Distribution of Neurokinin 2 and 3 Receptor mRNA in the Normal Equine Gastrointestinal Tract and Effect of Inflammation on Expression of Neurokinin 1, 2 and 3 Receptor mRNA in the Jejunum

by
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This study is an investigation of the distribution of neurokinin receptors in the equine gastrointestinal tract. The objectives of this research were to determine the relative distribution of neurokinin 2 (NK2) and 3 (NK3) receptor mRNA in the normal equine gastrointestinal tract, and also to determine changes in neurokinin 1 (NK1), NK2 and NK3 receptor mRNA expression after ischemia/reperfusion injury or intraluminal distension in the jejunum.

Samples from 9 regions in the gastrointestinal tract (duodenum, jejunum, ileum, caecum, right ventral colon, left ventral colon, pelvic flexure, right dorsal colon and left dorsal colon) were harvested from 5 mature healthy horses, euthanized for reasons unrelated to the gastrointestinal tract, for the study of NK2 and NK3 mRNA distribution in the normal intestinal tract. To evaluate the effect of inflammation on NK1, NK2 and NK3 receptor mRNA distribution, samples were taken from 6 horses whose jejunum underwent one of three treatments: control (sham-operated), intraluminal distension (ILD) or ischemic strangulation obstruction and subsequent reperfusion injury (ISO). NK2 and NK3 receptor primers were
designed and real-time PCR was used to quantify NK1 (primers previously described), NK2 and NK3 receptor mRNA expression in the treatment groups described above.

In healthy horses, NK2 mRNA expression in the small intestine was highest in the duodenum and lowest in the ileum. NK2 mRNA expression in the large intestine was highest in the caecum. NK3 mRNA expression was more variable between individuals than NK2 expression overall. No significant difference was found between concentrations of NK1 or NK3 receptor mRNA between control, ILD or ISO treatments. A trend was noted for NK1 mRNA to be lower in ILD treatments than control. For NK2 receptor mRNA, ILD and ISO values were significantly lower than that of control.

Tachykinin agonists and antagonists have shown therapeutic value in intestinal inflammation and motility disorders in laboratory animals and humans. Neurokinin receptor mRNA is present in the equine intestinal tract. Relative levels appear to be altered by inflammation, although the clinical significance of this finding needs to be further evaluated. The current study suggests that tachykinin therapy may have a potential utility in the medical treatment of equine post-operative ileus and equine colic, however further investigation into the physiology of neurokinin receptors in the horse is warranted.
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DECLARATION OF WORK PERFORMED

I declare that all the work reported in this thesis was performed by Dr. Christina E. W. Martin.
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INTRODUCTION

The distribution of neurokinin 2 (NK2) and neurokinin 3 (NK3) receptors in the equine gastrointestinal tract is unknown. Tachykinins, the natural ligands for neurokinin receptors, have been implicated in gastrointestinal inflammation and motility (61; 62; 63). Gastrointestinal disorders are serious and common conditions in the horse (71; 138) Tachykinin agonists and antagonists have been studied in both human and animal models for the potential treatment of inflammatory and motility disorders of the gut. In other species, NK2 and NK3 receptors are present in intestinal tissues (61). Tachykinins have been shown to affect intestinal motility, and provide protective effects in the case of intestinal inflammation (61; 62; 63). Characterizing the distribution of neurokinin receptors in equine tissues, both in normal conditions and in the case of ischemia and distension, would be useful information for the determination of the potential therapeutic uses of tachykinin agonists and antagonists in the horse.
HYPOTHESIS AND OBJECTIVES

This study was designed to test the hypothesis that NK2 and NK3 receptors are present in the equine intestinal tract, and that NK1, NK2 and NK3 receptors may be useful targets during equine colic, with the following objectives:

1) To determine the relative expression of NK2 and NK3 receptor mRNA in normal equine tissue throughout 9 regions of the intestinal tract.

2) To determine the relative expression of NK1, NK2 and NK3 receptor mRNA in the equine jejunum under normal (sham operated), ischemic strangulation obstruction, and intraluminal distension conditions.
1.2.1 Tachykinins

Tachykinins are a family of neuropeptides with the conserved carboxy (C)-terminal sequence: Phe-x-Gly-Leu-Met-NH$_2$, where ‘x’ signifies either a hydrophobic or aromatic group (22; 131). The mammalian tachykinins include Substance P (SP), Neurokinin A (NKA) and Neurokinin B (NKB).

The first tachykinin to be discovered was SP. It was extracted from intestinal and cerebral equine tissues and then tested on longitudinal smooth muscle samples from rabbit intestine (40). These samples were pre-treated with atropine, which decreases gastrointestinal secretion and motility, and it was found that their peripheral vasodilation and contractility subsequently increased after treatment with SP (40).

Eleven amino acids form SP (114), whereas NKA and NKB are decapeptides (24).

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<td>Substance P</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met NH$_2$</td>
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<tr>
<td>NKA</td>
<td>His-Lys-The-Asp-Ser-Phe-Val-Gly-Leu-Met NH$_2$</td>
</tr>
<tr>
<td>NKB</td>
<td>Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met NH$_2$</td>
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*Table 1:* Amino acid sequences for SP, NKA and NKB (24).

The preprotachykinin A gene (PPT-A) on chromosome 7 (22) encodes for SP and NKA. It is then differentially spliced to produce either SP or NKA. Preprotachykinin B (PPT-B) on chromosome 12 encodes for NKB (131).

Tachykinins act centrally or peripherally via NK receptors NK1, NK2 or NK3 (13). The natural ligand for SP is considered to be NK1, NKA the natural ligand for NK2 and NKB for NK3 (13). However, all three peptides are effective agonists for each receptor, according to the following rankings: NK1 receptor: SP>NKA>NKB; NK2 receptor: NKA>NKB>SP; NK3
receptor: NKB>NKA>SP (116). Homology of neurokinin receptors between species, as demonstrated with human and rat sequences, is highest for NK1 (90%), but also relatively high for NK2 and NK3 (85 and 88% respectively) (68).

NK receptors are G-protein linked and function by activating the IP$_3$-Ca$^{2+}$ intracellular signalling cascade (68; 100).

NK receptors consist of seven transmembrane segments (68) and site-directed mutagenesis has shown that the N-terminal sequence of the NK1 extracellular domain is required for binding to SP and related peptides (Fig. 1) (46).

![Proposed transmembrane model of NK receptor 1](image)

**Figure 1:** Proposed transmembrane model of NK receptor 1(103), where black circles represent amino acids that are conserved between all three receptors, sites labelled CHO represent potential glycosylation sites, S-S represent disulfide bonds and the zig-zag line represents potential site of covalent attachment of fatty acids.

It is known that once SP activates the NK1 receptor, both receptor and ligand are internalized via endocytosis (10; 53; 94), and that neutral endopeptidase (NEP) is the degradation enzyme for SP (106).
Tachykinins have been implicated in many physiological functions, including intestinal and bronchial smooth muscle contraction, neurotransmission, nociception, immune response and inflammatory processes (13; 22; 40; 131; 114).
1.2.2. The Role of Tachykinins in Gastrointestinal Inflammation

The role of tachykinins in the pathophysiology of gastrointestinal (GI) inflammation has been examined in both human, and lab animals (44; 45; 55; 62; 89; 114; 126; 75). One of the main goals of this research was to evaluate the therapeutic potential of selective antagonists in the treatment of inflammatory bowel diseases (IBD) (63; 77). In order to elucidate this concept, the primary method of study has been the use of antagonists as therapeutic agents to dampen or eliminate signs of experimental models of IBD (10; 17; 18; 26; 27; 36; 43; 52; 53; 92; 93; 94 95; 115; 129; 132; 136). In addition, quantification and localization of SP in tissues from human patients with IBD helped to increase understanding of its source and site of action (38; 98; 99). Tachykinins are also involved in allergic reactions (47) and hypersecretion (41; 42; 117; 118).
1.2.2.1. Models of Inflammation and The Benefit of Antagonists

a. Trinitrobenzene Sulfonic Acid (TNB) Model

An experimental model of colitis using trinitrobenzene sulfonic acid (TNB) in the rat, which produces transmural lesions (37), has been used to study the role of SP in GI inflammation (18; 36; 43; 52; 95; 136) with varying results. The administration of the NK1 antagonist SR 140 333 in rats thirty minutes prior to intraluminal influx of TNB led to no increase in histological damage and net weight of resected bowel compared to control rats, and was significantly less than that of rats treated with TNB alone (36). This suggests that the antagonist has a protective effect against the hyperemia and hemorrhagic damage (and thus weight increase) associated with inflammation. Myeloperoxidase (MPO) activity, (a measure of neutrophil infiltration), and intestinal permeability were also found to decrease with pre-treatment with SR 140 333 as well as with prior administration of the NK2 receptor antagonist SR 48 968 (95), indicating they have an anti-inflammatory effect. The use of NK1 antagonists RP 67 580 and CP 96 345 decreased granulocyte infiltration acutely (within the first twenty four hours) but, the same antagonists failed to decrease either MPO activity or tissue damage when continuously administered over seventy two hours (136). Contrarily, topical capsaicin administration, which stimulates SP release from primary sensory nerves, protected the rat colon from ulceration when given concurrently with TNB (52).

This effect, however, was lost after 23 hours, perhaps due either to limited potential for SP release from these nerves, or due to the protective action being mediated by some other mechanism (52). In another study in which rats were treated with TNB, a decrease in SP immunoreactivity as determined by radioimmunoassay in both acute (4 hours) and chronic (1
week) cases of colitis (43). This decrease in absolute amount of SP peptide suggests that SP may be affected by colitis, versus being involved in the cause. Affinity ($K_d$) of SP for its NK1 receptor was unchanged throughout the study, but there was an observed decrease in maximum binding site density ($B_{\text{max}}$) (43). The administration of an NK1 receptor antagonist (RP 67580) subcutaneously had no effect on intestinal injury in this case (43), presumably due to the down-regulation of receptors.

The effect of blocking the NK3 receptor on the development of inflammatory processes was also first investigated using the TNB model. In rats, SR 142 801, an NK3 receptor antagonist, was found to have no effect on the development of disease (95), but in a similar experiment with guinea pigs, intestinal permeability, MPO activity and histologic damage were all decreased. The NK1 receptor antagonist 14 0333 proved ineffective in guinea pigs (95), as did the the NK1 receptor antagonist RP 67580 (136), while successfully improving MPO activity (or tissue damage) in rats. This reinforces the importance of species differences in receptor pharmacology, a concept that has been investigated in rats and guinea pigs (3), but also in other species, including the dog and gerbil (79).

b. Acetic Acid and Dextran Sodium Sulfate (DSS) Models

An acetic acid model of recto-colitis, which is characterized by a transient (less than twenty-four hours) inflammation of the intestine that causes injury only to the mucosal layer, has also been used in the study of SP (26; 27). Both single and repeated doses of the NK1 receptor antagonist MEN 11467 (26) and NK2 receptor antagonist Nepadutant (27) were investigated in the treatment of colitis in guinea pigs. Single doses (given one hour before colitis was induced)
decreased tissue damage and plasma protein extravasation (26; 27). Repeated doses of MEN 11467 were also found to decrease tissue damage and MPO activity, but not plasma protein extravasation (26). Repeated doses of the NK2 antagonist did not improve the response over a single dose. Both findings suggest the involvement of NK1 and NK2 receptors in the acute phase of inflammation (26; 27).

