-threshold calcium channels (T-type) in the ICC is the proposed initiating mechanism for slow waves (Sanders, 1996; Farrugia, 1999). This causes depolarisation of the resting membrane potential which activates L-type calcium channels, amplifying the current. This pacemaker current spreads to neighbouring smooth muscle cells that are coupled via gap junctions. The depolarisation of membrane potential in the smooth muscle cells again activates L-type calcium channels. If threshold depolarisation is achieved, calcium action potentials are elicited, thus coupling the electrical activity to contraction (Sanders,

1996; Horowitz *et al.*, 1999). It is important to note that L-type calcium channels, but not T-type channels, are blocked by the drug nifedipine. Therefore, the addition of nifedipine to intestinal smooth muscle strips *in vitro* results in the cessation of contractile activity (Farrugia, 1999).

Slow waves have been recorded from a range of species such as the pig (Jiménez *et al.,* 1999), cat (Bortoff, 1965; Hara *et al.,* 1986), dog (Durdle *et al.,* 1983; El-Sharkawy, 1983; Hara *et al.,* 1986; Sanders and Smith, 1986a; Sanders and Smith, 1986b; Smith *et al.,* 1987a; Smith *et al.,* 1987b; Smith *et al.,* 1989; Huizinga *et al.,* 1990) and mouse (Ward *et al.,* 1994; Huizinga *et al.,* 1995; Torihashi *et al.,* 1995; Torihashi *et al.,* 1997; Ward *et al.,* 1997; Lee *et al.,* 1999). Most of the knowledge on intestinal electrophysiology comes from these animal studies but there also have been some *in vitro* experiments on human intestinal smooth muscle (Duthie and Kirk, 1978; Kubota *et al.,* 1983; Hara *et al.,* 1986; Rae *et al.,* 1998). There is good correlation between findings in human *in vitro* studies and the observed contractile properties *in vivo* (Taylor *et al.,* 1975; Ford *et al.,* 1995) showing that this approach is a useful reflection of whole body physiology. Pacemaking in the human intestine (Rae *et al.,* 1998) bears many similarities to other animal models such as the pig, cat and dog and indeed, intestine from animals such as the pig are very relevant for comparison with the human intestine (Thomsen *et al.,* 1997; Jiménez *et al.,* 1999).

The Sanders laboratory has developed a cross-sectional preparation of the intestinal muscle giving direct microelectrode access to specific sites in the muscle layers (Smith *et al.,* 1987a; Smith *et al.,* 1987b; Rae *et al.,* 1998). This system has the unique advantage over other systems that the ICC-evoked origin of electrical rhythmicity can be determined.

There have been numerous equine *in vivo* electrophysiological studies involving surgically-implanted extracellular electrodes (Davies and Gerring, 1983; Berry *et al.,* 1986; King and Gerring, 1989; Merritt *et al.,* 1989; Ross *et al.,* 1990). There have been only two reports using *in vitro* intracellular microelectrode recordings in equine intestine, indicating that this represents a novel approach. Rakestraw *et al.* (2000) characterised inhibitory neuromuscular transmission in equine jejunum describing

inhibitory junction potentials. The authors did not report the presence of any slow waves and the only spontaneous activity described was the presence of small membrane potential oscillations and action potentials. This spontaneous activity was abolished by the addition of nifedipine to the preparations. This is surprising because of the nifedipine-resistant component implicated in the initiation of slow waves (Bolton *et al.*, 1999). In the only other equine *in vitro* intracellular electrophysiological study, this time using caecum, no slow waves were recorded (Schneider *et al.*, 2000). No slow waves have been recorded in equine large intestinal *in vivo* studies.

There have been numerous equine *in vitro* studies of intestinal mechanical contractility (Malone *et al.*, 1996; Rakestraw *et al.*, 1996; Re *et al.*, 1997; Malone *et al.*, 1999; Van Hoogmoed *et al.*, 2000). Two studies have examined pharmacological responses in intestine taken from horses with grass sickness (Murray *et al.*, 1994; Murray *et al.*, 1997). This approach provides pharmacological and mechanical information but is very different to the intracellular methodology adopted in this thesis.

To date there have been very few studies of the *in vitro* electrical activities of diseased intestine. One study found that the aganglionic segements of colon in Hirschsprung's disease patients were electrically quiescent (Kubota *et al.,* 1983). Another study found that experimentally-induced inflammation modulated canine colonic myolectrical activity and was associated with ultrastructural damage to ICC and a reduction in ICC density (Lu *et al.,* 1997). Therefore, comparative *in vitro* electrophysiological studies in health and disease represent a novel approach in elaborating on the physiology and pathophysiology of intestinal function.

1.7 Aims of the thesis research programme

The aims of this study were:

- to characterise the morphology and neurochemical expression of the equine ENS using tissue culture and immunohistochemistry.

- to study the distribution of the ICC in the intestine in normal and grass sickness-affected horses.

- to perform a detailed *in vitro* investigation of the electrical properties and control of smooth muscle in both the healthy and diseased intestine.

- to test the hypothesis that impaired ICC-mediated control is responsible for intestinal dysfunction.

CHAPTER 2:IMMUNOHISTOCHEMICAL AND TISSUE CULTURE STUDIES OF THE ENTERIC NERVOUS SYSTEM

2.1 Aims

The purpose of this part of the study was to characterise the morphology and neurochemical expression of the equine ENS using tissue culture and immunohistochemistry. As an adjunct to this, experimentation on tissue from small mammals (guinea pig and rat) was used for comparison and the development of methodology.

Cell culture systems (dissociated and explant) were established along with fixed wholemount preparations and examined using immunohistochemistry. The ultimate aim was to utilise viability assays and electrophysiology to examine the cell systems and to determine the effects of challenges to the cultures with putative toxic agents such as serum from grass sickness cases and various neurotransmitters and toxins. This approach would establish an *in vitro* testing model for the autonomic neuropathy of grass sickness.

2.2 Materials and Methods

2.2.1 Tissue harvesting and preparation

2.2.1.1 Guinea pig studies

Adult female guinea pigs of body weight 250-400g were used. Animals were stunned with a blow to the head, exsanguinated, the abdomen incised and a loop of jejunum dissected out. The portion of intestine was opened along the mesenteric border and flushed thoroughly with phosphate-buffered saline (PBS, 0.1 M, pH 7.0) or Krebs solution. For tissue culture studies, the samples were placed in Krebs solution and for immunohistochemistry samples were fixed immediately.

2.2.1.2 Equine studies

Segments of ileum were removed fresh *post mortem* from adult horses destroyed humanely due to clinical conditions not involving the gastrointestinal tract (eg orthopaedic problems). The horses were euthanased at the Royal (Dick) School of Veterinary Studies Equine Hospital with 50ml of intravenously administered quinalbarbitone sodium BP (400 mg/ml)/ cinchocaine hydrochloride BP (25 mg/ml) (Somulose; Arnolds Veterinary Products, Shrewsbury, UK). For tissue culture experiments, the samples were placed immediately in Krebs solution and for immunohistochemical experiments samples were fixed immediately in Zamboni's solution (see below).

2.2.1.3 Rat studies

At the Open University, Milton Keynes, 7-day-old Wistar-derived rat pups (2-4 for each batch of cultures) were killed by decapitation. The abdomen was incised and the small intestine removed, rinsed and placed in Hank's balanced salt solution (HBSS).

2.2.2 Guinea pig ENS tissue culture

Small intestine was harvested from guinea pigs as described above, opened along the mesenteric border, flushed thoroughly and placed in warmed (37°C) sterile-filtered (sterile filters 0.2 μm; Supor Acrodisc; Gelman Sciences, Northampton, UK) Krebs solution of the following composition (mM): NaCl 120.7, KCl 5.9, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.5 and glucose 11.5 with 1 μM nifedipine (Sigma, Poole, UK), 100 μg/mL streptomycin (Sigma), 100 U/ml penicillin (Sigma) and 100 μg/ml gentamicin (Sigma). The intestine was pinned flat on silicone rubber (Sylgard; Merck, Glasgow, UK) mucosal surface uppermost. To obtain the submucous plexus (SMP), microdissection was performed under the view of a Nikon SMZ-2T stereoscopic microscope (x 63 maximal magnification). The mucosa was teased away from the submucosa and the SMP was then dissected from the underlying muscle layers with a scalpel (No. 10a blade). To obtain the myenteric

plexus (MP), preparations were pinned out in a similar fashion, the mucosa and submucosa peeled away and the circular muscle removed in strips with fine forceps leaving the MP attached to the longitudinal muscle layer.

The MP and SMP from six 3 cm long strips of jejunum were placed in separate (ie one for the SMP and one for the MP) sterile test tubes containing 1 ml of 1 mg/ml collagenase (Type XI; Sigma) and 10 μg/ml DNase (Sigma) at 37°C for 90 minutes. At this point, trypsin (Sigma) was added at a concentration of 0.125 mg/ml for 15 minutes. After this, 1 ml of culture medium was added {medium 199, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 2 mM L-glutamine and 10% foetal calf serum (all Sigma); 6 mg/ml D-glucose (Life Technologies, Paisley, UK)}. The tissues were then triturated 5x each through a 21 gauge and then a 25 gauge needle. This procedure broke up the tissue by applying enough shearing forces without producing any foaming of the suspension. The samples were then centrifuged at 1000 rpm (120g force) for 2 minutes and the supernatant removed. Either 200 μl or 500 μl of culture medium were added, the pellet of cells re-suspended and aliquots of 30,60,90 or 120 μl plated out on poly-L-lysine-coated (0.01%; Sigma) 13 mm diameter glass coverslips placed (3) in sterile culture dishes 100 mm in diameter (Nalgene; Merck, Glasgow, UK). The dishes were placed in a 37°C culture incubator (5% CO_2) and after 1 hour, the dishes were topped up with 2 mls of culture medium. Medium was changed every 24-48 hours and the cultures were maintained for up to 8 days before fixation and immunohistochemistry.

In order to establish explant cultures systems, SMP and MP (attached to longitudinal muscle) strips 3 cm in length were obtained and pinned on sterile Sylgard in glass Petri dishes. The MP explant cultures could also be described as being organotypic because more than just the plexus (but less than the entire organ) was cultured. Culture medium was added to these dishes and they were cultured as above.

2.2.3 Equine ENS tissue culture

Fresh ileal tissue samples were rinsed and placed in warmed (37°C) sterile-filtered Krebs solution. Microdissection was performed with the aid of a stereoscopic microscope by pinning out the gut on Sylgard mucosal surface down, and cutting the *muscularis externa* away from the underlying layers and the SMP from the mucosa with a No. 5 scalpel blade (Figure 6). The *muscularis externa* and SMP were placed in separate sterile test tubes containing 1 ml of 3.3 mg/ml collagenase (Type XI; Sigma) and 10 μg/ml DNase (Sigma) at 37°C for 4-5 hours. Trypsin (Sigma) was added at a concentration of 2.5 mg/ml for 15 minutes, after which 1 ml of culture medium was added (medium 199, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 2 mM L-glutamine and 10% foetal calf serum (all Sigma); 6 mg/ml D-glucose (Life Technologies, Paisley, UK)). The tissues were triturated, first through a sterile pipette (x5), then through a fine gauge (21g) needle (x5). Larger pieces of undigested tissue from the *muscularis externa* were removed prior to trituration. The suspension was then centrifuged at 1000 rpm (120g force) for 2 minutes and the supernatant removed. Either 200 μl or 500 μl of culture medium were added, the pellet of cells re-suspended and aliquots of 30, 60, 90 or 120 μl plated out on poly-L-lysine-coated (0.01%; Sigma) 13 mm diameter glass coverslips. Sterile culture dishes (100 mm diameter) containing these coverslips were placed in a 37°C culture incubator (5% CO_2) for 1 hour, after which 2 mls of culture medium were added. The medium was changed every 24 hours until fixation of the cultures after 5 days.

To establish explant culture systems (or organotypic) of the equine ENS, two to three cm long strips of SMP and *muscularis externa* were obtained from horses as described above and pinned on sterile Sylgard in glass Petri dishes. Culture medium was added to these dishes and they were incubated as described above.

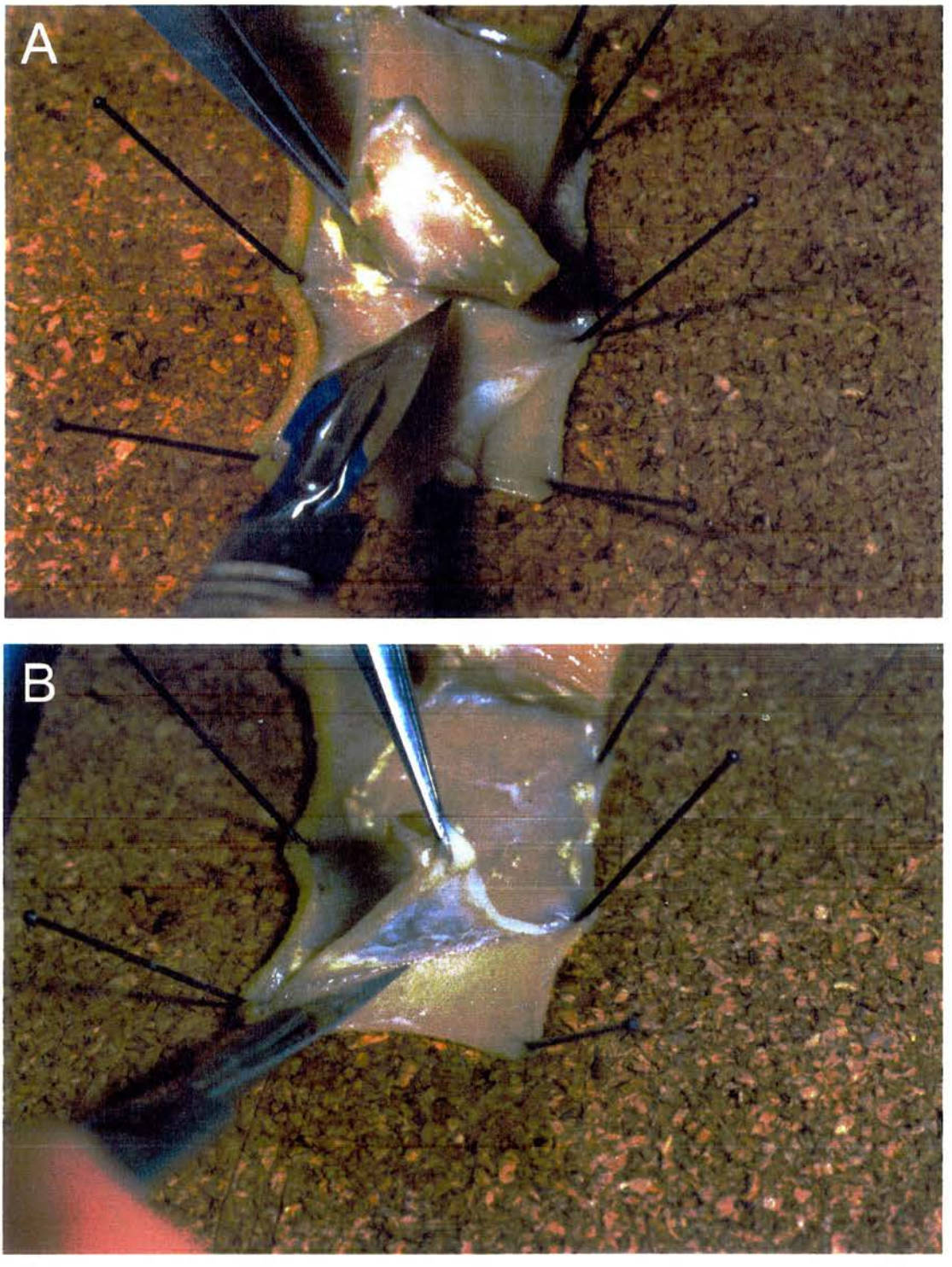


Figure 6. Dissection of the equine ileum. (A) Removal of the *muscularis externa* from the underlying layers. (B) Removal of the submucous plexus from the mucosa.

2.2.4 Rat ENS tissue culture

The intestinal samples were placed into Hank's balanced salt solution (HBSS; Sigma) containing 100 μg/ml streptomycin, 100 U/ml penicillin, 200 μg/ml gentamicin and 50 μg/ml metronidazole (all Sigma) and the mesentery removed with fine forceps. The rinsed intestine was placed in fresh HBSS and the *muscularis externa* peeled off with forceps in the manner of "removing a sock." The tissue was washed again in the HBSS-antibiotic rinse in Petri dishes and transferred into 1 mg/ml collagenase (CLSII, Worthington, UK) supplemented with 10 μg/ml DNase (Sigma) in HBSS buffered with Hepes (Sigma). The *muscularis externa* was cut into 2-3 mm strips which were incubated at 37°C (5% CO_2) for 30 minutes. The tissue was vortexed for approximately 20 seconds and incubated in fresh enzyme solution for a further 30 minutes. After a further 20-second period of vortexing, MP ganglia were harvested with a Gilson pipette (10-20 μl) with the assistance of a dissecting microscope. Gentle agitation with the pipette separated the plexus from the attached muscle. Harvested plexus was transferred to fresh HBSS and stored on ice. The plexus material was rinsed several times in calcium and magnesium-free balanced salt solution and then dissociated by incubation for 15 minutes at 37°C with trypsin-EDTA (Sigma; 0.5 mg/ml Trypsin), 10 μg/ml DNase (Sigma) and 10 mM Hepes (Sigma). The suspension was centrifuged at 900 rpm (100g force) for 2-3 minutes, and most of the supernatant discarded. 1 ml of 1 mg/ml trypsin inhibitor (Type I-S: from soybean; Sigma) was added to stop the trypsin reaction and the vial was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed, checking for any tissue loss at this stage and the centrifuged pellet re-suspended in 1 ml of defined culture medium (medium 199, 6 mg/ml glucose, 2 mM glutamine, 2 g/l sodium bicarbonate, 0.08% bovine serum albumin, 100 U/ml penicillin, 10 mM Hepes, 0.55 U/ml insulin, 5 μg/ml transferrin, 100 μM putrescine, $3x10^{-8}$ M sodium selenium, $2x10^{-8}$ M progesterone, 10% foetal calf serum and tissue culture water; all Sigma). The tissues were triturated through a 25 gauge needle (x5) and the cells counted in trypan blue (Sigma) using a haemocytometer. The dissociated cells were plated out in aliquots of 150 μl on poly-L-lysine coated (0.01%) 13 mm diameter coverslips in 24 well plates at a seeding density of $1.5x10^4$ viable cells per coverslip. After 1 hour

at 37°C, each well was topped up to 1 ml with defined culture medium (without foetal calf serum) and left to incubate overnight, with a medium change the next morning. The cultures were fixed at 48 hours with 4% buffered paraformaldehyde.

2.2.5 Immunohistochemical processing

2.2.5.1 Immunohistochemistry of explants and wholemount preparations (guinea pig and equine)

Wholemount preparations of the SMP were dissected (as described above) from tissues which had been fixed immediately after collection from the animal in 2% formaldehyde/15% picric acid (Zamboni's solution) for 15-20 hours at 4°C. After fixation, the tissues were placed in 80% ethanol to remove the picric acid and then dehydrated by sequential washing (10-minute periods) in 95% and 100% ethanol and xylene. The tissues were rehydrated via 100%, 95%, 80%, 50% ethanol solutions and stored in phosphate-buffered saline (PBS, 0.1 M, pH 7.0) containing 0.05% (w/v) sodium azide. After culturing, the explant/organotypic tissues were fixed and processed in the same way prior to immunohistochemistry.

A standard indirect immunofluorescence technique was used whereby the preparations were incubated in primary antisera (diluted in PBS and 0.3% Triton X-100) for 16-18 hours in humid chambers kept at room temperature. After rinsing in PBS (3 x 10 minutes), the tissues were exposed to secondary fluorescent antisera for 1.5-2 hours, washed in PBS as above and then mounted in a carbonate buffer medium (pH 8.6, 0.5 M) mixed 1:2 with glycerol. Antisera to a range of cell markers and neuropeptides were used (Table I). Control experiments were performed by omitting primary or secondary antisera. It should be noted that the antisera used in this study were raised against antigens that were originally isolated from species other than the horse. Therefore, statements used below such as "positive immunoreactivity for S100" would be more accurately given as "S100-like" immunoreactivity.

Immunofluorescence was viewed using 10x, 20x or 40x objectives on a Zeiss microscope equipped with phase contrast and epifluorescence optics. Provia Fujichrome film (400 ASA) was used for all photomicrography.

2.2.5.2 Immunohistochemistry of dissociated cell cultures (guinea pig and equine)

The fixation procedure for dissociated cell cultures involved a 30-minute immersion of the coverslips in Zamboni's solution at 4°C. After washing in PBS (6 x 5 minutes), the incubations with primary and secondary antibody were for periods of one hour each.

2.2.5.3 Immunohistochemistry of dissociated cell cultures (rat)

The rat dissociated MP cultures were processed for PGP 9.5-immunoreactivity (a general neuronal marker; Ultraclone, Isle of Wight, UK) using the avidin-biotin (ABC) method (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) with a diaminobenzidine substrate (DAB, BDH Laboratory Supplies, Poole, UK). This immunohistochemical technique is the same as that used in the evaluation of the ICC in this thesis. Therefore, the details of the technique will be described in Chapter 3.

Table I

Details of antibodies used in this study

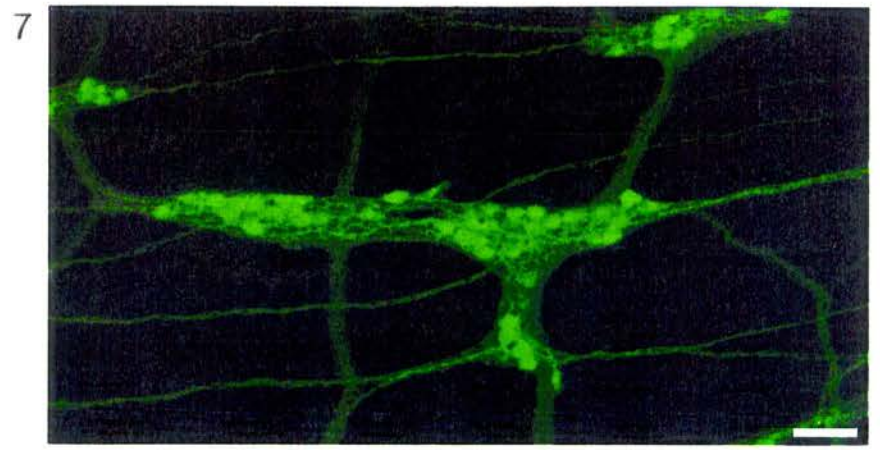
Antibodies	*Dilution in wholemounts and explants*	*Dilution in dissociated cell systems*	*Source*
Galanin	1:400	1:400	Serotec, Oxford, UK
Vasoactive intestinal polypeptide (VIP)	1:400	1:800	J. Walsh, UCLA
Neuropeptide Y (NPY)	1:400		P. Emson, Cambridge
Substance P	1:400		P. Emson, Cambridge
Nitric oxide synthase (NOS)	1:20	1:100	Affiniti, Exeter, UK
Microtubule-associated protein (MAP)	1:50		P. Brophy, Edinburgh
Protein gene product 9.5 (PGP 9.5)	1:20	1:100	Affiniti, Exeter, UK
Protein gene product 9.5 (PGP 9.5)		1:8000	Ultraclone, Isle of Wight, UK
Neurofilament M (NFM)	1:100	1:400	P. Brophy, Edinburgh
PAN-neurofilaments (PAN)	1:100	1:100	Affiniti, Exeter, UK
Calcium binding protein (D45)	1:100	1:200	P. Emson, Cambridge
Calcitonin gene-related peptide (CGRP)	1:400		Affiniti, Exeter, UK
Leu-enkephalin	1:200		Sera-Lab, Sussex, UK
S100	1:100	1:100	Sigma, Poole, UK
Glial fibrillary acidic protein (GFAP)		1:20	Boehringer Mannheim, East Sussex, UK
Cappel Fluorescein-5-isothiocyanate conjugated (CFITC) against rabbit	1:10	1:10	Dynatech, Sussex, UK
Cappel FITC conjugated (MFITC) against mouse	1:10	1:10	Precision Medical, Yorkshire, UK

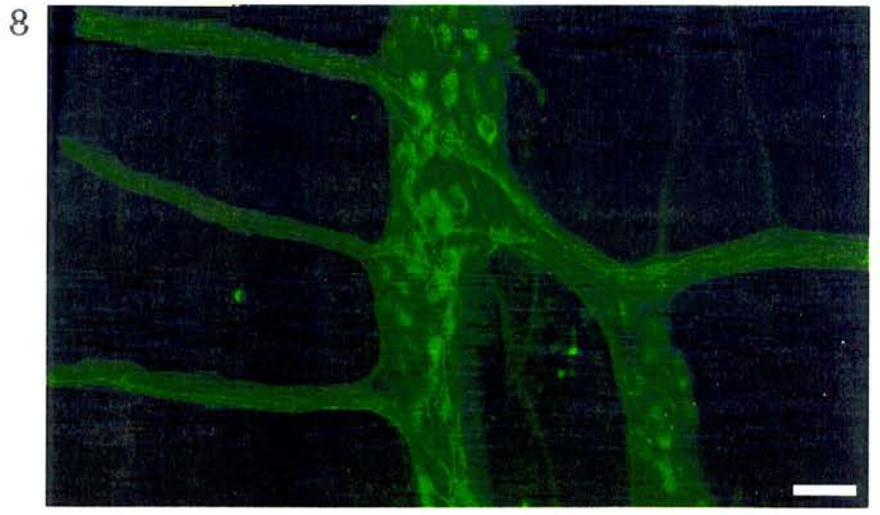
2.3 Results

2.3.1 Guinea pig ENS wholemount preparations

Wholemount preparations were obtained for both the MP and the SMP. Antibody penetration and consequent immunoreactivity in these wholemounts were satisfactory. Positive immunoreactivity was revealed with all the antibodies used except those raised against neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP). The most successful antibodies (ie with the greatest degree of repeatable positive immunoreactivity) were those raised against *Pan*-neurofilaments (*Pan*-N), neurofilament M (NFM), calcium binding protein (D45), vasoactive intestinal polypeptide (VIP) and S100 (an intracellular acidic protein that binds calcium ions).

The MP in the guinea pig was characterised by thick ganglia with major interconnecting strands (Figure 7). Nerve cell bodies were present in dense distribution in the ganglia and nerve fibres were seen in the interconnecting strands (Figures 8 and 9). The pattern in the SMP was of a more delicate web-like structure with thinner and smaller ganglia (Figure 10). The *Pan*-N antibody revealed immunoreactivity in the nerve cell bodies and in filaments in the axons and was considered to be a good general neuronal marker in both plexuses. The VIP antibody revealed immunoreactivity in certain neuronal cell bodies and in axonal varicosities (Figures 10 and 11). The S100 antibody revealed immunoreactivity in enteric glial cells in both the MP and SMP, as did the glial fibrillary acidic protein (GFAP) antibody. S100 was particularly useful for highlighting the architecture of the ganglia by showing immunoreactive glial cells surrounding non-immunoreactive neurons (Figures 12 and 13).





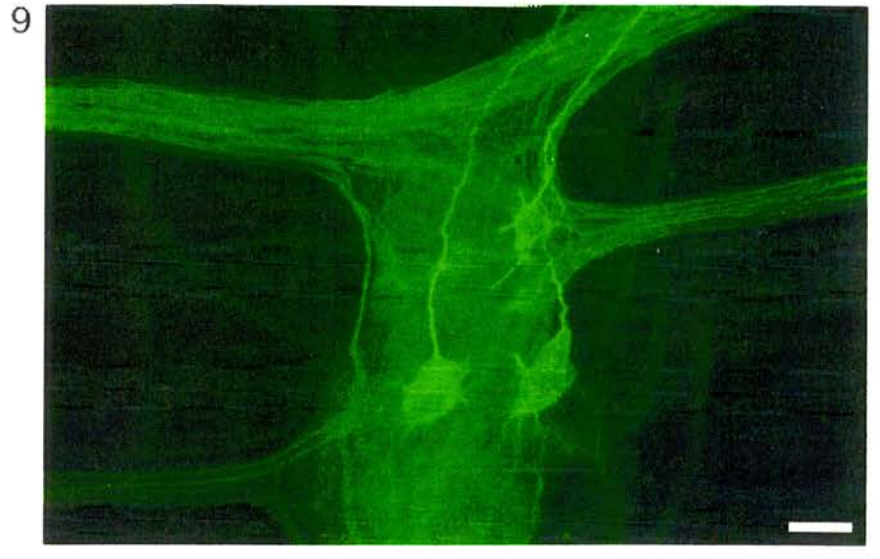
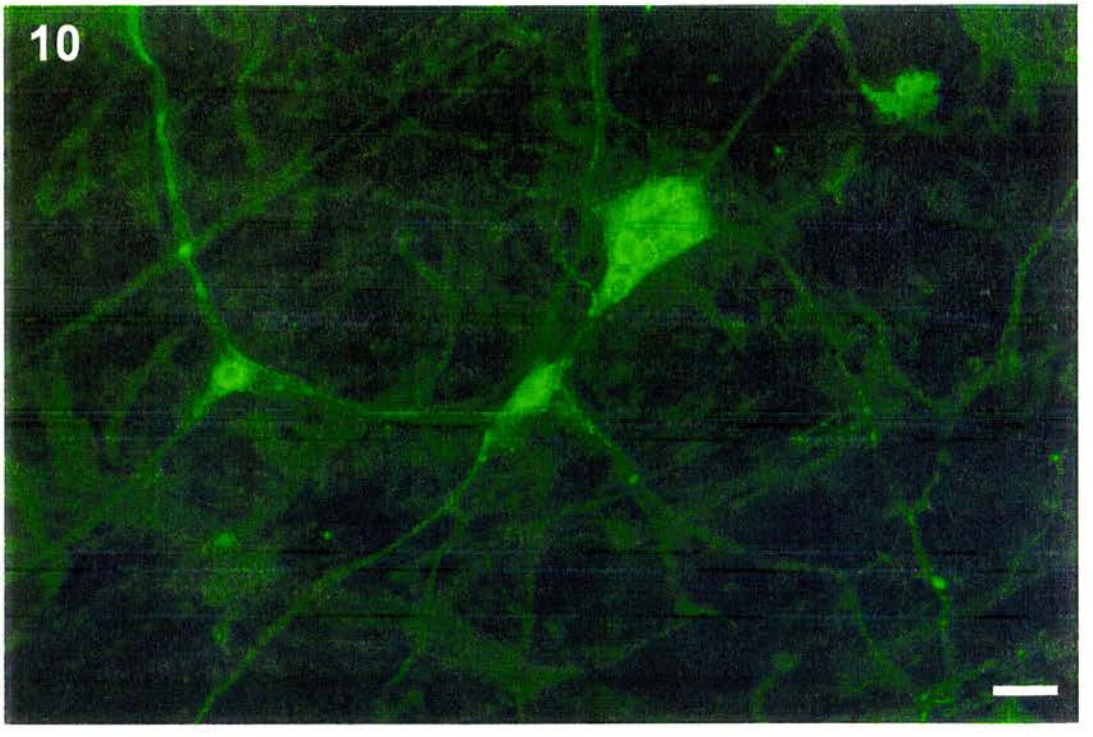


Figure 7. D45-immunoreactivity in nerve cells of the myenteric plexus of guinea pig jejunum. Bar = 100 µm.

Figure 8. *Pan*-N-immunoreactivity in nerve cells and fibres of the myenteric plexus of guinea pig jejunum. Bar = 50 µm.

Figure 9. NFM-immunoreactivity in nerve cells and fibres of the myenteric plexus of guinea pig jejeunum. Bar = 25 µm.



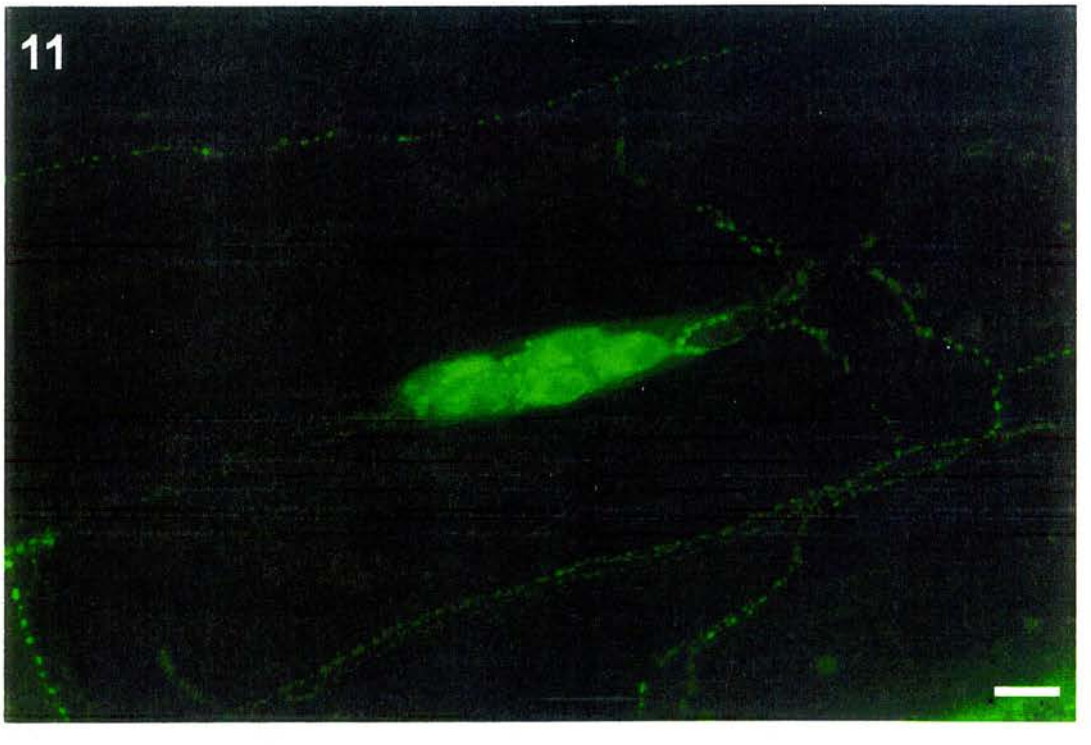
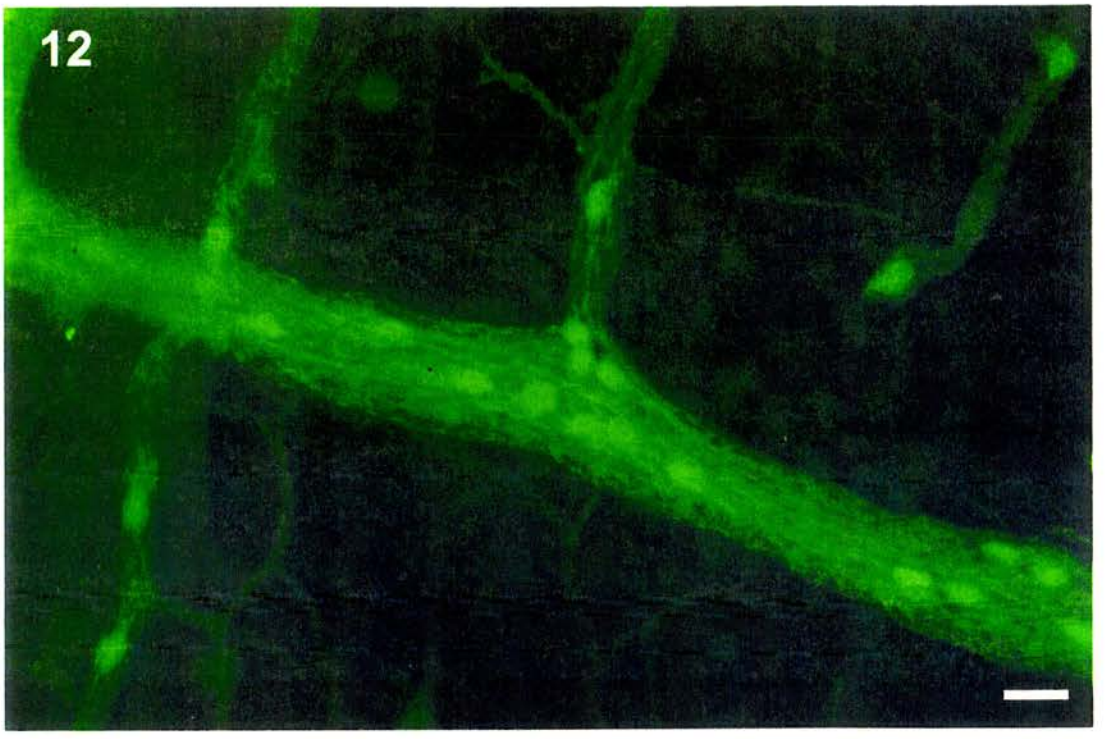


Figure 10. VIP-immunoreactivity in nerve cells and varicose fibres of the submucous plexus of guinea pig jejunum. Bar = 50 µm.