Dextran sodium sulfate (DSS), given orally, produces a similar GI inflammatory model to that of acetic acid, in that damage is limited to the mucosa (37). The NK1 antagonist CP 96345 was successful in decreasing MPO activity and histologic damage in rats when given intraperitoneally, concurrently with DSS (132). 8-iso-prostane (8IP), a measure of in vivo oxidative stress was also significantly decreased when CP 96345 was used (132), demonstrating that the antagonist diminishes cellular damage from neutrophil-derived oxidants.

c. Toxin A Model

Toxin A, an enterotoxin released by Clostridium difficile, has also served as a useful agent in creating models of inflammatory bowel disease for the study of SP (17; 92; 93; 115). Since it is known that once SP binds to its receptor both ligand and receptor are endocytosed (10; 53; 94), the amount of immunoreactive endosomes was measured in a model of Toxin A induced colitis in rats to determine the level and location of SP stimulation (92). Both the source and target of SP release were thus determined in the rat ileum, with the proposed mechanism of action illustrated in the following diagram (Fig. 2):
Figure 2: Postulated mechanism of neurogenic inflammation caused by SP (92). Signals from primary afferent nerves, activated by Toxin A in the lumen, travel towards the spinal cord where their cell bodies are found in the dorsal root ganglion. This action potential leads to the release of SP orthodromically in the spinal cord, transmitting pain sensation, and antidromically in the myenteric and submucosal plexuses where it is involved in intestinal inflammation.

This concept is supported by the fact that SP concentration increases in lumbar dorsal root ganglia after Toxin A is introduced in the rat ileum (17).

Extrinsic nerve denervation (93) and capsaicin administration (which ablates primary sensory nerves when injected neonatally) (92) were found to protect the ileum from pathological changes induced by Toxin A. This eliminates intrinsic enteric nerves as the primary mediators of Toxin-A induced inflammation, and further suggests that extrinsic primary sensory neurons take on this role (93). Both denervated loops of bowel (93) and those treated with the NK1 antagonist CJ 11 974 (92) showed decreased tissue damage compared to innervated and untreated samples.

The use of the SP antagonist 96 344 was found to inhibit the effects of Toxin A in a model of acute enterocolitis in the rat (115). MPO activity, histologic damage and fluid secretion were all decreased when the antagonist was administered prior to intraluminal influx of
Toxin A, but CP-96 344 was significantly less effective when administered 10 minutes after enterocolitis was established (115). Interestingly, CP-96 344 was found to completely silence the release of a specific mucosa mast cell protease (RMCP11) as measured by ELISA (115). This suggests a SP-mast cell interaction (115).

In addition, a Toxin A model of inflammation was used in rats to demonstrate a macrophage-SP interaction, by showing the release of SP from lamina propria macrophages during intestinal inflammation (17). Since the SP antagonist CP-96345 inhibited TNFα release from macrophages, it is also suggested that SP release may act in an autocrine or paracrine fashion on these inflammatory cells (17).

d. Cryptosporidium Parvum Model

A mouse model of IBD, established through the inoculation of mice with Cryptosporidium parvum, was used to investigate the effects of the neurokinin-1 receptor antagonist LY 303870 on IBD lesions (129). The NK-1 antagonist decreased severity of lesions if administered prior to the onset of IBD. Once IBD was already established, while it had no effect in the colon, LY 3033870 helped to decrease the severity of lesions in the caecum and small intestine. That this NK1 antagonist is not always useful once enteritis exists, is an important consideration for clinical application. In this study, mice remained chronically infected with C. parvum, suggesting that the effects of the NK-1 antagonist were not mediated by a direct effect on the parasite (129).
1.2.2.2. Measuring Substance P Levels in Human Tissues

Radioimmunoassay, *in situ* hybridization (98), real-time quantitative reverse transcription-PCR and immunoblot assays (99) have been used to investigate the level of SP in tissue from normal and IBD human patients. Immunostaining localized immunoreactive SP secretory granules in eosinophils of patients with IBD and also in some normal control samples (98). SP mRNA expression was found in lamina propria eosinophils, which suggests local synthesis (98). Levels of the degradation enzyme for SP, neutral endopeptidase (NEP), were found to be unchanged in inflammation using immunoblot assay on samples from Crohn’s disease (CD) and ulcerative colitis patients (99). This information suggests that the effect SP has on GI tissues is mediated by the amount of SP peptide produced and the number of associated receptors present, rather than changes in the rate of SP degradation. In addition, PPT-A expression was found to be increased in non-inflamed areas of CD patients (99). This, in turn, suggests that affected areas of the gut may have a positive feedback effect on healthy tissues, and subsequent gene expression therein.
1.2.2.3. Water flux – Determination of the Role of SP in Hypersecretion

Hypersecretion, or changes in the ion exchange and net fluid flow across the intestinal mucosa, is often a component of intestinal disease (54). Through various experimental techniques, using non-absorbable markers (41; 42) or Ussing chambers (117; 118), it has been demonstrated that both SP and NKA play a role in hypersecretion.

a. Non-absorbable Markers in Induced Hypersecretion

By measuring the effluent obtained from isolated rat colonic segments after constant rate infusion, net water flux was determined (positive values indicating absorption and negative indicating net secretion). Net water flux was then used as a measure of hypersecretion in an experimental model of rectal distension, established through the inflation of a balloon per rectum in rats (42). NK1 and NK2 antagonists (SR 140333 and MEN 10627, respectively) were successful in decreasing hypersecretion when administered locally. In contrast, NK2 and NK3 antagonists (SR 142801), when administered in the cerebral ventricles, diminished net water flux measurements. It was concluded that in hypersecretion due to intraluminal distension, the NK1 receptor is active locally, and the NK2 and NK3 receptors are active centrally (42).

The role of tachykinins in water secretion in vivo was also evaluated in an interleukin-1β (IL-1β) rat model of colonic secretion (41). The NK1 antagonist RP 67580 and the NK2 antagonist SR 48968 were found to block the effects of IL-1β, which are involved in hypersecretion during GI inflammation (41). Both the NK1 agonist GR-73,632 and NK2 agonist GR-64,349, but not an NK3 agonist, were also found to induce hypersecretion in rats (41). L-
NMA (N-methyl-L-arginine), a nitric oxide (NO) synthase inhibitor, inhibited both NK1 and NK2 agonist-induced hypersecretion, which suggests that tachykinins acting via these receptors mediate their secretory effects through NO (41).

b. Ussing Chambers – Electrophysiologic Studies

Ussing chambers, which measure the short circuit current ($I_{sc}$) and potential difference in tissues to indicate ion transport, have been used to describe SP induced secretion as well (117; 118). When SP was introduced serosally to rabbit colon sections (118) and human colon sections (117), the potential difference became more negative, indicating an increase in negative ions in the lumen (117; 118). The $I_{sc}$ increase observed was inhibited by a lack of chloride, the blockage of transport channels required for the movement of chloride ions, tetrodixin (an inhibitor of sodium channels in nerve cells) and lodoxamide (which inhibits the release of mediators from mucosal mast cells). These findings suggest that SP-induced secretion is chloride-dependent, involving nerves and mast cells (117; 118). An H1 receptor blocker was more effective than an H2 receptor blocker in inhibiting this response, but both suggest that SP causes the release of histamine (117; 118). The NK1 receptor antagonist CP 96345 also inhibited secretory changes, implicating this receptor in signalling for this response (117; 118).
1.2.2.4. Tachykinins and Food Allergy

There is some evidence to suggest that tachykinins are involved in hypersensitivity reactions to food. Daily intraperitoneal administration of NK1 and NK3 receptor antagonists (SR 140333 and SR 142801, respectively) decreased IgE and IgG serum levels in guinea pigs presensitized to cow milk protein (47). SP and NKB also seem to be involved in food allergy via different mechanisms, as SR 140333 was successful in decreasing mast cell numbers and hypersecretion induced by the antigen, while SR 142801 was not (47).

1.2.2.5. Summary:

Tachykinins are involved in the early stages of inflammation in the gastrointestinal tract. Pretreatment with tachykinin antagonists was significantly more protective than administration once enteritis was established. This may limit the potential therapeutic use of antagonists due to their poor protective effects if administered once clinical signs are evident. All three tachykinins have been implicated in inflammation, though their mechanisms of action may vary, especially when species differences are considered.

A variety of experimental techniques have elucidated the source, site of release, and pathway of SP-induced inflammation, food allergy and hypersecretion, which may involve macrophages, mast cells and eosinophils.
1.2.3  Inflammatory Bowel Diseases in the Horse

A review of inflammatory bowel diseases in the horse has identified four conditions of importance (122). These include: granulomatous enteritis (GE), multisystemic eosinophilic epitheliotropic disease (MEED), lymphocytic-plasmacytic enterocolitis (LPE) and idiopathic eosinophilic enterocolitis (EC).

1.2.3.1 Granulomatous enteritis (GE)

Granulomatous enteritis of unknown cause was first described in 1974 in a review of 10 cases (20). This condition causes chronic weight loss, occurs mostly in Standardbreds, and is characterized by distinct granulomas and diffuse infiltrates of inflammatory cells (82; 122). Gross characterization of tissue damage differed greatly, but during histological exam the ileum was generally most affected, with the lamina propria and submucosa being the most infiltrated with lymphocytes, macrophages or epithelioid cells (20; 82). Topography of the mucosal surface, as determined with scanning and electron microscopy, again varied greatly (83) but included villus distension and marked enterocyte loss. The pattern of mucosal ulceration in granulomatous enteritis has been found to be similar to Crohn’s disease in humans (20) and is associated with intrinsic abnormalities in the inflammatory response (82).
1.2.3.2 Lymphocytic-plasmacytic Enterocolitis (LPE)

LPE is a very rare disease that, in all reported cases, resulted in affected horses being euthanized (88; 122). Little can be concluded about the etiology of the disease based on the limited numbers of cases. However LPE was most often characterized by villous atrophy, diffuse lesions in the small (88) and large intestine (122), and edematous mesenteric lymph nodes (88) as well as infiltration of the lamina propria with lymphocytes and plasma cells (122).

1.2.3.3 Multisystemic Eosinophilic Epitheliotropic Disease (MEED)/ Idiopathic

Eosinophilic Enterocolitis (EC)

MEED is characterized by eosinophilic infiltration in locations other than just the gut, whereas ‘eosinophilic gastroenteritis’ and ‘eosinophilic enterocolitis’ are more specific forms of this disease where effects are limited to the gastrointestinal tract (7). Much like granulomatous enteritis, MEED and EC are thought to be more prevalent in Standardbreds (122). EC has been characterized as having eosinophil, lymphocyte, plasma cell and macrophage infiltration of the lamina propria and submucosa (7). While the etiology is unknown (7; 122; 123), the degranulation of mast cells and basophils triggered by type I hypersensitivity, leading to the release of eosinophilic chemotactants, has been suggested as a potential cause (122). Specifically, horses with EC differ from those with MEED in that they are more likely to survive with surgical intervention (123).
1.2.4. Ischemia/reperfusion Injury and Intraluminal Distension of the Equine Small Intestine

Strangulating obstruction is a common manifestation of an intestinal accident in the horse (138). It is characterized by occlusion of the intestinal lumen and its blood supply, most often due to strangulation by an internal hernia or abnormal band of tissue in the case of the small intestine (9). Ischemia, or decreased tissue perfusion, due to functional or mechanical obstruction of the vascular network (101) and intraluminal distension, where luminal contents and gas accumulate proximal to the lesion (1), are sequelae to strangulating obstruction injuries.