Figure 11. VIP-immunoreactivity in nerve cells and varicose fibres of the myenteric plexus of guinea pig jejunum. Bar = 25 µm.



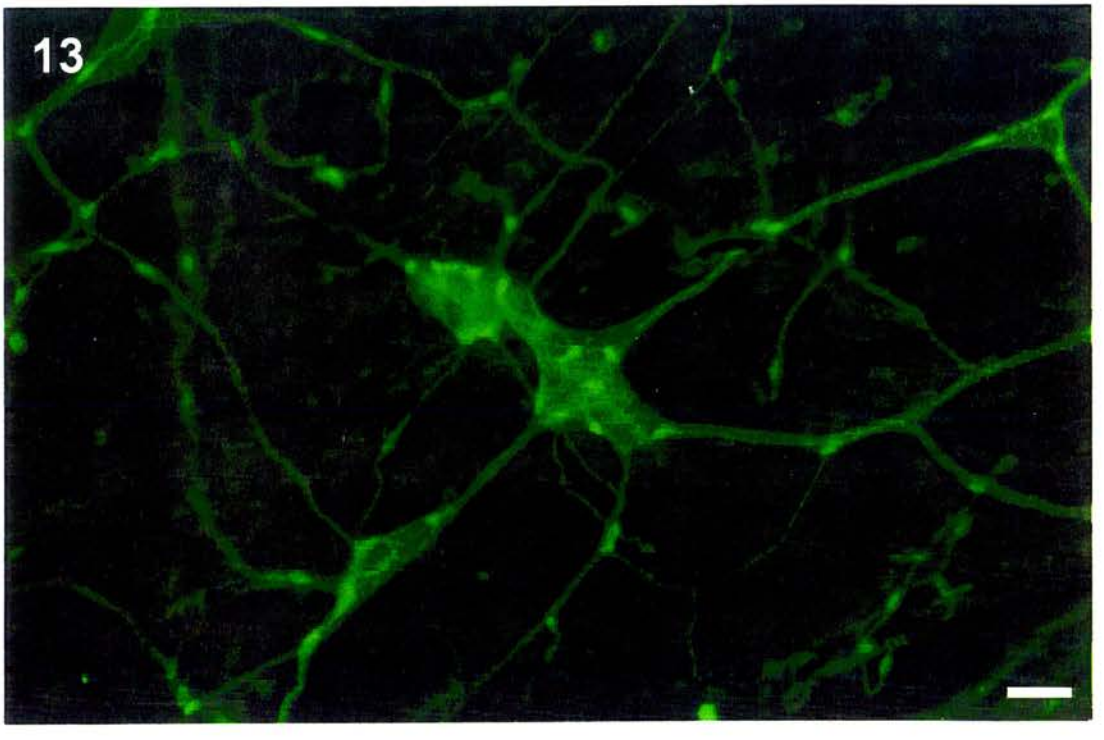


Figure 12. S100-immunoreactivity in enteric glial cells of the myenteric plexus of guinea pig jejunum. Bar = 25 µm.

Figure 13. S100-immunoreactivity in enteric glial cells of the submucous plexus of guinea pig jejunum. Bar = 50 µm.

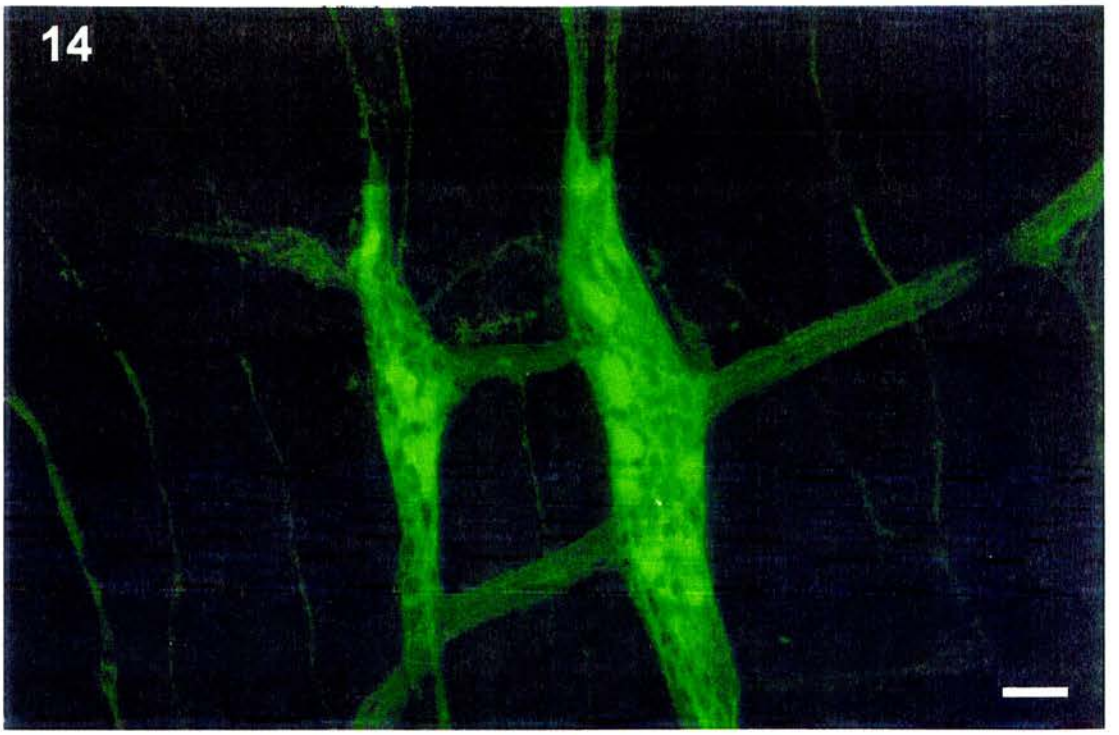
2.3.2 Guinea pig ENS cultures

2.3.2.1 Explant cultures

Explant cultures of both the MP and SMP were maintained for periods up to 5 days. The explants showed a pattern of immunoreactivity that was similar to the preparations that had not been cultured (Figures 14 and 15). Positive cellular immunoreactivity after varying periods *in vitro* was interpreted as being indicative of cellular viability. MP explants (attached including longitudinal muscle) showed signs of muscle contractions throughout the culture period.

2.3.2.2 Dissociated cell cultures

Dissociated cell culture systems were established using both the MP and SMP. These systems were maintained *in vitro* for periods up to 8 days. The neurons appeared to bed down in clumps (Figure 16) but some survived in isolation (Figure 17). There appeared to be a greater density of cells when systems were established using larger aliquots of the cellular suspension. For the range of antibodies, these cells showed similar patterns of immunoreactivity to the neurons in the preparations that had not been cultured. For example, VIP-immunoreactive neurons exhibited varicosities in their axons (Figure 18). After 2-3 days in culture, there was a marked proliferation of cells around these clumps. A proportion of these cells showed positive immunoreactivity for S100 (Figure 19A) and GFAP, making it most likely that these were enteric glial cells. The morphology of these cells was better appreciated using phase contrast microscopy (Figure 19B).



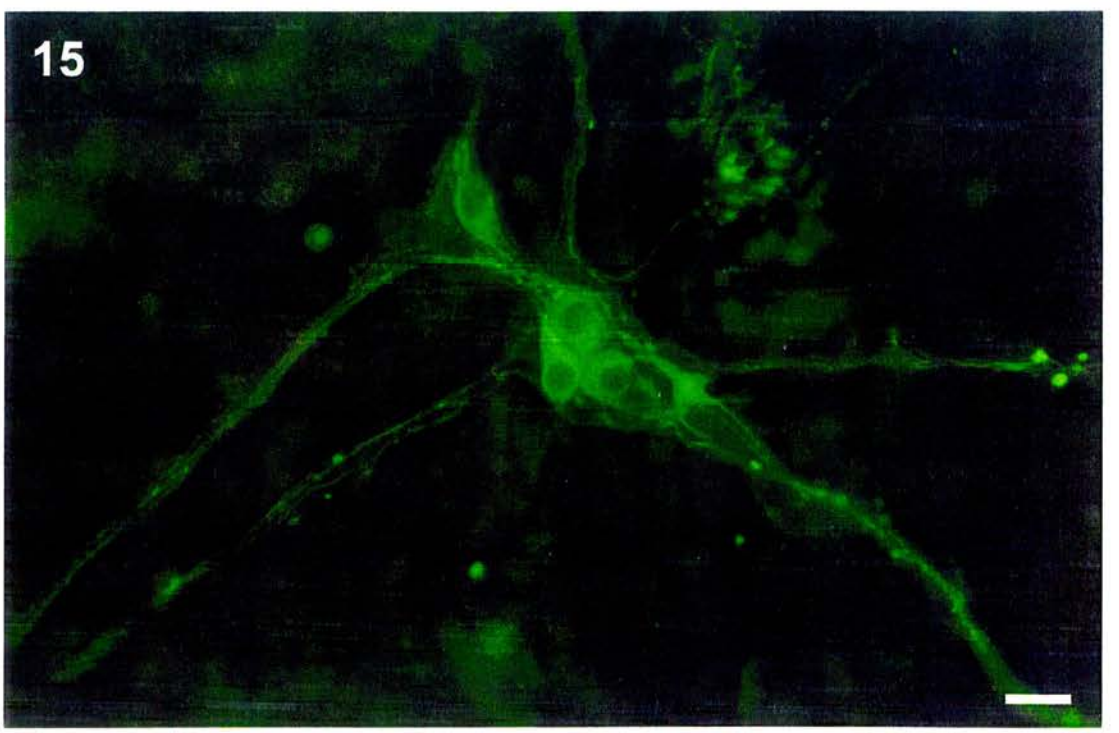


Figure 14. D45-immunoreactivity in nerve cells of the myenteric plexus of guinea pig jejunum (4-day cultured explant). Bar = 50 µm.

Figure 15. *Pan-N*-immunoreactivity in nerve cells and fibres of the submucous plexus of guinea pig jejunum (5-day cultured explant). Bar = 25 µm.

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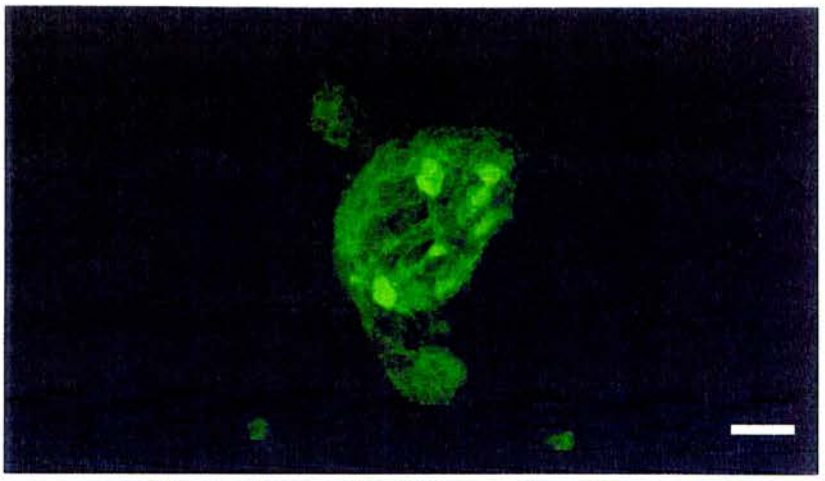

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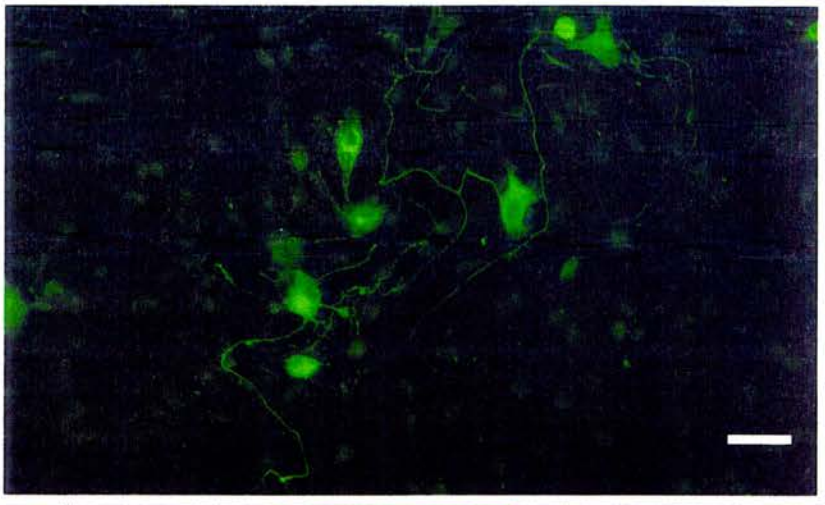

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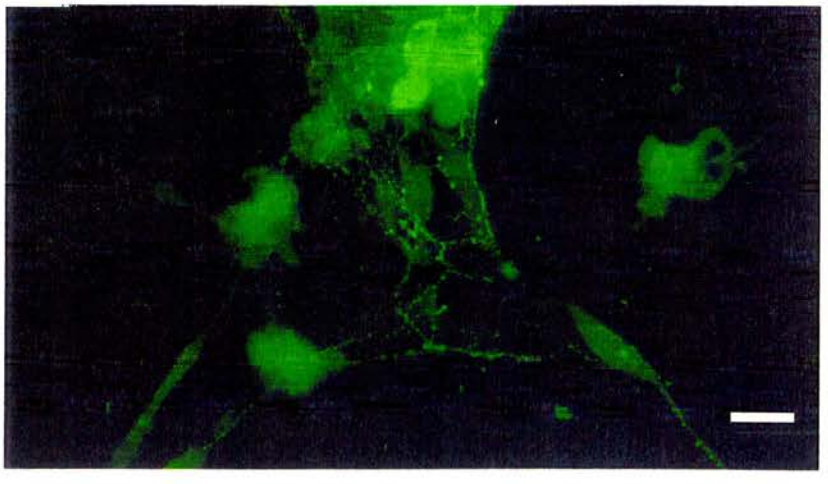


Figure 16. An aggregation of cells in a 2-day dissociated cell culture preparation from the myenteric plexus of guinea pig jejunum. D45-immunoreactivity is present in nerve cells. Bar = 50 µm.

Figure 17. Pan-N-immunoreactivity in nerve cells and fibres of the myenteric plexus of guinea pig jejunum. (6 days in dissociated cell culture). Bar = 50 µm.

Figure 18. VIP-immunoreactivity in nerve cells and varicose fibres of the submucous plexus of guinea pig jejunum. (4 days in dissociated cell culture). Bar = 25 µm.

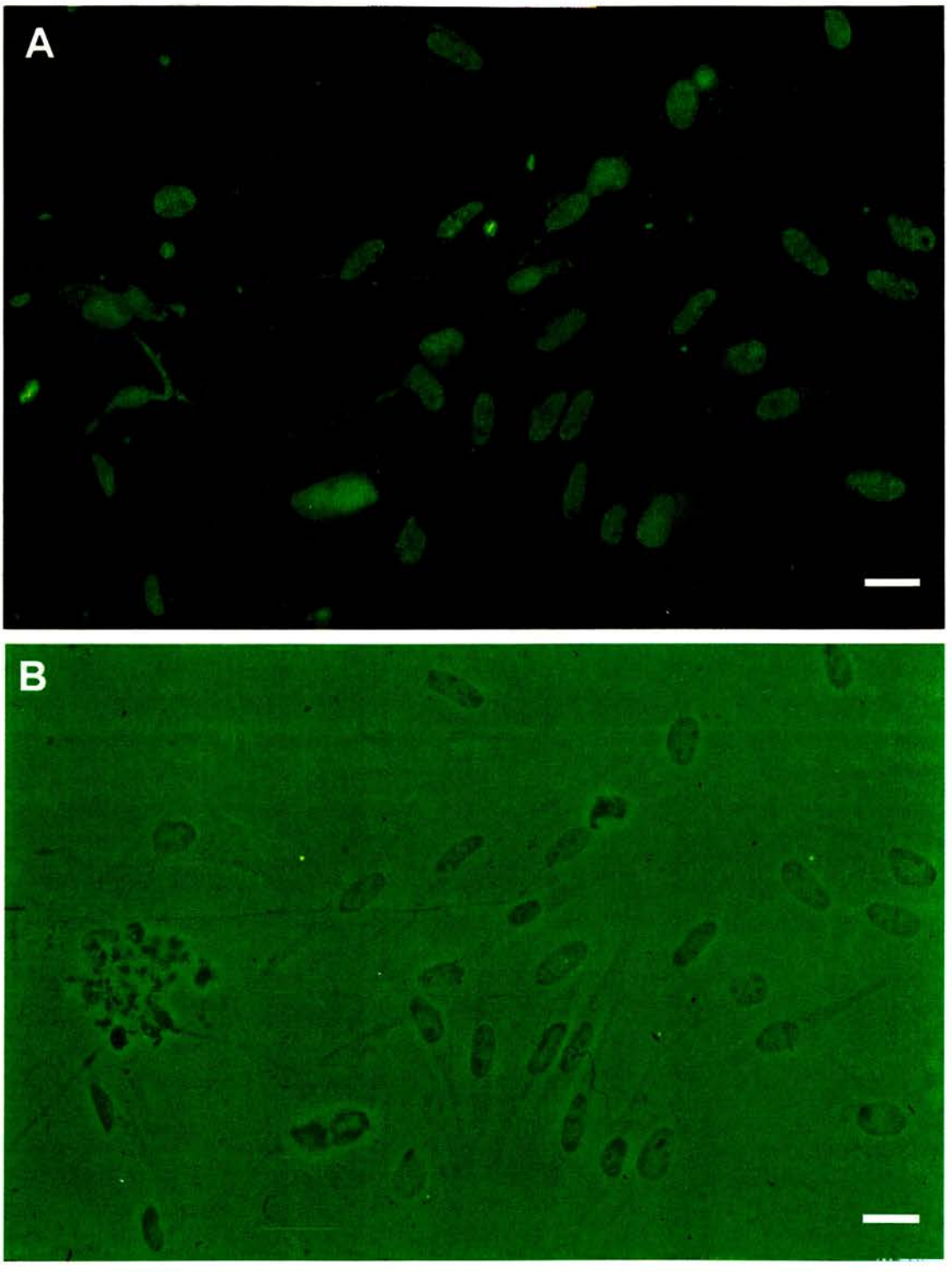


Figure 19. (A) S100-immunoreactivity in enteric glial cells of the submucous plexus of guinea pig jejunum. (5 days in dissociated cell culture). (B) Same field as (A) visualised in phase contrast. Bar = 25 µm.

2.3.3 Equine wholemount preparations

Wholemount preparations of the equine ENS were only successfully established using the SMP. There was a problem of antibody penetration in the preparations because of the marked thickness of the tissue. The ganglia had the appearance of "bunches of grapes." The most successful neuronal antibody was *Pan*-N which revealed immunoreactivity both in cell bodies and interconnecting strands (Figure 20). It was not possible to dissect out the MP from the muscle layers in the adult horse and the *muscularis externa* preparations were too thick to allow penetration of antibodies.

2.3.4 Equine ENS Cultures

2.3.4.1 Explant cultures

Explant cultures of the SMP and *muscularis externa* were maintained for 5 days. The SMP explants showed a pattern of neuronal immunoreactivity that was indistinguishable from the pattern seen in the preparations that had not been cultured. Positive cellular immunoreactivity in these 5-day-old cultures was interpreted as indicating the viability of these cells. It was not possible to examine the *muscularis externa* preparations immunohistochemically because of the marked thickness of the tissue. However, the explanted *muscularis externa* system was considered physiologically viable *in vitro* because there was still visible evidence of the muscle contracting 5 days after harvesting the tissue from the animal.

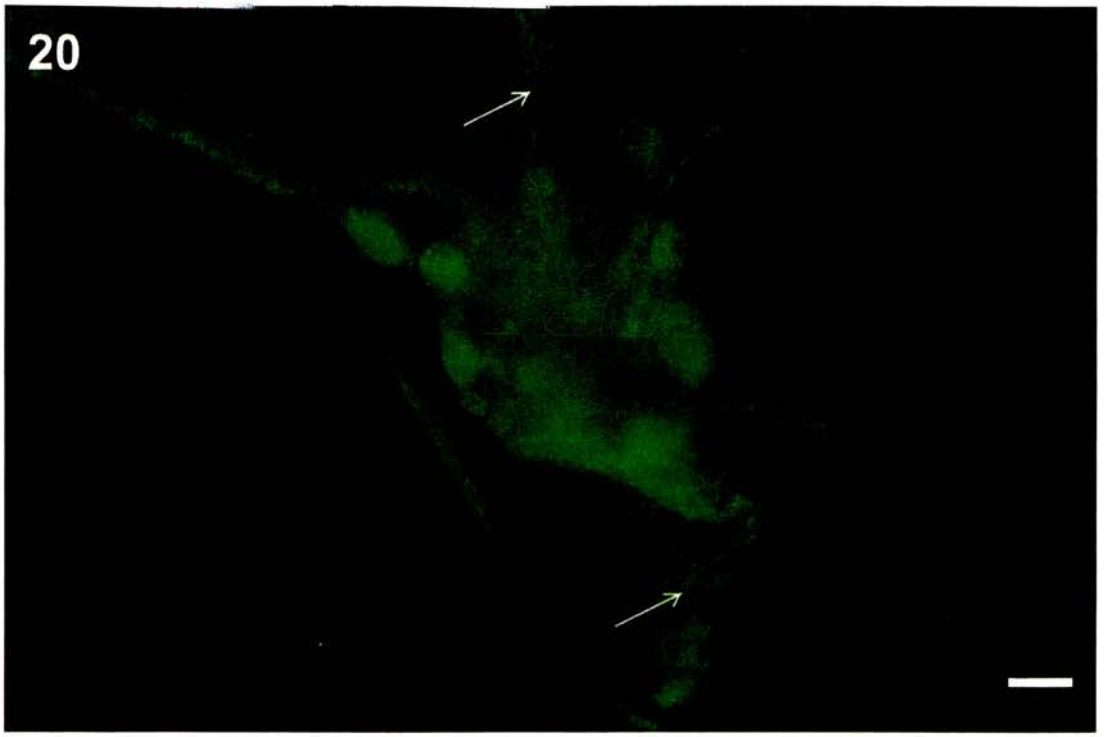
2.3.4.2 Dissociated cell cultures

At the time of the first change of culture medium (24 hours), it was clear that cells in the dissociated preparations had become attached to the coverslips. Although many individual cells could be seen, there were also up to six aggregations or clumps of cells present on each coverslip. This "bedding down" of the cells was only observed with suspensions derived from the SMP. Attempts to obtain a dissociated cell culture

from the *muscularis externa* were unsuccessful. After 2-3 days in culture, cell projections or processes became more evident and there was marked proliferation of cells around the clumps. The cultures were fixed at 5 days because of the increasing proliferation of these cells.

Immunoreactivity for the neuronal marker (*Pan*-N) was seen in the cytoplasm and processes of cells within the clumps (Figures 21 and 22A). This pattern of immunoreactivity was very similar to that seen in neurons of the submucous ganglia in wholemount preparations that had not been cultured.

The maximum number of *Pan*-N-immunoreactive cells counted on a coverslip was 50, with up to 15 in each clump of cells. The size of the aliquots of the cell suspensions plated on the coverslips did not seem to affect this seeded density. The cells that proliferated around the clumps did not show immunoreactivity for *Pan*-N (Figure 22) but many of these cells were immunoreactive for both S100 (Figure 23A) and GFAP. These non-neuronal cells were therefore considered to be enteric glial cells. The glial cell proliferation and cell morphology were best appreciated using phase contrast microscopy (Figure 23B). It should be noted that the field shown in Figure 23 was chosen from an area with fewer cells to illustrate better the cellular morphology of the enteric glia, and is by no means representative of the number of these swarming cells, which by far outnumbered the neuronal population.



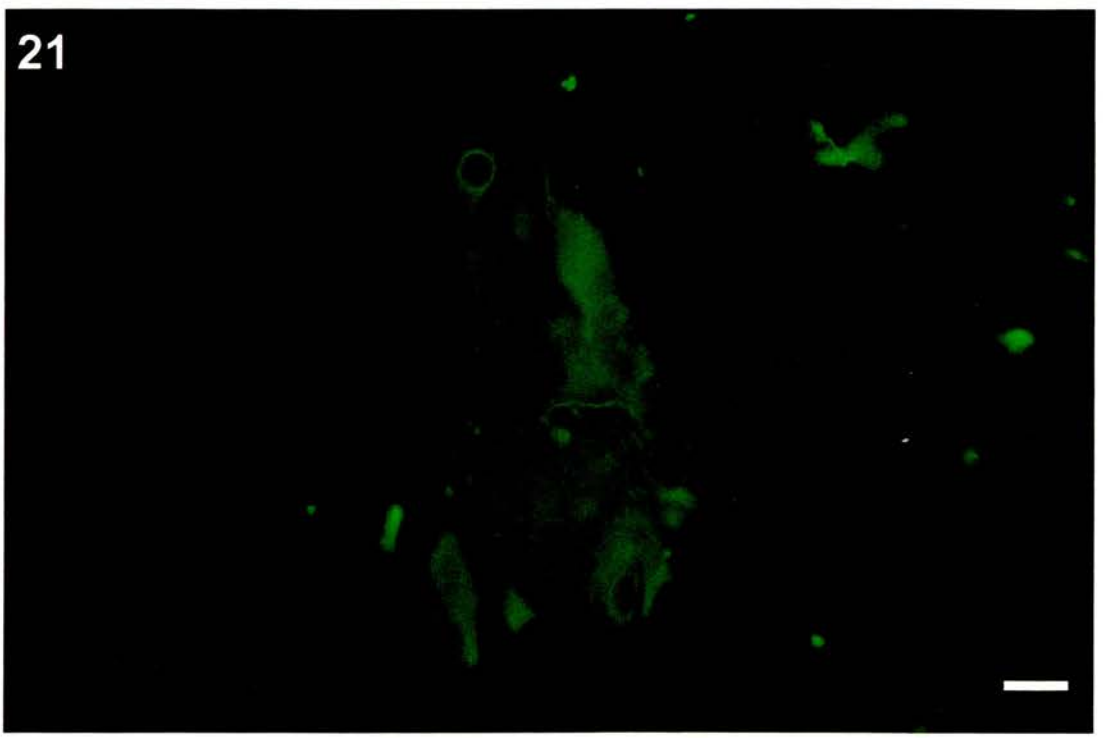


Figure 20. Pan-N-immunoreactivity in a ganglion of a wholemount preparation (not cultured) of the submucous plexus of equine ileum. Immunoreactivity can be seen both in cell bodies and interconnecting strands (arrows) extending away from the ganglion. Bar = 25 µm.

Figure 21. An aggregation of cells in a 5-day dissociated cell culture preparation from the submucous plexus of equine ileum. Pan-N-immunoreactivity is present in the cytoplasm and processes of the nerve cells. Bar = 25 µm.

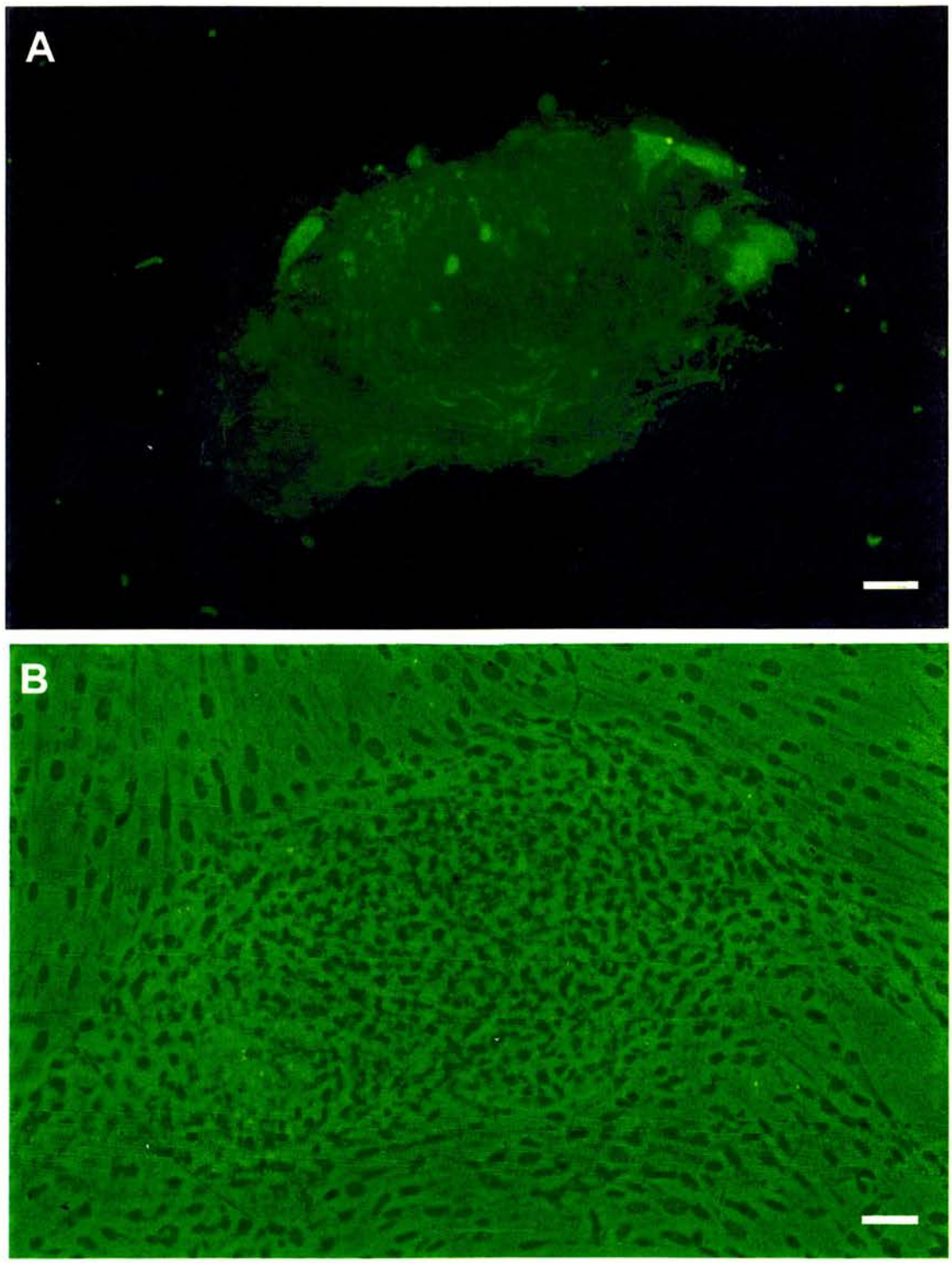


Figure 22. (A) An aggregation of cells in a 5-day dissociated cell culture preparation from the submucous plexus of equine ileum. Pan-N-immunoreactivity is present in the cytoplasm and processes of the nerve cells. (B) Same field as (A) viewed in phase contrast showing non-neuronal cell proliferation around the aggregation of cells. Bar = 50 µm.

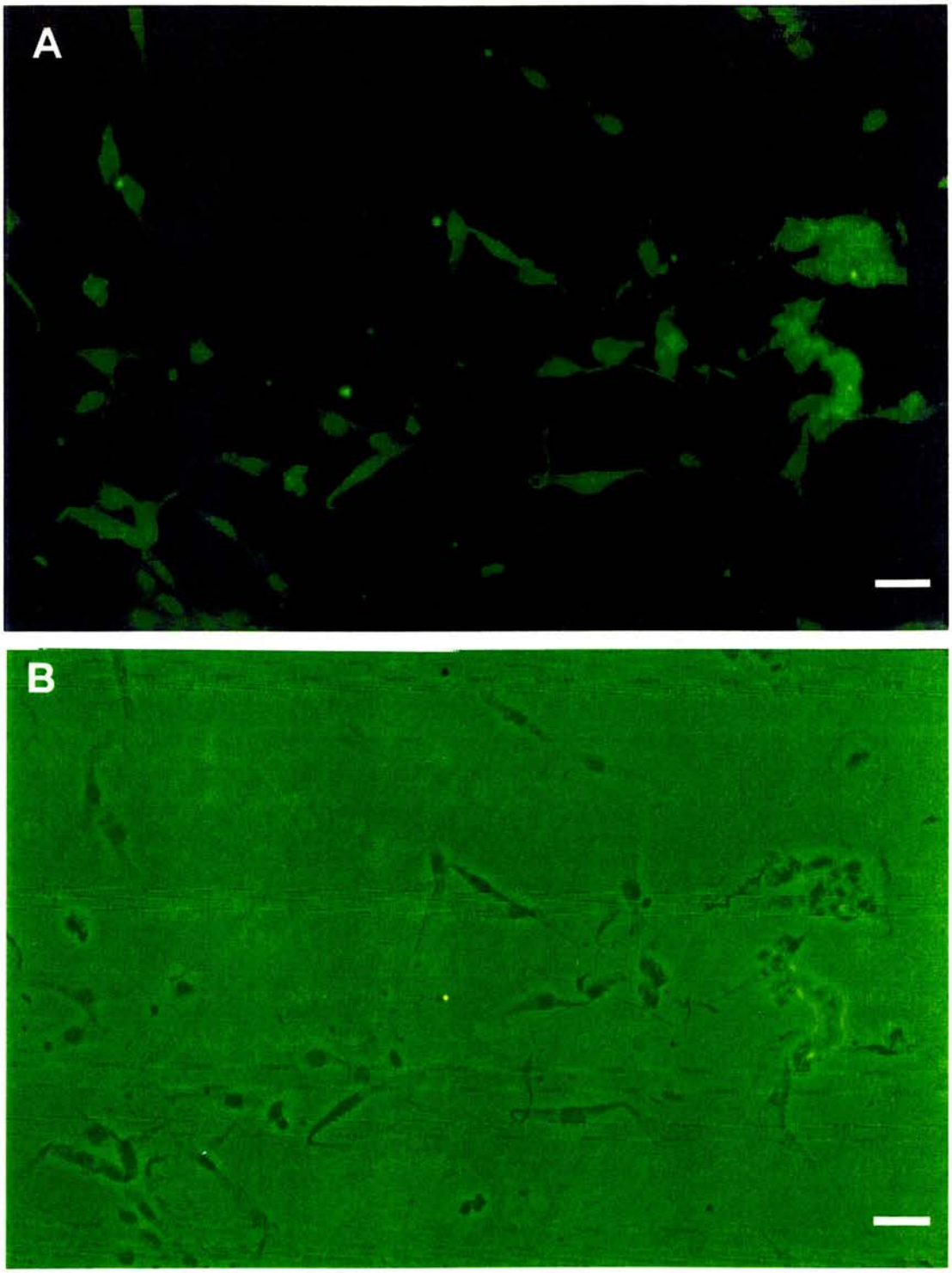


Figure 23. (A) S100-immunoreactivity in enteric glial cells of the submucous plexus of equine ileum (5 days in dissociated cell culture). (B) Same field as (A) visualised in phase contrast. Bar = 50 µm.

2.3.5 Rat ENS cultures

Dissociated cell cultures were established from rat MP and maintained for 2 days. The time of fixation of the cultures was arbitrary. The neurons appeared rounded with axons extending varying lengths from the cell body. The neurons were identified by their positive immunoreactivity for the general neuronal marker, PGP 9.5 (Figure 24). There were numerous non-neuronal cells in the culture system that were flatter in appearance and were not immunoreactive for PGP 9.5. The neuronal density was much higher than the densities achieved in the guinea pig or equine systems.

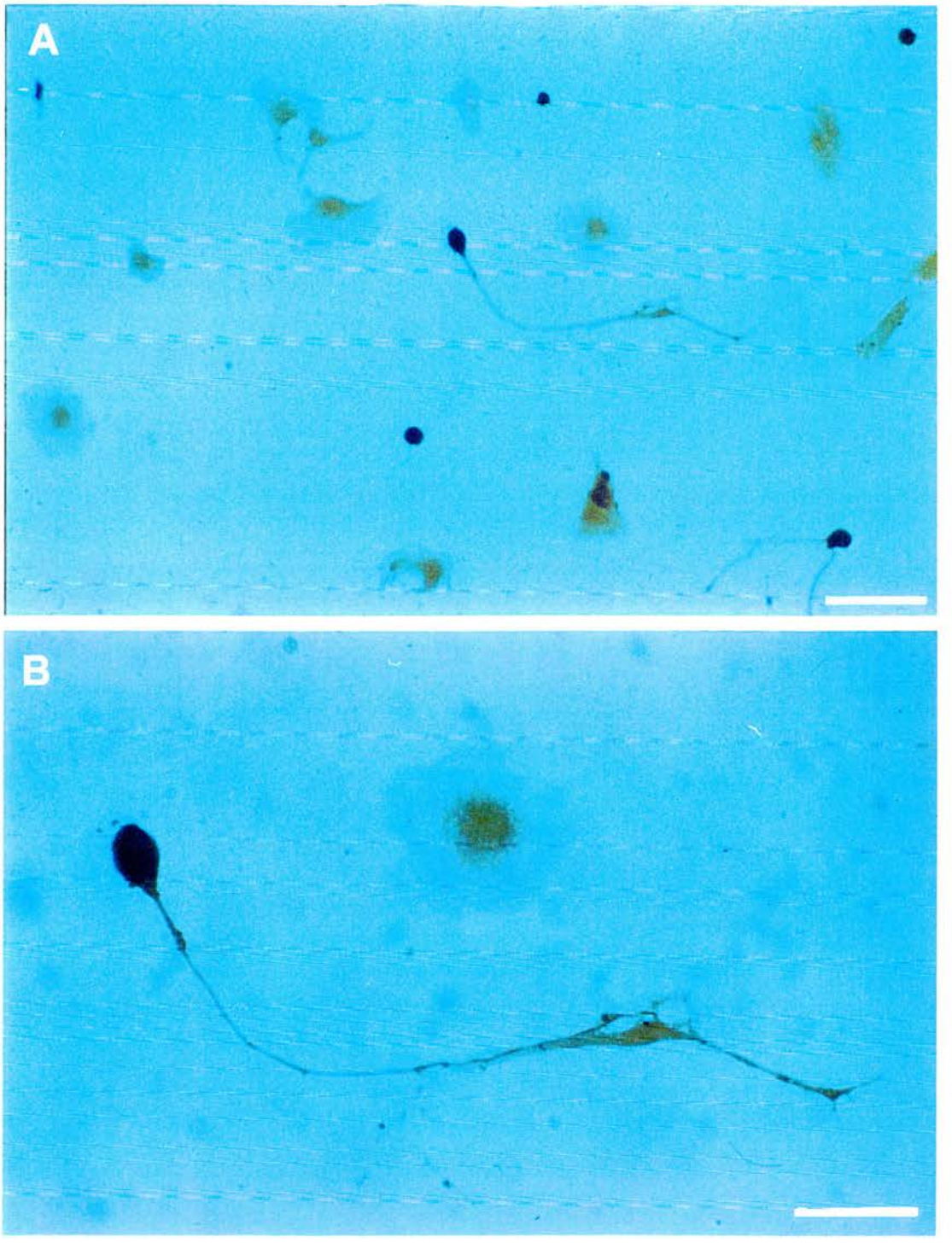


Figure 24. (A) PGP 9.5-immunoreactivity in neurons of the myenteric plexus from rat small intestine (2 days in dissociated cell culture). Bar = 50µm. (B) Same field as (A) at higher magnification. Bar = 25µm.

2.4 Discussion

This study aimed to establish immunohistochemical and tissue culture preparations of the equine ENS using small mammal models for comparison and the development of techniques. In the guinea pig, wholemount preparations of the MP and SMP were established and these were useful in examining the structure and characteristics of the ENS. It was also possible to establish *in vitro* preparations (explant and dissociated cell systems). The neuronal yield was relatively low in the dissociated cell systems.

In the horse, wholemounts were only possible using the SMP, and even in this system the problem of poor antibody penetration was evident. Sections may therefore be preferable when examining adult equine tissue (Cummings *et al.,* 1984; Kotze and Van Aswegen, 1990; Burns and Cummings, 1993), despite the loss of a degree of three-dimensional morphology.

It was only possible to establish equine dissociated culture preparations using the SMP. Explant preparations were possible both in the SMP and *muscularis externa*, although in the latter it was not possible to dissect out and examine the MP. There was good correlation between the neuronal morphology in the cultured preparations and the preparations that had not been cultured. To the best of my knowledge, this is the first report of equine enteric neurons being grown successfully in tissue culture. The ileum was chosen for this study because it has been shown to be the region of intestine most severely affected in grass sickness (Scholes *et al.*, 1993a) and this is the disease for which this *in vitro* model was being designed.

In the present study, neurons and enteric glia were identified in the cultures which were fixed at 5 days because of the increasing proliferation of non-neuronal cells. The proliferation of the non-neuronal cells appeared to be much greater in the horse compared to the guinea pig. Many of the non-neuronal cells were glial and it is likely, when comparing this work with other studies, that the remaining cells were fibroblasts (Saffrey *et al.*, 1992a; Hanani, 1993). In this study, the equine enteric glia, or the supporting cells of the ENS, showed positive immunoreactivity for both GFAP

and S100 which concurs with findings in other species (Jessen and Mirsky, 1980; Bannerman *et al.*, 1987; Gershon and Rothman, 1991). It may be possible to extend the culture period in future studies because the numbers of these non-neuronal cells can be manipulated in culture. Further work will be necessary however, to increase the *in vitro* yield of neurons in our equine system from the present maximum of only 50 neurons per coverslip.

The rat dissociated ENS cultures established at the Open University showed that neonatal small mammal models were superior to the equine and adult guinea pig experiments in this study. This was largely due to the increased accessibility to the plexus in the intestine and the consequent increase in cellular yield. A comparison of 50 neurons per coverslip in the equine system to $1.5x10^4$ total viable cells per coverslip in the rat system highlights the poor yield from the large animal system. This means that the intestine of the adult horse is not suitable for the establishment of a repeatable and quantifiable ENS culture model that would be amenable to analysis and *in vitro* manipulation. This is unfortunate, as this was the aim in examining the equine ENS in the grass sickness setting. An alternative approach of using foetal and neonatal equine tissues could be suggested, but this would not be feasible because of the limited supply of such tissues.

This study has shown some of the *in vitro* and immunohistochemical characteristics of the equine ENS. However, the difficulties in accessing and harvesting the neurons indicate that further work will be necessary in order to establish an *in vitro* model amenable to the quantitative analysis of diseases such as grass sickness. The rat system of the Saffrey laboratory could be useful in examining putative toxins involved in grass sickness even though it is in a different species to the horse. It was decided at this point however, because of the poor neuronal yield from the equine system, that the tissue culture approach would not be pursued further.

CHAPTER 3:IMMUNOHISTOCHEMICAL EVALUATION OF THE INTERSTITIAL CELLS OF CAJAL IN NORMAL AND GRASS SICKNESS-AFFECTED HORSES

3.1 Introduction and aims

The emphasis of the thesis at this point switched to examining the interstitial cells of Cajal (ICC) and the electrical properties of the intestine that these cells generate. It was felt that this approach would lead to the desired *in vitro* equine system for examining further the intestine in health and in disease.

The purpose of this part of the study was to examine the distribution and characteristics of the interstitial cells of Cajal (ICC) in the equine gastrointestinal tract. The next stage was to examine the ICC in equine grass sickness to determine if there were any changes that might explain the development of intestinal stasis in affected horses.

c-Kit immunohistochemistry was used to examine the ICC in normal horses and a semi-quantitative grading system employed to assess any differences between normal and diseased animals.

3.2 Materials and Methods

3.2.1 Immunohistochemical study of ICC in the normal equine gastrointestinal tract

Gastrointestinal samples were taken within one hour of death from 27 "normal" (control) horses euthanased because of clinical conditions not involving the gastrointestinal tract (eg orthopaedic problems). The horses ranged from 16 weeks to 36 years of age (mean, 10.3 years; median, 8 years). The animals were euthanased by intravenous administration of quinalbarbitone sodium BP (400 mg/ml)/cinchocaine hydrochloride BP (25 mg/ml) (Somulose; Arnolds Veterinary Products, UK). In four of the horses, samples were taken from 13 anatomically-defined sites from the stomach through to the small colon (Table II). In three horses, samples were taken

from the duodenum, ileum and pelvic flexure, in eight from the ileum and pelvic flexure, in ten from the ileum alone and in two from the jejunum only.

Table II
Anatomical positions of the sample sites in the equine gastrointestinal tract

REGION	SAMPLE SITE
1. Stomach	Midpoint of greater curvature
2. Stomach	Pyloric canal
3. Duodenum	Midpoint of descending duodenum
4. Jejunum	Midpoint of the small intestine
5. Ileum	Level with the midpoint of the ileocaecal fold
6. Caecum	Midpoint of body including medial taenial band
7. Caecum	Midpoint of the caecal base
8. Right ventral colon	Midpoint, including medial free band
9. Left ventral colon	Midpoint, including medial free band
10.Pelvic flexure	Apex
11.Left dorsal colon	Midpoint, antemesenteric aspect
12.Right dorsal colon	Midpoint, including dorsomedial taenial band
13.Small colon	Midpoint, including free taenial band

All tissues were rinsed with phosphate-buffered saline (PBS, 0.1 M, pH 7.0) and placed immediately in 10% phosphate-buffered formalin (pH 7.4) and fixed for at least 24 hours. After rinsing in distilled water, the tissues were cryo-protected in graded sucrose solutions (10% and 30% sucrose in PBS) and then frozen rapidly in isopentane pre-cooled in liquid nitrogen. Before sectioning, the tissue blocks were oriented to allow either transverse or longitudinal sections of the entire intestinal wall to be cut. Cryostat sections were cut at thicknesses of 10μm-30μm and mounted on Tespa (3-aminopropyltriethoxysilane)-coated slides (Sigma, Poole, UK) and allowed to air-dry overnight.

After a thorough wash with PBS, the sections were incubated for 30 minutes in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Non-specific antibody binding was blocked by a 60-minute incubation in 1% goat serum in PBS. Sections were then incubated overnight at 4°C in humid chambers in a

rabbit-raised polyclonal antiserum to c-Kit (Ab-1), (Oncogene Research Products, Cambridge, MA, USA) at a concentration of 1μg/ml. After washing with PBS, the sections were then incubated at room temperature for 60 minutes in a biotin-conjugated goat anti-rabbit immunoglobulin at a concentration of 1:200. Immunoreactivity was revealed with the avidin-biotin (ABC) method (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) using a diaminobenzidine substrate (DAB, BDH Laboratory Supplies, Poole, UK). Sections were dehydrated in ethanol, cleared in xylene, and then mounted in Depex (Merck, Glasgow, UK). Immunohistochemical controls were prepared in a similar manner, omitting the primary antibody or replacing it with normal rabbit serum. This led to the complete absence of immunolabelling. In some experiments, frozen sections were stained with toluidine blue to demonstrate metachromatically-stained mast cells. It should be noted that the antiserum to c-Kit used in this study was raised against antigens that were originally isolated from species (human) other than the horse. Therefore, statements used below such as "positive immunoreactivity for c-Kit" would be more accurately given as "c-Kit-like"-immunoreactivity.

3.2.2 Semi-quantitative evaluation of normal compared to diseased tissue

For this part of the study, comparisons were made between intestine taken from normal (control) horses and from horses suffering from grass sickness. Ileal samples were taken from 24 normal horses ranging in age from 6 weeks to 36 years (mean, 9.6 years; median, 7.8 years) and compared with ileal samples taken from 28 horses with grass sickness (11 acute, 9 subacute, 7 chronic and 1 chronic recovered with a subsequent acute episode) ranging in age from 8 months to 18 years (mean, 5.9 years; median, 5 years). Samples of pelvic flexure (colon) were collected from 13 normal horses ranging in age from 6 weeks to 36 years (mean, 10 years; median, 7.5 years) and compared with pelvic flexure samples taken from 10 horses with grass sickness (4 acute, 4 subacute and 2 chronic) ranging in age from 2 to 9 years (mean, 5.4 years; median, 5 years). The samples were taken from a similar anatomical location for each animal as outlined in Table II: ileum was taken level with the midpoint of the ileocaecal fold and the pelvic flexure at its apex. The pelvic flexure

in the horse is the junction of the left ventral colon with the left dorsal colon. The classification of categories of grass sickness (acute, subacute and chronic) was made on clinical grounds according to the nomenclature used by the University of Edinburgh equine clinicians (Doxey *et al.*, 1991). The diagnosis of grass sickness was confirmed in all cases by histopathological examination of the ileum and autonomic ganglia.

The samples were processed for c-Kit immunohistochemistry as described above and the 10 μm sections were assessed by two independent observers. The myenteric region (MP) and circular muscle layers were assigned grades according to the density of c-Kit-immunoreactivity. ICC density (assessing immunoreactivity both in cell bodies and processes) was therefore subjectively classified as absent (0), sparse (1), moderate (2) and abundant (3){see Figures 35-37 in the results section for representative grades}. The grading for each animal was based on the scrutiny of at least four adjacent 10 μm sections of tissue. In the event of disagreement of classification between the two observers, a consensus grade was assigned after a joint review of the sections.

Mann-Whitney tests corrected for ties were used to compare results between normal and diseased animals and between AGS and CGS categories of disease. Kruskal-Wallis tests corrected for ties were used for comparison of > 2 groups (for example, categories of grass sickness). Comparison of the results from the two independent observers was performed using a one sample Wilcoxon test. For all tests, a probability of less than 0.05 was considered to be significant. Results were reported as means ± S.E.M. and medians.

3.3 Results

3.3.1 ICC distribution and characteristics in normal horses

Cells possessing c-Kit-immunoreactivity (c-Kit-I) were identified in several locations throughout the gastrointestinal tract of all the horses studied. Spindle- or triangular-

shaped cells within the intermuscular space between the circular and longitudinal muscle layers at the level of the myenteric plexus (MP), stellate or bipolar cells located within the circular or inner smooth muscle layer (Figures 25 and 26), and round cells in the submucosa were all identified as being c-Kit-immunoreactive cells. The cells in the intermuscular space usually had 1-2 processes whilst the intramuscular cells had 2-5 processes. The different orientation of the sections showed that the cells had a preferential position in one plane and direction, giving them a long axis parallel to the direction of the fibres of the neighbouring smooth muscle. The round cells with c-Kit-I in the submucosa (Figure 27) in all regions of the gastrointestinal tract were recognised as mast cells (Galli *et al.,* 1993) on the grounds of their differing (round) morphology and by comparing experimental sections with toluidine blue-stained sections in which they showed distinct metachromatically-stained granules (Figure 28).

3.3.1.1 Stomach

Many stellate cells and portions of cellular processes with c-Kit-I were found in the inner muscle layer of the greater curvature of the stomach (Figure 29). Occasional immunoreactive cellular processes were seen in the outer muscle layer. There was evidence of immunoreactivity along the border of the inner and outer muscle layers (Figure 30). A similar pattern was seen in the pyloric canal region of the stomach.

3.3.1.2 Small intestine

Cells with c-Kit-I were found in highest numbers in the intermuscular space between the circular and longitudinal muscle layers of the *muscularis externa* in the region of the MP (Figure 31). These cells formed dense networks which sometimes extended a short distance into the longitudinal muscle layer. Some bipolar and stellate cells were found throughout the circular muscle layer, especially in the inner third (Figures 32 and 33). The same patterns of distribution were found in the duodenum, jejunum and ileum but the density of cells with c-Kit-I in the region of the MP appeared greatest

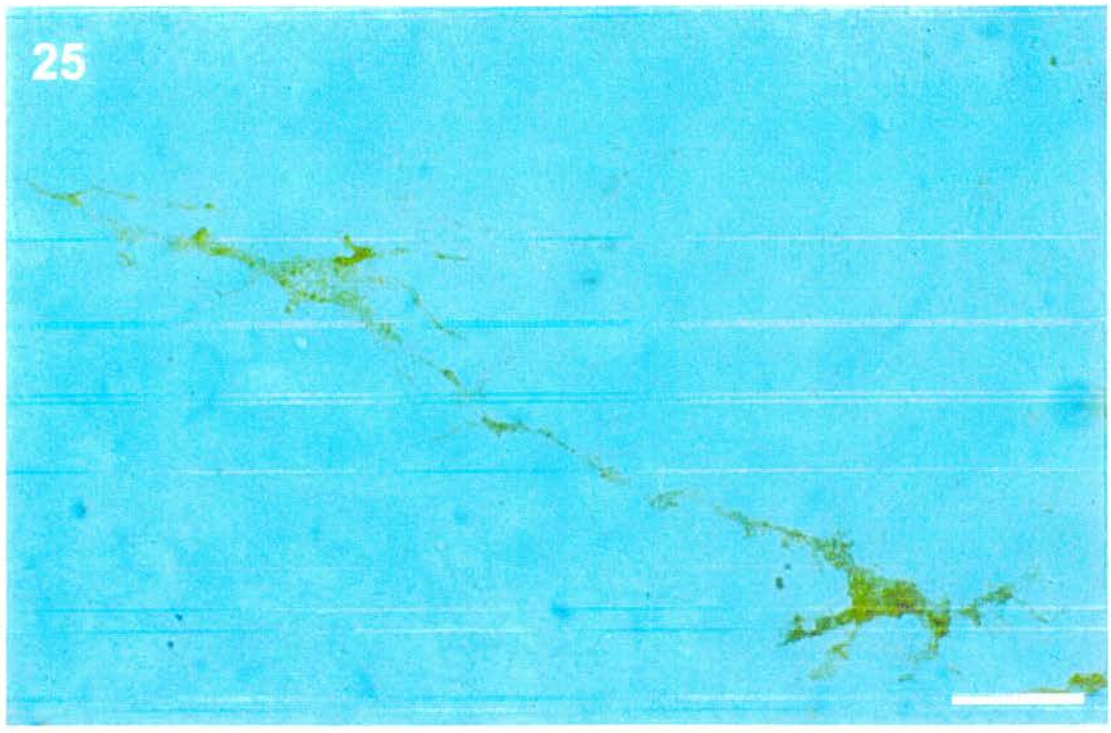
in the ileum, with traces of immunoreactivity spreading further into the longitudinal muscle layer.

3.3.1.3 Caecum

In contrast to the small intestine, large numbers of stellate and bipolar cells with c-Kit-I were found throughout the circular muscle layer of the caecum (Figure 34). Furthermore, very few cells were seen at the region of the MP, producing a delicate lace-like pattern that was different to the denser pattern seen in the small intestine. Occasional traces of immunoreactivity were seen in cellular processes in the longitudinal muscle layer. The pattern of distribution of c-Kit-immunoreactive cells was identical in both the mid-caecum and caecal base, but the density of cells was far greater in the MP and circular muscle regions in the mid-caecum than in the caecal base.

3.3.1.4 Colon

Many stellate and bipolar cells and cell processes with c-Kit-I were seen throughout the circular muscle layer. As in the caecum, the density of cells in the circular muscle layer was much greater than in any region of the small intestine. A similar lace-like pattern of immunoreactivity to that seen in the caecum was seen in the region of the MP and this was very different to the dense branching network seen in the small intestine. There were occasional cell processes found in the longitudinal muscle layer. In sections taken to include taenial bands there appeared to be more cells in the circular muscle and an increased density of the MP pattern underneath the taenial bands. Identical patterns of distribution were seen in the right ventral colon, left ventral colon, pelvic flexure, left dorsal colon, right dorsal colon and small colon. However, the density of the cells in the circular muscle of the pelvic flexure appeared greater than in other parts of the colon. Furthermore, the density of the network in the region of the MP of the right ventral colon appeared to be less than in other parts of the colon.



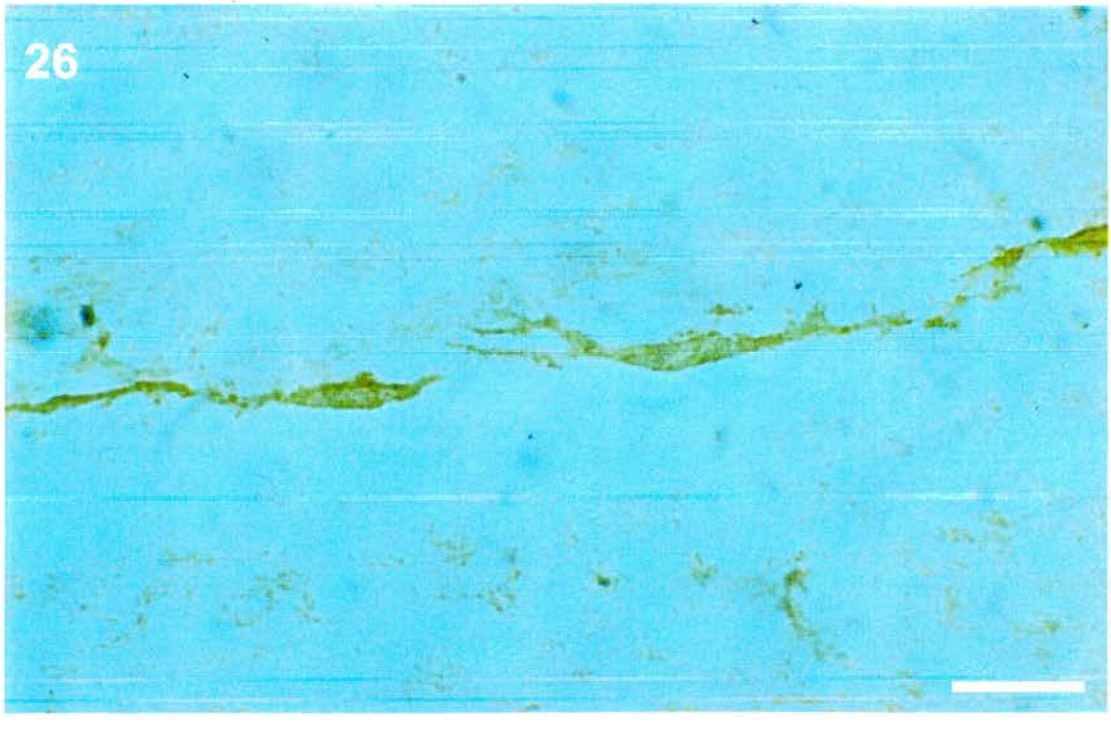
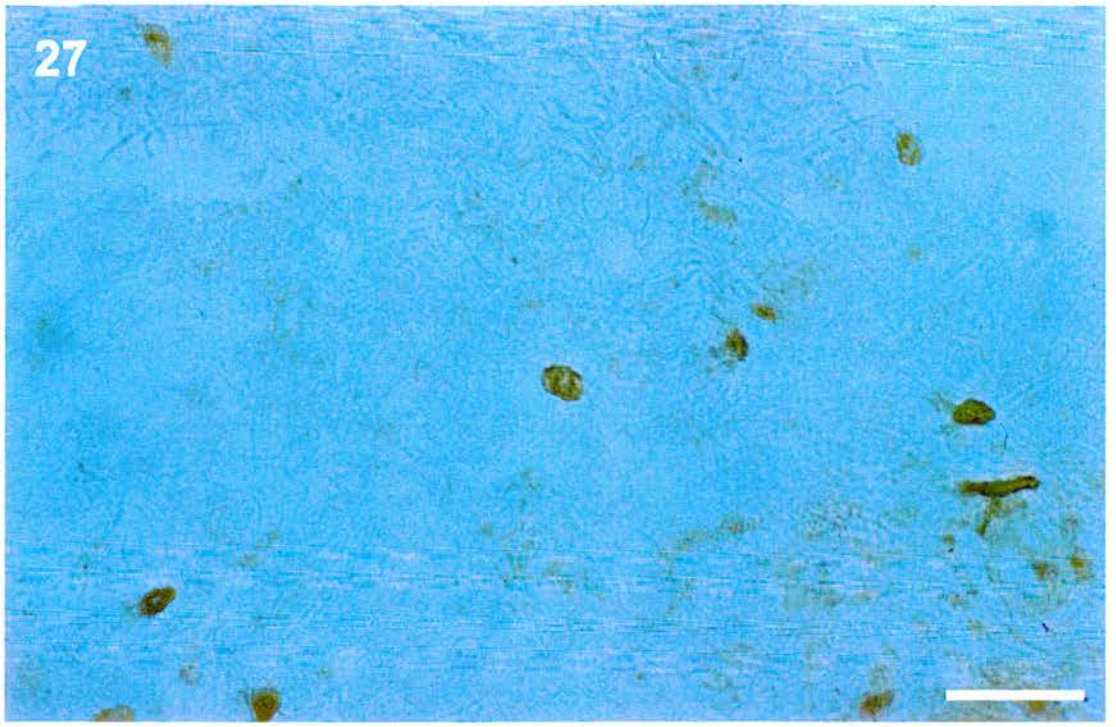


Figure 25. Stellate-shaped ICC with c-Kit-immunoreactivity within the circular muscle layer of normal equine left dorsal colon. Bar = 25µm.

Figure 26. Bipolar-shaped ICC with c-Kit-immunoreactivity within the circular muscle layer of normal equine left ventral colon. Bar = 25µm.



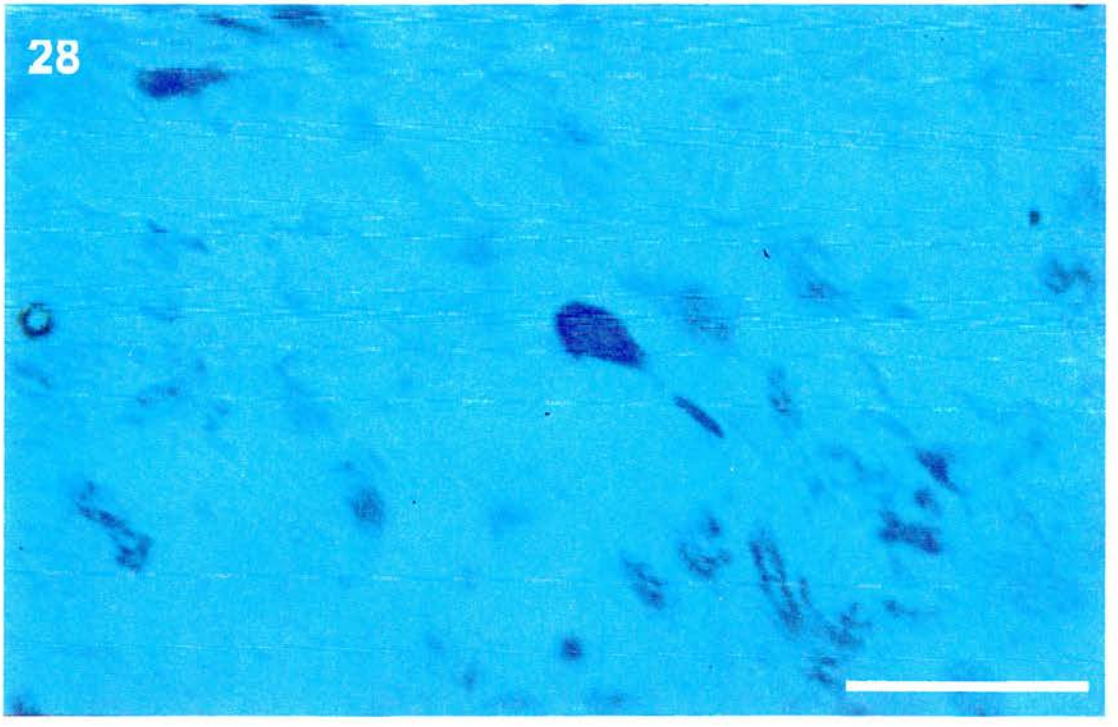


Figure 27. Round-shaped mast cells with c-Kit-immunoreactivity in the submucosal region of normal equine ileum. Bar = 50µm.

Figure 28. Mast cell in the submucosal region of normal equine ileum. Toluidine blue stain showing metachromatically-stained granules. Bar = 25µm.

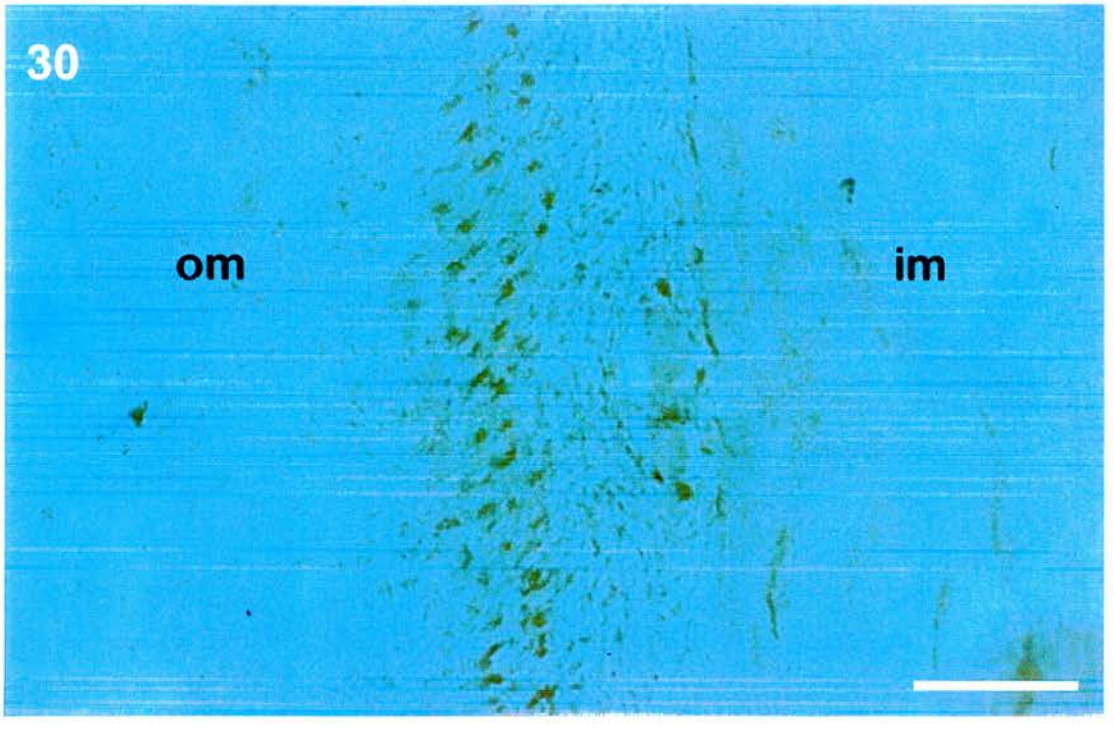


Figure 29. Stellate-shaped ICC with c-Kit-immunoreactivity throughout the inner muscle layer of the greater curvature of normal equine stomach. Bar = 100µm.

Figure 30. c-Kit-immunoreactivity in the border region between the inner (im) and outer (om) muscle layers of the greater curvature of normal equine stomach. Bar = 100µm.

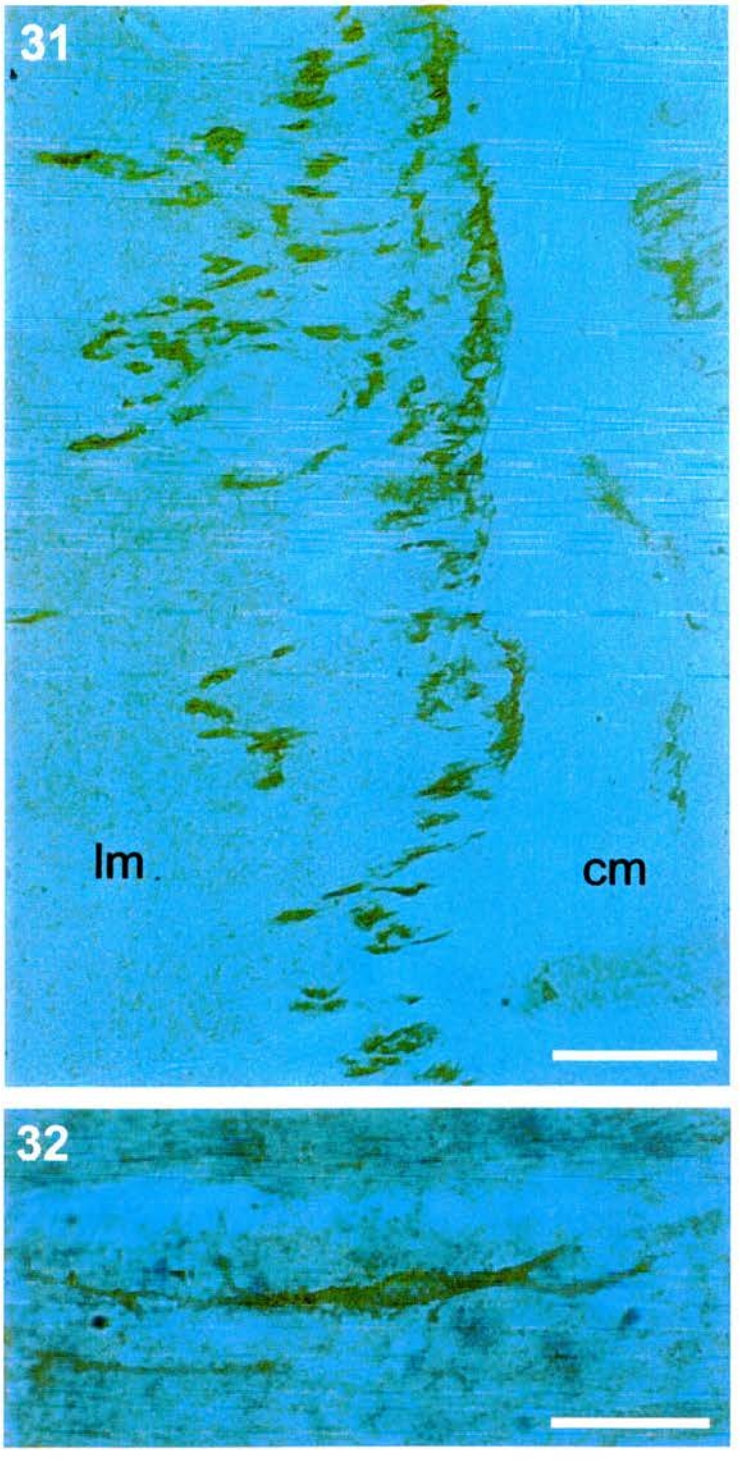
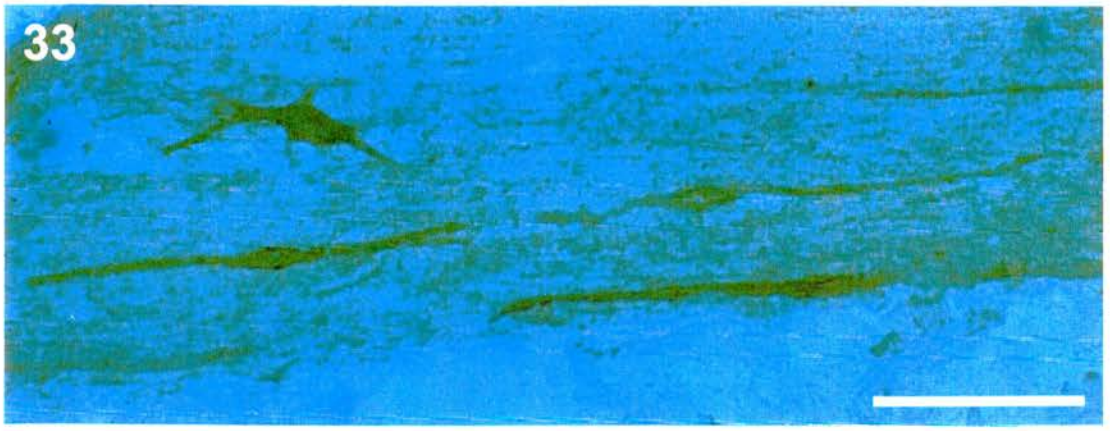


Figure 31. ICC with c-Kit-immunoreactivity within the intermuscular space between the circular (cm) and longitudinal (lm) muscle layers at the level of the myenteric plexus of normal equine ileum. Bar = 100µm.

Figure 32. A bipolar-shaped ICC with c-Kit-immunoreactivity within the circular muscle layer of normal equine duodenum. Bar = 25µm.



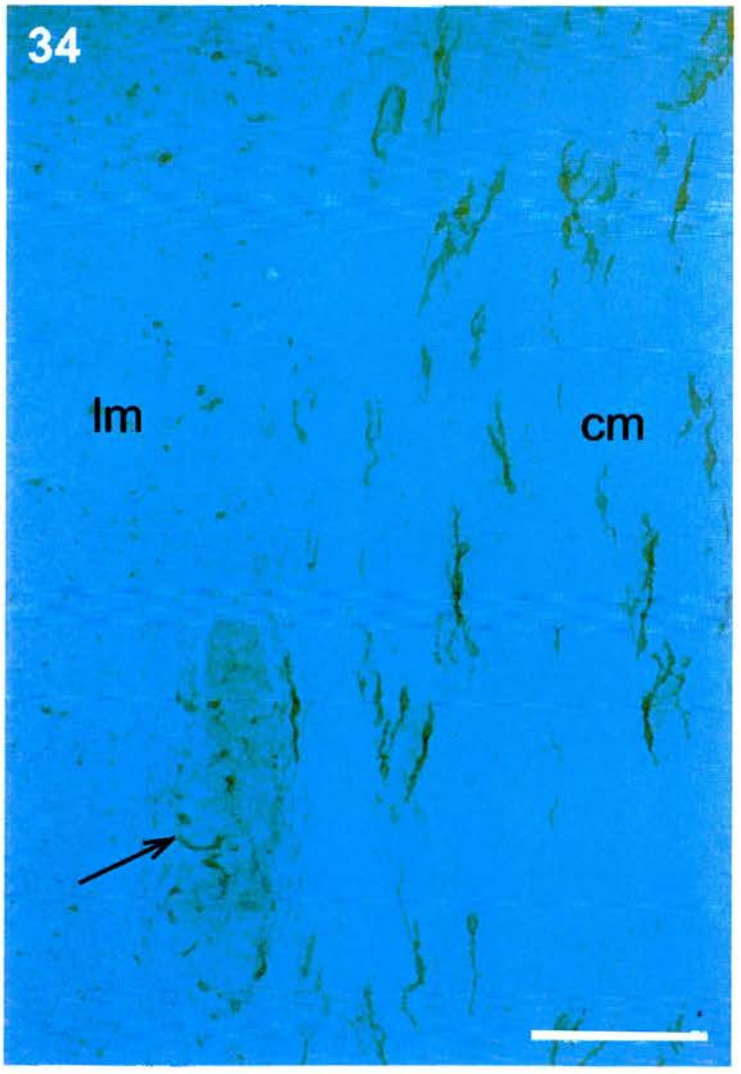


Figure 33. Bipolar- and stellate-shaped ICC with c-Kit-immunoreactivity within the circular muscle layer of normal equine duodenum. Bar = 50µm.