1.2.4.1. Ischemia/reperfusion:

Lack of perfusion is a source of potential of GI tissue injury since cellular energy stores are depleted and metabolic demands of the tissue exceed oxygen delivery (101). However, the restoration of blood flow to a region thus affected seems to actually exacerbate damage (101). Both mechanisms, ischemia and reperfusion, have been the focus of considerable study (4; 31; 30; 28; 70; 91; 133).

After ischemia and subsequent reperfusion in the equine jejunum, the following morphological changes have been observed: villus retraction (4; 133) with the central arteriole being short and convoluted (28), interstitial edema (4; 28; 133) and endothelial cell swelling, mesothelial cell loss (31), and disrupted enterocyte attachments to the basement membrane and cell-to-cell adhesions (4). Increased vascular permeability (31) and infiltration of leukocytes are also observed (28; 31). These changes are consistent with acute inflammation and lead to increased permeability of the intestine which results in systemic endotoxemia (1; 31).
Cellular receptors on intestinal smooth muscle have also been evaluated using ischemia/reperfusion as a model of inflammation. Motilin receptors were found to be decreased relative to control samples (70), whereas receptor function remained intact. It was also concluded that ischemia induces more damage in the jejunum compared to the ascending colon (31). Smooth muscle strips from bowel proximal and distal to ischemic bowel showed decreased contractility compared to controls (91). Immunohistochemistry revealed that there was no change in immunoreactivity for SP, adrenergic and cholinergic neurons and NO synthase (91) between normal and tissues proximal and distal to ischemic/reperfused regions.

An important consideration in ischemia/reperfusion models are the differences seen between ‘complete’, where artery and vein are completely occluded (70) and ‘low flow’ models, where either arterial flow is decreased to 25% of baseline (30) or only the vein is occluded (133). These have also been termed “ischemic strangulating obstruction” and “hemorrhagic strangulating obstruction” (9) respectively. When the two are compared grossly, ‘completely’ ischemic tissues appeared pale and void of edema or haemorrhage, whereas ‘low flow’ tissues developed progressive edema, hemorrhage and congestion (133). Histologically, however, results were similar for both conditions (133). When a ‘low flow’ model was used, a significant increase in vascular permeability, leukocyte infiltration and edema was noted (30), similar to ‘complete’ models (4; 28; 31).
1.2.4.2. Distension:

The consequences of intraluminal distension (ILD) in the equine jejunum have been examined in several studies (1; 29; 32; 70; 105). When equine small intestine was distended to 25 cmH₂O intraluminal pressure, shortened villi (29; 105), extended crypts, mesothelial cell loss (105), neutrophil infiltration (32) and edema in the seromuscular layer were observed (29). Edema and haemorrhage of the serosa, muscosa and submucosa were also noted (32; 105). When subsequent decompression for 60 minutes was performed, these lesions progressively worsened (29). Similarly, when jejunal segments were distended for one and four hours, respectively, edema was observed in the lamina propria and central lacteals (1). Grossly, however, all distended samples appeared normal (1) and contractility of the segments returned immediately after decompression (1). Distension caused a decrease in seromuscular perfusion, which did not completely return to its normal state upon decompression (29). Vascular resistance was increased during distension (32; 105), but returned to baseline upon decompression. Elevated venous pressure and dilated lymphatic vessels were also seen (32). A “low flow” type of ischemia, as described above, was also observed when mesenteric blood flow, oxygen delivery and oxygen consumption were measured in distended jejunal tissues (32).

In those cases where distension was compared to ischemia/reperfusion, serosal injury was found to be more severe in the distended segments over the same time period, with the most damage to the serosa seen in decompressed samples (32). There is conflicting evidence concerning histologically-evident damage to structures. Some have noted that there is no degeneration of mitochondria, disruption of the basement membrane or epithelial cells during ischemia (1). On the other hand, disruption of the basement membrane has been reported (32), and attributed to mechanical stretching.
1.2.4.3. Summary:

The changes seen in both ischemia/reperfusion and intraluminal distension are consistent with acute inflammatory changes. The process by which these changes lead to tissue injury is thought to be through the generation of reactive oxygen species (ROS) when oxygen from arterial blood is reintroduced to a region where substrates from the metabolism of cellular ATP have accumulated (101). The ‘bystander effect’ created by activation of infiltrative neutrophils is also thought to play a significant role in injury (101). For this reason, both ischemia/reperfusion and intraluminal distension were used as models of inflammation in the equine intestinal tract for the present study. It is clinically useful to better understand the pathologic changes in receptors in some of the most common forms of lesions seen in this species in order to potentially improve therapeutic protocols.
1.2.5. **Normal Motility of the Equine Gastrointestinal Tract**

There are two types of motility in the gastrointestinal tract: propulsive and mixing patterns (56). Peristalsis occurs in the aboral direction in response to distension stimuli (56). Mixing or segmentation activity varies in different regions of the gut, and consists of constricting contractions that aid in mixing of chyme (56). Similarly, there are two types of electrical wave patterns in the GIT: slow waves and spiking waves (9). Slow waves represent the baseline rhythm of the gut, and spiking waves are the actual action potentials generating depolarization of the muscle cells (9). Spiking waves usually occur superimposed on the slow wave pattern (104).

Gastrointestinal motility is controlled by neural, hormonal and myogenic mechanisms (9). There are 2 nerve plexuses in the gut that control motility, the submucosal (inner) and myenteric (outer) plexuses (57). The submucosal plexus modulates contraction of the inner submucosal muscle of each segment, whereas the myenteric mediates overall motility, since it is comprised of interconnecting neurons along the length of the GIT (57). These enteric neurons release many factors, functions of which are still not well understood (14). It is known that nitric oxide is found in inhibitory motor neurons and that acetylcholine is found in excitatory motor neurons (14). Norepinephrine and epinephrine are known inhibitory factors (33). Parasympathetic and sympathetic innervations modulate GIT motility (73). Parasympathetic activity generally increases gut functions and activation of the enteric nervous system, whereas increased sympathetic activity has the opposite effect (73).
1.2.5.1. The MMC

The migrating motor complex (MMC) is the pattern of motility in the stomach and small intestine seen in the fasted state of most animals (104), but it is always present in the horse (71). It consists of three phases: phase I, or the quiescent phase, where little or no spiking activity is observed, phase II that is characterized by irregular spiking, and phase III or the activity front (81). Phase II has a mixing function, and phase III, which propagates down the gut, rapidly voids the lumen of its contents (104).

1.2.5.2. Interstitial Cells of Cajal

Interstitial Cells of Cajal (ICC) are intermediate or post-junctional cells, through which enteric neurons influence intestinal motility (137). There is close contact between ICCs, muscle cells and nerve terminals (137). It has been shown that ICCs mediate inputs from enteric neurons and, as pacemakers, generate slow waves in the longitudinal smooth muscles (130).
1.2.6. **The Role of Tachykinins in Gastrointestinal Motility**

When Substance P was first discovered in 1931, one of the main effects it was found to have was stimulation of tone and rhythm in isolated intestine pre-treated with atropine (40). These contractions were seen to come on more slowly than those induced by acetylcholine, whose affect is abolished by atropine (40). Since that time, the role that tachykinins play in gastrointestinal motility has been extensively studied, though it is not as of yet completely understood (78). Many experimental studies have investigated the effect that either agonists or antagonists to the 3 neurokinin receptors have on isolated smooth muscle samples from various species (5; 6; 11; 12; 23; 49; 96; 102; 108; 109; 110; 111; 112; 120; 125; 135). These contractile effects in relation to adrenergic and cholinergic activity and other effector molecules have been studied (16; 74; 109; 110; 111; 121) in an effort to map out their mechanisms of action.

The complex mode of action of tachykinins on gut motility has been further revealed through the discovery of both excitatory and also inhibitory effects (74; 109; 110; 111). More recently, investigations into the alteration of tachykinin motor activity in pathological states such as ileus (39; 134), stress (65; 107), irritation or inflammation (2; 35; 51; 76; 96) have surfaced. This has been a promising area of research with the potential for the development of therapeutics. Several *in vivo* projects have been performed in humans (87) and lab animals (16; 19; 36; 50), demonstrating the effects of tachykinins in a whole organism.
1.2.6.1. Isometric Studies:

Isometric studies, which involve mounting tissue samples in an organ bath perfused with oxygenated solution and measuring mechanical activity using pressure transducers (102), have been useful in determining the effects of tachykinins on smooth muscle of the gut. Mostly, studies have been performed on samples from animals – [rats (102; 112; 125), mice (12), guinea pigs (5; 6; 11; 135), rabbits (108; 109; 110), and pigs (120)], but some studies have also examined human specimens (23; 49; 96; 111).

In general, NK2 receptor agonists were found to have a positive contractile response on isolated circular muscle strips (23; 49; 96; 108; 111; 112; 125). These contractile effects have been demonstrated in both small intestinal segments (5; 6; 111; 120) and large intestinal samples (11; 12; 23; 49; 96; 108; 109; 110; 111; 112; 125; 135). Both the natural agonist NKA (23; 49; 111) and a multitude of synthetic agonists (49; 96; 102; 108; 111; 125) have been shown to have this effect. While both NK1 and NK3 receptor agonists have also been investigated, the response to SP, NKB and synthetic agonists in this case, have proven to have either no (96), or a significantly decreased (23; 49) effect when compared to NK2 agonists. In fact, NKA was found to be 370 times more potent than SP when tested on human colon isolated muscle strips (49). Interestingly however, when an NK1 and NK3 antagonist were simultaneously added to isolated guinea pig ileal smooth muscle pretreated with capsaicin (which induces small intestinal contractions), an inhibitory effect was observed (6). The further addition of an NK2 antagonist did not increase this effect (6). Contrarily, MEN 11420, an NK2 receptor antagonist, was found to decrease propulsive activity induced by distension in the guinea pig isolated distal colon, with NK1 and NK3 antagonists having little effect (135). In the
proximal colon of the rat, an NK1 antagonist and an NK2 antagonist had additive inhibitory
effects on motility induced by electrical field stimulation (125).

When the MMC was examined in the mouse colon, tachykinin antagonists (NK1 receptor
antagonist SR 140333 and NK2 receptor antagonist SR 48968) reduced the amplitude, integral
and duration of slow MMC contractions, but did not affect fast contractions (12). MEN 10627,
an NK2 receptor antagonist, was also found to inhibit spontaneous spiking without affecting
basal tone in the proximal colon of the rat, with SR 48968 producing similar effects in this study
(102). Both these findings suggest that tachykinins may be involved in the generation of MMCs
(12), since slow MMC contractions determine rhythmicity and spontaneous spiking determines
smooth muscle depolarization.

A relationship between tachykininergic and cholinergic pathways in the regulation of
smooth muscle contraction in the intestine has been suggested (64; 135). The fact that blocking
all three neurokinin receptors was able to inhibit distension-induced propulsion in the distal
colon by 50%, implies that there are both tachykinin-dependent and tachykinin-independent
components of motility (135). In fact, acetylcholine and SP have been co-localized in the cells
bodies of palatine nerves using immunofluorescence, suggesting that they may also be co-
released (59).
1.2.6.2. **Inhibitory Effects:**

That tachykinins have an excitatory effect on gastrointestinal motility is well established. However, inhibitory effects have also been reported. Both *in vivo* (74) and isometric studies (109; 110; 111) have investigated the inhibitory effects of the NK1 (74; 110), NK2 (108) and NK3 (109) receptors.

NK1 receptors antagonists MEN 10930 (110) and SR 140333 (74; 110) have been used in the rabbit isolated distal colon (110) and the guinea pig small intestine (74) for this purpose. In the rabbit, both MEN 10930 and SR 140 333 enhanced propulsion in an isometric study (110). Septide, an NK1 agonist, inhibited motility (110). When pretreated with an NO-synthase inhibitor, septide conversely had a prokinetic effect (110). Inhibitory effects were also seen due to the NK1 agonist GR 73, 632 (74). These were repressed by tetrodoxin (TTX) (which inhibits nervous conduction), suggesting that this mechanism is neurogenic in origin (74). The excitatory effects of the agonist, however, were TTX-resistant (74). Therefore, these results suggest that NK1 receptor excitatory effects are neurogenic, acting through NO, and that inhibitory effects are myogenic (74; 110).

Similar results were seen with NK2 and NK3 receptors (108; 109). Low concentrations of two NK2 receptor antagonists (MEN 10627 and SR 48968) and two NK3 receptor antagonists (SR 142801 and SB 222200) resulted in decreased motility in strips of colonic circular muscle stimulated with sub-maximal distension (108). Conversely, higher concentrations of both hastened propulsion (108; 109).

When atropine was given, the inhibitory effects of the NK2 receptor antagonists were enhanced, however, when an NO-synthase inhibitor was given, the excitatory effects of this antagonist were decreased (108). This suggests that NK2 receptors have inhibitory effects,
acting through an NO mechanism, and that excitatory effects occur through synergism with muscarinic receptors.

At low concentrations, senktide, an NK3 receptor agonist, inhibited motility due to submaximal and maximal distension stimuli, but at higher concentrations increased motility due to submaximal stimulation (109). An NO-synthase inhibitor enhanced the excitatory effects of the agonist, and hexamethonium (a selective nicotinic receptor blocker) markedly enhanced the inhibitory effects of the agonist (109). When hexamethonium was combined with the NK3 receptor antagonist SR 142801, propulsion was greatly inhibited. These results argue for the presence of a subset of NK3 receptors having an inhibitory effect acting through NO, and an excitatory effect involving nicotinic receptors (109).

1.2.6.3. Motility and Pathology:

a) Post-Operative Ileus:

The role of tachykinins in postoperative ileus (POI), defined as impairment of bowel motility after abdominal surgery (60), has been investigated. Two studies (39; 134), support the involvement of tachykinins in the inhibition of gastrointestinal motility post-surgically. In both cases, rat models were used to assess the effect of a non-selective SP antagonist (SP-ar) (39) and an NK2 receptor antagonist (MEN 11420, or Napadutant) (134) on the return to regular spiking activity after intestinal atony. A non-dose-dependent, minor effect was seen when SP-ar was used, as seen through a slight reduction in return of MMCs and a 23% faster return of motility, as measured with a marker (39). At low doses, Napadutant had similar effects on jejunal spiking (134), with a treatment fifteen minutes before surgery resulting in significantly faster return of
motility by 36 and 39% (134). MEN 11420 was also found to decrease time for return of phase III of MMCs (134).

b) Stress:

The effect that neurokinins have on hypermotility due to stress, as measured by rate of fecal output in both rat (65) and gerbil models (107), has also been examined. Natural SP and an SP agonist [pGlu^6]SP_6-1 (107) were both found to increase fecal output. An NK1 receptor antagonist RP 67580 (65) and Substance P antagonist TAK-637 (107) significantly decreased fecal pellet output in response to restraint stress. Surprisingly however, the NK2 receptor antagonist SR 48968 had no effect (65) on gastrointestinal transit time in this model. Treatment with capsaicin, which causes loss of intestinal sensory nerves, had no effect on transit time (65; 107).

c) Inflammation:

The contractile effects of tachykinins in inflamed tissues, compared to healthy samples have been explored in both lab animal specimens (35; 51; 76), and in human tissues (2; 96). In isolated ileum (2) and colon (2; 96) the response to NK2 receptor agonists Nle10-NKA(4-10) (2) and [βAla^8]NKA(4-10)] (96) was reduced in ulcerative colitis, Crohn’s disease (2) and irritable bowel disease (96). Contrarily, responses were increased in ICC (idiopathic chronic constipation) (96) and in a ricin model of inflammatory bowel disease in rabbits (51). In a rodent model of inflammation, doses of NK2 receptor antagonists that have no effect on hypermotility induced in normal tissues, decreased propulsion induced by [βAla^8]NKA(4-10) in rats with acetic acid-induced recto-colitis (76). This is suggestive of a potential pharmacologic use of tachykinins in conditions with altered motility and local inflammation.
There is evidence to support that this response is not simply due to damage to the overall contractility of the inflamed samples, but instead due to specific receptor changes. This is supported by the observation that contractility in response to a muscarinic agonist, carbachol, remained identical in normal and inflamed tissues (96). In addition, in a TNBS model of colitis (see chapter on inflammation for details), the contractile response to SP was decreased, but this decrease developed more slowly than the decrease in response to acetylcholine stimulation (35).

1.2.6.4. In Vivo Studies:

In vivo studies, both in lab animals (16; 19; 36; 50) and in people (85; 87), are an important component of tachykinin research in that they assess the potential for practical therapeutic use. Work done has touched on aspects explored in vitro, and also on other parameters such as their effects on the cardiovascular and respiratory systems in anaesthetized and unanaesthetized patients (50).

Specifically, when motility recordings were taken from the proximal small intestine of thirty five healthy and awake human subjects, the NK2 receptor agonist NKA was found to cause irregular contractions at high doses (85). These contractions, when characterized, had frequency similar to phase II contractions of the MMC and amplitude similar to that of phase III (85). Measurements of myoelectric activity in the rat intestine also showed that NKA was the most potent tachykinin in inducing phase II like spiking activity (86). At lower doses, NKA dose-dependently increased amplitude and frequency of small intestinal contractions, increasing the fraction of phase II of the MMC (85).
As was seen with isometric studies, NKA stimulated motility (50). NKA (and other synthetic NK2 agonists) had a more significant effect on motility than Substance P (or other synthetic NK1 agonists), while both were found to have some effect in humans (85) and rats (86; 121).

The mechanism by which NKA induces motility in the small intestine was investigated in vivo in rats, by administering a nitric oxide (NO) synthesis inhibitor (L-NMA)(121). When NO synthesis was inhibited, a lower dose of NKA was required in order to produce the same contractile effects seen under normal conditions (121). It was thus suggested that NO must inhibit the contractile effects of NKA (121). Similarly, the mode of action was further elucidated by the observation that acetic acid-induced hypermotility (a model of irritation in the colon) was entirely inhibited by atropine, and partially reduced by Nepadutant (an NK2 receptor antagonist)(16). This indicates that cholinergic reflexes may be enhanced by NK2 receptors (16).

In addition to digital recordings of pressure changes, as indicators of motility, radiochromium has been used in rats as an indicator of transit time in response to tachykinins stimulation (19). When NKA and SP were administered intraperitoneally, acute effects on transit time were found to cause stasis proximally, and accelerate transit in the distal parts of the intestine (19). An overall peristaltic effect of NKA and SP (19) was found following calculations of the geometric centre of intestinal transit determined by the relative radioactivity of intestinal and gastric segments.

The ability of NK antagonists to affect the increased motility induced by irritation, distension (16) or inflammation (36) has been evaluated in vivo as well. In a model of chronic irritable bowel disease, TNB was used to induce colitis in rats (36). Colonic smooth muscle
samples taken from these animals at day 14 showed an increased contractility in those treated with the NK1 receptor antagonist SR 140333 compared to control (36). Similarly, Nepadutant (an NK2 receptor antagonist) was found to decrease acetic-acid-induced irritation hypermotility (16) and reliably reverse the effects of NKA without affecting the baseline MMC in the dog and rat intestine (50) \textit{in vivo}. Another synthetic antagonist (MEN 11420) was found to have the same effect (87), showing that NK2 receptors are not involved in the interdigestive rhythm (86). Nepadutant did not seem to have any effect on hypermotility induced by distension (16).

\textbf{1.2.6.5. Summary:}

Overall, research to date reflects a complex mechanism of action of tachykinins in the gastrointestinal tract (61). Both excitatory myogenic and inhibitory neurogenic mechanisms have been described. Differences in individual and species receptor concentrations and distributions on both smooth muscle and nerves have significance in terms of the expected contractile response. It is also important to recall that receptor specificity of natural agonists is not absolute, and may contribute to the variation (87).

Alterations in the influence that tachykinins have on motility in the face of pathological conditions including: stress-induced hypermotility, post-operative ileus and inflammation are important to consider. These alterations provide information for potential therapeutic targeting of these receptors in the clinical setting. \textit{In vivo} studies are also an important source of models for future work in the equine circumstance. Particularly, studies involving measurement of myoelectric activity are promising (86), since electrointestinography has been shown to be a useful non-invasive tool in equine research (72).
1.2.7. Ileus: Pathophysiology and Relevance

Ileus has been defined as “the functional inhibition of propulsive intestine activity irrespective of its pathology” (80). Postoperative ileus (POI), can be defined as “transient impairment of bowel motility after abdominal surgery or other injury” (60), “especially where intestinal manipulation was involved, that is not caused by mechanical obstruction” (97). POI is an important clinical condition in both horses and people, which contributes to delayed recovery and may also be a crucial factor in survivability post-surgically (71; 104). Prevalence for POI of 10 to 47% has been reported in horses, with a mortality rate ranging from 10 to 86% (97), making this common condition one of great interest in equine medicine.

1.2.7.1. Risk Factors:

Several retrospective studies have examined the risk factors associated with POI in horses (21; 84; 119). A breed predisposition (Arab horses) was only noted in one of these studies (119). Decrease in motility was associated with horses older than 5 years old who had undergone orthopaedic surgery of 1 hour or more in duration (84).

While signalment, duration of clinical signs prior to anaesthesia, as well as the amount of nasogastric reflux obtained during hospitalization did not significantly differ between those horses that developed POI and those that did not, some clinicopathologic parameters seem to play an important role in predisposition to ileus (21). In a study involving 251 horses at the Texas A&M University Large Animal Clinic, 47 of which developed POI, packed cell volume (PCV), concentration of white blood cells, total protein, and total calcium were all significantly
greater in cases of POI (21). Similarly, review of 69 cases of ileus associated an increased PCV, serum protein and albumin with an increased likelihood of developing POI (119).

Length of anaesthesia and lesion localization in the small intestine were correlated with increased risk of POI (119). Similarly, it was found that hypovolemia and small intestinal obstructions could be associated with increased risk of POI (21). Researchers also found that performing anastomoses or resections of the small intestine increased the likelihood of developing POI, and performing an enterotomy significantly decreased this likelihood (119).

1.2.7.2. Etiology:

Numerous etiologies have been suggested for the development of POI, including release of inflammatory mediators (66), sympathetic hyperactivity (48; 60; 71) and endotoxemia (69; 71). A study using a rat model of ileus examined the effects of various degrees of surgical manipulation of the tissue on the development of POI (66). It was found that, compared to laparotomy alone, manipulation of the bowel resulted in increased activation of resident macrophages and recruitment of polymorphonuclear cells (66). In addition, when the bowel was compressed, more leukocytes migrated to the site (66). The decrease in contractile activity seen in circular muscle post-surgically was found to be less severe prior to leukocyte infiltration (66).