Figure 34. Stellate- and bipolar-shaped ICC with c-Kit-immunoreactivity throughout the circular (cm) muscle layer of the body of normal equine caecum. Note the lace-like pattern of immunoreactivity (arrow) at the level of the myenteric plexus. lm = longitudinal muscle. Bar = 100µm.

3.3.2 ICC in grass sickness cases: comparison with normals

In both normal and diseased tissues, ICC were evident with similar distribution and morphological characteristics in the ileum and pelvic flexure to those described above. In horses with grass sickness (acute, subacute and chronic), ICC were present to varying degrees, indicating that there was not a total absence of these cells. Figures illustrating the semi-quantitative grading scale are shown (Figures 35-37). However, differences were noted between the diseased animals and normal animals (Figures 38-41; Tables III and IV) and these are described below. When comparing the consistency of scoring between the 2 observers, no significant difference was present ($P = 0.211$) showing that there was good inter-observer agreement.

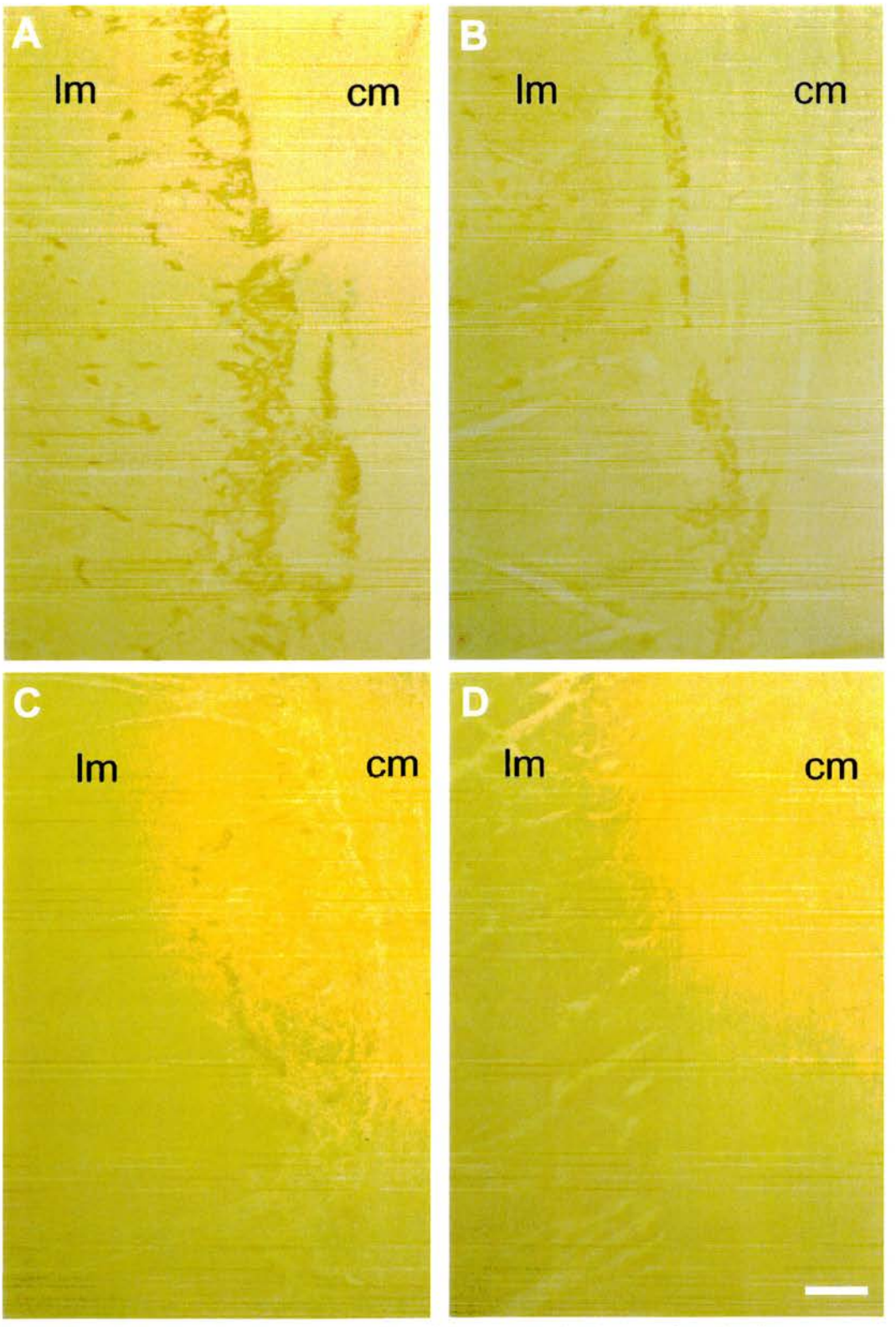


Figure 35. ICC density grades in the region of the myenteric plexus of equine ileum.
lm = longitudinal muscle; cm = circular muscle.
(A) Grade 3 in normal ileum.
(B) Grade 2 in normal ileum.
(C) Grade 1 in ileum from a horse with acute grass sickness.
(D) Grade 0 in ileum from a horse with acute grass sickness. Bar = 100 μm.

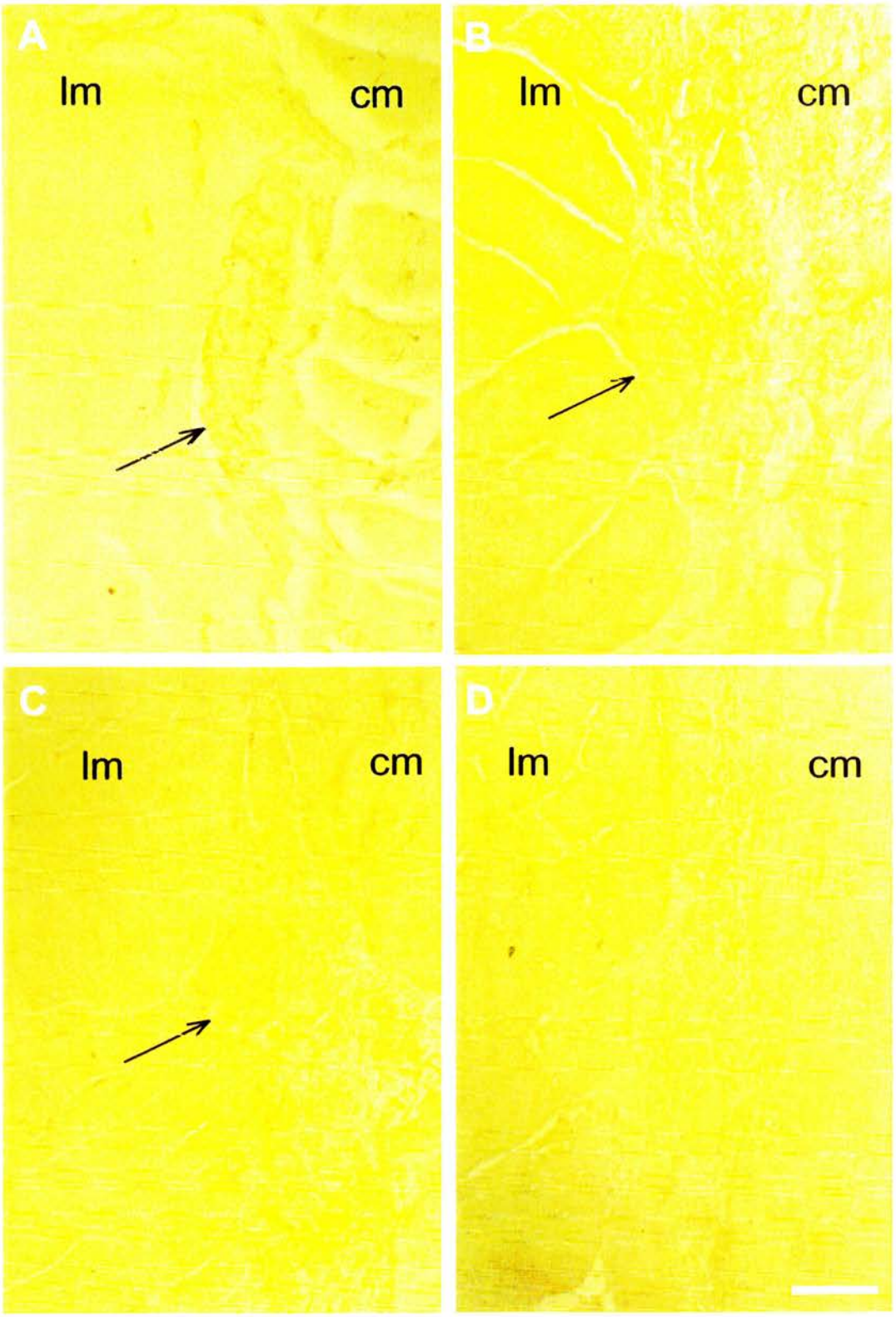


Figure 36. ICC density grades in the region of the myenteric plexus (arrow) of equine pelvic flexure. lm = longitudinal muscle; cm = circular muscle.
(A) Grade 3 in normal pelvic flexure.
(B) Grade 2 in normal pelvic flexure.
(C) Grade 1 in pelvic flexure from a horse with subacute grass sickness.
(D) Grade 0 in pelvic flexure from a horse with subacute grass sickness. Bar = 100μm.

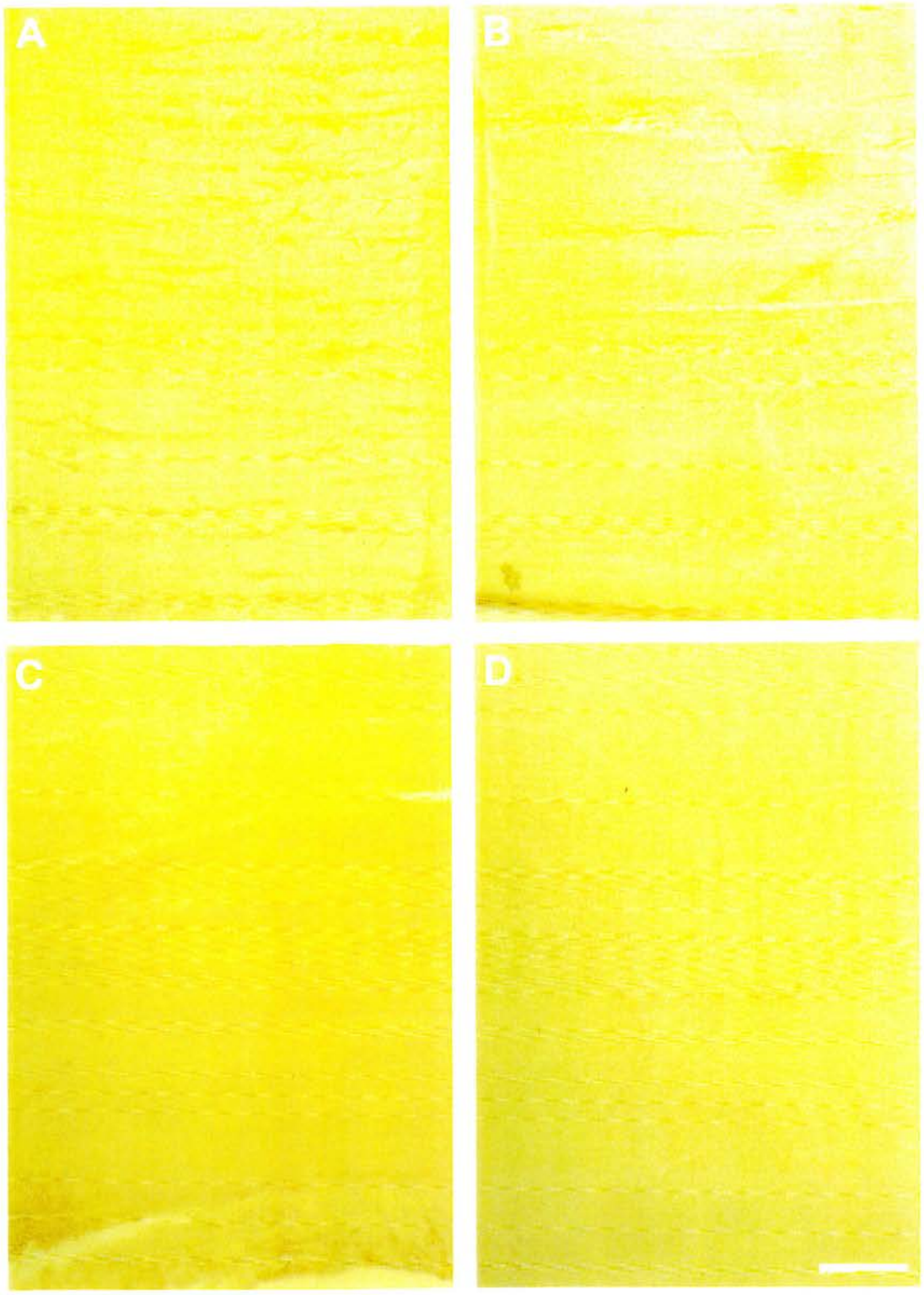


Figure 37. ICC density grades in the circular muscle layer of equine intestine.
(A) Grade 3 in normal pelvic flexure.
(B) Grade 2 in normal pelvic flexure.
(C) Grade 1 in pelvic flexure from a horse with acute grass sickness.
(D) Grade 0 in ileum from a horse with subacute grass sickness. Bar = 100µm.

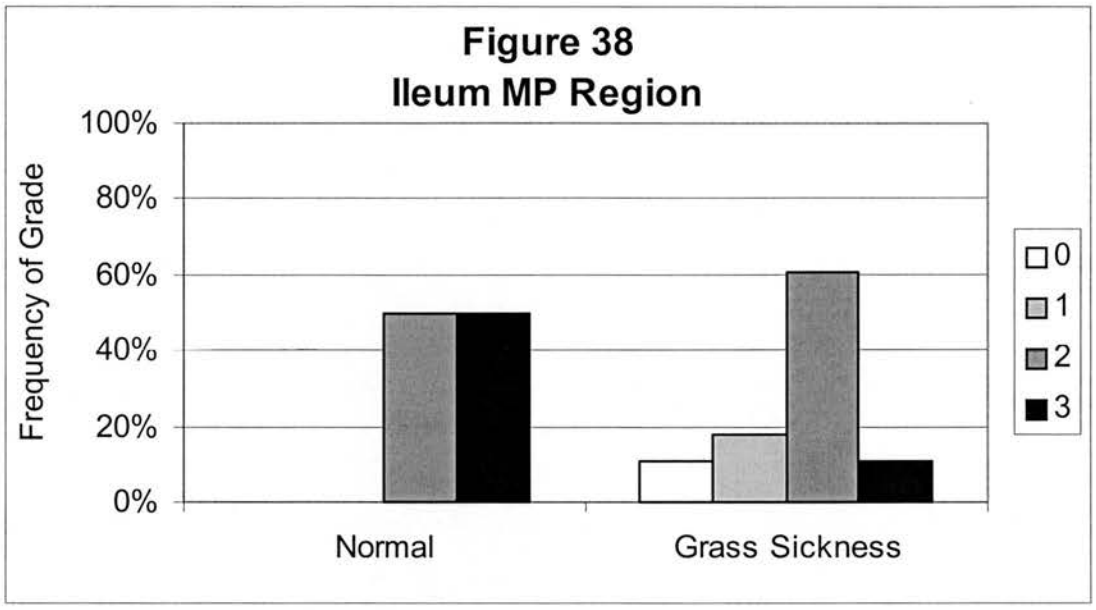
3.3.2.1 Ileum

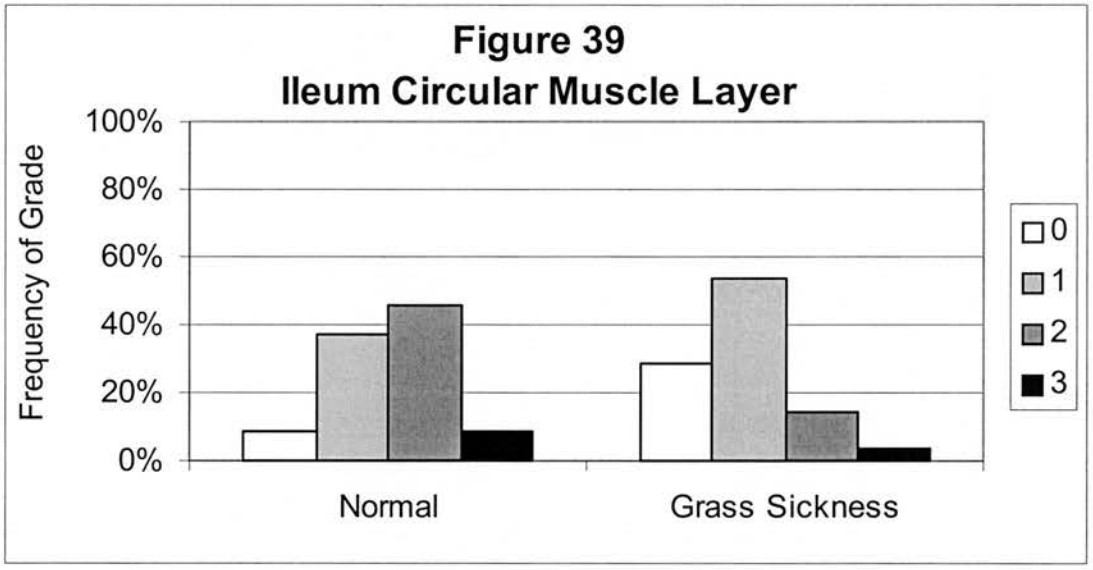
The ICC density grades for both regions of the ileum in normal and diseased animals are summarised in Table III. In the MP region of normal horses, the density of ICC was significantly greater than in the horses with grass sickness ($P = 0.0001$)[Figure 38]. Similarly, there was a reduction in the ICC density in the circular muscle (CM) layer of diseased animals compared to normals ($P = 0.0028$) [Figure 39]. The ICC density in the MP region was not significantly different between acute and chronic grass sickness ($P = 0.1133$), nor was there a significant difference when comparing all 3 categories of the disease ($P = 0.315$). In contrast however, there was a significant difference in the ICC density in the CM region among the categories of disease ($P = 0.045$) with AGS animals having a lower density than CGS animals ($P = 0.012$).

Table III
ICC density grades in equine ileum

	Normals	Total grass sickness	AGS	SGS	CGS
Number of animals	24	*28	11	9	7
MP grade ± S.E.M (median)	2.50 ± 0.10 (2.50)	1.71 ± 0.15 (2.00)	1.55 ± 0.25 (2.00)	1.89 ± 0.26 (2.00)	2.00 ± 0.22 (2.00)
CM grade ± S.E.M (median)	1.54 ± 0.16 (2.00)	0.93 ± 0.14 (1.00)	0.64 ± 0.20 (1.00)	0.89 ± 0.20 (1.00)	1.57 ± 0.30 (1.00)

*Includes 1 chronic recovered case that had a subsequent acute episode





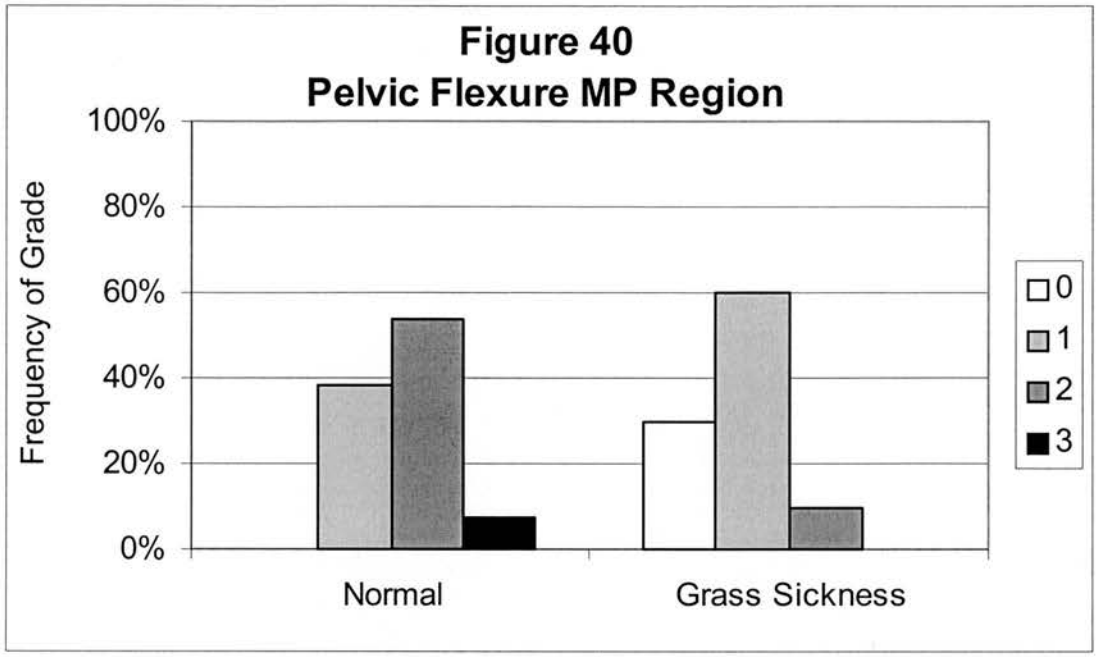
Figures 38 and 39. Interstitial cells of Cajal density in the ileum of normal (n = 24) and grass sickness-affected (n = 28) horses. Figure 38 illustrates the myenteric plexus (MP) density and Figure 39 the circular muscle (CM) density. Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) or abundant (3).

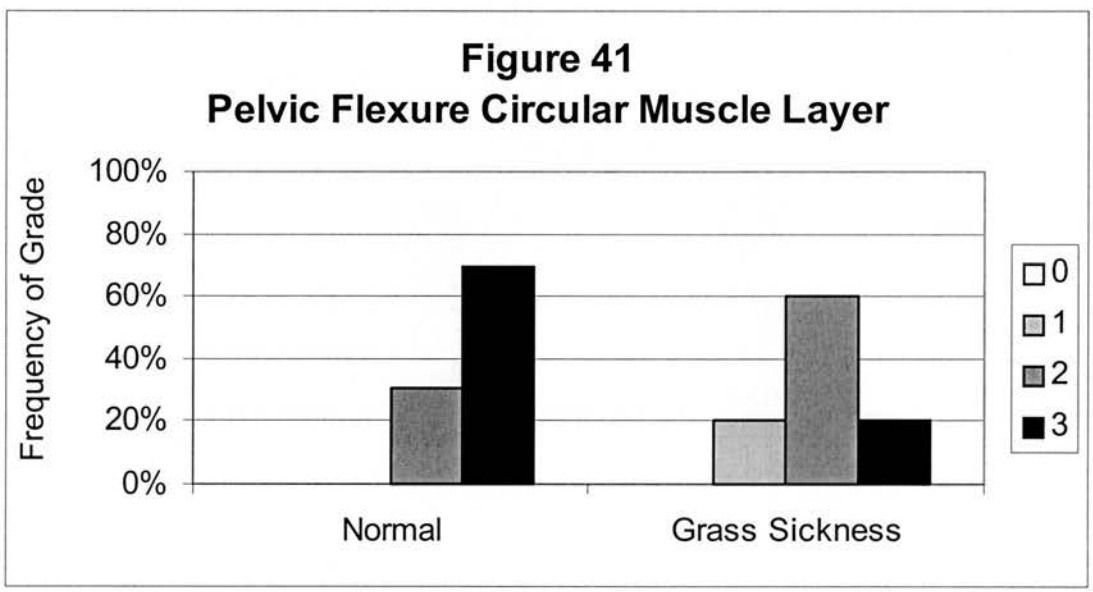
3.3.2.2 Pelvic flexure

The ICC density grades for both regions of the pelvic flexure in normal and diseased animals are summarised in Table IV. In the MP region, the ICC density was significantly greater in the normal horses compared to the horses with grass sickness (P = 0.0029) [Figure 40]. Similarly in the CM layer, there was also a greater ICC density in normal horses compared to horses with grass sickness (P = 0.0072) [Figure 41]. Unlike the CM region in the ileum, no obvious differences were noted in the pelvic flexure between the grades recorded in the different categories of the disease in either the MP (P = 0.187) or the CM region (P = 0.430).

Table IV
ICC density grades in equine pelvic flexure

	Normals	Total grass sickness	AGS	SGS	CGS
Number of animals	13	10	4	4	2
MP grade ± S.E.M (median)	1.69 ± 0.17 (2.00)	0.80 ± 0.20 (1.00)	1.25 ± 0.25 (1.00)	0.50 ± 0.29 (0.50)	0.50 ± 0.50 (0.50)
CM grade ± S.E.M (median)	2.69 ± 0.13 (3.00)	2.00 ± 0.21 (2.00)	2.00 ± 0.00 (2.00)	1.75 ± 0.48 (1.50)	2.50 ± 0.50 (2.50)





Figures 40 and 41 Interstitial cells of Cajal density in the pelvic flexure of normal (n = 13) and grass sickness-affected (n = 10) horses. Figure 40 illustrates the myenteric plexus (MP) density and Figure 41 the circular muscle (CM) density. Frequency grade (%) represents the proportion of specimens that were assigned to a particular density grade: absent (0), sparse (1), moderate (2) or abundant (3).

3.4 Discussion

This immunohistochemical study has demonstrated the distribution and characteristics of c-Kit-immunoreactive cells in the normal equine gastrointestinal tract. Previous studies in the mouse (Ward *et al.,* 1994; Huizinga *et al.,* 1995; Torihashi *et al.,* 1995; Burns *et al.,* 1996; Torihashi *et al.,* 1997), guinea pig (Komuro and Zhou, 1996; Burns *et al.,* 1997; Seki *et al.,* 1998), rat (Isozaki *et al.,* 1995, Ekblad *et al.,* 1998) and human (Vanderwinden *et al.,* 1996a; Vanderwinden *et al.,* 1996b; Isozaki *et al.,* 1997; Hagger *et al.,* 1998a; Hagger *et al.,* 1998b; Rømert and Mikkelsen, 1998) have shown that anti-c-Kit antibodies specifically label several classes of ICC. The morphology and location of the c-Kit-immunoreactive cells seen in the *muscularis externa* in this study make it most likely that they are the ICC of the equine gastrointestinal tract.

The present study has shown a major difference in the distribution patterns of the ICC in the small intestine compared to that of the large intestine. In the small intestine c-Kit-I was concentrated in the MP region whereas in the large intestine it was mainly seen throughout the circular muscle layer. Given the postulated functions of the ICC, this may reflect a regional difference in the origin of slow wave activity, bearing in mind that the dominant source of slow waves in the stomach and small intestine is the myenteric region, whilst in the large intestine it is the circular muscle layer (Sanders, 1996).

There is considerable variation in the distribution of ICC in man and in other mammals (Christensen *et al.,* 1992; Hagger *et al.,* 1997). A classification scheme for the different types of ICC that may be seen has been published (Sanders, 1996). These cell types are ICC in the myenteric region (stomach, small bowel, colon), ICC at the submucosal surface of the circular muscle of the colon, ICC of the deep muscularis plexus of the small intestine, and intramuscular ICC in the circular and longitudinal muscle layers. In this equine study, no specific accumulations of ICC were seen either in the area of the deep muscularis plexus of the small intestine or the submucosal aspect of the colonic circular muscle layer. However, there did appear to

be greater numbers of cells observed in the inner third of the circular muscle layer of the small intestine. In this study, sectioning of tissues in different planes has shown that the ICC tend to have their long axes parallel to the direction of the muscle fibres. This correlates well with findings of other workers (Rømert and Mikkelsen, 1998).

The highest densities of ICC were observed in the ileum, pelvic flexure and body of the caecum. It is therefore tempting to speculate as to whether these areas are notable with regard to control of gut motility in the horse. There have been many pacemaker sites suggested in the equine gastrointestinal system. These include the gastric antrum (Gerring, 1991; Fenger *et al.,* 1998), the longitudinal muscle of the proximal duodenum (Phaneuf and Ruckebusch, 1983), the pelvic flexure (Sellers *et al.,* 1979; Sellers *et al.,* 1982; Clark, 1990), the caecal body (Clark, 1990), the caecal apex (Ross *et al.,* 1989), the caecal base (Gerring, 1991), the right ventral colon (Ross *et al.,* 1990) and the transverse colon (Schusser and White, 1997). Much of this information must be qualified in that there is great difficulty and complexity in the interpretation of equine intestinal myoelectrical activity with regard to pacemaker function (Merritt *et al.,* 1995).

Further work is needed to confirm whether the density of ICC in the horse parallels the areas where there is a prominence of slow wave activity as has been shown in other animals (Christensen *et al.,* 1992). This study has shown that there are ICC in many locations in the equine gastrointestinal tract. Therefore, it is possible that the coordination of activity exerted by ICC is by means of a complicated and diffuse network rather than by discrete pacemaker sites. Furthermore, additional work is necessary to determine what proportion of equine ICC are mediators of neurotransmission rather than pacemakers. It is interesting that the distribution of cells in the caecal base was more similar to the right ventral colon than the body of the caecum. This may reflect the fact that in embryological terms the caecal base in the horse is actually the initial part of the right ventral colon rather than a true part of the caecum (Dyce *et al.,* 1996).

Studies of ICC will certainly be very important in understanding equine gastrointestinal disease because the cells have been implicated in many diseases,

particularly in humans. The semi-quantitative analysis in this study examined the differences between ICC density in normal animals compared to animals suffering from grass sickness. In horses with grass sickness, ICC were reduced in both the MP and circular muscle regions of the ileum and the pelvic flexure. The ileum was chosen because it is the region of intestine most severely affected in grass sickness (Scholes *et al.*, 1993a) and has a high density of ICC in normal animals. The pelvic flexure was chosen as a representative segment of the colon because it has a high density of ICC and it is an important region clinically since it is an area where impactions develop frequently. The decrease in ICC density is interesting because it may implicate these cells, in conjunction with the enteric neurons, in the development of intestinal stasis. It is unclear which cells are affected first in the disease and this needs further investigation. The remaining ICC need to be examined for any signs of ultrastructural damage and this would require a detailed electron microscopy study of the ICC in normal and grass sickness-affected animals. It is possible that if neurons are damaged by the putative neurotoxin then ICC may also be damaged either directly or indirectly. For instance, ICC with intimate contact with dying neurons may be damaged merely by their close association. In Figure 35C, c-Kit-immunoreactivity in the region of the MP is reduced (grade 1) in a case of AGS. The delicate pattern of immunoreactivity surrounds the area where enteric neurons would have been located before the disease and it is possible that as the neurons died, so too did the number of ICC decrease in the neighbouring area.

It is interesting that in the ileum the ICC are decreased in the circular muscle layer to a greater degree in AGS compared to CGS. This was not the case in the pelvic flexure and this may be a reflection of the ileum being a predilection area for grass sickness ENS pathology or it may simply be due to the smaller sample size of the categories of disease in the pelvic flexure study. Perhaps, the scale of the decrease in ICC in the ileum contributes to the severity of the clinical picture. If ICC are preserved to a greater extent in less severely-affected horses, then this may help explain the better gastrointestinal function in these animals. The category of chronic cases that have survived may be important in this respect. Only one of these animals is included in this study and it may be unusual because it died of a suspected

"second" episode of grass sickness which may explain its low grades in the MP (0) and circular muscle (0). There have been very few animals of this type that have become available for study. Indeed only recently, Doxey *et al.* (2000) reported histological findings in four such animals. Unfortunately, in the study for this thesis, tissues were not collected freshly in some of these recovered CGS animals and hence they were unsuitable for immunohistochemical examination because of *post mortem* deterioration. Further work is necessary on more animals that have survived CGS and died subsequently years later of unrelated causes to determine if the levels of ICC are greater than in CGS animals that did not survive.

A possible criticism of this study is that it does not address the influence of age on the density of the ICC. It has been suggested that the expression of c-Kit wanes with age in the mouse (Torihashi *et al.*, 1995). In human studies however, there is evidence of c-Kit expression by the ICC in young and old patients, even up to 90 years of age (Hagger *et al.*, 1998a; Hagger *et al.*, 1998b) and that the distribution of ICC is similar at different ages (Vanderwinden *et al.*, 1996b). In this study, there was no evidence of differing ICC density at differing ages and there was obvious c-Kit-immunoreactivity even in the oldest horse in the study (36 years). It may also be noted that the grass sickness animals were on average younger than the normal horses ($P = 0.0492$ for ileum samples; no significant difference in age in the pelvic flexure samples, $P = 0.1185$). This may reflect the fact that the disease is predominantly seen in the 2-7 year age group (Gilmour and Jolly, 1974). The finding that the normal horses in this study had a higher ICC density (detected by c-Kit expression) suggests that c-Kit continues to be expressed with increasing age in the horse and is a valuable indicator of ICC presence and density. Age-matching is difficult in these clinically-derived equine studies because of the small sample sizes involved. This area needs further work and a study on the ontogeny of the ICC in the horse and any age-related factors would be indicated.

Whilst this immunohistochemical study does have its limitations, it nevertheless suggests that the ICC may be involved in the disease. Further work would address the functional implications of this by examining the electrical properties of the intestine in health and disease. This is the subject of the next chapter.

CHAPTER 4:ELECTROPHYSIOLOGICAL STUDIES ON GASTROINTESTINAL SMOOTH MUSCLE IN HEALTH AND DISEASE

4.1 Aims

The aims of this part of the study were to perform an *in vitro* investigation of the electrical properties of smooth muscle in both the healthy and diseased intestine. This functional approach aimed to test the hypothesis that impaired ICC-mediated control is responsible for intestinal dysfunction.

Tissue from normal and grass sickness-affected horses were used for experiments. In addition, tissue from abattoir-slaughtered pigs were used for comparison and for the development of techniques.

4.2 Materials and Methods

4.2.1 Normal equine ileum

Portions of ileum (level with the midpoint of the ileocaecal fold) were taken fresh *post mortem* from 11 "normal" (control) horses euthanased for conditions not involving the alimentary tract. The horses ranged from 1 to 25 years of age (mean, 8.3 ± 7.5 years; median, 7.0 years). The animals were euthanased by intravenous administration of quinalbarbitone sodium BP (400 mg/ml)/cinchocaine hydrochloride BP (25 mg/ml) (Somulose; Arnolds Veterinary Products, UK). The ileal samples were placed immediately in modified Krebs solution of the following composition (mM): NaCl 120.7, KCl 5.9, Na HCO_3 25, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.5 and glucose 11.5. The segment of ileum was opened along the mesenteric border with scissors and rinsed in fresh Krebs. The gut was pinned out on cork board mucosal surface down and a 1 mm thick cross-sectional preparation cut with a double-bladed knife (Figures 42 and 43). The direction of the cut enabled the preparation of sections orientated either parallel to the circular or to the longitudinal muscle fibres. This cross-sectional preparation of the intestinal muscle (Figure 44) gave direct

microelectrode access to specific sites in the muscle layers (Smith *et al.* 1987a). The cross-sectional preparation was pinned out in a tissue bath, superfused with oxygenated Krebs (95% O_2-5% CO_2) and maintained at 36-37°C (Figure 45) for experimental periods of up to 8 hours. An equilibration period of 60 minutes elapsed before the commencement of recordings. In some experiments tissues were left overnight in Krebs solution at 4°C to assess whether recordings could be made after extended tissue storage.