Since increasing increments of surgical manipulation resulted in greater leukocyte infiltration as well as decreasing contractile activity, this implicates the acute inflammatory response in the manifestation of POI (66). This causal relationship was further elucidated in the 1999 study by the same group of researchers using RT-PCR and immunohistochemistry (66). In support of their previous work (66), they demonstrated that P-selectin and ICAM-1 (intercellular adhesion molecule 1), which both mediate leukocyte extravasation through the endothelium,
were increased after surgical manipulation of the bowel (66). In fact, antibodies against these adhesion molecules increased circular muscle contractile response post-operatively (66).

In an equine model of ileus, that involved exteriorising and rubbing jejunum to create serosal lesions, motility in response to various prokinetic drugs was measured in eight ponies via the migration of spheres through the jejunum, and also by electrical and mechanical recordings of motility (48). This study showed that blocking β-adrenoreceptors was not effective in reducing the signs of ileus, that alpha2-adrenoceptor blockage was effective, and that stimulation of cholinergic receptors had some effect (48). Metoclopramide, a 5-hydroxytryptamine ligand as well as an alpha2-receptor blocker and dopamine antagonist, had a significant prokinetic effect. This suggests that dopamine is involved in the development of ileus, since the other effects of metoclopramide, also produced by other antagonists used in the study, did not amount to the same response (48).

In addition to sympathetic and inflammatory involvement, endotoxemia has been implicated in causing POI. When electrical and mechanical recordings of intestinal motility were recorded in six ponies after the administration of endotoxin, severe disruption in propulsive activity was noted (69). However, the fact that this has been correlated to the release of PGE2, which is an inflammatory mediator, may be an argument for inflammation itself as the root cause (71).
1.2.7.3. Recognition and Treatment:

Early recognition of POI and appropriate treatment are crucial in the successful management of these patients. The most common clinical signs of POI include - depression, abdominal pain, nasogastric reflux, decreased or absent borborygmi, an increased heart rate, hemoconcentration and decreased plasma chloride and potassium levels (58).

A review of 37 cases of POI (as measured by reduced fecal output) in horses undergoing surgery unrelated to the gastrointestinal tract, found that 12% of horses that developed colic had less than three defecations in the immediate 24-hour post-operative period (84). While not all cases of reduced fecal output developed colic, this still remains an important parameter to monitor post-operatively (84).

Preventing risk factors, discussed above, as well as supportive therapy and the use of prokinetic agents are useful in the treatment of POI. Several reviews of the clinical use of prokinetics have been published (34; 60; 71). The usefulness of adrenergic receptor antagonists, cholinergic agonists, benzamides, dopaminergic antagonist, macrolide antimicrobials, opiate antagonists and agonists, somatostatin analogues and local anaesthetics in the prokinetic treatment of POI has been investigated (34; 71). Bethanechol, neostigmine and erythromycin seem to be most effective for large intestinal problems, whereas metoclopramide, cisapride and local anesthetics are used for abnormalities in small intestinal motility (71).
1.2.7.4. **Summary:**

Overall, though a significant amount of research has been completed on lab animals, people and horses concerning the development of POI, a more complete understanding is still required. Etiology is complex, and seems to involve the inhibitory effect of inflammatory mediators on intestinal motility as well as sympathetic hyperactivity. The management of ileus, both through early recognition and appropriate pharmacological treatment, has been thoroughly described and reviewed. However, POI remains a significant cause of morbidity and mortality in equine cases and more research into causation and potential therapeutics is warranted.
1.2.8. Tachykinin Research and the Equine Gastrointestinal Tract

Considering that Substance P was initially discovered in isolates of equine intestinal tissue \(^{40}\), it is surprising that there have not been more extensive studies on the role of tachykinins in the gastrointestinal system of this species. For the most part, research has focused on localizing Substance P immunoreactivity in different regions of the gut \((15; 25; 113)\). Relatively few studies have evaluated the type of tachykinin receptors present and their relative expressions \((127; 128)\), pathological changes in receptors \((15; 128)\) and smooth muscle response to tachykinins in vitro \((8; 90)\). Only one study has involved an in vivo trial \((124)\).

1.2.8.1. Immunoreactivity of Substance P in the Equine Gastrointestinal Tract

Several studies have evaluated substance P (SP) immunoreactivity in the equine gastrointestinal tract by examining samples from the left dorsal colon \((25)\), the pelvic flexure \((15)\), and the jejunum \((113)\). In all cases, it was found that immunoreactivity was high in the nerve fibres of the myenteric plexus \((15; 25; 113)\). SP reactivity was also found in varicose networks surrounding the myenteric plexus \((15; 25)\), around glands in the submucosa, and also surrounding arterial vessels \((15)\). Interconnecting nerve strands (intra- and interganglionic) also showed reactivity \((15; 25; 113)\).

Mucosal cells \((25)\) and neuronal cell bodies of the myenteric plexus in the pelvic flexure and the jejunum were not immunoreactive to SP \((25; 113)\). However, 60% of perikarya in the submucosal plexus of the pelvic flexure showed SP reactivity \((25)\), and there was a weak reactivity in cells of the submucosal plexus in samples from the jejunum \((113)\).
In the left dorsal colon, SP immunoreactivity was found in all layers of the bowel wall, with more intense reactivity in cell bodies of submucosal ganglia than myenteric ganglia (15). Reactive nerve fibres from the myenteric plexus seemed to supply the circular muscle of the tunica muscularis, and those from the submucosal plexus seemed to supply the muscularis mucosae (15). There is a consensus that SP reactivity is sparse in longitudinal muscle samples when compared to that of circular muscle (25; 15).

1.2.8.2. Studies for Determination of NK Receptor Distribution in Equine Gastrointestinal Tract

Two studies have previously investigated the relative distribution of neurokinin receptors in the equine gastrointestinal tract. One examined all three receptors (NK1, NK2 and NK3) in the pelvic flexure using autoradiography (128) and the other, through the use of RT-PCR, quantified the distribution of NK1 receptor mRNA in all regions of the intestinal tract (127). When NK1 receptor mRNA was examined in smooth muscle samples from nine different regions of the equine GI tract (127), its distribution was found to differ. The highest levels of expression of NK1 mRNA were found in the duodenum, right ventral colon and left ventral colon. In the large intestine, moderate levels were seen in the caecum, and expression was lowest in the pelvic flexure, left and right dorsal colon. Results from the small intestine showed that duodenum had a significantly higher proportion of NK1R mRNA than the jejunum and ileum (127).

The region of the pelvic flexure was observed more specifically, through the use of autoradiography and a tachykinin receptor ligand binding (128). Relative receptor concentrations
were determined by measuring the binding of the ligand in the presence or absence of an NK1 receptor agonist ([Sar9, Met (O2)11]-substance P), an NK2 receptor agonist ([Nle10]-neurokinin A (4-10)) and an NK3 receptor agonist (senktide) (128). NK1 receptors were found to predominate over both NK3 and NK2 receptors (128). Overall, binding was also found to be more significant in the circular smooth muscle layer than the longitudinal smooth muscle layer of the pelvic flexure (128), which is in agreement with the observation that the longitudinal smooth muscle is less sensitive to tachykinins than circular smooth muscle (8; 15; 25; 90).

1.2.8.3. Pathological Receptor Changes

Amitraz, an antiparasitic triazapentadiene which induces intestinal stasis and results in colic signs (abdominal pain), was administered to ponies in order to determine if there would be a difference in SP immunoreactivity between this impaction colic model and control animals (25). No significant difference was found (25). Similarly, samples from 8 ponies did not show a significant change in receptor expression post ischemic injury (60 minutes at a 720 degree torsion) when compared to samples taken pre-injury (128).

1.2.8.4. Isometric Studies on Isolated Muscle Preparations

The contractile response of isolated longitudinal and circular muscle preparations to SP (8; 90), NKA, NKB (8) and to an NK1 receptor antagonist (CP-96,345) (90) have been examined in samples of equine tissues.
As expected from immunocytochemical results, SP had a positive effect on contraction of equine gastrointestinal smooth muscle (8; 90). Circular muscle was found to be more responsive to tachykinin stimulation than longitudinal muscle strips (8; 90); with large intestinal samples being less sensitive overall than those from the small intestine (8). When the relative contractile response of longitudinal and circular muscle strips to SP, NKA and NKB was measured, NKB was found to have the most profound contractile effects on the small intestine (8). All tachykinins had a slower onset of action, and lower contractile response in the descending colon when compared to the small intestine (8).

Electrical field stimulation (EFS) has also been used to study the contractile effects of SP in equine smooth muscle mounts (90). Depolarization of nerves, but not muscle, occurs at intensities of 30 and 70mV via EFS. An NK1R blocker was found to alter the contractile effect of EFS at these settings, which supports the involvement of SP’s release from myenteric neuron in modulating contraction (90). However, this effect was only seen at high intensity stimulation (70mV), which represents a model of high luminal distension (more tension). The authors therefore suggest that SP may act to modulate the reflex pathway, or to increase cholinergic activity (90). It is also suggested that SP may act to relax smooth muscle in the equine gut (90). Since CP-96,345 increased the EFS-induced off response in all longitudinal muscle and in already contracted circular muscle, SP may help to prevent spasm or closure of the lumen (90).

1.2.8.5. In Vivo Studies of Responses to Substance P

Only one study has evaluated the in vivo effects of SP on gastrointestinal motility in the horse (124). Direct injection of SP through indwelling colic artery catheters in Shetland-type
ponies, was followed by assessment of motility and relative blood flow using intestinal catheters measuring intraluminal pressure and volume transit-time blood flowmeters (124). Vasodilation to the region occurred first, followed by an increase in contractility with effects seen at a SP mean blood concentration of $9 \times 10^{-10}$M. However, SP was not effective in reducing the colic-impaction effects of pre-treatment with amitraz (124).
1.2.8.6. Summary:

It is clear that additional work is warranted concerning tachykinins and their receptors in the equine intestinal tract. It is believed that circular smooth muscle is likely to be more reactive to tachykinin stimulation than longitudinal muscle, and, based on the innervation patterns, SP probably acts as a neurotransmitter to modulate motility and vasodilation. In all likelihood, tachykinin stimulation increases contractility and motility even when pathology is present. However, a better understanding of neurokinin receptor distribution, function and response to agonists and antagonists in the horse would help elucidate their potential therapeutic effect.

Most tachykinin research to date has utilized rodent models. However, a few studies have investigated tachykinins in the gut of other species (120, 121). For example, congruity as well as differences have been noted between findings in guinea pig and porcine samples (120). This reinforces the need for species-specific research.