Measurements of membrane potential were made from smooth muscle cells in both the circular and longitudinal layers using intracellular glass microelectrodes manufactured using a Sutter Instruments P97 electrode puller. The microelectrodes were filled with 2 M KCl and had resistances in the range 25-50 MΩ. The set-up for the electrophysiological experiments is shown in Figure 46. A calibrating graticule in the eyepiece of the dissecting microscope was used to determine the exact position of impaled cells relative to the thickness of the muscle layer. A percentage scale was employed for both muscle layers. For the circular muscle layer, 0% referred to the muscle at the submucosal border and 100% to that at the myenteric border. For the longitudinal muscle layer, 0% referred to the muscle at the myenteric border and 100% to that at the serosal border.

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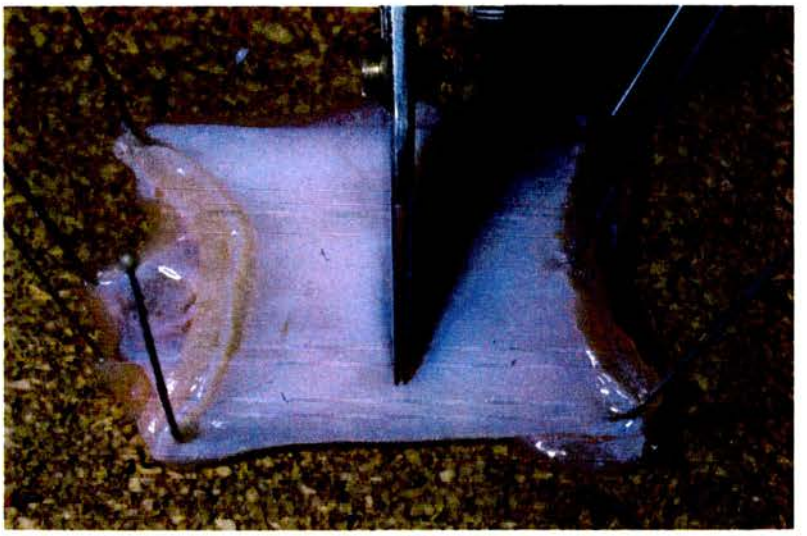

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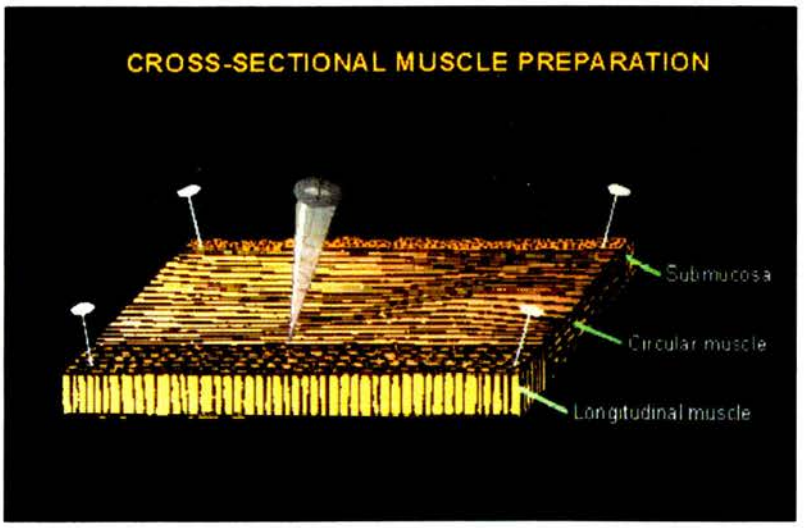
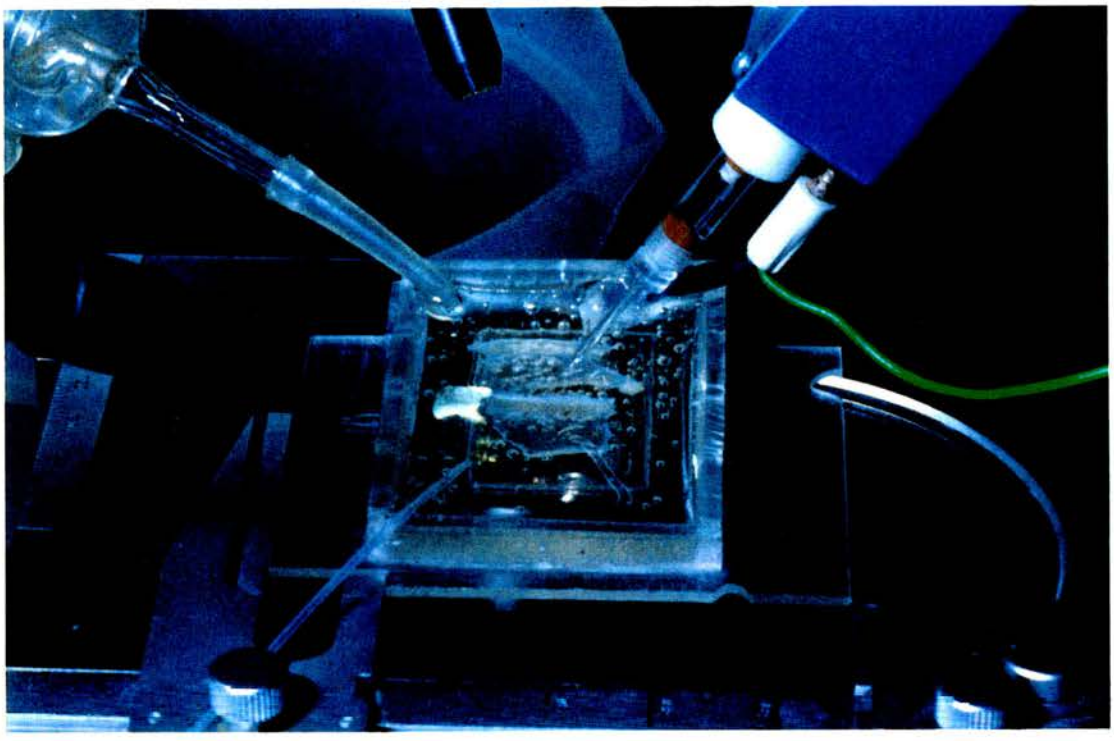


Figures 42 & 43. Cutting of the cross-sectional muscle preparation of equine ileum using a double-bladed knife.

Figure 44. Cross-sectional muscle preparation of equine ileum.
Adapted and reproduced with kind permission from K.M. Sanders.



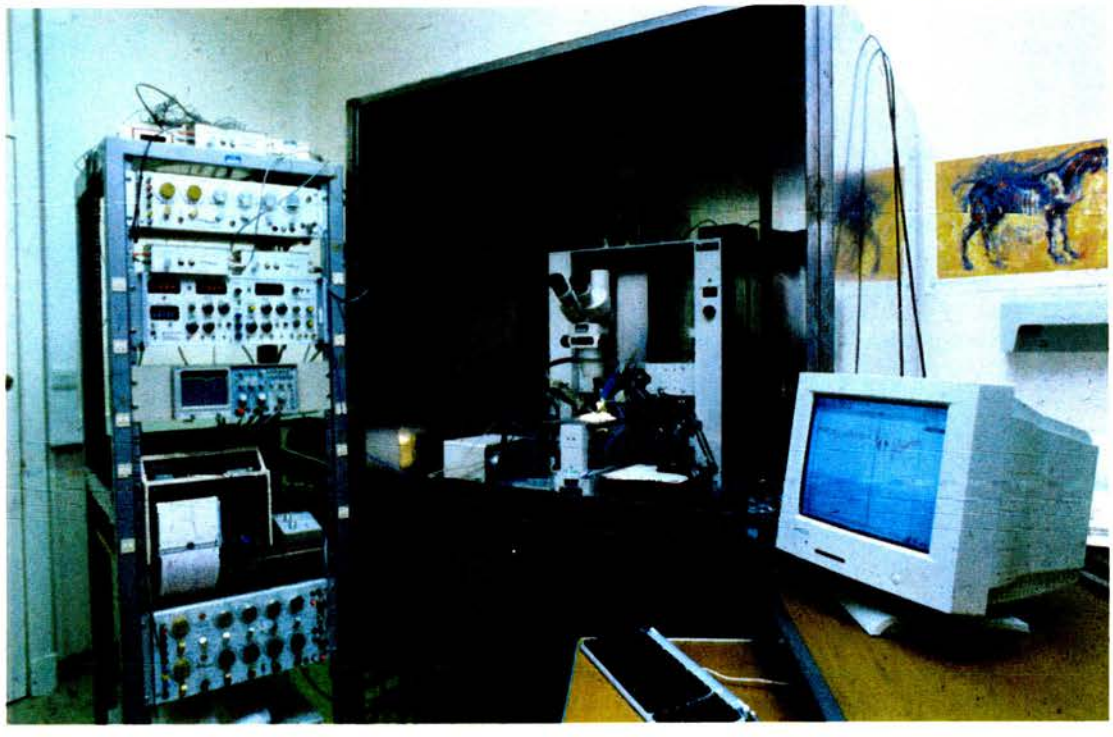


Figure 45. Cross-sectional muscle preparation of equine ileum pinned out in a tissue bath.

Figure 46. Set-up for electrophysiological experiments.

All data were captured and stored using an AD Instruments PowerLab 8SP acquisition system interfaced to a PowerMacintosh G4 computer. The software package Chart was used for analysis of resting potentials, amplitude, frequency and duration of slow wave oscillations and other waveforms. The resting membrane potential (RMP) was measured at the most negative reading for an impalement. For instance, in a cell with an oscillating potential due to slow wave activity, the reading was taken at the "base" of the slow waves. Recordings from cellular impalements were considered acceptable if there was a sharp initial drop in voltage and if the RMP remained stable. The intestinal electrical activity and its modulation were examined by the addition of pharmacological agents to the superfusion fluid. These included the L-type calcium channel blocker nifedipine (1 μM; Sigma, Poole, UK) and the neuronal blocker (sodium channel blocker) tetrodotoxin (TTX, 1 μM; Tetrodotoxin Crystalline 3X, Sankyo Co. Ltd, Tokyo, Japan).

4.2.2 Normal porcine ileum

Fresh ileal samples (level with the midpoint of the ileocaecal fold) were collected from 3 young healthy pigs slaughtered at a local abattoir. The processing of the tissue was the same as the equine methods described above.

4.2.3 Ileum from grass sickness-affected horses

Portions of ileum (level with the midpoint of the ileocaecal fold) were taken fresh *post mortem* from 6 horses euthanased with grass sickness (3 acute, 1 subacute and 2 chronic). The clinical diagnosis was confirmed by histopathological examination in all cases. The horses ranged from 4 to 9 years of age (mean, 6.6 ± 1.9 years; median, 7.0 years). The animals were euthanased by intravenous administration of quinalbarbitone sodium BP (400 mg/ml)/cinchocaine hydrochloride BP (25 mg/ml) (Somulose; Arnolds Veterinary Products, UK). The processing of the tissue for electrophysiological experiments was the same as described above.

Mann-Whitney tests corrected for ties were used to analyse and compare results. Relationships between measured values were examined using linear regression analysis. For all tests, a probability of less than 0.05 was considered to be significant. Data were expressed as means ± S.E.M and medians.

4.3 Results

4.3.1 Normal porcine ileum

Intracellular recordings of membrane potential were made from the *muscularis externa* of the porcine ileum. High frequency spiking activity was observed in the circular muscle layer with a mean frequency of 44.4 ± 10.5 cycles per minute and amplitude of 17.8 ± 2.4 mV (n = 5 cells from 1 animal). Contraction of the preparation in the tissue bath was observed. Both this visible contractile activity and the spiking activity were abolished by the addition of 1 μM nifedipine. Mean resting membrane potential (RMP) recorded from circular smooth muscle cells was –59.2 ± 3.3 mV (n = 17 cells from 3 animals). Slow waves were recorded in the circular (Figure 47) but not the longitudinal muscle layer. The slow waves had a mean amplitude of 6.5 ± 1.1 mV (range, 1-15), frequency 9.5 ± 0.2 cycles per minute (range, 8-10) and duration 5.7 ± 0.2 s (range, 4.4-7.0; n = 17 cells from 3 animals). Slow waves were present with and without nifedipine in the superfusion fluid. In some cells, a degree of rhythmical waxing and waning of the slow wave amplitude was noted (n = 4 cells from 1 animal). Attempts to record from tissue that had been stored at 4°C were unsuccessful, suggesting that prolonged storage was detrimental to tissue viability.

When analysing the recordings with respect to position in the circular muscle layer (n = 17 cells from 3 animals), it was found that neither amplitude ($R^2 = 0.0703$; $P > 0.1$) nor RMP ($R^2 = 0.0387$; $P > 0.1$) were affected significantly by the distance along the width of the section (Figures 48 and 49). RMP however, did have an effect ($R^2 = 0.39$; $P < 0.01$) on slow wave amplitude (Figure 50).

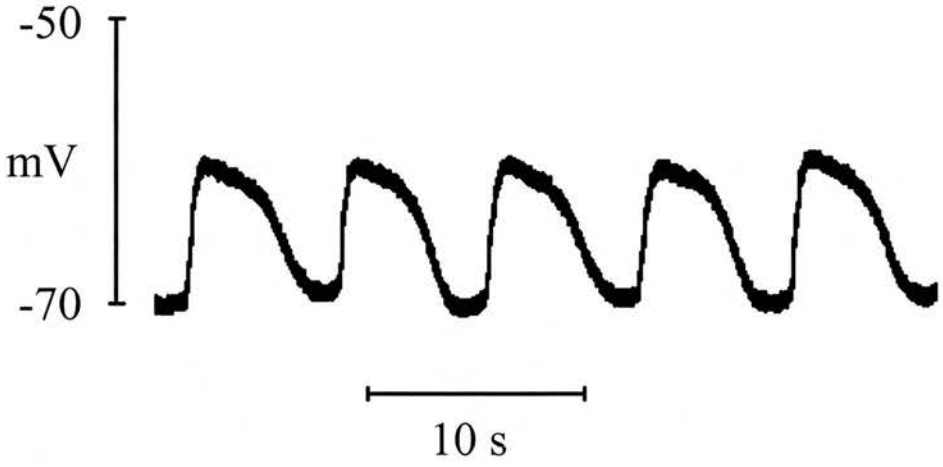


Figure 47. Intracellular recording from circular smooth muscle of the porcine ileum showing 5 slow waves.

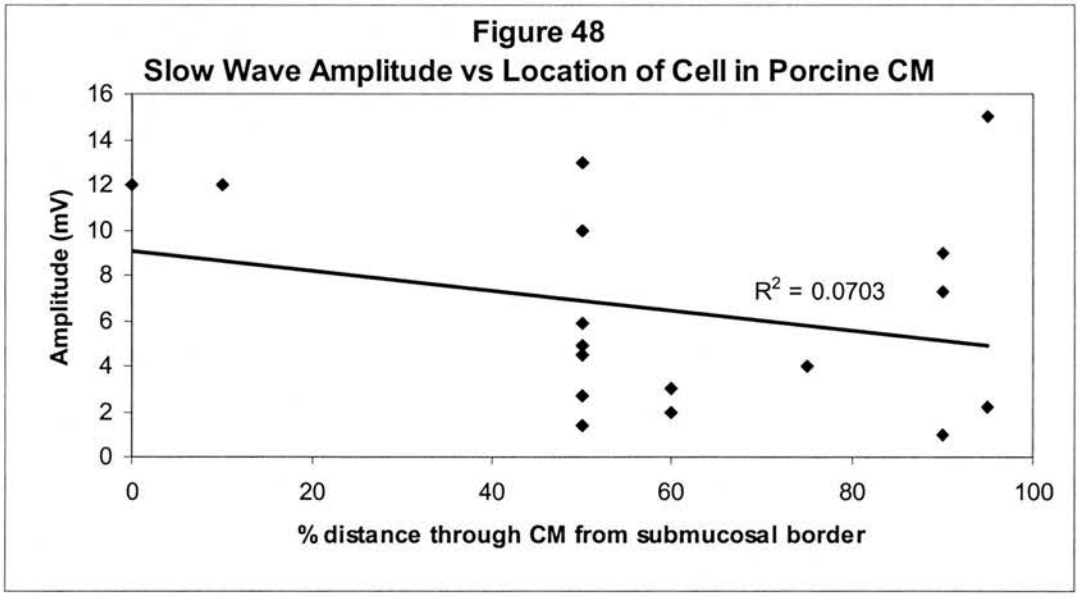


Figure 48. Relationship of slow wave amplitude to the position of the impaled cell through the thickness of the circular muscle (CM) in porcine ileum.

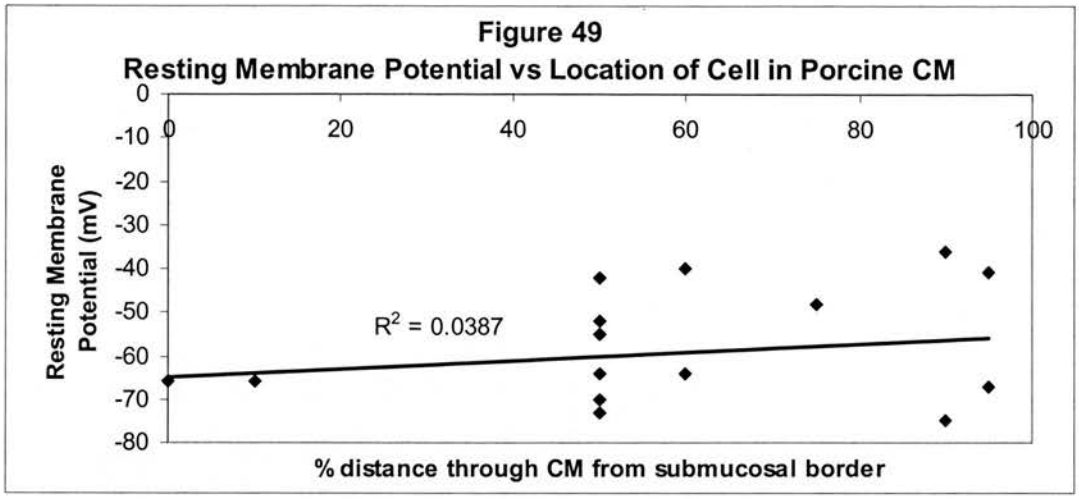


Figure 49. Relationship of resting membrane potential (RMP) to the position of the impaled cell through the thickness of the circular muscle (CM) in porcine ileum.

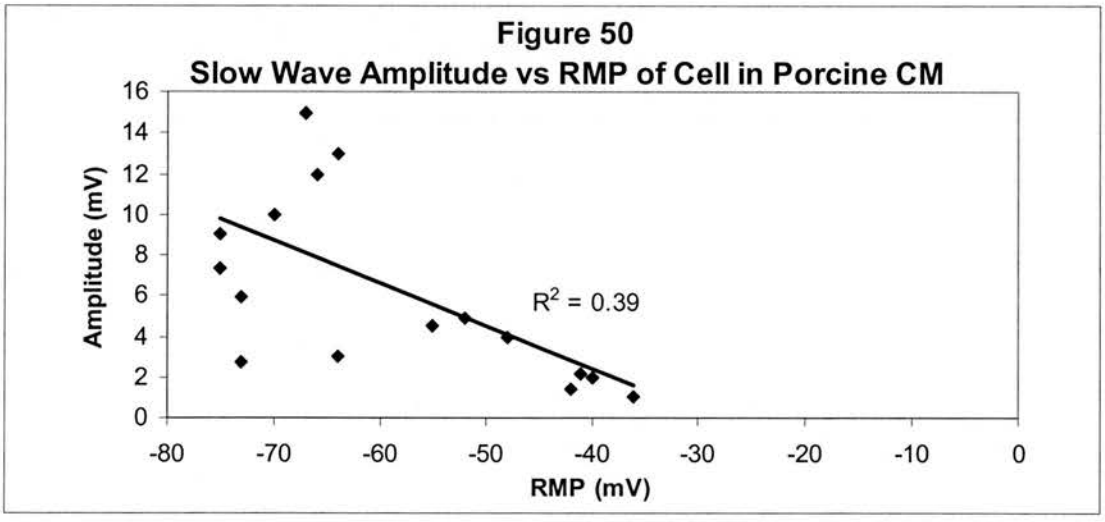


Figure 50. Relationship of slow wave amplitude to resting membrane potential (RMP) in the circular muscle (CM) in porcine ileum.

4.3.2 Normal equine ileum

Intracellular microelectrode recordings were made from both the circular and longitudinal muscle layers of the normal equine ileum. Slow waves were recorded in all of the horses (n = 60 cells in 11 animals). Mean RMP of smooth muscle cells in the *muscularis externa* was –51.8 ± 1.1 mV (range, -33.0 to –70.5; n = 64 cells in 11 animals). Slow waves had a mean amplitude of 4.8 ± 0.4 mV (range, 1.3-16.5), a frequency of 9.0 ± 0.1 cycles per minute (range, 5-11) and a duration of 5.6 ± 0.1 s (range, 3.0-8.4) [n = 60 cells in 11 animals]. The characteristics of the slow waves in the longitudinal and circular smooth muscle layers are summarised in Table V. In comparing the slow waves in the two layers, there were no significant differences in amplitude (P = 0.4462), RMP (P = 0.3493) and frequency (P = 0.5416) but the duration was greater in the longitudinal muscle layer (P = 0.0137).

Table V
Characteristics of slow waves recorded from the *muscularis externa* of normal equine ileum (mean values are given ± S.E.M)

Muscle Layer	Number of cells	Amplitude mV (median)	RMP mV (median)	Frequency per minute (median)	Duration s (median)
Longitudinal	52	4.8 ± 0.4 (4.5)	-51.9 ± 1.2 (-51.6)	9.0 ± 0.1 (9.0)	5.8 ± 0.2 (5.6)
Circular	8	4.8 ± 1.4 (3.5)	-51.1 ± 2.2 (-49.5)	9.0 ± 0.7 (9.5)	4.7 ± 0.4 (5.0)

Slow waves were characterised by a fast upstroke and a slower downstroke phase (Figure 51). In some cells, spikes (action potentials, 7-49 mV in amplitude) were superimposed on top of the slow waves (Figure 52; n = 9 cells in 4 animals). Both this spiking activity and any contractile activity observed in the preparations were abolished in the presence of 1 μM nifedipine. Slow waves were preserved in the presence of nifedipine in the superfusion fluid. Indeed, nifedipine was added in most experiments to abolish spiking activity and concomitant muscle contractions in order

to facilitate stable cellular impalements. The characteristics of slow waves recorded in the presence and absence of nifedipine are summarised in Table VI. Nifedipine had no significant effect on amplitude ($P = 0.4783$) and RMP ($P = 0.5845$) but slow waves recorded in the presence of nifedipine had a slower frequency ($P = 0.0034$) and a longer duration ($P = 0.0024$) than those recorded in the absence of the drug. Recordings were not possible in the tissue that had been stored at 4°C suggesting again that prolonged storage was detrimental to tissue viability.

Table VI

Slow wave characteristics recorded from the *muscularis externa* of normal equine ileum in the presence and absence of 1 µM nifedipine (means are given ± S.E.M)

Nifedipine	*Number of cells*	Amplitude mV *(median)*	RMP mV *(median)*	Frequency per minute *(median)*	Duration s *(median)*
Present	45	4.7 ± 0.4 (4.1)	-52.7 ± 1.2 (-53.0)	8.9 ± 0.1 (9.0)	5.9 ± 0.1 (5.6)
Absent	15	5.0 ± 0.7 (4.5)	-52.5 ± 2.0 (-50.0)	9.5 ± 0.3 (10.0)	4.8 ± 0.2 (5.0)

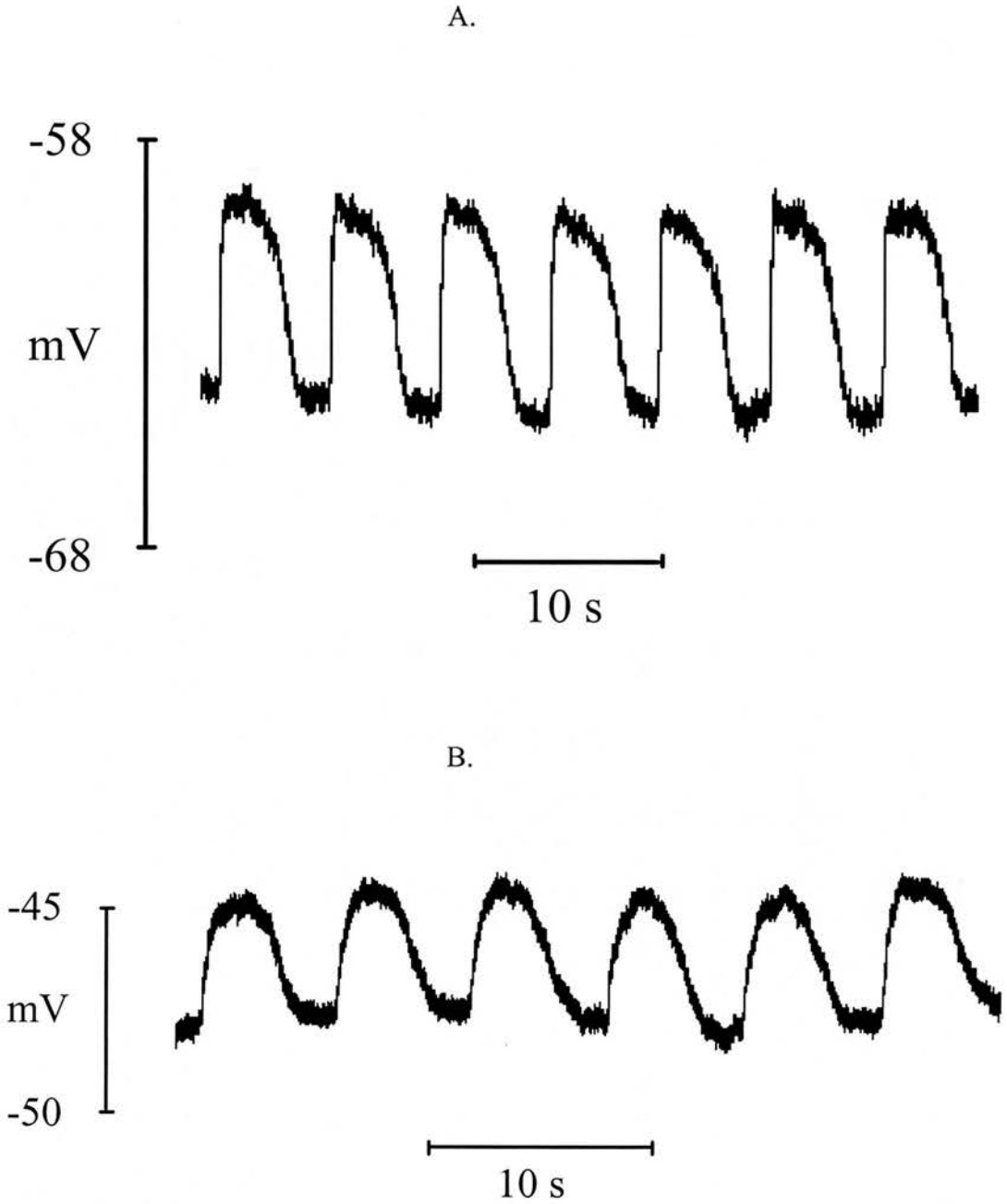


Figure 51. Slow waves in longitudinal [A] and circular [B] smooth muscle of normal equine ileum. Both recordings in the presence of 1 μM nifedipine.

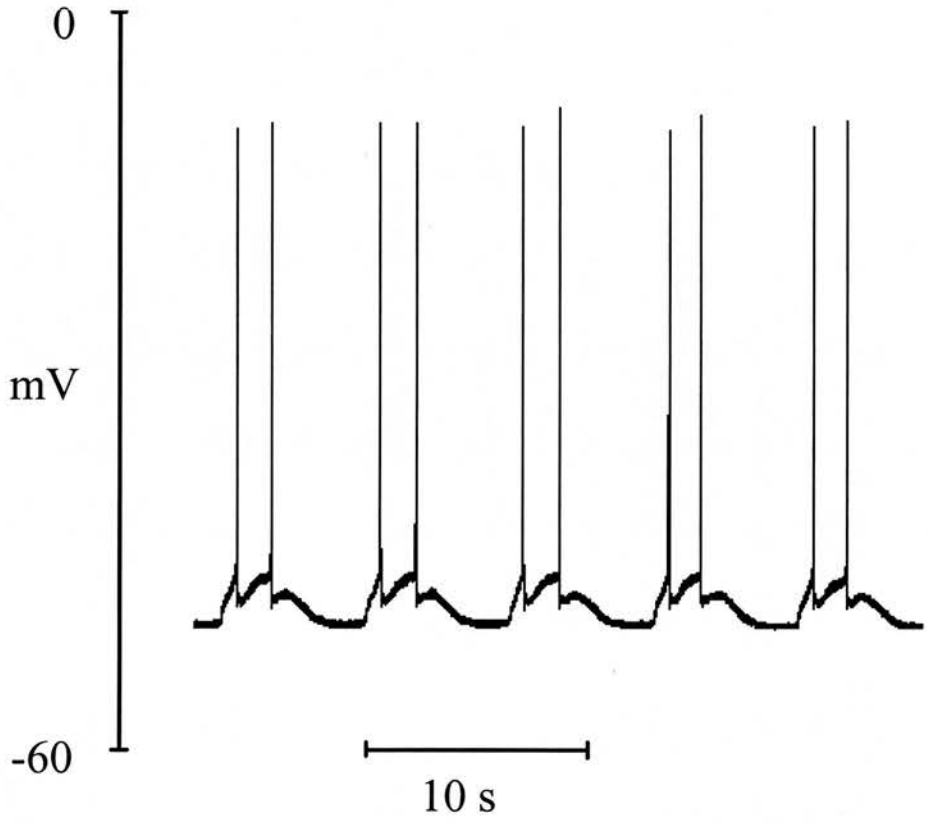
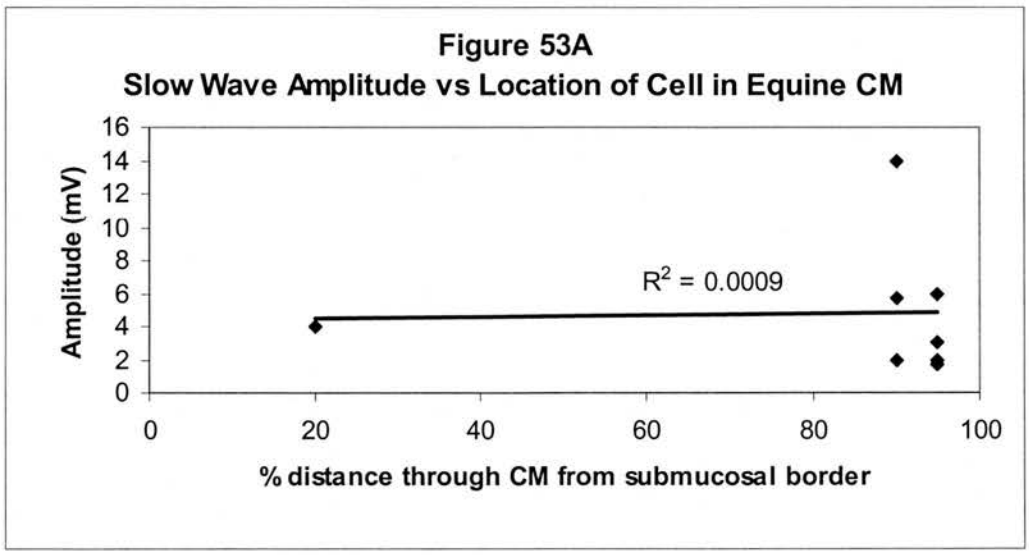


Figure 52. Intracellular recording from longitudinal smooth muscle of normal equine ileum showing slow waves and associated spontaneous action potentials.

When analysing the recordings with respect to position in the circular muscle layer, it was found that neither amplitude ($R^2 = 0.0009$; $P > 0.1$) nor RMP ($R^2 = 0.0276$; $P > 0.1$) were affected significantly by the distance along the width of the section (Figures 53A and 54A). In the longitudinal muscle layer, amplitude was not affected significantly by distance along the width of the section ($R^2 = 0.0305$; $P > 0.1$; Figure 53B) but there did appear to be a decline in RMP (to more positive values) with increasing distance away from the MP region ($R^2 = 0.1653$; $P < 0.01$ Figure 54B). In addition, RMP had no significant effect on slow wave amplitude in the *muscularis externa* ($R^2 = 0.008$; $P > 0.1$; Figure 55). Therefore the calculation of mean values for the slow wave measurements throughout the muscle thickness was justified, although the RMP values should be interpreted with caution because of the apparent correlation with distance. Despite the finding that position had no major effect on RMP and amplitude, there was a suggestion of a decay in the slow wave signal at extreme distances from the MP region (eg 90-100%) [Figure 56].

In many cells, a waxing and waning behaviour of the slow waves was noted occurring every 9 – 15 slow waves ($n = 23$ cells in 6 animals; Figure 57). The amplitude increased and decreased rhythmically by between 1 and 9 mV. The RMP also oscillated by between 0.5 and 4.6 mV. The RMP was greater (more negative) at the greater amplitudes in the cyclical oscillations.



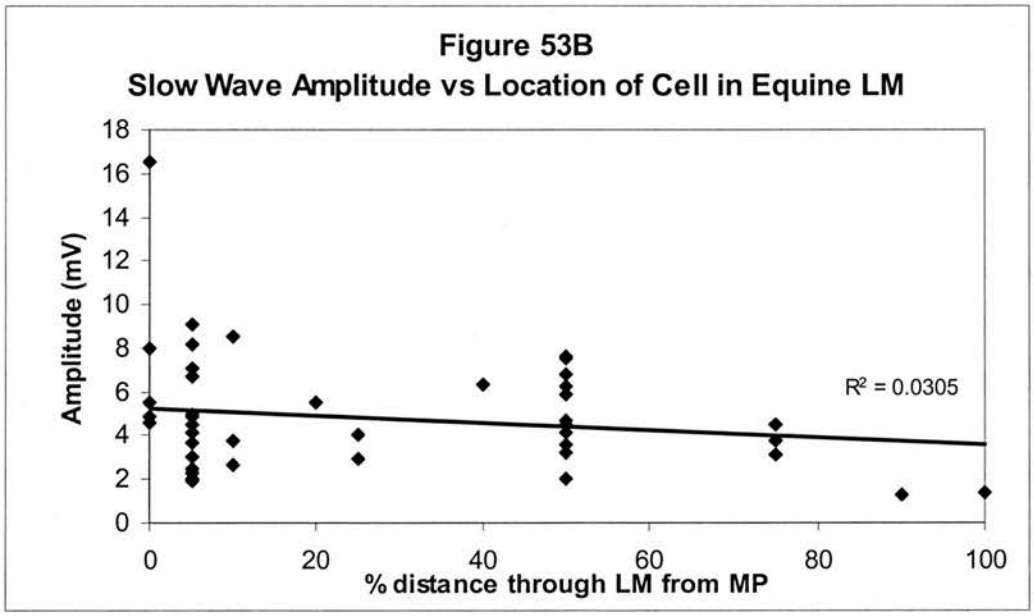
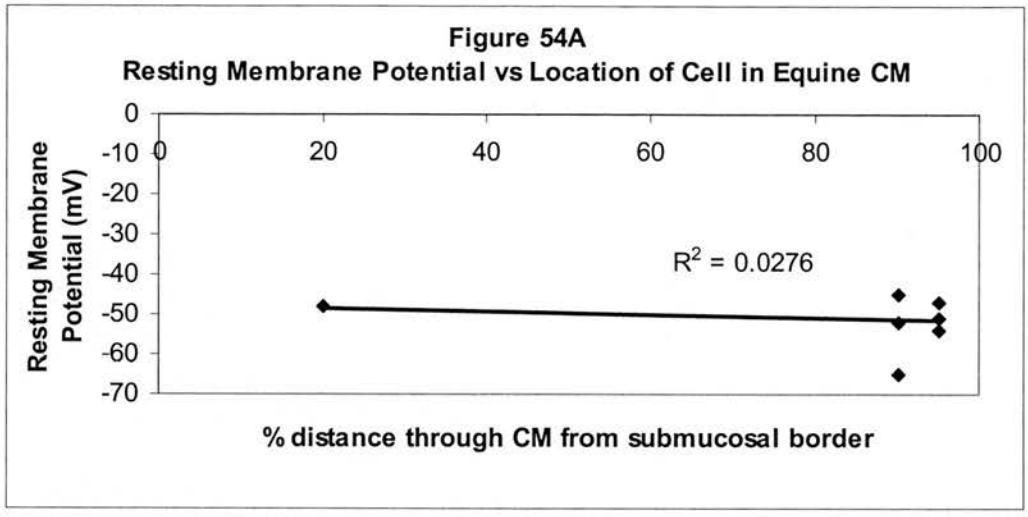


Figure 53. Relationship of slow wave amplitude to the position of the impaled cell through the thickness of the circular muscle (CM) [Figure 53A] and longitudinal muscle (LM)[Figure 53B] in equine ileum. MP = myenteric plexus.