While an NK2 antagonist was able to inhibit basal peristaltic activity in the guinea pig small intestine (135), the same was not found for the porcine ileum (120). An NK1 antagonist (CP99994), was however able to abolish aboral motility in porcine ileal segments infused with atropine (120). That this NK1 antagonist was able to block atropine-resistant peristalsis and thus cause ileus, suggests that SP (the NK1 receptor’s natural ligand), may be useful in increasing motility in some sections of the gut. Tachykinin agonists may be useful in the treatment of ileus, especially when cholinergic neurons have been impaired, but more species-specific equine studies are required to further elucidate this potential.
REFERENCES


22) Conlon, JM. The tachykinin peptide family, with particular emphasis on mammalian
tachykinins and tachykinin receptor agonists. In: Holzer P, ed. Tachykinins. Germany:
Springer, 2004; 25 - 62

Ferla. In vitro characterization of tachykinin NK2-receptors modulating motor responses of

24) Cuello AC. Peptides as neuromodulators in primary sensory neurons. Neuropharmacol
1987; 26 (7b): 971 - 979

25) Cummings JF, Sellers AF and JE Lowe. Distribution of substance-P-like immunoreactivity
in the enteric neurons of the large colon of normal and amitraz-treated ponies - an

Effect of MEN 11467, a new tachykinin NK1 receptor antagonist, in acute rectocolitis

Protective effect of the tachykinin NK2 receptor antagonist nepadutant in acute rectocolitis

1683 - 1692


57) Hansen MB. The enteric nervous system I: organisation and classification. *Pharm Toxicol* 2003; 92(3): 105 - 113


64) Holzer P and CA Maggi. Synergistic role of muscarinic acetylcholine and tachykinin NK-2 receptors in intestinal peristalsis. *Arch Pharmacol* 1994; 349: 194 - 201


130) Sanders KM. A case for interstitial cells of cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterol* 1996; 111: 492 - 515


Distribution of neurokinin 2 and 3 receptor mRNA in the normal equine gastrointestinal tract and effect of inflammation on neurokinin 1, 2, and 3 receptor mRNA in the equine jejumum

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Abstract

Objectives – To quantify neurokinin 2 and 3 receptor mRNA from nine regions throughout the equine intestinal tract, and to evaluate the influence of jejunal inflammation on neurokinin 1, 2, and 3 receptor mRNA.

Sample Population – Specimens were harvested from 5 adult horses euthanized for reasons unrelated to gastrointestinal disease for the study of normal distribution of neurokinin receptor mRNA. Jejunal segments from 6 healthy adult horses subjected to intraluminal distension or ischemia/reperfusion injury were harvested to study the influence of inflammation on neurokinin 1, 2, and 3 receptor mRNA expression.

Procedure – RNA was isolated from normal tissues and also from tissues that underwent either a sham operation (control), 120 minutes of ischemic strangulating obstruction (ISO) or 120 minutes of intraluminal distension (ILD) as part of an inflammatory model. RNA was reverse transcribed into cDNA. NK2 and NK3 primers were designed and mRNA was quantified using real-time PCR for all experimental groups.

Results – NK2 and NK3 receptor mRNA were not uniformly distributed throughout the equine intestinal tract. Expression of NK2 receptor mRNA was highest for the duodenum and the body of the caecum. NK3 mRNA expression had high variability. In the inflammatory model, no statistical difference was noted between treatment groups for NK1 or NK3 receptor mRNA. NK2 receptor mRNA expression decreased for ILD and ISO, when compared to control.

Conclusions and Clinical Relevance – The description of neurokinin receptor mRNA distribution throughout the equine intestinal tract is an important initial step towards determining potential clinical therapy using tachykinin agonists and antagonists.
Abbreviations

GIT – Gastrointestinal tract

G-protein – Guanine nucleotide-binding protein

Introduction

Tachykinins are a group of neuropeptides that includes Substance P (SP), Neurokinin A (NKA), Neurokinin B (NKB), Neuropeptide γ (NPγ), Endokinin C, Endokinin D and Hemokinin 1 (HK1) (1). NPK and NPγ are variants with NH2 terminal extensions on NKA, which have similar actions as NKA (1). Endokinin C and D have weak bioactivity and HK 1 has an unknown function (1). Research to date, has thus shown SP, NKA and NKB to be the physiologically relevant mammalian tachykinins. Tachykinins act via binding to one of three G-protein-coupled neurokinin receptors: NK1, NK2 and NK3. All three tachykinins will bind to all three receptors, however, SP has the strongest affinity for NK1 and NKA has the highest affinity for NK2; NKB has the strongest affinity for the NK3 receptor (2).

SP, NKA and NKB all play a role in the development of intestinal inflammation (3). A variety of experimental models of inflammation in laboratory animals and humans have been used to demonstrate the potential therapeutic benefit of neurokinin antagonists in the management of inflammatory bowel disease symptoms (4). Both natural and synthetic NK1 (5) and NK2 (6) antagonists have been shown to decrease tissue damage in experimental gastrointestinal inflammation. NK3 antagonists, while ineffective in rats, significantly decreased intestinal permeability, myeloperoxidase activity (a measure of neutrophil infiltration) and histologic damage in guinea pigs (6). This is an example of the potential for crucial species
differences in NK receptor function. It is also important to note that tachykinins seem to be involved in the early stages of inflammation, with antagonists being less protective when examined after seventy-two hours compared the first twenty-four (7). This suggests that their therapeutic benefit may be the highest in the first twenty-four hours.

NK receptors 1, 2 and 3 play a role in modulation of gastrointestinal motility, with a suggested involvement in the generation of Migrating Motor Complexes (8). Initially, SP was found to have a positive contractile effect on intestinal smooth muscle (9). Further study revealed NKA as the most potent tachykinin agonist to affect contractility (10). The complex mode of action that tachykinins exhibit, however, includes both excitatory myogenic and inhibitory neurogenic effects (11; 12; 13; 14). The distribution of receptors in both muscle and nerves therefore is expected to have a significant impact on the expected contractile response.

Little information is available on tachykinins and their receptors in horses. Most studies have evaluated SP immunoreactivity (15; 16; 17). One study examined the distribution of NK receptors in the pelvic flexure (18) and another quantified NK1 receptors but not NK2 or 3 receptors in all regions of the intestinal tract (19). A positive contractile effect to NKA and NKB was observed in the equine duodenum and the ileum, with circular muscle being more responsive than longitudinal (20). It was also concluded that SP acts as a neurotransmitter in the equine jejunum, based on the presence of SP immunoreactivity in the myenteric plexus (16) and the release of SP from myenteric neurons (21).

Ileus, or the cessation of aboral motility, has been reported to have a mortality rate as high as 80 percent when it occurs postoperatively in horses (22). The release of inflammatory mediators is one of the proposed causative factors for the development of ileus (23, 24), however its etiology is complex and not completely understood. Improvement in prokinetic drug therapy
for this condition is needed since post-operative ileus remains an important cause of complications and mortality in equine medicine.

The purpose of this study was to evaluate the distribution of neurokinin receptors 2 and 3 in smooth muscle of various anatomic regions in the equine intestinal tract by quantifying mRNA expression using real-time PCR. Further, we sought to evaluate the effect of inflammation on the distribution of mRNA expression of neurokinin receptors 1, 2 and 3 in the equine jejunum.
Materials and Methods

Sample collection

a. Distribution of NK2 and NK3 receptors

Intestinal samples were collected from 5 mature horses euthanized for reasons unrelated to gastrointestinal disease within 1 hour of euthanasia, as previously described (19). Full thickness samples from the antimesenteric side of the intestine were harvested from the following regions: duodenum (D), jejunum (J), ileum (I), body of the caecum (C), right ventral colon (RV), left ventral colon (LV), pelvic flexure (PF), right dorsal colon (RD) and left dorsal colon (LD). Samples were rinsed with saline, and tissue freezing compound\(^\text{1}\) was added. These samples were placed in liquid nitrogen, then stored at -80°C for further processing. Small pieces of longitudinal and circular muscle was later dissected on dry ice from each sample and stored in 100 mg aliquots at -80°C until RNA was extracted.

b. Inflammatory model

Six mature healthy horses were used for the study of changes in NK1, NK2 and NK3 receptor distribution in the jejunum after ischemia and subsequent reperfusion (ISO) or intraluminal distension (ILD). Anaesthetic and surgical procedures have been previously described (25). Briefly, all horses were sedated with xylazine\(^\text{2}\) and anaesthetized with guaifenesin\(^\text{3}\) and ketamine\(^\text{4}\). General anaesthesia was maintained with halothane in oxygen with intermittent positive pressure ventilation. All horses were positioned in dorsal recumbency for the procedure. A routine ventral midline laparotomy incision was made, and six jejunal segments were identified, beginning 100 cm distal to the duodenocolic ligament. These segments were each approximately 25 cm in length, supplied by a separate jejunal arcade artery and vein, and

\(^{1}\) Tissue-Tek O.C.T.; Bayer Corporation, Pittsburgh, Pennsylvania, USA

\(^{2}\) Rompun, Bayer Animal health, Etobicoke, ON, Canada

\(^{3}\) Guiafenesin powder, Rodia, Mississauga, ON, Canada

\(^{4}\) Bioniche Animal Health, Belleville, ON, Canada
separated by a dividing 25 cm jejunal segment. Two segments were randomly designated ILD, two as ISO and two as control segments. ILD was created by occluding the lumen at each end of the segment with Penrose drains, and infusing sterile Lactated Ringer’s solution into the lumen via an intraluminal 18 gauge catheter. Pressures, measured by a pressure transducer, were maintained at 25 cmH₂O for 120 minutes. ISO was created by occluding major mesenteric vessels with rubber-shod haemostats. Collateral vessels were also occluded with rubber-shod Doyen clamps. Ischemia was maintained for 60 minutes, followed by 60 minutes of reperfusion. Control segments were marked with non-absorbable suture. Full thickness 10 x 10 cm samples were harvested from the antimesenteric region in the centre of each segment. Samples were rinsed with saline, and smooth muscle was dissected free from surrounding tissue. The smooth muscle samples were then rinsed a second time, wrapped in tinfoil, placed in liquid nitrogen, and stored at -70°C until RNA was extracted.

**RNA Isolation**

*a. Distribution of receptors*

RNA isolation of samples has been previously described (19). Briefly, smooth muscle samples were minced, then homogenized in 1 mL of isolation reagent and 0.5 mL of 2.4 mm zirconia beads using a cell disrupter. Samples were centrifuged and the supernatant was placed in a new tube. RNA was isolated according to the manufacturer’s instructions (routine guanidine-chloroform-phenol extraction).

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5 Lactated Ringer’s solution, Baxter, Mississauga, ON, Canada
6 Tripure Isolation Reagent; Roche Diagnostics Ltd., Basel, Switzerland
7 BioSpec Products Inc, Bartlesville, Oklahoma, USA
8 Mini BeadBeater; BioSpec Products Inc, Bartlesville, Oklahoma, USA
b. Inflammatory model

RNA isolation of samples has been previously described (25). Briefly, smooth muscle samples were minced and homogenized in 10 volumes of buffer (250mM sucrose and 50mM tris-HCl; pH 7.4) by a homogenizer\textsuperscript{9}. Samples were then centrifuged and washed 4 times, before being resuspended in 5 volumes of 50mM tris-HCl buffer (25). RNA was isolated according to the manufacturer’s instructions.

Removal of Contaminating DNA

DNase Inactive Reagent\textsuperscript{10} was used to remove trace genomic DNA contamination for samples used in the determination of receptor distribution. Ambion DNA-free\textsuperscript{11} was used on samples for the inflammatory model studies. Spectrophotometry was used to determine purity and concentrations of RNA samples (OD 260/280 ratio), and gel electrophoresis was used to determine whether RNA was intact.