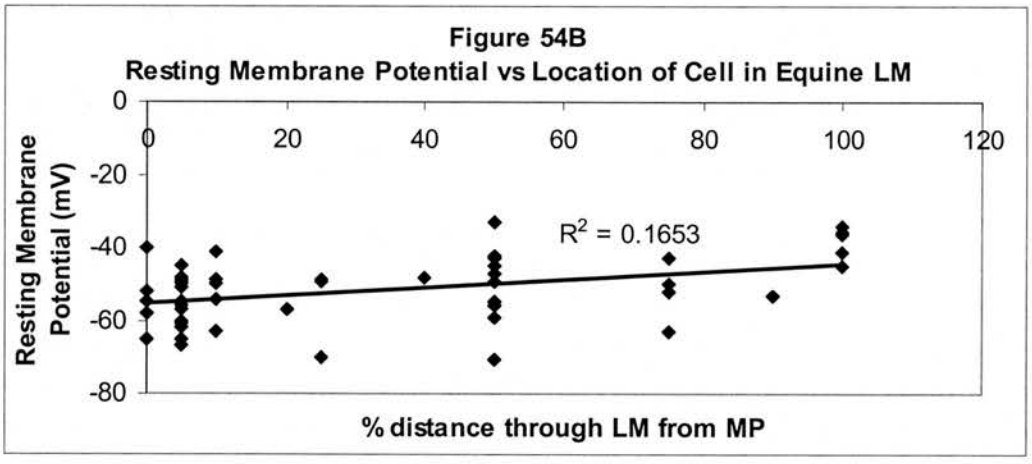


Figure 54. Relationship of resting membrane potential (RMP) to the position of the impaled cell through the thickness of the circular muscle (CM)[Figure 54A] and longitudinal muscle (LM)[Figure 54B] in equine ileum. MP = myenteric plexus.

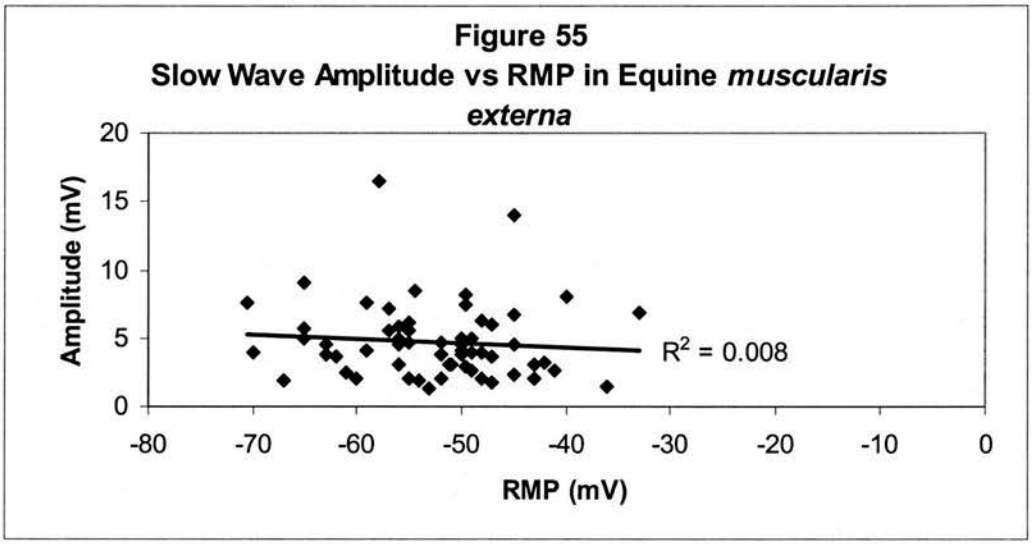


Figure 55. Relationship of slow wave amplitude to resting membrane potential (RMP) in the *muscularis externa* in equine ileum.

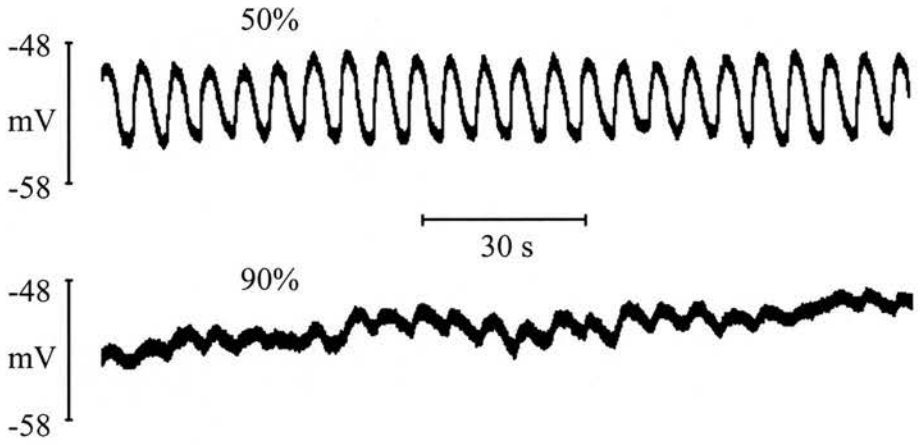


Figure 56. Two segments of intracellular recordings from the same preparation of longitudinal smooth muscle of normal equine ileum. The upper trace shows slow wave activity recorded at a distance of 50% through the muscle layer from the myenteric border. The lower trace shows the decay of the slow wave signal at 90% through the muscle. Nifedipine was present at 1 μM.

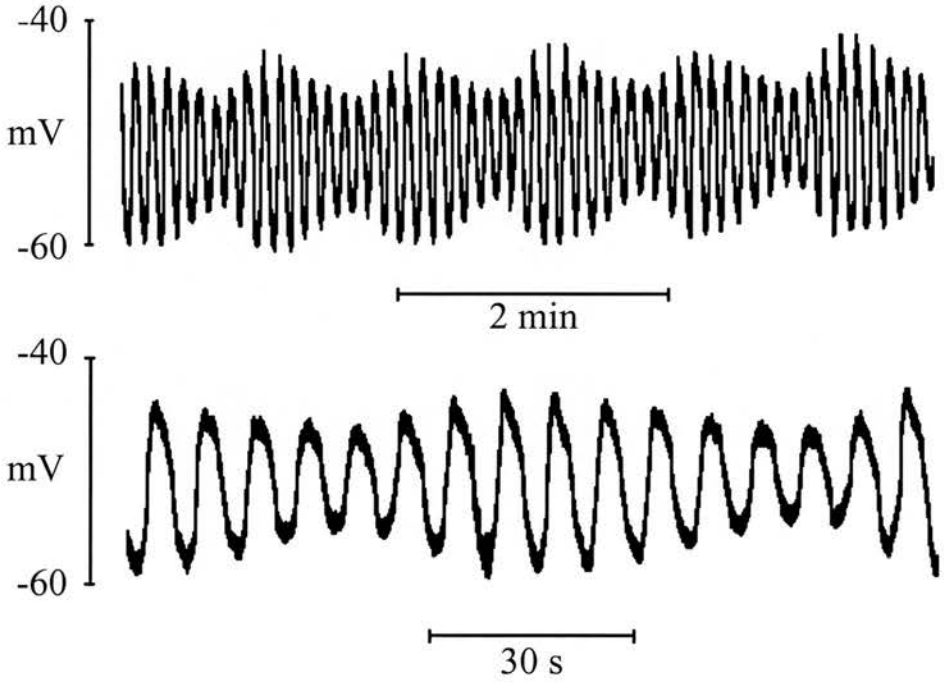


Figure 57. Intracellular recording from longitudinal smooth muscle of normal equine ileum showing cyclical "waxing and waning" of the slow wave amplitude. The two segments of trace are from the same cell. Nifedipine was present at 1 μM.

Slow wave activity was preserved and unchanged in the presence of 1 μM tetrodotoxin (Figure 58) showing that these waves were non-neural in origin (n = 8 cells in 3 animals). The waxing and waning behaviour was also preserved in the presence of tetrodotoxin.

4.3.3 Horses with grass sickness

In 4 of the 6 horses with grass sickness (2 acute, 2 chronic) slow wave activity was recorded (n = 12 cells in 4 animals). Slow waves were observed in both the circular and longitudinal muscle layers (Figures 59 and 60). Mean RMP of smooth muscle cells in the *muscularis externa* was –51.9 ± 1.6 mV (range, -44.8 to –64.0; n = 12 cells in 4 animals). Slow waves recorded in the *muscularis externa* had a mean amplitude of 4.7 ± 0.5 mV (range, 2.2-7.8), a frequency of 7.3 ± 0.1 cycles per minute (range, 7-8) and a duration of 7.4 ± 0.2 s (range, 6.0-8.0)[n = 12 cells in 4 animals]. The slow wave characteristics recorded in the normal and grass sickness-affected ileum are summarised in (Table VII). When comparing the slow wave characteristics recorded in normal and grass sickness animals, there were no significant differences in amplitude (P = 0.6232) and RMP (P = 0.6072). However in grass sickness horses, the slow wave frequency was lower (P < 0.0001) and the duration longer (P < 0.0001) than in normal animals.

Table VII
Slow wave characteristics recorded from the *muscularis externa* of equine normal and grass sickness-affected ileum (means are given ± S.E.M)

Category of ileum	*Number of cells*	Amplitude mV *(median)*	RMP mV *(median)*	Frequency per minute *(median)*	Duration s *(median)*
Normal	60	4.8 ± 0.4 (4.3)	-51.8 ± 1.1 (-51.1)	9.0 ± 0.1 (9.0)	5.6 ± 0.1 (5.6)
Grass sickness	12	4.7 ± 0.5 (4.9)	-51.9 ± 1.6 (-50.3)	7.3 ± 0.1 (7.0)	7.4 ± 0.2 (7.6)

Waxing and waning behaviour was also noted in many cells in grass sickness-affected ileum (n = 10 cells in 4 animals). In one experiment, after recordings made

in the presence of nifedipine, the drug was removed from the superfusion fluid. This resulted in the recorded waveform becoming biphasic with a large and a small component (Figure 61). Both components appeared to wax and wane synchronously. The smaller (latter) component appeared to wax and wane more markedly than the early (aborted) component. This biphasic waveform was abolished when nifedipine was re-added to the superfusion fluid and "normal" slow waves were then recorded.

No slow waves were recorded in the remaining 2 animals (1 acute, 1 subacute). There were signs of contractility in the tissue preparations but no concomitant electrical activity could be recorded.

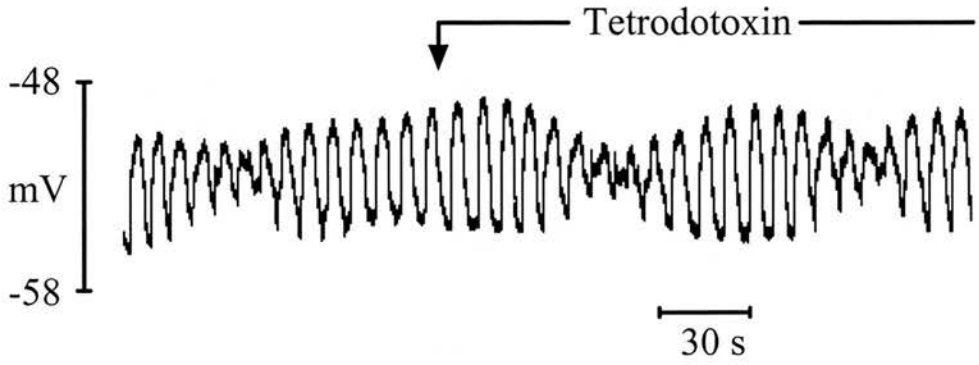


Figure 58. Slow wave activity recorded from the longitudinal smooth muscle of normal equine ileum before and after the addition of tetrodotoxin [1 μM] to the superfusion fluid. Nifedipine [1 μM] was present throughout the experiment.

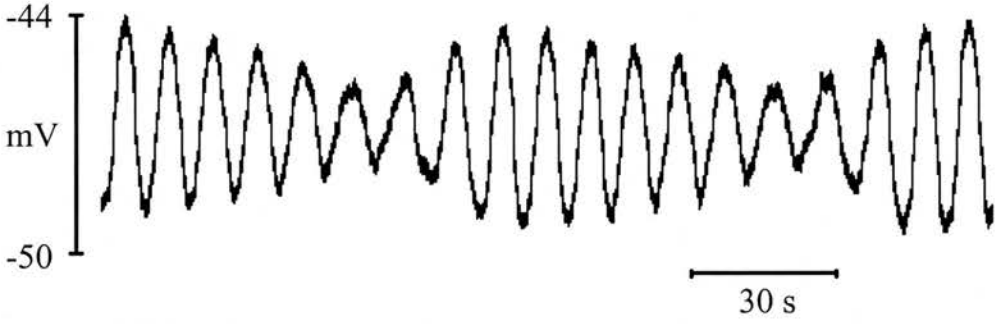


Figure 59. Slow wave activity recorded from the circular smooth muscle of ileum from a horse with acute grass sickness. Nifedipine [1 μM] was present.

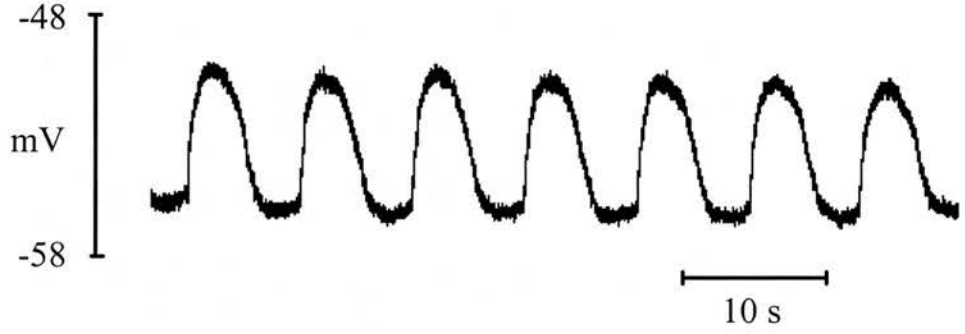


Figure 60. Slow wave activity recorded from the longitudinal smooth muscle of ileum from a horse with acute grass sickness. Nifedipine [1 μM] was present.

Figure 61. Biphasic waveform activity recorded from the longitudinal smooth muscle of ileum from a horse with chronic grass sickness. No nifedipine was present.

4.4 Discussion

The porcine experiments were useful for comparison and the development of techniques. The findings in this study were similar to those reported by other workers (Jiménez *et al.*, 1999). Slow waves were recorded only in the circular muscle layer of the porcine ileum and this is in agreement with the study by Jiménez *et al.* (1999). The interpretation of the porcine results in this thesis must be tempered by the small sample size of cellular impalements. For example, Figures 48 and 49 show data that may have been skewed by just two impalements of cells at a low percentage distance through the circular muscle. Given the ready availability of abattoir tissue, there is clearly scope for further investigation of electrical events in the porcine intestine. This should include a more exhaustive electrophysiological study of the presence or absence of slow waves in longitudinal muscle.

The longitudinal and circular smooth muscle layers of the equine ileum showed prominent slow wave activity. Indeed, this study would appear to be the first to demonstrate the presence of slow waves *in vitro* in equine intestine. Rakestraw *et al.* (2000) demonstrated small membrane potential oscillations in equine jejunum but these oscillations were abolished by nifedipine unlike the slow waves in this study. The addition of tetrodotoxin had no effect on the slow wave activity indicating that they were non-neural in origin. It seems reasonable to presume that the initiators of slow waves in the equine intestine are the interstitial cells of Cajal, as has been shown in other species (Ward *et al.*, 1997; Koh *et al.,* 1998; Thomsen *et al.,* 1998; Lee *et al.,* 1999). The results from this equine *in vitro* study correlate well with the *in vivo* results of other equine studies involving surgically-implanted extracellular electrodes (Davies and Gerring, 1983; Berry *et al.,* 1986; King and Gerring, 1989; Merritt *et al.,* 1989; Ross *et al.,* 1990).

The amplitude of the slow waves did not appear to be affected significantly by the position of the impaled cell relative to the site of the supposed pacemakers (in the MP region in the small intestine) whereas there did appear to be a correlation

between RMP and position in the longitudinal muscle. These findings are not in complete agreement with the work of Smith *et al.* (1987a) who found that both the RMP and slow wave amplitude decreased through the thickness of the circular muscle in canine colon away from the pacemaker region at the submucosal border. In the equine ileum, there was no such decrease in amplitude but there was the suggestion that the slow wave signal decayed in approximately the last 10% away from the MP region. It is possible that the electrotonic coupling between smooth muscle cells is more efficient in the horse than in the dog. This would allow the slow wave signal to travel more efficiently across the muscle layer and only decrease at the very outer limit away from the pacemaker region. If this is the case, it reflects a functional adaptation in the much thicker and more expansive intestine of the larger mammal.

The waxing and waning of slow wave amplitude was an interesting phenomenon seen in both the pig and the horse. It has not been commonly reported in the literature but Bortoff (1965) observed this behaviour in an *in vitro* study of feline jejunum. He suggested that the waxing and waning was due to two populations of pacemaker cells discharging at slightly different frequencies. The fact that the waxing and waning in the equine ileum was not blocked by tetrodotoxin suggests that it is not a nerve-mediated phenomenon. Further work is necessary to increase our understanding of these events. The biphasic slow waves seen in one diseased animal were converted to a normal slow wave morphology by nifedipine. It is possible that the cells were trying to "fire" and generate action potentials on top of the slow waves (aborted spikes). The nifedipine blocks the action potential mechanism by blocking L-type calcium channels and once this was done, "normal" slow wave activity ensued.

Nifedipine did not abolish slow wave activity but it is interesting to note that it did have an effect on some of the slow wave characteristics. In the presence of the drug, slow waves had a lower frequency and a longer duration than slow waves recorded in the absence of the drug, whilst amplitude and RMP were unaffected. This suggests that although the initiating pacemaker mechanism in the ICC is unaffected by the drug, there may be an effect on the determination of the frequency and duration by blockade of the L-type calcium channels. This subtle differential effect of nifedipine

on slow wave characteristics needs further investigation. It was difficult to achieve stable impalements in the absence of nifedipine because the spiking activity and concomitant muscle contractions dislodge the microelectrode from the cells. To examine the effects of nifedipine therefore, it may be possible to reduce the force of contractions by using wortmannin which inhibits myosin light chain kinase, whilst still preserving underlying electrical events. This has been successfully achieved in other studies (Rae *et al.*, 1998).

A possible criticism of this study is the unknown effect of the barbiturate used for euthanasia of the horses, in addition to any drugs that were being used in the animals' treatment regimens, on the *in vitro* properties of the ICC and smooth muscle. Murray (1995) showed that if barbiturates were added to *in vitro* equine preparations at the concentration of a lethal dose, they could be washed off again in minutes without permanently affecting the rate of background contractions. The equilibration period (60 minutes) in this study make it most likely that any drugs will have been washed off and will not have any residual effect. The ideal situation would have been to have horses that had been shot and without previous drug treatments. This was not possible for two reasons in this clinical study of large animals. At the University of Edinburgh, all horses are euthanased by lethal injection and the horses in the sample are clinical hospital cases rather than experimental animals. Furthermore, much of the reported *in vitro* work on experimental animals was performed on tissues taken from animals anaesthetised or euthanased with barbiturate drugs and there appeared to be no detrimental effect on the physiology of the tissue (Bortoff, 1965; Sanders and Smith, 1986a; Sanders and Smith, 1986b; Smith *et al.,* 1987a; Smith *et al.,* 1987b; Jiménez *et al.*, 1999). Human studies using tissue taken during surgical procedures under anaesthesia also reported no untoward effects related to the drugs administered to the clinical patients (Duthie and Kirk, 1978; Rae *et al.,* 1998).

In grass sickness, slow wave activity was preserved in 4 of the 6 animals studied. The fact that 2 of these were chronic and 2 acute cases suggests that the severity of the disease does not influence the slow wave activity. This is a fascinating finding because despite the fact that neural elements have been destroyed in the disease, the

ICC-mediated pacemaker function remains intact. This has implications for the development of pharmacological strategies in tackling gut dysmotility syndromes. The slow waves in grass sickness-affected ileum had lower frequencies and longer durations. This could be due to the decrease in ICC reported in the previous chapter or could be an effect of the decrease in enteric neurons observed in the disease. The fact that tetrodotoxin did not appear to have any effect on slow wave characteristics suggests that the latter explanation is unlikely and that the decline in ICC in grass sickness may be the most likely reason. It may be that the ICC that determine the frequency and duration of slow waves are decreased or that their function may be damaged in some way by the disease.

The remaining 2 animals with grass sickness did not show any slow wave activity. This could be either due to the fact that the source of electrical activity may have been damaged in the disease or because of problems in experimental technique. Tissues from these 2 animals were used very early on in the development of the electrophysiological experimental protocol and it may be that slow waves were present in the preparations but were not detected.

CHAPTER 5:DISCUSSION AND CONCLUSIONS

5.1 General Discussion

5.1.1 Tissue culture studies

The tissue culture experiments in this thesis highlighted the difficulties of working with equine tissue. Experiments were successful using guinea pig, rat and equine tissue but, of these systems, the rat MP culture methodology employed in the Saffrey laboratory was the most useful. Problems encountered using equine intestine included difficulties accessing the plexuses, poor penetration of antibodies in immunohistochemical experiments using wholemounts, poor neuronal yield and the overgrowth of non-neuronal cells.

Whilst it was considered inappropiate to pursue in the present study, the yield of neurons and successful growth *in vitro* can be improved by the addition of various trophic factors such as basic fibroblast growth factor, bFGF, (Schäfer *et al.*, 1995) and glial cell-line-derived neurotrophic factor (Schäfer and Mestres, 1999) to the culture systems.

Furthermore, the numbers of non-neuronal cells can be manipulated in culture. For example, glial proliferation can be minimised by using serum-free culture medium (Schäfer *et al.,* 1995) and fibroblasts can be decreased in number by the use of mitotic inhibitors such as cytosine arabinoside (Bannerman *et al.,* 1988b). Fibroblasts can also be selectively destroyed by treatment with anti-thy 1.1 antibody followed by the addition of guinea pig serum as a complement source (Sawant-Mane *et al.*, 1994). Serum-free chemically-defined medium provides a more controlled environment but serum-containing medium allows the maintenance of neurons at a lower density in addition to providing a detoxifying effect, allowing growth of neurons on substrata previously toxic to neurons (Higgins *et al.*, 1991). Nishi (1996) reported that the mitogenicity of the culture medium can be controlled by using horse serum instead of foetal calf serum. There is debate as to whether neurons grow

better in isolation from glia (Saffrey *et al.,* 1992a) or whether they are healthier in direct contact with glia (Saffrey *et al.,* 1991). Accordingly, cultures can be enriched for neurons or glia by using complement mediated cytolysis of glia or neurons (Bannerman *et al.,* 1988b).

Enteric glial cells have been the subject of much research. They are different cells to the non-myelinating Schwann cells of the peripheral nervous system but both are derived embryologically from the neural crest and only diverge relatively late in ontogeny (Gershon and Rothman, 1991). Both types of cell are known to show immunoreactivity for glial fibrillary acidic protein, GFAP, (Jessen and Mirsky, 1980) and S100 (Gershon and Rothman, 1991) which is an intracellular acidic protein that binds calcium ions (Bannerman *et al.,* 1988a).

This study has found that equine enteric glia also show immunoreactivity for S100 and GFAP. Nada and Kawana (1988) have a differing view from Gershon and Rothman (1991) on the classification of the supporting cells of the rat ENS. They found that immunoreactivity to GFAP was almost exclusively found in cells associated with the MP and a small number of cells within the SMP. The use of S100 protein antisera resulted in the visualisation of all the supportive elements of the ENS. They suggested that there are 2 types of supporting cells in the ENS: glial cells (positive for GFAP and S100) that possibly are associated with the parasympathetic preganglionic fibres directly derived from the CNS, and Schwann cells (positive for S100) that are descended from the neural crest. There is debate as to whether the use of S100 antibody reveals immunofluorescence in the nucleus (Almqvist *et al.,* 1994; Kobayashi *et al.,* 1986) or the cytoplasm of the cells (Hanani *et al.,* 1989). In this equine study, the immunofluorescence observed was in the nuclei of the enteric glia.

The electrophysiological characteristics of enteric glial cells have been described (Maudlej and Hanani, 1992) and it is believed that these cells may share some characteristics in common with CNS astrocytes (Hanani *et al.,* 1989). This latter work immunohistochemically identified the glia on the basis of the size and disposition of the nuclei and processes which formed a basket-like network around non-fluorescent spaces (neurons). Glia in peripheral ganglia (sympathetic,

parasympathetic and sensory) are flattened satellite cells whereas enteric glia are process-bearing cells, morphologically similar to astrocytes. The antigenic profile of enteric glia includes vimentin, GFAP and S100 (Bannerman *et al.* 1987). The enteric glia warrant further investigation in the horse in both health and disease to determine their involvement, if any, in the pathophysiology of gut dysfunction.

An alternative to ENS cultures is the culture of neurons from autonomic ganglia. Neurons from the autonomic ganglia of small mammals such as guinea pigs (Matsumoto *et al.,* 1993) and rats (Lein *et al.,* 1995) have been cultured. The former report showed that these neurons retain their *in vivo* characteristics *in vitro.* In studies using prenatal or perinatal rat superior cervical ganglia the neurons retain their *in vivo* plasticity in culture conditions. It was shown that cell morphology, receptor profile and neurotransmitter phenotype may change in response to alterations in tissue culture environment; these changes tend to be uniform (more than 80% neurons affected) and large in magnitude (10-100 fold changes) (Higgins *et al.*, 1991).

There has been some preliminary work published documenting the development of cultures of equine autonomic ganglia cells that have been genetically-altered to immortalise the cell lines (John *et al.,* 1997a). This approach may be useful in examining the aetiology of grass sickness with the cautionary note that genetically-altered neurons may have different properties to the neurons in the animal and, almost certainly, little genetic pleomorphism. An alternative approach would be to culture neurons directly in dissociated cell systems taken from fresh equine ganglia, such as the cranial cervical ganglion. This would have the advantage over equine ENS cultures because the cells are far more accessible and this may increase the neuronal yield. Preliminary experiments using this approach have encountered the problem of overgrowth of fibroblasts (I. Griffiths, personal communication) but this could be overcome by methods described above.

One of the aims of the tissue culture experiments was to eventually challenge the cultures with putative toxic agents to clarify the neurotoxic mechanisms involved in grass sickness. Tissue cultures can be challenged by various toxic insults and the

effects on the cells can be measured in a number of ways. Gurwell and Hauser (1993) added morphine to mouse glial cultures and measured the effect with cell counts using a haemocytometer and a cell viability assay. Vige *et al.* (1993) antagonised L-glutamate-induced toxicity in rat cortical neurons with N^G-Nitro-L-Arginine and assessed neuronal injury qualitatively with phase contrast microscopy and quantitatively with a lactate dehydrogenase (LDH, which is released with cell damage) assay. Other methods of assessing viability and cell damage include: examining the markers of apoptosis with the TUNEL reaction and DNA fragmentation (Hossain *et al.,* 1996; Papassotiropoulos *et al.,* 1996), measuring the number of cells per mg of tissue in cases such as Schwann cell cultures from peripheral nerves (Levi *et al.,* 1994), morphological assessment with video image analysis (Malgrange *et al.,* 1994) and the MTT (a tetrazolium salt) cell viability assay (Malgrange *et al.,* 1994; Gavrilovic *et al.,* 1995). Mulderry (1994) used two methods to check cell viability of adult rat sensory neurons in culture: phase-contrast optics and ethidium-DNA fluorescence (indicating an intact plasma membrane). Froissard *et al.* (1997) showed that the addition of glutamate to neurons in culture caused cell death and that this effect could be abolished with the addition of cystine and *N*-acetylcysteine to the system. Anti-cancer drugs (such as cisplatin, vincristine and taxol) clinically induce toxic sensory and autonomic neuropathies. Nerve growth factor prevents and reverses this in adult rat superior cervical ganglia cultures (Hayakawa *et al.,* 1994). Using an explant culture system measuring neurite outgrowth and regeneration, the authors described a useful *in vitro* model of toxic autonomic neuropathy. VIP has been shown to increase neuronal survival in dissociated spinal cord cultures by acting as an astroglial mitogen and a secretagogue for neuronal survival-promoting activity (Brenneman *et al.*, 1990). The authors suggest that neurotransmitters and neuromodulators, in addition to their recognised action on synaptic transmission, may regulate glial-derived neurotrophic substances by modifying the number and function of supporting cells. This has direct implications in grass sickness where there is, as described earlier, an increase in the number of supporting cells and a decrease in the number of neurons showing positive immunoreactivity for neurotransmitters such as VIP (Hodson *et al.,* 1982; Sabate *et al.,* 1983; Bishop *et al.,* 1984; Scholes *et al.,* 1993a). Broussard *et al.* (1993)

examined the mitogenic potential of various growth factors (acidic fibroblast growth factor, bFGF and forskolin) on cultured neonatal guinea pig enteric glia. The effects on DNA synthesis were determined by incorporation of the thymidine analogue, bromodeoxyuridine. More than 95% of sympathetic neurons die in culture in the absence of nerve growth factor and manipulating this component in the medium can be useful (Higgins *et al.*, 1991). Following ischaemia (removal of oxygen and glucose for 4 hours), it has been shown that cultured rat embryo CNS astrocytes show an increase in somatic GFAP-immunoreactivity (Goldberg *et al.*, 1987). Protection against the ischaemia with a medium low in sodium and calcium and high in potassium results in the absence of this enhancement of GFAP-immunoreactivity. Serum antibody from patients with Guillain-Barré syndrome mediates complement-mediated cytolysis (detected by Trypan blue vital dye exclusion) of rat Schwann cells in culture. This susceptibility reflects Schwann cell phenotype and maturity with less differentiated cells being the most susceptible (Sawant-Mane *et al.*, 1994). This model could potentially be applied to the challenging of cultured neurons and supporting cells with serum from grass sickness cases. It will be important to aim for a repeatable culture system in the horse and to examine how neurons behave in culture before and after challenges. The results in the literature are quite varied, but some authors report that only 35% of adult neurons die in culture whereas over 90% of neurons from neonatal animals do not survive in culture (Hanani, 1993). The equine ENS cultures described in this thesis unfortunately need more work to enable them to be used for quantifiable analysis of toxic challenges. There has been some preliminary work in challenging equine immortalised autonomic neurons in culture with fungal agents (Robb *et al.,* 1997; Hoey and John, 1998), plasma from grass sickness cases (John *et al.* 1997a; John *et al.* 1997b; Hoey and John, 1998) and soluble extract of an autonomic ganglion from a case of grass sickness (Hoey and John, 1998). These studies showed that extracts of *Fusarium* fungus, grass sickness plasma and ganglion extract were all toxic to the neurons in tissue culture. This area of research offers much promise, whilst still bearing in mind the cautionary note of the uncertainty concerning the validity of using immortalised cells.

5.1.2 Immunohistochemical studies of the interstitial cells of Cajal

This study has shown the distribution of ICC in the equine gastrointestinal tract. Also, it has shown that the ICC are decreased to varying degrees in grass sickness suggesting that the cells may be involved in some way in the pathophysiology of the disease.

The normal immunohistochemical study raised the issue of the ICC distribution and density at different ages. Although it was felt that there was no obvious change in c-Kit expression at different ages, a definitive study of the ontogeny and post-natal development of the ICC in the horse is indicated. It has been suggested that there is a common developmental origin of ICC and the longitudinal smooth muscle layers in the mammalian small intestine and that *kit* expression is necessary for the postnatal development of ICC (Klüppel *et al.*, 1998). There have been some recent studies on the ontogeny of ICC in the human intestine (Kenny *et al.*, 1999; Wester *et al.*, 1999). ICC are present from an early stage in human gut development and the morphology and network structure is age-dependent during early life up to approximately 2 years of age where the distribution is similar to adults. Therefore, neonatal studies of ICC (eg in Hirschsprung's disease) need to be carefully controlled and preferably supported by electrophysiological or molecular biological data (Kenny *et al.*, 1999). This is particularly pertinent to this equine study where immunohistochemical data on the ICC can be put into context by functional electrophysiological experiments.

The ICC have been implicated in many diseases of the gastrointestinal tract, particularly in humans. In infantile hypertrophic pyloric stenosis, ICC are almost entirely absent in the affected pylorus (Langer *et al.*, 1995; Vanderwinden *et al.*, 1996a). In Hirschsprung's disease, an intestinal aganglionosis similar to the lethal white syndrome in foals, the density of ICC is markedly decreased in the aganglionic segment (Vanderwinden *et al.*, 1996b). There are alterations in the ultrastructure of ICC in ulcerative colitis suggesting that they may be involved in some way in the motility disturbances seen in this disease (Rumessen, 1996). A deficiency of ICC is seen in patients with a myopathic form of chronic idiopathic intestinal pseudo-obstruction (Isozaki *et al.*, 1997). Defects in the populations of ICC have been

reported in children with anorectal malformations (Kenny *et al.*, 1998a). A loss of ICC in a case of megacolon in an adult has been reported (Faussone-Pellegrini *et al.*, 1999). In patients with slow transit constipation, there is a significant decrease in the volume of ICC of the colon (He *et al.*, 2000) and it has also been shown that there is a deficiency of ICC in the colon of patients with Chagas' disease (Hagger *et al.*, 2000). In experimentally induced ileal hypertrophy in rats, there is a decreased number of ICC in the hypertrophied segment (Ekblad *et al.*, 1998). Studies of ICC may be important to help in the diagnosis of certain diseases (Hagger *et al.*, 1997). Indeed, this may be extended to making ICC a target for pharmacological intervention in the treatment of gastrointestinal disease (Huizinga *et al.*, 1997; Vanderwinden and Vanderhaeghen, 1998). Therefore the findings in this study of a decrease in the ICC in cases of grass sickness with a suggestion of a relationship with the severity of the disease are very interesting. Ultrastructural studies would be indicated to determine if the remaining ICC are damaged in any way, although the presence of slow waves in grass sickness cases suggests that the ICC are functional. Ward *et al.* (2000a) noted that the loss of cholinergic neural responses in smooth muscle was due to the loss of synaptic contacts between neurons and ICC (which were absent in the W/W^{v} mutant mice) in the circular muscle. Perhaps in grass sickness, the loss of synaptic contact between neurons and ICC, either because of neuronal death or a decrease in ICC, is crucial in the subsequent disruption of normal gut motility.

Further work is necessary on the ICC and neurons in recovered cases of grass sickness. Kitamura *et al.* (1998) presented preliminary data on the changes in immunoreactive neural elements in the three different categories of the disease (acute, subacute and chronic). Once this work is completed, it will stand as a useful comparison for subsequent studies on recovered chronic cases.

5.1.3 Electrophysiological studies

The electrophysiological part of the thesis described some of the electrical properties of porcine and equine intestinal smooth muscle. The pig is a useful model, not only

because of the abundant supply of abattoir-derived tissue, but because intestine from the pig is generally considered as the most appropiate for comparison with the human (Thomsen *et al.,* 1997; Jiménez *et al.,* 1999).

The equine study showed abundant slow wave activity in both normal and grass sickness-affected horses. The preservation of slow wave activity in grass sickness despite the immunohistochemical results indicating a decline in ICC is interesting. The remaining ICC would therefore appear to be functional and this offers hope in the development of treatments for the disease. Furthermore, the Sanders laboratory has made a recent exciting discovery concerning ICC plasticity in a murine experimental model of ileal pseudo-obstruction. They have demonstrated that the ICC phenotype and slow wave generation is plastic and can diminish in pathophysiological states. However, the ICC phenotype and slow wave activity can reappear following removal of pathophysiological stimuli, in this case a surgically-applied clip around the ileum (S. Ward, personal communication).

Further experiments are necessary on normal equine ileum to better characterise the electrical properties. Electrical field stimulation and focal stimulation can be used to excite nerve fibres within the *in vitro* preparations (Katayama *et al.*, 1986). The neurotransmitters released will be expected to have direct effects on the smooth muscle cell membrane by evoking either excitatory or inhibitory junction potentials. They may also influence the slow wave characteristics directly or indirectly (via ICC). The effects of enteric nerve stimulation can then be assessed in both normal and diseased intestine. Analysis of the possible effects of enteric neurotransmitters involves the use of antagonists for muscarinic, nicotinic and adrenergic receptors. In view of the putative role of nitric oxide in the intestine (Rakestraw *et al.*, 1996; Keef *at al.*, 1997), the nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine can be used to investigate the involvement of the nitrergic neurons.

The physiological role of the ICC can be addressed by examining the relationship between the electrical properties of individual cells in both the longitudinal and circular smooth muscle layers and the distribution of ICC as revealed by c-Kit immunohistochemistry and recombinant DNA technology. Knowledge of the ICC

distribution from this study can be used as the basis for ablation experiments in which the hypothesised pacemaker regions can be removed by microdissection. The effect of specifically-sited ablations on the slow wave activity allows the origin of pacemaker activity to be determined in different tissues (Smith *et al.*, 1987b). Once the normal physiology is better understood, direct pathological comparisons can be made both within and between different species. The studies can also expand to include different parts of the gastrointestinal tract. For instance, the equine colon is an area that warrants investigation because of its clinical importance.

5.1.4 Recent developments in gastrointestinal motility research

Double labelling and confocal microscopic techniques have recently been applied to ICC research and have opened up interesting avenues of gastrointestinal research (Torihashi *et al.,* 1999b, Ward *et al.* 1999b; Ward *et al.* 2000a). For instance, these techniques have been applied to a study where blockade of Kit signalling was shown to induce a change of ICC to a smooth muscle phenotype (Torihashi *et al.* 1999b). Therefore these established techniques will facilitate quantitative and qualitative comparison between normal and diseased tissues in addition to improving our understanding of gut physiology.

Recombinant DNA technology has allowed closer analysis of gastrointestinal function. The c-Kit expression of ICC can be examined using recombinant DNA technology. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) has been performed on total RNA prepared from intestinal smooth muscle using established methods in the Sanders laboratory (Ward *et al.* 1999b). Primers for *c-kit* have been used in other studies (Giebel and Spritz, 1991; Teng *et al.,* 1998) and northern, southern and western blot analyses can be utilised. The expression of the c-Kit protein in the ICC of normal tissues and clinical cases can now be compared. A conserved house-keeping gene such as β-actin can be used as a comparative marker. Interference with downstream c-Kit signalling by inhibition of phosphatidylinositol 3′-kinase (PI3-kinase) may cause a loss of ICC (Ward *et al.,* 1999a). Therefore it is now possible to examine putative mechanisms involved in motility disorders by

investigating whether dysfunction relates to genetic predisposition (problems with the *c-kit* gene) or postnatal c-Kit signalling. Also, the expertise exists in the Sanders laboratory to examine stem cell factor (SCF) (the ligand for c-Kit) expression in the intestine. The use of neonatal tissue in a range of species will be critical to the molecular biological research as it offers a window into the developmental components of intestinal dysmotility.

Information about the genetic basis of gastrointestinal diseases is expanding rapidly. For example, gastrointestinal stromal tumours are increasingly being recognised in human medicine and there is evidence to suggest that a novel gain-of-function mutation of the *c-kit* gene may be involved (Nakahara *et al.*, 1998). The acquisition of intestinal tissues in this study will allow the creation of a database for future genetic studies. The store of equine tissues can be used in studies of genes such as *c-kit*, *ret* and *endothelin*. In the Sanders laboratory, work is underway on examining ICC in glial cell line-derived neurotrophic factor (GDNF) knockout mice which lack enteric neurons (Ward *et al.,* 1999b). In addition to genetic factors, the effects of inflammatory mediators and environmental factors on the ICC need to be examined in the future.

There is currently great interest in the ionic basis of the ICC pacemaker currents and their intracellular regulation. Ward *et al.* (2000b) have recently suggested that integrated calcium handling by endoplasmic reticulum and mitochondria is necessary for electrical pacemaking in the gastrointestinal tract. It has also been shown that, in murine gastric smooth muscle, the expression of inositol triphosphate receptors may be causally related to the generation of slow waves but not to the generation of action potentials. A lack of these receptors attenuates cholinergic excitatory and nitrergic inhibitory responses but has no effect on the response to noradrenaline (Suzuki *et al.*, 2000). This could be an important area of research in gut dysfunction and the results may be interesting in cases of grass sickness.

5.2 Conclusions

This study has shown that it is possible to grow equine enteric neurons successfully in tissue culture. The most useful equine system was the dissociated cell preparation derived from the submucous plexus. However, technical difficulties mean that if the work on an *in vitro* model for grass sickness were to continue, then the best approach would be to use a small mammal model or cells harvested from equine autonomic ganglia.

The immunohistochemical evaluation of the ICC has shown that there is a rich distribution of these cells throughout the normal equine gastrointestinal tract. The ICC distribution shows regional differences that might be related to function. ICC are present in the ileum and pelvic flexure of horses with grass sickness but in reduced densities. This has possible implications for the development of the intestinal dysmotility observed in the disease.

The normal equine ileum has prominent slow wave activity as determined by intracellular microelectrode recordings. Slow wave activity is preserved in most horses with grass sickness suggesting that although the enteric neurons are damaged, the ICC-mediated control of electrical activity remains intact. This has potential future therapeutic implications.

5.3 Future Directions

The work from this thesis has stimulated various avenues for possible investigation. I would like to investigate the electrical properties of normal equine small and large intestine. This will include the use of pharmacological and electrical manipulations of the tissue preparations. This will then provide the basis for work investigating the equine intestine in disease states such as the various colic syndromes and postoperative ileus.

Further work in the horse is also indicated examining immunohistochemically the ICC, neural and glial elements, for example using double labelling to understand

better the anatomy and physiology of these cells. A study of the ontogeny of the ICC in the horse is indicated. This can then be used for a comparison in neonatal disease situations such as lethal white foal syndrome. A supply of neonatal equine tissues is already being collected for this purpose at the University of Florida, USA.

The work on the ICC needs to explore the techniques employed in the Sanders laboratory involving advanced electrophysiology, recombinant DNA technology, ICC culture techniques and work on cellular ion channels.

Once the work on the horse has been completed this will provide a basis for a comparative study on the physiology and pathophysiology of intestinal motility. This can encompass investigation of normal porcine tissue, diseased feline tissue (megacolon) and eventually surgically resected human tissue. Ultimately, it is hoped that these studies will address the relationships between the components of motility control in health and disease, namely the neural, muscular or pacemaker (ICC) cells.

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APPENDIX

Publications and presentations arising from work described in this thesis.

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An immunohistochemical study of interstitial cells of Cajal (ICC) in the equine gastrointestinal tract

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SUMMARY

The interstitial cells of Cajal (ICC) are c-kit immunoreactive cells of the gastrointestinal tract which are suggested to have a role in the control of intestinal motility. Cells with c-kit immunoreactivity have not been previously described in the gastrointestinal tract of the horse. Immunoreactivity for c-kit was revealed using immunohistochemical labelling with an anti-c-kit polyclonal antibody. Sections of normal gastrointestinal tissue were examined from 13 anatomically defined sites from stomach to small colon taken from horses free from gastrointestinal disease. Three types of c-kit immunoreactive cells were identified: spindle-shaped cells in the region of the myenteric plexus, stellate or bipolar cells in the circular muscle layer, and round cells in the submucosa. The round cells were shown to be mast cells with the use of toluidine blue staining, whereas the other c-kit immunoreactive cells did not exhibit metachromasia and were classified as ICC. This study will serve as a basis for future pathological studies in the horse.

THE interstitial cells of Cajal (ICC) are a group of cells in the gastrointestinal tract which were first described by Santiago Ramón y Cajal (Cajal 1893, Cajal 1911). The ICC are currently attracting much interest because of their putative role in the control of intestinal motility (Hagger et al 1997). These cells form a network in close association with the smooth muscle of the intestine (Vanderwinden et al 1996a) and they are considered by some authors as being the pacemakers and mediators of neurotransmission in the gastrointestinal tract (Langton et al 1989, Torihashi et al 1995, Sanders 1996). Furthermore, it has been confirmed recently that ICC generate a rhythmic pacemaker current (Thomsen et al 1998).

ICC have been shown to express the proto-oncogene c-kit, which encodes a receptor tyrosine kinase (Ward et al 1994, Huizinga et al 1995). Antibodies raised against c-kit are now available for the immunohistochemical labelling of ICC (Ward et al 1994). The distribution of ICC has been examined in humans and some animals including the guinea pig, mouse, rat, cat, dog, rabbit, ferret, opossum and pig, and there does appear to be some interspecies variation (Christensen et al 1992, Burns et al 1997, Hagger et al 1997, Henry et al 1998, Rømert and Mikkelson 1998). There have been no studies of the ICC in the horse.

Abnormalities of the ICC have been implicated in diseases of the human gastrointestinal system such as Hirschsprung's disease (Vanderwinden et al 1996b), hypertrophic pyloric stenosis (Langer et al 1995, Vanderwinden et al 1996a), ulcerative colitis (Rumessen 1996) and chronic idiopathic intestinal pseudo-obstruction (Isozaki et al 1997). Gastrointestinal diseases, including obstructive and motility disorders, are very common in the horse. As a basis for future investigations of pathological states, the present study examined the distribution and characteristics of the c-kit immunoreactive cells of the normal equine gastrointestinal tract, from the stomach through to the small colon.

MATERIALS AND METHODS

Gastrointestinal samples were taken within one hour of death from seven horses euthanased because of clinical conditions not involving the gastrointestinal tract (eg orthopaedic problems). The horses were euthanased by intravenous administration of quinalbarbitone sodium BP (400 mg ml^{-1})/cinchocaine hydrochloride BP (25 mg ml^{-1}) (Somnulose; Arnolds Veterinary Products, UK). In four of the horses, samples were taken from 13 anatomically defined sites from the stomach through to the small colon (Table 1). In the remaining three horses, samples were taken from the duodenum, ileum and pelvic flexure. All tissues were rinsed with phosphate-buffered saline (PBS, 0·1M, pH 7·0) and placed immediately in 10 per cent phosphate-buffered formalin (pH 7·4) and fixed for at least 24 hours. After rinsing in distilled water, the tissues were cryoprotected in graded sucrose solutions (10 per cent and 30 per cent sucrose in PBS) and then frozen rapidly in isopentane pre-cooled in liquid nitrogen. Before sectioning, the tissue blocks were oriented to allow either transverse or longitudinal sections of the entire intestinal wall to be cut. Cryostat sections were cut at thicknesses of 10 µm to 30 µm and

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TABLE 1: Anatomical positions of the sample sites in the equine gastrointestinal tract

Region	Sample site
1. Stomach	Midpoint of greater curvature
2. Stomach	Pyloric canal
3. Duodenum	Midpoint of descending duodenum
4. Jejunum	Midpoint of the small intestine
5. Ileum	Level with the midpoint of the ileocaecal fold
6. Caecum	Midpoint of body including medial taenial band
7. Caecum	Midpoint of the caecal base
8. Right ventral colon	Midpoint, including medial free band
9. Left ventral colon	Midpoint, including medial free band
10. Pelvic flexure	Apex
11. Left dorsal colon	Midpoint, antimesenteric aspect
12. Right dorsal colon	Midpoint, including dorsomedial taenial band
13. Small colon	Midpoint, including free taenial band

mounted on Tespa (3-aminopropyltriethoxysilane) coated slides (Sigma, Poole, UK) and allowed to air-dry overnight. After a thorough wash with PBS, the sections were incubated for 30 minutes in 0·3 per cent hydrogen peroxide in methanol to quench endogenous peroxidase activity. Non-specific antibody binding was blocked by a 60 minute incubation in 1 per cent goat serum in PBS. Sections were then incubated overnight at 4°C in humid chambers in a rabbit-raised polyclonal antiserum to c-kit(Ab-1), (Oncogene Research Products, Cambridge, MA, USA) at a concentration of 1 μg ml^{-1}. After washing with PBS, the sections were then incubated at room temperature for 60 minutes in a biotin-conjugated goat anti-rabbit immunoglobulin at a concentration of 1:200. Immunoreactivity was revealed with the avidin-biotin (ABC) method (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) using a diaminobenzidine substrate (DAB, BDH Laboratory Supplies, Poole, UK). Sections were dehydrated in ethanol, cleared in xylene, and then mounted in Depex (Merck, Glasgow, UK). Immunohistochemical controls were prepared in a similar manner, omitting the primary antibody or replacing it with normal rabbit serum. This led to the complete absence of immunolabelling. In some experiments, adjacent frozen sections were stained with toluidine blue to demonstrate metachromatically stained mast cells. As an alternative to the ABC method, a standard indirect immunofluorescence technique was used for some tissues. In this case, the sections were incubated in the primary antiserum at a concentration of 10 μg ml^{-1} for 16 to 18 hours in humid chambers, rinsed in PBS and then incubated in FITC-conjugated goat anti-rabbit antiserum (Cappel; Dynatech Laboratories, Billinghurst, Sussex, UK) at a dilution of 1:10 for 2 hours. To confirm the properties of the primary antibody, indirect immunofluorescence tests were also carried out on wholemount preparations of guinea pig jejunum using well established methods (Llewellyn-Smith et al 1985).

RESULTS

Cells possessing c-kit-like immunoreactivity (c-kit-LI) were identified in several locations throughout the gastrointestinal tract of all the horses studied. Spindle- or triangular-shaped cells within the intermuscular space between the circular and longitudinal muscle layers at the level of the myenteric plexus (MP), stellate or bipolar cells located within the circular or inner smooth muscle layer (Fig 1), and round cells in the submucosa were all identified as being c-kit-LI cells. The cells in the intermuscular space usually had 1 to 2 processes whilst the intramuscular cells had 2 to 5 processes. The different orientation of the sections showed that the cells had a preferential position in one plane and direction, giving them a long axis parallel to the direction of the fibres of the neighbouring smooth muscle. The round cells with c-kit-LI in the submucosa in all regions of the gastrointestinal tract were recognised as mast cells (Galli et al 1993) by comparing experimental sections with adjacent toluidine blue sections in which they showed distinct metachromatically stained granules.

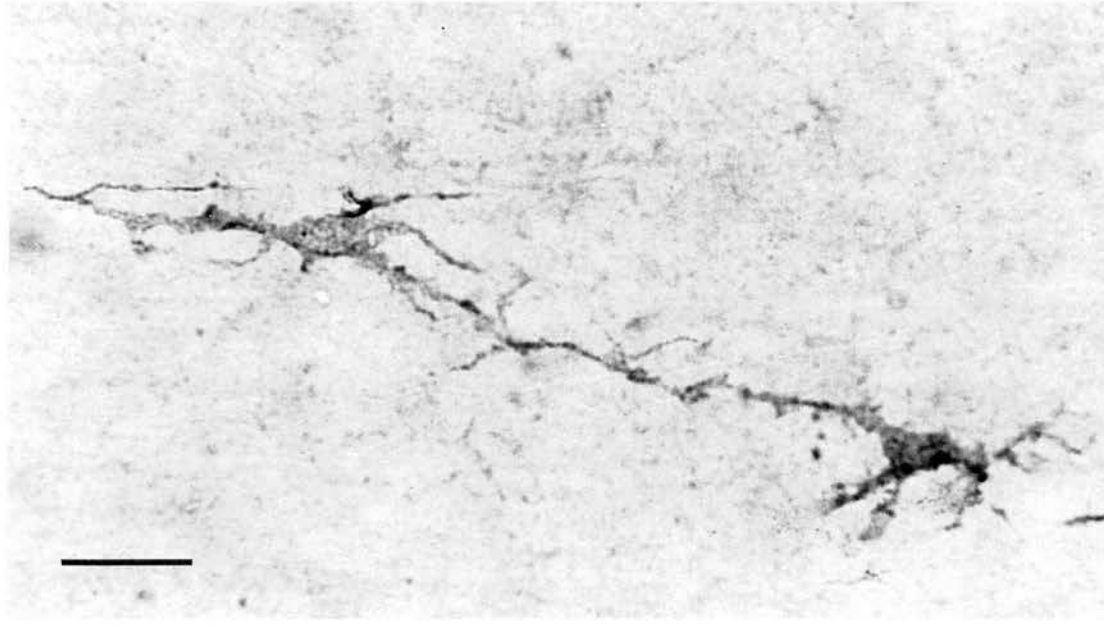

FIG 1: Stellate-shaped cells with c-kit-LI within the circular muscle of the left dorsal colon (Bar = 25 μm).

FIG 2: Stellate-shaped cells with c-kit-LI throughout the inner muscle layer of the greater curvature of the stomach (Bar = 100 μm).

FIG 3: c-kit-LI in the border region between the inner and outer muscle layers of the greater curvature of the stomach (Bar = 50 μm).

Stomach

Many stellate cells and portions of cellular processes with c-kit-LI were found in the inner muscle layer of the greater curvature of the stomach (Fig 2). Occasional immunoreactive cellular processes were seen in the outer muscle layer. There was evidence of immunoreactivity along the border of the inner and outer muscle layers (Fig 3). A similar pattern was seen in the pyloric canal region of the stomach.

Small intestine

Cells with c-kit-LI were found in highest numbers in the intermuscular space between the circular and longitudinal muscle layers of the muscularis externa in the region of the MP (Fig 4). These cells formed dense networks which sometimes extended a short distance into the longitudinal muscle layer. Some stellate and bipolar cells were found throughout the circular muscle layer, especially in the inner third

FIG 4: Cells with c-kit-LI within the intermuscular space of the ileum between the circular (cm) and longitudinal (lm) muscle layers at the level of the myenteric plexus (Bar = 50 μm).

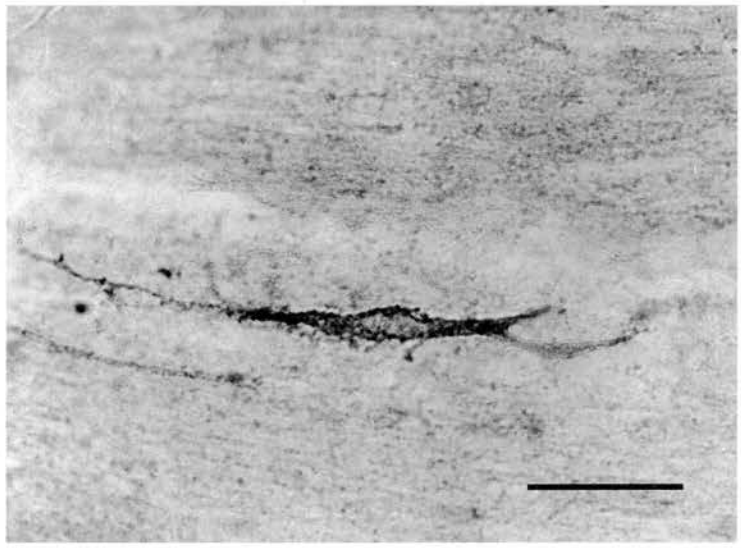

FIG 5: Bipolar-shaped cell with c-kit-LI within the circular muscle layer of the duodenum (Bar = 25 μm).

(Fig 5). The same patterns of distribution were found in the duodenum, jejunum and ileum but the density of cells with c-kit-LI in the region of the MP appeared greatest in the ileum, with traces of immunoreactivity spreading further into the longitudinal muscle layer.

Caecum

In contrast to the small intestine, large numbers of stellate and bipolar cells with c-kit-LI were found throughout the circular muscle layer (Fig 6). Furthermore, very few cells were seen at the region of the MP, producing a delicate lace-like pattern that was different to the denser pattern seen in the small intestine. Occasional traces of immunoreactivity were seen in cellular processes in the longitudinal muscle layer. The pattern of distribution of c-kit-like immunoreactive cells was identical in both the mid-caecum and caecal base, but the density of cells was far greater in the MP and circular muscle regions in the mid-caecum than in the caecal base.

Colon

Many stellate and bipolar cells and cell processes with c-kit-LI were seen throughout the circular muscle layer. As in the caecum, the density of cells was much greater than in any region of the small intestine. A similar lace-like pattern of immunoreactivity to that seen in the caecum was seen in the region of the MP and this was very different to the dense branching network seen in the small intestine. There were occasional cell processes found in the longitudinal muscle layer. In sections taken to include taenial bands there appeared to be more cells in the circular muscle and an increased density of the MP pattern underneath the taenial

FIG 6: Stellate- and bipolar-shaped cells with c-kit-LI throughout the circular muscle layer of the body of the caecum. Note the lace-like pattern of immunoreactivity (arrow) at the level of the myenteric plexus (Bar = 50 μm).

bands. Identical patterns of distribution were seen in the right ventral colon (RVC), left ventral colon (LVC), pelvic flexure (PF), left dorsal colon (LDC), right dorsal colon (RDC) and small colon (SC). However, the density of the cells in the circular muscle of the PF appeared greater than in other parts of the colon. Furthermore, the density of the network in the region of the MP of the RVC appeared to be less than in other parts of the colon.

Immunofluorescence studies

The equine sections processed using the indirect immunofluorescence method showed a similar morphology and distribution of the c-kit-like immunoreactive cells to the ABC processed sections. In the guinea pig jejunum, cells with c-kit-LI were found in the wholemount preparations of the MP but not in the submucous plexus. The cells in the MP possessed triangular or oval nuclei with multiple cellular processes which formed a well defined and branching network around the myenteric ganglia. This correlates well with results from other studies in the guinea pig (Burns et al 1997) and hence the data are not shown here.

DISCUSSION

This immunohistochemical study has demonstrated the distribution and characteristics of c-kit immunoreactive cells in the normal equine gastrointestinal tract. Previous studies in the mouse (Ward et al 1994, Huizinga et al 1995, Torihashi et al 1995, Burns et al 1996, Torihashi et al 1997), guinea pig (Komuro and Zhou 1996, Burns et al 1997, Seki et al 1998), rat (Isozaki et al 1995, Ekblad et al 1998) and human (Vanderwinden et al 1996a, Vanderwinden et al 1996b, Isozaki et al 1997, Rømert and Mikkelsen1998) have shown that anti-c-kit antibodies specifically label several classes of ICC. The morphology and location of the c-kit-LI cells seen in the muscularis externa in this study make it most likely that they are the ICC of the equine gastrointestinal tract.

The ICC are postulated to have three roles in the gastrointestinal tract: they act as pacemaker cells generating slow waves, they facilitate propagation of electrical events and they mediate neurotransmission (Sanders 1996). Slow waves are rhythmical oscillations of the smooth muscle membrane potential (Weisbrodt 1974). Contractile activity occurs when spike bursts are superimposed on slow waves. Slow wave activity is the rate limiting step for peristaltic activity (Hagger et al 1997). It has been shown in vitro that ICC are the cells that generate this rhythmic pacemaker current (Ward et al 1997, Thomsen et al 1998). Without slow wave activity no normal patterns of peristalsis develop and there is an increased transit time of ingesta (Huizinga et al 1996). The ICC express the c-kit proto-oncogene and it has been shown that when anti-c-kit antibodies are injected into mice this results in disruption of gut motility, a reduction of ICC and a loss of electrical rhythmicity (Torihashi et al 1995). The development of ICC and generation of electrical intestinal rhythmicity are blocked in W/W^v mice which have a mutation of the c-kit gene (Ward et al 1994). Furthermore, Ws/Ws mutant rats lacking a functional c-kit gene have disturbed intestinal movement which is attributed to a lack of ICC (Isozaki et al 1995).

The present study has shown a major difference in the distribution patterns of the ICC in the small intestine compared to that of the large intestine. In the small intestine c-kit-LI was concentrated in the MP region whereas in the large intestine it was mainly seen throughout the circular muscle layer.

Given the postulated functions of the ICC, this may reflect a regional difference in the origin of slow wave activity, bearing in mind that the dominant source of slow waves in the stomach and small intestine is the myenteric region, whilst in the large intestine it is the circular muscle layer (Sanders 1996). There is considerable variation in the distribution of ICC in man and in other mammals (Christensen et al 1992, Hagger et al 1997). A classification scheme for the different types of ICC that may be seen has been published (Sanders 1996). These cell types are ICC in the myenteric region (stomach, small bowel, colon), ICC at the submucosal surface of the circular muscle of the colon, ICC of the deep muscularis plexus of the small intestine, and intramuscular ICC in the circular and longitudinal muscle layers. In this equine study no specific accumulations of ICC were seen either in the area of the deep muscularis plexus of the small intestine or the submucosal aspect of the colonic circular muscle layer. However, there did appear to be greater numbers of cells observed in the inner third of the circular muscle layer of the small intestine. ICC have been described as being interposed between nerves and muscle cells where they form gap junctions with each other and nearby smooth muscle cells (Langton et al 1989). They are innervated by enteric neurons and can send extensions into both smooth muscle layers, between muscle cells and into connective tissue septa (Rumessen and Thuneberg 1991). In this study, sectioning of tissues in different planes has shown that the ICC tend to have their long axes parallel to the direction of the muscle fibres. This correlates well with findings of other workers (Rømert and Mikkelsen 1998).

The highest densities of ICC were observed in the ileum, pelvic flexure and body of the caecum. It is therefore tempting to speculate as to whether these areas are notable with regard to control of gut motility in the horse. There have been many pacemaker sites suggested in the equine gastrointestinal system. These include the gastric antrum (Gerring 1991, Fenger et al 1998), the longitudinal muscle of the proximal duodenum (Phaneuf and Ruckebusch 1983), the pelvic flexure (Sellers et al 1979, Sellers et al 1982, Clark 1990), the caecal body (Clark 1990), the caecal apex (Ross et al 1989), the caecal base (Gerring 1991), the right ventral colon (Ross et al 1990) and the transverse colon (Schusser and White 1997). Much of this information must be qualified in that there is great difficulty and complexity in the interpretation of equine intestinal myoelectrical activity with regard to pacemaker function (Merritt et al 1995). Further work is needed to confirm whether the density of ICC in the horse parallels the areas where there is a prominence of slow wave activity as has been shown in other animals (Christensen et al 1992). This study has shown that there are ICC in many locations in the equine gastrointestinal tract. Therefore, it is possible that the coordination of activity exerted by ICC is by means of a complicated and diffuse network rather than by discrete pacemaker sites. It is interesting that the distribution of cells in the caecal base was more similar to the right ventral colon than the body of the caecum. This may reflect the fact that in embryological terms the caecal base in the horse is actually the initial part of the right ventral colon rather than a true part of the caecum (Dyce et al 1996).

Studies of ICC will certainly be very important in understanding equine gastrointestinal disease because the cells have been implicated in many diseases, particularly in humans. Indeed, if ICC are important motility regulating cells, it is more than likely that they are involved, either primarily or secondarily, in the pathogenesis of some gastrointestinal disease processes in which there is a prominent element of motility disturbance (Rumessen et al 1993). In infantile hypertrophic pyloric stenosis ICC are almost entirely absent in the affected pylorus (Langer et al 1995, Vanderwinden et al 1996a). In Hirschsprung's disease, an intestinal aganglionosis similar to the lethal white syndrome in foals, the density of ICC is markedly decreased in the aganglionic segment (Vanderwinden et al 1996b). There are alterations in the ultrastructure of ICC in ulcerative colitis suggesting that they may be involved in some way in the motility disturbances seen in this disease (Rumessen 1996). A deficiency of ICC is seen in patients with a myopathic form of chronic idiopathic intestinal pseudo-obstruction (Isozaki et al 1997). In experimentally induced ileal hypertrophy in rats there is a decreased number of ICC in the hypertrophied segment (Ekblad et al 1998). Studies of ICC may be important to help in the diagnosis of certain diseases (Hagger et al 1997). Indeed, this may be extended to making ICC a target for pharmacological intervention in the treatment of gastrointestinal disease (Huizinga et al 1997, Vanderwinden and Vanderhaeghen 1998).

In conclusion, this study will serve as a basis for studies of ICC in disease states of the equine intestine such as equine dysautonomia and other common obstructive and motility problems.

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Tissue Culture of the Enteric Nervous System from Equine Ileum

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ABSTRACT

Ileal samples were harvested fresh from euthanized adult horses. The tissues were microdissected to prepare wholemount preparations for immunohistochemistry and for either explant or dissociated culture systems of the enteric nervous system. Explant culture systems were established using whole-mounts of either the submucous plexus or the muscularis externa (including the myenteric plexus). Dissociated cell cultures could only be obtained from the submucous plexus. Culture systems were maintained for up to 5 days. Immunoreactivity for a neuronal marker (*Pan*-N) and for glial cell markers (GFAP and S100) indicated the presence of both neurons and enteric glia in the tissue culture preparations.

This is the first report of equine enteric neurons being grown in tissue culture. Further refinements to the techniques will be required before this *in vitro* model can be used for quantitative analysis.

Keywords: culture, enteric nervous system, glia, horse, ileum, immunohistochemistry, neuron, submucous plexus

Abbreviations: ENS, enteric nervous system; FITC, fluorescein 5-isothiocyanate; GFAP, glial fibrillary acidic protein; MP, myenteric plexus; *Pan*-N, *Pan*-neurofilaments; PBS, phosphate-buffered saline; SMP, submucous plexus

INTRODUCTION

The enteric nervous system (ENS) has a central role in the control of most gastrointestinal functions (Furness and Costa, 1987). In humans, there are numerous diseases that affect the ENS, such as oesophageal achalasia, Chagas' disease (American trypanosomosis), familial dysautonomia, intestinal aganglionosis (Hirschsprung's disease) and chronic idiopathic intestinal pseudo-obstruction (Heaton *et al.*, 1988). In horses, there are important diseases of the ENS that are poorly understood at the cellular level, such as equine dysautonomia (grass sickness) (Milne, 1997), myenteric ganglionitis (Burns *et al.*, 1990) and ileocolonic aganglionosis (lethal white foal syndrome) (Hultgren, 1982). The ENS consists of a complicated three-dimensional network of neurons organized into the submucous plexus (SMP) and the myenteric plexus (MP). The SMP lies in the region between the mucosa and the two main muscle layers of the intestinal wall (muscularis externa). The MP lies between the circular and

longitudinal intestinal muscle layers of the muscularis externa. The MP is concerned primarily with motility, while the SMP is concerned with transepithelial ion transport, mucosal blood flow and secretomotor functions (Furness and Bornstein, 1995). Much of the understanding of the ENS comes from work on the guinea-pig but the equine ENS has been shown to consist of a MP and a two-layered SMP (internal and external SMPs) (Pearson, 1994).

The development of a technique for culturing explants of the MP and SMP from the guinea-pig ENS by microdissection and enzymatic methods has led to a greater understanding of ENS physiology (Jessen *et al.*, 1978, 1983). Three types of culture are possible: organotypic (the whole intestine is cultured), explant (intact plexuses) and dissociated cell cultures (Willard and Nishi, 1989). Organotypic cultures have an advantage over dissociated cultures, and to a lesser extent over explant cultures, in that the ultrastructural features of the ENS are kept intact (Song *et al.*, 1995). However, dissociated cultures are potentially valuable because the neurons continue to express many of their differentiated properties (Nishi and Willard, 1985). Refinements to the methodology for ENS tissue culture in the rat have increased the amounts of myenteric plexus that can be obtained (Schäfer *et al.*, 1997). Such cultures are useful both for correlating the morphological, biophysical, pharmacological and synaptic properties of neurons and for testing the ability of altered environmental conditions to change these properties (Nishi and Willard, 1985). Indeed, recent research has been examining the effects of various trophic factors on ENS cultures, with the aim of increasing our understanding of the development of the ENS (Schäfer *et al.*, 1998; Schäfer and Mestres, 1999). Thus, culture systems offer considerable potential for the development of *in vitro* models for diseases of the ENS. Hanani (1993) and Schäfer and Mestres (1997) have successfully cultured myenteric neurons from the human large intestine, but there appear to be no previous reports of equine enteric neurons being grown in tissue culture.

The aim of this study was to develop a tissue culture system for the equine ENS in order ultimately to establish an *in vitro* testing model for diseases such as grass sickness.

MATERIALS AND METHODS

Dissociated cell cultures

Portions of the ileum (level with the proximal end of the ileocaecal fold, as described by Scholes and colleagues, 1993) were removed fresh *post mortem* from adult horses destroyed humanely due to clinical conditions not involving the gastrointestinal tract (e.g. orthopaedic problems). The horses were euthanized at a veterinary hospital with 50 ml of intravenously administered quinalbarbitone sodium BP (400 mg/ml)/ cinchocaine hydrochloride BP (25 mg/ml) (Somulose; Arnolds Veterinary Products, Shrewsbury, UK). The tissue samples were rinsed and placed in warmed (37°C) sterile-filtered (sterile filters 0.2 μm; Super Acrodisc; Gelman Sciences, Northampton, UK) Krebs solution of the following composition (mmol/L): NaCl 120.7; KCl 5.9; $NaHCO_3$

25; $MgSO_4$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.5 and glucose 11.5 with 1 μmol/L nifedipine (Sigma, Poole, UK), 100 μg/ml streptomycin (Sigma), 100 U/ml penicillin (Sigma) and 100 μg/ml gentamicin (Sigma). Microdissection was performed with the aid of a Nikon SMZ-2T stereoscopic microscope (×63 maximal magnification) by pinning the gut out on Sylgard (Merck, Glasgow, UK), mucosal surface down, and cutting the muscularis externa away from the underlying layers and the SMP from the mucosa with a scalpel blade (no. 5). The muscularis externa and SMP were placed in separate sterile test tubes containing 1 ml of 3.3 mg/ml collagenase (type XI; Sigma) and 10 μg/ml DNase (Sigma) at 37°C for 4–5 h. Trypsin (Sigma) was added at a concentration of 2.5 mg/ml for 15 min, after which 1 ml of culture medium was added (medium 199, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 2 mmol/L L-glutamine and 10% fetal calf serum (all Sigma), 6 mg/ml D-glucose (Life Technologies, Paisley, UK)). The tissues were triturated, first through a sterile pipette (× 5), then through a fine gauge (21G) needle (× 5). Larger pieces of undigested tissue from the muscularis externa were removed prior to trituration. The suspension was then centrifuged at 120*g* for 2 min and the supernatant was removed. Either 200 μl or 500 μl of culture medium was added, the pellet of cells was resuspended, and aliquots of 30, 60, 90 or 120 μl were plated on poly-L-lysine-coated (0.01%; Sigma) 13-mm-diameter glass coverslips. Sterile culture dishes (100 mm diameter) containing these coverslips were placed in a 37°C culture incubator (5% CO_2) for 1 h, after which 2 ml of culture medium was added. The medium was changed every 24 h until fixation of the cultures after 5 days.

Explant cultures

Strips of SMP and of muscularis externa 20–30 mm long were obtained from horses as described above and pinned on sterile Sylgard in glass Petri dishes. Culture medium was added to these dishes and they were incubated as described above.

Immunohistochemistry of explants and wholemount preparations

Wholemount preparations of the SMP were dissected (as above) from tissues that had been fixed immediately after collection from the animal in 2% formaldehyde/15% picric acid (Zamboni's solution) for 15–20 h at 4°C. After fixation, the tissues were placed in 80% ethanol to remove the picric acid and then dehydrated by sequential washing (10 min periods) in 95% ethanol, 100% ethanol and then in xylene. The tissues were then rehydrated via 100%, 95%, 80% and 50% ethanol solutions and stored in phosphate-buffered saline (PBS, 0.1 mol/L, pH 7.0) containing 0.05% (w/v) sodium azide. After culturing, the explant tissues were fixed and processed in the same way prior to immunohistochemistry.

A standard indirect immunofluorescence technique was used whereby the preparations were incubated in primary antisera (diluted in PBS and 0.3% Triton X-100) for 16–18 h in humid chambers kept at room temperature. After rinsing in PBS (3 × 10 min), the tissues were exposed to secondary fluorescent antisera for 1.5–2 h, washed in

PBS as above, and then mounted in a carbonate buffer medium (pH 8.6, 0.5 mol/L) mixed 1:2 with glycerol. The primary antisera used were anti-*Pan*-neurofilaments (anti-*Pan*-N; raised in rabbit; Affinity, Exeter, UK; 1:100 dilution), anti-S100 (an intracellular acidic protein that binds calcium ions; raised in rabbit; Sigma; 1:100 dilution) and anti-GFAP (glial fibrillary acidic protein; raised in mouse; Boehringer Mannheim, Lewes, UK; 1:20 dilution). The secondary antisera used were fluorescein 5-isothiocyanate (FITC)-conjugated affinity-purified goat antibody to rabbit IgG (Dynatech, Billinghurst, UK; 1:10 dilution) and FITC-conjugated affinity-purified goat antibody to mouse IgG (Precision Medical, Pickering, UK; 1:10 dilution). It should be noted that the primary antisera used in this study were raised against antigens that were originally isolated from species other than the horse. Therefore, statements such as 'positive immunoreactivity for S100' would be more accurately given as 'S100-like' immunoreactivity.

Immunofluorescence was viewed using ×10, ×20 or ×40 objectives on a Zeiss microscope equipped with phase-contrast and epifluorescence optics. Provia Fujichrome film (400 ASA) was used for all photomicrography.

Immunohistochemistry of dissociated cell cultures

The fixation procedure for dissociated cell cultures involved a 30 min immersion of the coverslips in Zamboni's solution at 4°C. After washing in PBS (6 × 5 min), the incubations with primary and secondary antibody were for periods of 1 h each.

RESULTS

At the time of the first change of culture medium (24 h), it was clear that cells in the dissociated preparations had become attached to the coverslips. Although many individual cells could be seen, there were also up to six aggregations or clumps of cells present on each coverslip. This 'bedding down' of the cells was only observed with suspensions derived from the SMP. Attempts to obtain a dissociated cell culture from the muscularis externa were unsuccessful. After 2–3 days in culture, cell projections or processes became more evident and there was marked proliferation of cells around the clumps. The cultures were fixed at 5 days because of the increasing proliferation of these cells.