First-strand cDNA synthesis

The method of first-stand cDNA synthesis has been previously described (19). Briefly, 6µg RNA from each sample was reverse transcribed in a reaction volume of 75µL (including M-MLV reverse transcriptase 5x buffer, KCl, dNTPs, random primers\textsuperscript{11}, R NA sin and M-MLV reverse transcriptase). A thermal cycler (Progene)\textsuperscript{12} was then used (to incubate at 70°C, 37°C, heat at 99°C and cool the samples at 5°C). Repeating this process for each sample, without reverse transcriptase, confirmed appropriate removal of contaminating DNA. For the inflammatory model, a synthesis system\textsuperscript{13} was used generate cDNA from 3µg of RNA from each sample. All samples were then stored at -80°C until analysis.

\textsuperscript{9} Polytron homogenizer, Kinematica AG, Lita, Switzerland
\textsuperscript{10} Ambion Inc, Austin, Texas, USA
\textsuperscript{11} Ambion DNA – free, Roche Molecular Biochemicals, Laval, QC, Canada
\textsuperscript{12} Invitrogen Corp, Carlsbad, California, USA
\textsuperscript{13} Techne Inc, Burlington, New Jersey, USA
**Primer design**

NK1 receptor primers had previously been designed (19). NK2 and NK3 primers were designed using GenBank information and Primer\textsuperscript{14} software, and based on the bovine NK2 and NK3 sequences. Conditions for real-time PCR were optimized using a LightCycler apparatus\textsuperscript{15}, following preliminary experiments with a thermal cycler (T-Gradient Thermoblock)\textsuperscript{16}.

**Standard Curve Construction**

Standard curves were prepared from cDNA pooled from each sample. For each curve, four dilutions (1, 1/10, 1/100 and 1/1000) were used and each was run 6 times. Standard curves for β-actin, NK1, NK2 and NK3 receptors were then used to generate coefficient files, which were used in the quantification of mRNA expression. Pooled cDNA samples were used as a calibrator.

**Real-time Quantitative PCR**

Primers were used to quantify equine cDNA in samples using the LightCycler\textsuperscript{17} for Real-Time PCR. Conditions were: activation at 95°C for 2 min, followed by 50 amplification cycles (95°C/15 s; 55°C/25 s; 72°C/35 s), acquisition of fluorescence (75°C/1 s), melting (55–95°C with a temperature transition rate of 0.1°C per second and continuous fluorescence measurement) and cooling to 40°C. A reaction volume of 20μL was used and SYBR Green I\textsuperscript{18} identified product. Samples were run in triplicate for β-actin (previously determined to be a suitable house-keeping gene (Solinger, 2008), and target genes NK1, NK2 and NK3. A positive control (calibrator cDNA) and negative control (water) were included in each run.

Analysis was performed using LightCycler Relative Quantification Version 1.0\textsuperscript{19} computer software. The amount of target cDNA (NK1, NK2 or NK3 receptor) in a sample was

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\textsuperscript{14} BioSpec Products Inc, Bartlesville, Oklahoma, USA
\textsuperscript{15} Roche Diagnostics Ltd., Basel, Switzerland
\textsuperscript{16} Biometra Corp, Göttingen, Germany
\textsuperscript{17} Roche Diagnostics Ltd., Basel, Switzerland
\textsuperscript{18} Invitrogen Corp, Carlsbad, California, USA
\textsuperscript{19} Roche Diagnostics Ltd., Basel, Switzerland
compared to the amount of reference cDNA (β-actin) and a ratio was generated using the coefficient files.

**Statistical Analysis**

A generalized linear mixed-model (ANOVA) accounting for the random effect of horse was employed. NK1, NK2 and NK3 receptors were analyzed for significant differences between bowel segments and in the second study between control, ISO and ILD. Factors included in the model were bowel section or ISL/ILD treatment group. The assumptions of the ANOVA were assessed by comprehensive residual analyses. A Shapiro-Wilk test, a Kolmogorov-Smirnov test, a Cramer-von Mises test, and an Anderson-Darling test were conducted to assess overall normality. Residuals were plotted against predicted values and explanatory variables to determine patterns in the data that suggest outliers, unequal variance or other problems. If the overall F-test was significant, post hoc Tukey-Kramer tests were applied. Significance was set at p ≤ 0.05. (SAS Institute Inc. 2004. SAS OnlineDOC (R) 9.1.3. Cary, NC: SAS Institute Inc)
Results

Generation of PCR product with NK2 and NK3 primers

In order to determine the quality of primers designed, gel electrophoresis was used to confirm the generation of a single product of predicted length (not shown). The absence of primer-dimer compounds was also verified in this way. The NK2 and NK3 primers designed for this study, which were based on bovine NK2 and NK3 sequences, as well as the housekeeping gene β-actin and NK1 receptor sequences previously determined (19) are presented in Table 1.

NK2 Receptor mRNA Expression

Mean ratios of NK2 mRNA gene expression to β-actin gene expression, normalized to a calibrator sample, are reported in Figure 1. NK2 receptors were not evenly distributed throughout the equine intestinal tract. A significant difference (as determined by 2-way ANOVA) was evident between intestinal segments (p ≤ 0.0003). In the small intestine, the duodenum showed the highest level of NK2 receptors. Tukey-Kramer adjustment for comparison of means showed that duodenal NK2 mRNA concentration was significantly higher than that in the ileum (p = 0.0107). Four of the five horses in the study showed higher levels of NK2 receptors in the duodenum than other regions of the small intestine. NK2 receptor levels were next highest in the jejunum, and followed the ileum. Four of five horses showed the lowest small intestinal concentrations of NK2 receptors in the ileum.

In the large intestine, the caecum showed the highest mean level of NK2 mRNA expression. Tukey-Kramer adjustment showed that levels of NK2 receptors in the caecum were significantly higher than the jejunum (p = 0.0039), ileum (p ≤ 0.0001), left ventral colon (p =
0.0066), pelvic flexure (p = 0.0269), left dorsal colon (p = 0.0194), and right dorsal colon (p = 0.0121).

**NK3 Receptor mRNA Expression**

Mean ratios of NK3 mRNA gene expression to β-actin gene expression, normalized to a calibrator sample, are reported in Figure 2. Similarly to NK2 receptor mRNA expression, NK3 expression was not uniform throughout the equine intestinal tract. However, in contrast to the NK2 receptor results, no significant differences in NK3 expression was evident between the segments.

NK3 receptors did show overall lower values than NK2 receptors. NK3 receptor expression also appeared more variable between individual horses. Two of five horses showed very low, to undetectable concentrations of NK3 mRNA throughout the intestinal tract. One horse showed higher overall values in the large intestine than the other four horses.

**Inflammatory Model**

Mean ratios of change in NK1, NK2 and NK3 receptor mRNA gene expression to β-actin gene expression, normalized to a calibrator sample, in response to ILD or ISO are reported in Figure 3. For NK1 receptors, overall ANOVA analysis did not reveal any statistically significant differences between treatment groups (p = 0.1947). However, pair-wise comparison revealed a trend for ILD NK1 mRNA expression to be lower than that of control (p = 0.075).

For NK2 receptor analysis 1 outlier was removed. An overall ANOVA for NK2 receptor expression showed a significant difference in the inflammatory model (p = 0.0216). Tukey-Kramer analysis showed that NK2 receptors were higher in control samples than ILD (p =
0.021). A trend was also noted, for control to be higher than ISO \( (p = 0.1200) \). No significant differences were observed in NK3 mRNA expression between control, ischemic or distension treatments \( (p \leq 0.6244) \).
**Discussion**

The present study is the first to report distribution patterns for NK2 and NK3 tachykinin receptor mRNA throughout the equine intestinal tract. This is an initial step in the evaluation of the roles that neurokinins play in equine enteric physiology and pathology. Previously, the distribution of NK1 receptor mRNA in 9 regions of the equine intestinal tract was described (19). NK receptors have also been quantified for the equine PF (18). However, it is surprising that tachykinins and their neurokinin receptors have not been more thoroughly examined in the horse, when considering that Substance P was first extracted from equine intestinal tissues in 1931 (9). While tachykinin agonists and antagonists have been extensively studied in laboratory animals and in humans for the treatment of inflammatory bowel diseases and abnormal motility patterns, exploration of this topic in the equine species is still in its infancy.

Abnormal motility and inflammatory changes in the gastrointestinal tract are common problems in equine medicine (28). Acute inflammatory changes characterize most cases of colic, due both to ischemia/reperfusion and intraluminal distension that occur secondary to strangulating obstruction injuries (29). Acute inflammation during colic is also thought to contribute to post-operative ileus (23; 24). Research to date in other species supports the benefit of NK antagonists in acute inflammation, with the most protective effects seen as pre-treatments or within the first 24 hours (7). Tachykinin antagonists have been shown to be protective against enteritis by decreasing intra-luminal fluid hypersecretion (30, 31) and decreasing TNFα release from macrophages (32). Less tissue (33, 34) and histologic (31, 35) damage due to enteritis have been documented in the presence of neurokinin antagonists.

In addition to protective properties, NK therapy has shown therapeutic benefit for abnormal motility. For example, in rats, SP and NK2 receptor antagonists have been useful in
restoring regular motility after postoperative intestinal atony (36; 37). NK2 agonists seem to have the strongest contractile effect on smooth muscle of the small and large intestine (13). Previous reports have shown a positive contractile response of isolated smooth muscle strips harvested from the equine intestinal tract to NKA, NKB (20) and SP (20; 21). Circular muscle has also proven to be more responsive than longitudinal smooth muscle sections (20; 21; 18).

In the current study, the duodenum showed consistently high levels of NK2 receptor mRNA in the small intestine. In a similar study, NK1 receptor mRNA was also found to have high levels in the duodenum (19). Other equine enteric receptors have also shown high levels in the duodenum (26). While not statistically different from each other, levels of NK2 mRNA were next highest in the jejunum, followed by the ileum. This suggests that tachykinin agonists and antagonists would have their greatest effect in the proximal small intestine, as receptor concentration appears to decrease aborally. In the small intestine, the most common GIT pathologies in the horse include: ileal impaction, adhesions, proximal enteritis/jejunitis, intussusception, volvulus (involving the ileum in most cases), internal incarceration, pedunculated lipomas and inguinal herniation (46). Often, inflammatory and motility abnormalities affect the distal small intestine most severely (46). Tachykinins have been implicated in both phase II (the mixing phase) and the overall generation of MMCs (migrating motor complex) of the GIT (38, 39). For example, in rats a SP antagonist returned MMCs and subsequent motility 23% faster than control after intestinal atony (40). At lower doses, NKA has been shown to dose-dependently increase amplitude and frequency of small intestinal contractions of humans, increasing the fraction of phase II of the MMC (38). In the small intestine, Nepadutant (an NK2 antagonist) when given 15 minutes prior to GIT surgery, decreased post-operative time for return to motility by 39% in the rat jejunum (41). So, it is
anticipated that, while receptor mRNA expression is highest orally, their effect may continue aborally and also affect the small intestine more generally.