Immunoreactivity for the neuronal marker (*Pan*-N) was seen in the cytoplasm and processes of cells within the clumps (Figure 1). This pattern of immunoreactivity was very similar to that seen in neurons of the submucous ganglia in wholemount preparations that had not been cultured (Figure 2).

The maximum number of *Pan*-N-immunoreactive cells counted on a coverslip was 50, with up to 15 in each clump of cells. The size of the aliquots of the cell suspensions plated on the coverslips did not seem to affect this seeded density. The cells that proliferated around the clumps did not show immunoreactivity for *Pan*-N, but many of these cells were immunoreactive for both S100 (Figure 3A) and GFAP. These non-

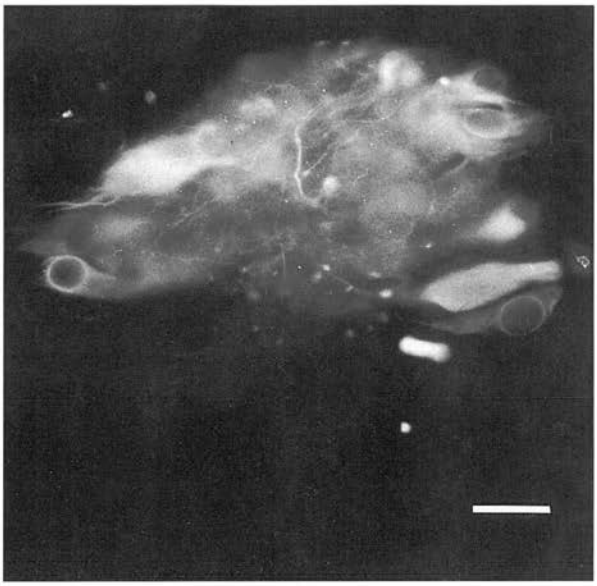

Figure 1. An aggregation of cells in a 5-day dissociated cell culture preparation from the submucous plexus of the equine ileum. Immunoreactivity for *Pan*-N is present in the cytoplasm and processes of the cells. Bar = 25 μm

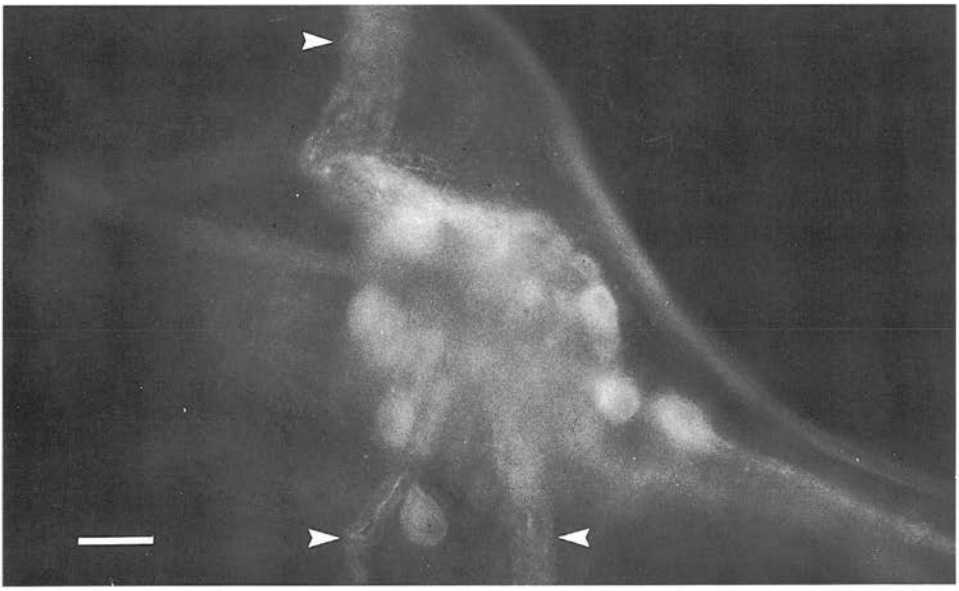

Figure 2. *Pan*-N-immunoreactivity in a ganglion in a wholemount preparation (not cultured) of the submucous plexus of the equine ileum. Immunoreactivity can be seen both in cell bodies and in interconnecting strands (arrowheads) extending away from the ganglion. Bar = 25 μm

neuronal cells were therefore considered to be enteric glial cells. The glial cell proliferation and cell morphology were best appreciated using phase-contrast microscopy (Figure 3B). It should be noted that the field shown in Figure 3 was chosen from an area with fewer cells to better illustrate the cellular morphology of the enteric glia and is by no means representative of the number of these swarming cells, which by far outnumbered the neuronal population.

Explant cultures of the SMP and muscularis externa were maintained for 5 days. The SMP explants showed a pattern of neuronal immunoreactivity that was indistinguishable from that seen in the preparations that had not been cultured (Figure 2). Positive cellular immunoreactivity in these 5-day-old cultures was interpreted as indicating the viability of these cells. It was not possible to examine the muscularis externa preparations immunohistochemically because of the thickness of the tissue. However, the explanted muscularis externa system was considered physiologically viable *in vitro* because there was still visible evidence of the muscle contracting 5 days after harvesting the tissue from the animal.

DISCUSSION

To the best of our knowledge this is the first report of equine enteric neurons being grown successfully in tissue culture. The ileum was chosen for this study because it has been shown to be the region of intestine most severely affected in grass sickness (Scholes *et al.*, 1993) and this is the disease for which this *in vitro* model is being designed. Only SMP cultures were possible in the horse, which is in contrast to small-mammal models where both MP and SMP cultures have been described (Jessen *et al.*, 1983; Saffrey *et al.*, 1991). In the present study, neurons and enteric glia were identified in the cultures, which were fixed at 5 days because of the increasing proliferation of non-neuronal cells. Many of the non-neuronal cells were glial and it is likely, when comparing this work with other studies, that the remaining cells were fibroblasts (Saffrey *et al.*, 1992; Hanani, 1993). It may be possible to extend the culture period in future studies because the numbers of these non-neuronal cells can be manipulated in culture. For instance, glial proliferation can be minimized by using a serum-free culture medium (Schäfer *et al.*, 1995) and fibroblasts can be decreased by the use of mitotic inhibitors, such as cytosine arabinoside (Bannerman *et al.*, 1988). Alternatively, both fibroblasts (Sawant-Mane *et al.*, 1994) and glia (Bannerman *et al.*, 1988) can be selectively destroyed by using complement-mediated lysis. There is, however, debate as to whether neurons grow better in isolation from glia (Saffrey *et al.*, 1992) or are healthier in direct contact with glia (Saffrey *et al.*, 1991). Further work will be necessary to increase the *in vitro* yield of neurons in our equine system from the present maximum of 50 neurons per coverslip.

In this study, the equine enteric glia, or the supportive cells of the ENS, showed positive immunoreactivity for both GFAP and S100. Enteric glia are different to the non-myelinating Schwann cells of the peripheral nervous system, but both are derived embryologically from the neural crest and diverge only relatively late in ontogeny (Gershon and Rothman, 1991). Both types of cell are immunohistochemically positive

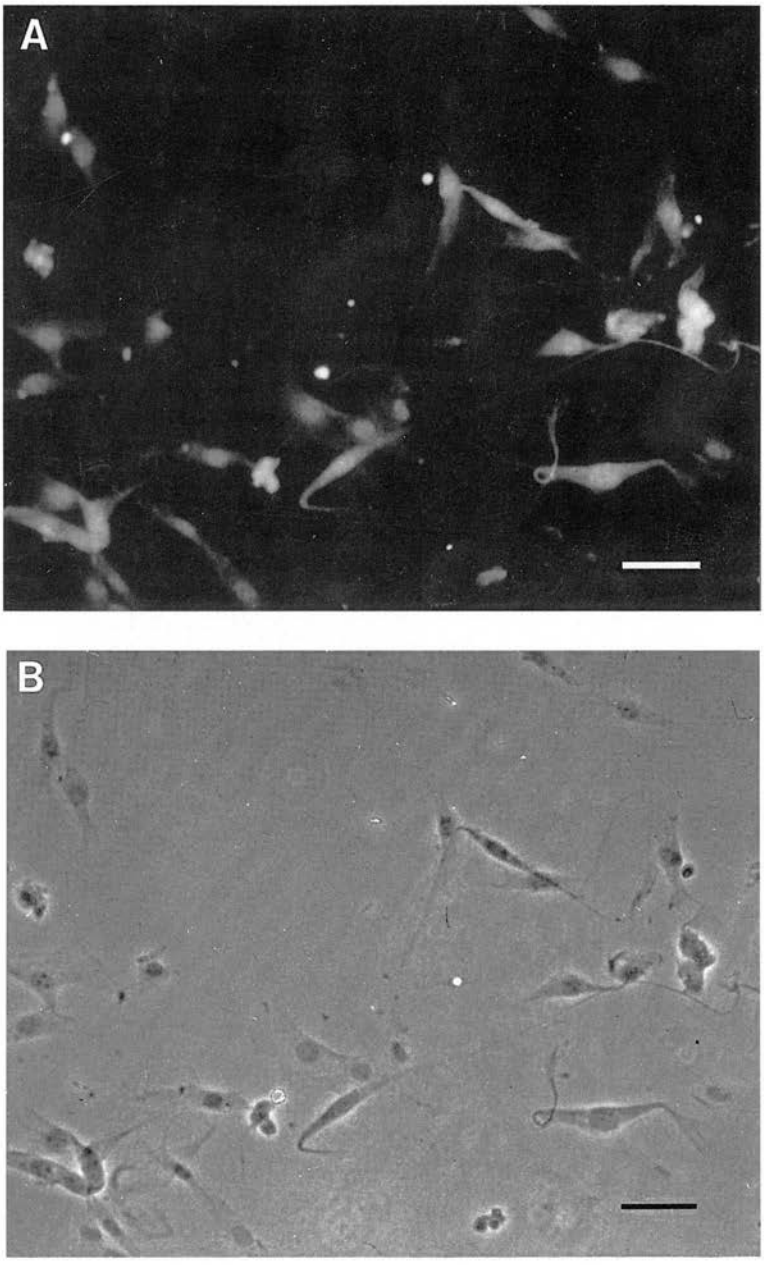

Figure 3. (A) S100-immunoreactivity in cells from the submucous plexus of the equine ileum (5 days in dissociated cell culture). (B) Same field as (A) visualized in phase contrast. Bar = 50 μm

for GFAP and S100 (Jessen and Mirsky, 1980; Gershon and Rothman, 1991; Bannerman *et al.*, 1987).

Wholemount preparations of the ENS are more suitable than sections for evaluation in human diseases such as Hirschsprung's disease and neuronal intestinal dysplasia because the three-dimensional morphology can be appreciated more readily (Krammer *et al.*, 1993). These authors commented on the disadvantages of working with adult human intestine, highlighting difficulties both with the preparation of the wholemounts and with the penetration of antibodies. These problems are also evident when working with intestine from the adult horse, which is also very thick. The two equine studies using wholemounts overcame such problems by using either foal tissue (Pearson, 1994) or enzymatic digestion of smooth muscle (Burns and Cummings, 1991). Sections may therefore be preferable when examining adult equine tissue, despite the loss of a degree of three-dimensional morphology (Cummings *et al.*, 1984; Kotze and Van Aswegen, 1990; Burns and Cummings, 1993; Hudson *et al.*, 1999).

This study has shown some of the *in vitro* and immunohistochemical characteristics of the equine ENS. However, the difficulties in accessing and harvesting the neurons indicate that further work will be necessary in order to establish an *in vitro* model amenable to the quantitative analysis of diseases such as grass sickness.

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CELLULAR ASPECTS OF EQUINE DYSAUTONOMIA

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Equine dysautonomia (ED; grass sickness) is a common, frequently fatal disease of horses characterised by dysfunction of the gastrointestinal tract. Neurons of the enteric nervous system (ENS) are primarily and most severely targeted by the putative neurotoxin causing the disease. The ENS of the horse has been shown, using wholemount preparations in the foal, to comprise a myenteric plexus and two (external and internal) submucous plexuses (Pearson 1994). In other species, the microdissection of the components of the ENS has been important in the development of tissue culture systems. The aims of this study are to characterise the morphology and neurochemical expression of the equine ENS, with special reference to grass sickness, using tissue culture and immunohistochemistry. Ultimately it is hoped that the culture system could represent an *in vitro* testing model to elaborate on the unknown aetiology of grass sickness.

Intestinal samples were harvested from euthanased horses (with and without grass sickness, destroyed on humane grounds) at the R(D)SVS. The tissues were microdissected to prepare both wholemount preparations for immunohistochemistry and explant and dissociated culture systems of the ENS. Immunoreactivity was assessed using a standard indirect immunofluorescence technique.

A selection of primary antibodies was used and the most successful ones in the horse proved to be anti-VIP (vasoactive intestinal polypeptide), anti-CGRP (calcitonin gene-related peptide), anti-D45 (calcium binding protein), anti-PAN and anti-NFM (neurofilaments), anti-GFAP and anti-S100 (glial and Schwann cell markers). Wholemount preparations were very difficult to prepare in the adult equine intestine and positive results were only found using the submucous plexuses.

Both explant and dissociated cell ENS culture systems were successful in the horse, but only in the submucous plexus. This is in contrast to small mammal models where enteric neurons have been grown in both myenteric and submucous systems. This reflects the difficulty in accessing the myenteric plexus in the adult horse. Neurons have been successfully grown in culture for 5 days and there is the potential to make this period longer. Refinement is necessary to improve the neuronal yield. Three main cell types are evident in dissociated culture: neurons, enteric glia and fibroblasts. The neurons appear to be bedded down in clumps. The non-neuronal cells proliferate markedly after 2-3 days in culture and this may need to be controlled by the addition of mitotic inhibitors to the culture system. Double labelling experiments reveal that the enteric glial cells are very similar to the Schwann cells of the peripheral nervous system.

This is the first report of equine enteric neurons being grown in tissue culture. The difficulties in accessing and harvesting these neurons in the equine intestine mean that further work is necessary in order to produce a repeatable, quantifiable *in vitro* model for the autonomic neuropathy of grass sickness.

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CCK-A receptors. It seems likely that cefaclor, like peptone, releases CCK from gut endocrine cells, as also suggested by studies in ST_1 cells (Nemoz Gaillard *et al.* 1998).

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Dual effect of ATP on mesenteric afferent nerve discharge in the anaesthetized rat

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The selective P2X receptor agonist, α,β-methylene-ATP ($\alpha\beta$meATP), evokes excitation of intestinal afferent nerves (Kirkup *et al.* 1999). However, the effects of ATP on the terminals of these sensory fibres are not known. Thus in the present study we assessed the effects of the endogenous nucleotide on this population of afferent nerves. Furthermore, we examined whether the effects of ATP were sensitive to P1 (xanthine amine congener; XAC) or P2 (pyridoxalphosphate-6-azophenyl-2,4′-disulphonic acid (PPADS) and suramin) receptor antagonists.

Experiments were performed with male Wistar rats (330–450 g), anaesthetized with an intraperitoneal injection of pentobarbitone sodium (60 mg kg^{-1}). Anaesthesia was maintained by intravenous (I.V.) infusion of pentobarbitone (0·5–1·0 mg kg^{-1} min^{-1}). Animals were killed at the end of an experiment by an anaesthetic overdose. Mesenteric afferent nerve activity was recorded as previously described (Kirkup *et al.* 1998) except that a cannula was inserted, via the femoral artery, up to the coeliac axis to permit close intra-arterial (I.A.) injection of ATP. Data are means ± S.E.M. from 3–6 rats and were analysed using the Mann–Whitney test.

ATP (0·1–10 mg kg^{-1}, I.A.) induced dose-dependent increases in afferent nerve activity and intrajejunal pressure, effects that did not reach a maximum over the dose range tested. The effects of ATP on afferent nerves comprised an early (< 2 s after injection), intense burst of firing, followed by a less pronounced later increase (> 2 s after injection), which coincided with elevated intrajejunal pressure. Pre-treatment with PPADS (20 mg kg^{-1}, I.V.) but not suramin (80 mg kg^{-1}, I.V.) for 5 min prior to ATP administration, inhibited the peak early peak effect of ATP (1 mg kg^{-1}, I.A.) on afferent nerve activity and on intrajejunal pressure (Table 1). The later peak burst of afferent activity evoked by ATP was unaffected by PPADS. In contrast, pre-treatment with XAC (30 mg kg^{-1}, I.V.) for 5 min before ATP, did not affect the early peak early discharge evoked by ATP, but attenuated the later peak burst of afferent nerve activity and the ATP-evoked rises in intrajejunal pressure.

Table 1. Effect of vehicle, PPADS, suramin and XAC on the early and late peak increase in mesenteric afferent nerve activity and peak increase in jejunal pressure (JP) evoked by ATP

	Percentage of control response		
	Early	Late	JP
Vehicle	121 ± 22	100 ± 12	108 ± 9
PPADS	38 ± 6**	87 ± 9	68 ± 10*
Suramin	98 ± 15	112 ± 12	80 ± 18
XAC	99 ± 3	36 ± 7*	49 ± 10*

* $P < 0.05$, ** $P < 0.01$ *vs.* vehicle-treated group.

In conclusion, the effect of ATP on mesenteric afferent nerves appears to consist of a direct action on the nerve endings (early effects) followed by a later effect which, in part, reflects discharge evoked by activation of adenosine receptors (Kirkup *et al.* 1998). Furthermore, ecto-nucleotidease inhibition might explain the differential sensitivities of ATP and $\alpha\beta$meATP to PPADS and suramin (see Kirkup *et al.* 1999).

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Immunohistochemical identification of interstitial cells of Cajal in the equine gastrointestinal tract

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Gastrointestinal diseases, including obstructive and motility disorders such as grass sickness (Cottrell *et al.* 1999), are very common in the horse. Interstitial cells of Cajal (ICCs) are the c-Kit-immunoreactive cells responsible for the generation of pacemaker activity in gastrointestinal smooth muscle (Ward *et al.* 1997; Thomsen *et al.* 1998). Impairment of this ICC-mediated control has been implicated in several motility disorders in both humans and small mammals (Ward *et al.* 1994; Vanderwinden *et al.* 1996). This study therefore is the first to investigate ICCs in the horse.

Tissues were obtained at post-mortem from seven horses free from gastrointestinal disease but euthanased at a veterinary hospital for humane reasons. Samples were taken

at multiple anatomically defined sites from stomach to small colon. After tissue fixation in 10% phosphate-buffered formalin, 10 μm cryostat sections were processed using standard immunohistochemical techniques and the avidin-biotin-peroxidase method. The primary antiserum used was an affinity-purified polyclonal antibody raised against c-Kit protein (Oncogene Research Products, Cambridge, USA).

Specific immunoreactivity for c-Kit was detected in all sites and three types of immunoreactive cell were identified. These were spindle-shaped cells in the region of the myenteric plexus with occasional cell processes extending into the longitudinal muscle, stellate- or bipolar-shaped cells in the circular muscle layer and round cells in the submucosa. The round cells were shown to be mast cells with the use of toluidine blue staining, whereas the other c-Kit-immunoreactive cells did not exhibit metachromasia and were therefore classified as ICCs.

In the duodenum, jejunum and ileum, ICCs were found predominantly in the region of the myenteric plexus and to a lesser extent in the circular muscle. In contrast, in the large intestine (large colon, caecum, small colon) most cells were seen throughout the circular muscle and very few ICCs were seen in the myenteric plexus area. The implication of these distributions with regard to the pacemaker function of ICCs is at present unclear. These observations on healthy tissues will serve as the basis for future studies of gastrointestinal disease in the horse.

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Mucosal cell proliferation is an unreliable index of intestinal tumour formation in *Min* mice

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Mutations in the *Apc* gene are an early event in the majority of human colorectal tumours. The *Min* (multiple intestinal neoplasia) mouse, which has an autosomal dominant stop mutation at codon 850 of *Apc* and develops several intestinal adenomas (Su *et al.* 1992), is a valuable model for studies of dietary chemoprevention of intestinal tumourigenesis.

At weaning heterozygous male and female *Min* mice were allocated at random to one of four experimental diets for 90 days (20 mice per diet). The basal (B) diet was modified by the addition of 100 g guar gum (GG), raw potato starch (RPS) or Hylon VII per kilogram (HYL) at the expense of maize starch. At the end of the experimental period mice were killed by CO_2 inhalation, a midline laparotomy was performed and the whole alimentary tract from stomach to the anus was removed. The small intestine, caecum and colon were opened longitudinally and examined macroscopically for the presence of tumours. Tumour size and location were recorded. Crypt cell proliferation (CCP) was measured by the vincristine metaphase arrest method (Mathers *et al.* 1993) at five sites throughout the intestine. Apoptosis was measured by conventional H&E staining of hemi-crypts at corresponding locations.

Small intestine tumour multiplicity was increased significantly by GG ($P = 0{\cdot}016$, Dunnett's t test) ($B = 11{\cdot}3 \pm 2{\cdot}75$, $GG = 25{\cdot}3 \pm 5{\cdot}01$, $RPS = 13{\cdot}9 \pm 2{\cdot}58$, $HYL = 17{\cdot}6 \pm 3{\cdot}07$ (mean ± S.E.M.)). With all treatments the large majority of tumours were in the small intestine. There was no evidence that diet had any effect on CCP at any site within the small or large bowels. However, GG feeding reduced apoptotic index in the terminal ileum to about half the value for the basal diet. These results suggest that downregulation of apoptosis in GG-fed animals may contribute to the increased tumour multiplicity seen with this treatment, and that CCP in the histologically normal mucosa does not appear to be a reliable biomarker of tumour risk in this animal model.

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Evidence for UDP-stimulated chloride secretion across murine intestinal epithelium

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Chloride (Cl^-) secretion across the intestinal epithelium may occur via a calcium-activated Cl^- channel (CaCC) (Barrett, 1997). Activation of luminal nucleotide receptors increases $[Ca^{2+}]_i$, which in turn results in activation of the CaCC and so induces an increase in Cl^- secretion. Previous work in this area has focused on purine receptors mediating this response. Here we tested for the presence of a

190 Withdrawn

191 AN IMMUNOHISTOCHEMICAL AND *IN VITRO* STUDY OF THE EQUINE ENTERIC NERVOUS SYSTEM

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The aims of this study were to characterise the morphology and neurochemical expression of the equine enteric nervous system (ENS) using tissue culture and immunohistochemistry, with a view to investigating autonomic neuropathies such as grass sickness.

Intestinal samples were harvested fresh from euthanased horses free from gastrointestinal disease. The tissues were microdissected to prepare both wholemount preparations for immunohistochemistry and explant and dissociated culture systems of the ENS. Immunoreactivities for cell markers and neuropeptides were revealed using a standard indirect immunofluorescence technique. These included: anti-VIP (vasoactive intestinal polypeptide), anti-CGRP (calcitonin gene-related peptide), anti-D45 (calcium binding protein), anti-PAN and anti-NFM (neurofilaments), anti-GFAP and anti-S100 (glial and Schwann cell markers). Wholemount preparations were very difficult to prepare in the adult equine intestine and positive results were only achieved using the submucous plexuses.

Both explant and dissociated cell ENS culture systems were successful in the horse, but only those derived from the submucous plexus. This is in contrast to small mammal models where enteric neurons have been cultured from both myenteric and submucous plexuses. This reflects the difficulty in accessing the myenteric plexus in the adult horse. Neurons were grown successfully in culture for 5 days and there is the potential to make this period longer. Three main cell types were evident in dissociated culture: neurons, enteric glia and fibroblasts. Neurons appeared to be bedded down in clumps and non-neuronal cells proliferated markedly after 2–3 days in culture. Double labelling experiments revealed that the enteric glial cells are very similar to the Schwann cells of the peripheral nervous system.

This is the first report of equine enteric neurons being grown in tissue culture. The difficulties in accessing and harvesting these neurons in the equine intestine mean that further work is necessary in order to produce a repeatable, quantifiable *in vitro* model.

192 PLASMA MAGNESIUM CONCENTRATIONS IN HORSES WITH GASTROINTESTINAL TRACT DISEASE.

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Magnesium (Mg), an important intracellular cation, has numerous physiologic roles, but the most critical ones are associated with production and utilization of adenosine triphosphate (ATP), function of the sodium-potassium ATPase pump, and regulation of intracellular calcium. Hypomagnesemia has been reported to be common in critically ill human patients. Hypomagnesemia often accompanies other electrolyte abnormalities. The purpose of this study was to survey a population of 75 horses suffering from gastrointestinal disease to determine the incidence of plasma magnesium abnormalities. A total of 90 horses were evaluated and divided into 5 groups: small intestinal strangulation (n=27), large colon torsion (n=14), enterits (n=15), colitis (n=19) and clinically normal horses (n=15). Total plasma magnesium was determined using a colorimetric procedure.

Thirty-three horses with gastrointestinal disease (44%) were hypomagnesemic at hospital admission. Hypomagnesemia was present in the following percentages: small intestine strangulation (63%), large colon torsion (14%), enterits (33%) and colitis (47%). Eight of the 75 horses (11%) were hypermagnesemic. All clinically normal horses were normomagnesemic and had electrolytes within the normal ranges. Hypokalemia was present in 30% of hypomagnesemic horses, whereas hypokalemia was present in 20% of normomagnesemic horses with gastrointestinal disease. Hypochloremia occurred in 75% of hypermagnesemic horses, compared with 18% in normomagnesemic horses. These results suggest that Mg abnormalities are important in the critically ill horses suffering from gastrointestinal abnormalities.

193 IMMUNOPHENOTYE OF LEUKEMIA IN 3 HORSES.

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The prognosis and management of leukemia depend in part on the lineage of the leukemic cells. Immunophenotyping using monoclonal antibodies (mAbs) specific for leukocyte differentiation antigens permits definitive identification of cell lineage. The cells from 3 horses diagnosed with lymphocytic leukemia on the basis of peripheral leukocyte cytology or bone marrow histopathology were studied using a panel of monoclonal antibodies and FACS analysis. All horses were subsequently euthanized, and immunohistochemical staining of neoplastic tissues was performed.

By FACS analysis the cells in all 3 cases were confirmed as leukocytes (EqCD11a/18 +, EqCD44 +). However, based on expression of EqCD13 the cells in one of the horses proved to be myeloid, rather than lymphocytic. The two remaining leukemias were both lymphoid (EqCD13 -, EqWC1 +). One of these neoplasms was poorly differentiated, but likely of B cell origin (MHC II+). The other leukemia was of T cell origin (EqCD2 +, EqCD3+, EqCD5+) and on further evaluation was found to be EqCD4 +/EqCD8 -. In addition, up to 20% of the EqCD4+ cells were found to be EqCD3-, a phenotype not previously observed in the horse. This latter observation was confirmed by 2- color flow cytometry. Immunohistochemical staining of neoplastic tissues using the same panel of mAbs permitted visual identification of the distribution of neoplastic cells within affected lymphoid organs.

This study demonstrates the utility of mAbs in combination with FACS analysis and immunohistochemistry in defining the lineage and distribution of leukemic cells in horses. Correlation of immunophenotype with biologic behavior and response to therapy may enhance the management of leukemia in the horse.

The use of transrectal ultrasonography to monitor the effects of romifidine o gastrointestinal motility in the horse

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A technique of transrectal ultrasonography was developed and then used to investigate the effect of romifidine 80 and 12 µg/kg bwt on gastrointestinal function. Motility of the small intestine, caecum and left dorsal colon was assessed in 6 horse following injection of romifidine or a saline control, using a blinded cross-over study design. Measurements were taken at 1 30, 60, 120, 180 and 240 min after drug administration. The transrectal technique proved suitable for monitoring intestina function. The mean (± s.e.) number of contractions per min was 4.5 (± 0.5) for the caecum, 3.9 (± 0.5) for the left dorsal colo and 9.7 (± 0.9) for the small intestine prior to treatment. There were no significant changes in motility in the saline contr group. Administration of romifidine 80 and 120 µg/kg bwt was, however, associated with a marked depression i gastrointestinal motility. The number of normal contractions significantly decreased and abnormal (nonfunctiona contractions were observed. The decrease in normal contractions was significant for up to 30 min in the large colon an caecum and up to 60 min in the small intestine following romifidine. In general, 120 µg/kg romifidine produced a significant greater degree and duration of depression of motility than 80 µg/kg. The frequency of the abnormal contractions varie between the doses and regions of intestine. In the small intestine and large colon abnormal contractions persisted for longer period of time following 120 µg/kg romifidine; in the caecum abnormal contractions were observed for a similar tim with both doses of romifidine. This study demonstrates that transrectal ultrasonography is a useful technique for assessin changes in gastrointestinal motility, and that romifidine produced a dose-dependent depression of gastrointestinal functio for up to 60 min.

Interstitial cells of Cajal (ICC) in the equine gastrointestinal tract

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The interstitial cells of Cajal (ICC) are *c-kit* immunoreactive cells of the gastrointestinal tract responsible for the generation o electrical pacemaker activity in smooth muscle. The morphology and distribution of these cells have not been describe previously in the horse. The aim of this study was to establish the distribution of ICC in the normal equine gastrointestinal trac

The interstitial cells of Cajal were identified by immunohistochemical labelling with an anti-*c-kit* polyclonal antibod Sections of normal gastrointestinal tissue were examined from up to 13 anatomically defined sites from stomach to sma colon taken from 7 horses free from gastrointestinal disease. Three types of *c-kit* immunoreactive cells were identifie spindle-shaped ICC in the region of the myenteric plexus, stellate- or bipolar-shaped ICC in the circular smooth muscle laye and round cells in the submucosa. The round cells were shown to be mast cells with the use of toluidine blue staining whereas the other *c-kit* immunoreactive cells did not exhibit metachromasia. In the small intestine, ICC were foun predominantly in the region of the myenteric plexus and, to a lesser extent, in the circular muscle layer. This was in contras to the large intestine where the cells were seen mainly throughout the circular muscle layer. This study will serve as a bas for future pathological studies in the horse.

Caecal amputation within the right ventral colon for surgical treatment o nonreducible caecocolic intussusception in 8 horses

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The purpose of this study was to review the medical records of 8 horses having undergone caecal amputation via a righ ventral colon (RVC) enterotomy for treatment of a nonreducible caecocolic intussusception. There were 2 stallions, 3 geldin and 3 females and 8 different breeds represented. Median age was 2 (1–8) years. Degree of pain ranged from mild (n = 6) t severe (n = 2) and duration ranged from 6.5 h to several months. Median abdominal fluid protein and WBC were 3 g/ (2.5–4.8) and 10,000 cell/ul (1000–22,500), respectively. A tentative diagnosis was made based on rectal examination in horses. The large colon was exteriorised and evacuated of its contents via a pelvic flexure enterotomy. Contamination wa minimised by careful packing: a sterile plastic bag (n = 5) was sutured to the RVC and an enterotomy centred over th intussusceptum was performed. Attempts made to reduce the intussusception manually from within the RVC failed Invaginated caecum was pulled further into the RVC and ligated with either umbilical tape (n = 2) or suture (n = 6) belo below the amputation site; the caecum was then amputated and the remainder was reduced. Additional caecum was resecte and oversewn. Tapeworms were present in 5 horses. An abdominal cavity drain was used in 3 horses and maintained fo 24–48 h. Broad spectrum antibiotics (median, 7; range, 3–14 days) and flunixin meglumine (4.5; 2–7 days) were administere Median surgical time was 180 (135–300) min. Fever developed in 7 days, beginning on Day 3 (1–7) and lasted 3.5 (0.75–8) day Horses were hospitalised for 12 (6–21) days. Two horses developed an incisional infection. All horses survived to hospit discharge. One horse died 3 months post operatively, but all others survived long term. Despite appreciable unavoidab contamination during surgery, this method provides a reasonable chance for successful treatment of horses wit nonreducible caecocolic intussusception.

Interstitial cells of Cajal and slow wave activity in the equine gastrointestinal tract.

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INTRODUCTION

Intestinal motility disorders may represent a dysfunction of the neural, muscular or pacemaker components (interstitial cells of Cajal, ICC) of bowel control. Equine dysautonomia (grass sickness) is a common, often fatal disease of horses characterised by severe motility abnormalities in the gastrointestinal tract and profound neuronal lesions in the enteric nervous system.[1] The aims of this work were to investigate the electrical properties of smooth muscle in healthy and diseased intestine and to study the distribution of ICC in the equine intestine using c-Kit immunohistochemistry.

1. Gomez, D.F. McGorum, B.C. & Pearson, G.T. (1999) *Neurogastroenterol. Mot.* 11, 79-92

METHODS

Electrophysiology
Slice preparations of equine small intestine were superfused with modified Krebs-Henseleit solution and maintained at 36-37°C for 3-6 hours. Intracellular recordings of membrane potential were made from both longitudinal and circular smooth muscle cells in both normal and grass sickness-affected tissues. Glass microelectrodes were filled with 2M KCl and had resistances in the range 25-50 MΩ. Drugs were applied to the tissues by changes of the superfusion fluid.

Immunohistochemistry
Tissues from the stomach, small and large intestines of horses were fixed and processed for cryostat sectioning (10-30 μm thickness).[2] A polyclonal rabbit-raised antiserum against c-Kit (Oncogene Research Products, Cambridge MA) was used for the immunohistochemical labelling of ICC. The c-Kit immunoreactivity was visualised using the avidin-biotin (ABC) method.

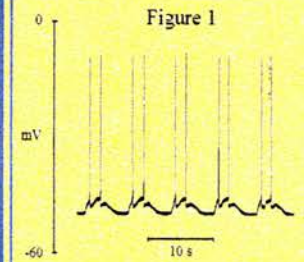
2. Hudson, NPH, Pearson, GT, Kitamura, N & Mayhew, IG (1999) *Research in Veterinary Science* 66, 265-271

RESULTS

Electrophysiology
- intracellular recording from muscularis externa of equine ileum
- resting membrane potentials -40mV to -70mV
- slow waves recorded in both circular and longitudinal muscle [***Figures 1-3***]
- slow wave properties:
  a) 2-20 mV amplitude; 8-12 Hz frequency
  b) unaffected by nifedipine or tetrodotoxin [1 μM]
  c) nifedipine-sensitive spontaneous action potentials [***Figure 1***]
  d) waxing & waning (TTX-insensitive) [***Figure 2***]
- slow waves present in 4 of 6 grass sickness-affected preparations [***Figure 3***]

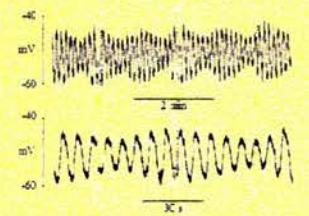
Immunohistochemistry
- specific immunoreactivity for c-Kit revealed distribution of ICC cells and processes
- ICC seen throughout normal equine gastrointestinal tract (stomach to colon)
- two general types of ICC identified:
  a) spindle-shaped cells in myenteric plexus region that have some cellular processes extending into the longitudinal muscle
  b) stellate/ bipolar-shaped cells in the circular muscle layer [***Figure 4***]
- ICC distribution:
  a) small intestine (duodenum, jejunum and ileum): ICC predominantly in myenteric region and to a lesser extent in the circular muscle [***Figure 5A***]
  b) large intestine (colon, caecum): ICC throughout the circular muscle with very few in the myenteric region [***Figure 5B***]
- semi-quantitative analysis of ICC in grass sickness-affected tissues is in progress

Figure 1



Intracellular recording from longitudinal smooth muscle of normal equine ileum showing slow waves and associated spontaneous action potentials.

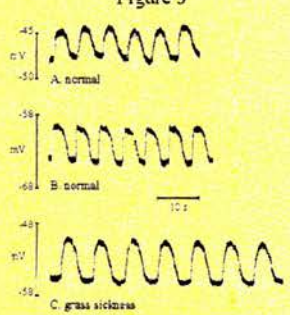
Figure 2



Intracellular recording from longitudinal smooth muscle of normal equine ileum showing cyclical 'waxing and waning' of the slow wave amplitude. The two segments of trace are from the same cell and nifedipine (1 μM) was present in the superfusion fluid.

A horse with chronic grass sickness at the University of Edinburgh equine hospital.

Figure 3



Slow waves in circular (A) and longitudinal (B) smooth muscle of normal equine ileum and the longitudinal layer of the ileum from a horse with acute grass sickness (C). All traces in the presence of 1 μM nifedipine.

Figure 4

c-Kit immunoreactivity of stellate-shaped ICC in the circular smooth muscle layer of equine colon. Calibration bar: 25 μm.

Figure 5

Small intestine Large intestine

Comparison of c-Kit immunoreactivity distribution in the small and large intestines of the horse. cm: circular muscle, lm: longitudinal muscle. Calibration bars: A 250 μm, B 100 μm.

CONCLUSIONS

- Smooth muscle in the equine intestine has prominent slow wave activity and has a rich distribution of interstitial cells of Cajal.
- The ICC distribution shows regional differences that might be related to function.
- Slow waves appear unaffected in the motility disorder grass sickness.

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