In the large intestine, the caecum showed the highest levels of NK2 mRNA expression. High levels of NK1 receptor mRNA in the right and left ventral colons, and moderate levels in the caecum have also been reported (19). It is reasonable to expect then that the caecum would respond well to therapy targeting neurokinin receptors. Motilin receptors, to which the prokinetic agent erythromycin lactobionate binds, were also found to be at high levels in the caecum of the horse (26). Caecal disease in the horse includes: impaction, rupture, infarction, torsion, abscess/adhesion, tumour, and infarction (42). While caecal disease is not common in the horse (consisting 3.7% of reported surgical referrals (42)), case management can be challenging (43). Tachykinin therapy may be a viable tool in cases of colic involving the caecum, many of which are managed medically (43). NK3 receptor mRNA levels proved to be more variable between individual horse samples than that of NK2. An individual showed very high concentrations, while others showed low to undetectable levels in both the small and large intestine. Standard error was high between individual samples in the present study as well. Low detectable amounts of mRNA may account for this problem. A larger sample size may be useful in determining the true role of NK3 in the equine intestinal tract. Perhaps NK3 expression is greater in different layers of the intestinal lining, as the smooth muscular layer was only evaluated here. NK3 therapy may also have a greater effect if given centrally versus locally, as has been noted in the rat colon (30). Based on the current study, it is expected that they would have low therapeutic value, or at least as an unreliable therapeutic target. Potential use may be as adjunctive therapy in some individuals. Species differences have been noted in the relative importance of one receptor
compared to another in a particular pathological process (27, 6). The current study may support
NK3 as a less physiologically relevant receptor than NK2 and NK1 for the horse.

Changes seen in models of ILD and ISO have been previously described, and are
consistent with acute inflammatory changes (44). It was found that NK1 receptor mRNA tended
to decrease after distension. Similarly, NK2 receptor mRNA concentrations were lower for ILD
and ISO when compared to control/sham operated samples. Other equine enteric receptors have
shown similar trends; motilin receptors were seen to decrease by 10% after intraluminal
distension (25). Also similar to motilin receptors, the decrease in NK2 receptor mRNA was more
pronounced after 2 hours of ILD, compared to 120 minutes of ischemia and subsequent
reperfusion. Distension has a greater effect on the seromuscular layer (45), when compared to
ischemia/reperfusion. Since seromuscular layers were evaluated here, the present results are thus
not unexpected. The relative importance of this decrease is in NK mRNA expression is
unknown. The efficacy of NK2 agonist therapy may be hampered in postoperative ileus due to
the decreased expression of NK2 receptors in the jejunum following intraluminal distension and
ischemia.

In contrast to the current study, SP, as measured by immunoreactivity, was found to be
unchanged in a model of impaction colic (15) and ischemic injury (18). Studies evaluating
electrical activity in the GIT, such as isometric studies (48) or vivo studies using
electrogastrography (47, 49), may be useful in determining the true biologic significance of this
decrease in receptor concentration. It is important to note, for all the results described here, that
the levels reported are for receptor mRNA which, although obviously important in determining
receptor level, may not always correlate directly with functional receptor expression.
The present study confirms the presence of NK2 receptor mRNA in the smooth muscle of the equine intestinal tract. Distribution of NK2 receptor mRNA was not uniform throughout the intestinal tract, with highest concentrations in the small intestine being in the duodenum, while the caecum had the highest levels of the large intestine. Additional study is indicated to determine the true role of NK3 receptors in the horse; current analysis suggests that they may not play an important physiological role. Tachykinin agonists and antagonists may be of therapeutic value in the horse for both mediating motility post-operatively in the case of ileus, and decreasing the destructive effects of inflammation; however, additional research concerning ligand and receptor physiology in the horse is warranted. Receptor mRNA concentrations were affected by intraluminal distension and ischemia/reperfusion injury, however the consequence of this decrease in concentration requires further investigation. Tachykinins have a complex mechanism of action in the gastrointestinal tract (4), and thus additional research and in vivo studies in the horse are indicated.
### Gene Forward Primer Nucleotide Sequence | Reverse Primer Nucleotide Sequence
---|---
**β-actin** | 5' CTTCCAGCCCTCCTTCC 3' | 5' GTCCCACCGACAGC 3'
**NK1** | 5' ACGGGTCACGCAGATGT 3' | 5' GGGCTACTACTCCACCACAGA 3'
**NK2** | 5' CTTGAGAGCAACACCACAGG 3' | 5' TGGCTGCGATAGACGAAGTT 3'
**NK3** | 5' ATTTGCTGGGTGCCCTTC 3' | 5' CTCTTCTCCGACTGGATGTG 3'

*Table 1*: Primers used to quantify receptor gene expression using Real Time PCR.
Figure 1: Mean NK2 receptor mRNA receptor expression from 5 horses, as a ratio to β-actin expression and normalized to a calibrator sample, for 9 regions of the intestinal: duodenum (D), jejunum (J), ileum (I), body of the caecum (C), right ventral colon (RV), left ventral colon (LV), pelvic flexure (PF), right dorsal colon (RD) and left dorsal colon (LD). * and □ – indicates findings that were significantly different from each other.
Figure 2: Mean NK3 receptor mRNA receptor expression from 5 horses, as a ratio to β-actin expression and normalized to a calibrator sample, for 9 regions of the intestinal tract: duodenum (D), jejunum (J), ileum (I), body of the caecum (C), right ventral colon (RV), left ventral colon (LV), pelvic flexure (PF), right dorsal colon (RD) and left dorsal colon (LD).
**Figure 3:** Mean NK1, NK2 and NK3 receptor mRNA expression from the jejunum of 6 horses, as a ratio to β-actin expression and normalized to a calibrator sample for Control (C), Ischemia reperfusion (ISO) and Intraluminal distention (ILD) segments. * and □ - indicates segments that were significantly different from each other.
References:


CONCLUSIONS AND FUTURE STUDY

While homology for neurokinin receptors between species appears to be high (1), important species differences have been noted in response to agonists and antagonists. For example, both NK1 and NK3 receptor antagonists have shown varying success in affecting the development of GIT disease in the rat when compared to the guinea pig (2, 3). Species dissimilarities in receptor pharmacology have been investigated not only in lab animal species (4), but also in the dog (5) and the pig (6). While many tachykinin properties can be extrapolated from other species and applied to the horse, the importance of equine-specific study is evident. Neurokinin receptor localization is an important first step in determining the therapeutic potential of tachykinins in the horse.

This research provides evidence that NK2 and NK3 receptors are present in the equine intestinal. Receptor mRNA was localized in smooth muscle tissue throughout 9 regions of the intestinal tract. Tachykinin release and mode of action in the gut is complex and many studies have investigated their proposed function in human and lab animal tissues (7, 8). Neurokinins act centrally and locally (9), and their effect is often facilitated by release of other mediators such as nitric oxide (10) or histamine (7, 8). A positive feedback mechanism on local gene expression has also been suggested (11). Tachykinins have been implicated in excitatory myogenic response, but also in inhibitory neurogenic effect on GIT motility (12, 13, 14, 15). For this reason, the distribution of neurokinin receptors in nerve complexes in addition to muscular tissue is expected to further elucidate their physiologic function in the horse.
NK receptors are G-protein linked, and function by activating the IP$_3$-Ca$^{2+}$ intracellular signaling cascade (16, 17). Both receptor and ligand are internalized via endocytosis once binding occurs (18, 19, 20). The present study evaluated NK receptor mRNA expression in the normal equine intestinal tract, and also in the case of inflammation. In a rat model of colitis (21), affinity of SP for NK1 receptors did not decrease when a decrease in maximum binding sites was seen (21). Similarly, the affinity of motilin for motilin receptors in the horse was not affected by inflammation (22). Additional study, however, is warranted to assess affinity of tachykinin ligands for their receptors in the face of pathology in equine tissue. The NK2 receptor mRNA decrease noted here, subsequent to ISO and ILD, may hamper the therapeutic potential of tachykinins in the treatment of ileus in the horse.

A protective effect of tachykinin antagonists has been shown in models of enteritis. This was evident when antagonists were used as pretreatments prior to the development of inflammation through decreased hyperemia, hemorrhage (27), neutrophil infiltration, and intestinal permeability (2). These beneficial effects however, seem to be lost after the first 24 hours. This was seen as the same antagonist failed to be protective when given continuously over 72 hours (3). Furthermore, repeated doses show no improved protection over a single initial dose (28). Early recognition is important in the treatment of POI (29). POI stages after laparotomy have been described as: the period of reduced intestinal motility (days 1-2 post-op), the unstable period – in which motility was partially recovered, (days 3-7) and the full recovery period (days 8-31) (26). The duration of POI has also been described as 1 to 7 days (30) or 1 to 9 days (31). While NK antagonists have sometimes failed to be protective past the acute phases of inflammation, an NK1 antagonist, LY 3033870 did help decrease the severity of GIT lesions in the caecum and small intestine once IBD had already been established in a mouse model (32).
Since tachykinin receptors were shown here to be highest in the duodenum and the caecum, they may serve as useful targets for enduring pathology. Long-term study of tachykinins in the equine intestinal tract is warranted in order to evaluate their potential therapeutic use in cases of POI and colic.

Electrointestinography (EIG) involves the use of transdermal measurements of GIT motility with surface electrodes that record electrical activity of smooth muscle. This non-invasive, objective method has proven to be a viable method in dogs (23) and horses (24, 25, 26) for the assessment of GIT motility. When EIG recordings were taken for the equine caecum and large colon after saline, erythromycin lactobionate and detomidine IV administration, it was determined that EIG recordings were significantly correlated with ultrasound readings (24). EIG is also useful in providing more detail than auscultation or ultrasonography in horses, since it assesses contractility, as opposed to contraction or migrating complexes (26). EIG has been used to evaluate a prokinetic agent, mosapride, in clinical trials (25), which serves as a practical model for the non-invasive study of tachykinins in the horse.
REFERENCES


APPENDIX

Figure 1: Mean NK2 receptor mRNA receptor expression from 5 horses, as a ratio to β-actin expression and normalized to a calibrator sample, for 9 regions of the intestinal: duodenum (D), jejunum (J), ileum (I), body of the caecum (C), right ventral colon (RV), left ventral colon (LV), pelvic flexure (PF), right dorsal colon (RD) and left dorsal colon (LD).

<table>
<thead>
<tr>
<th>NK2</th>
<th>MEAN RATIO</th>
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<tbody>
<tr>
<td>D</td>
<td>1.7431</td>
<td>0.1699</td>
</tr>
<tr>
<td>J</td>
<td>1.2200</td>
<td>0.1539</td>
</tr>
<tr>
<td>I</td>
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<td>0.1539</td>
</tr>
<tr>
<td>C</td>
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<td>0.1699</td>
</tr>
<tr>
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</tr>
<tr>
<td>LV</td>
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<td>0.1699</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>RD</td>
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Figure 2: Mean NK3 receptor mRNA receptor expression from 5 horses, as a ratio to β-actin expression and normalized to a calibrator sample, for 9 regions of the intestinal tract: duodenum (D), jejunum (J), ileum (I), body of the caecum (C), right ventral colon (RV), left ventral colon (LV), pelvic flexure (PF), right dorsal colon (RD) and left dorsal colon (LD).

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<tr>
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<tr>
<td>RD</td>
<td>0.636</td>
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</table>
Figure 3: Mean NK1, NK2 and NK3 receptor mRNA expression from the jejunum of 6 horses, as a ratio to β-actin expression and normalized to a calibrator sample for Control (C), Ischemia reperfusion (ISO) and Intraluminal distention (ILD) segments.

<table>
<thead>
<tr>
<th>RECEPTOR</th>
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<tbody>
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<td>NK1</td>
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<td></td>
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<tr>
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</tr>
<tr>
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