PORCINE MODEL OF INFLAMMATORY-MEDIATED VISCERAL HYPERSENSITIVITY

By

LINDA CHRISTINE SANCHEZ

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This dissertation is dedicated to the many animals who have contributed to my education.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
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By

Linda Christine Sanchez

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Chair: A.M. Merritt
Major Department: Veterinary Medicine

Irritable bowel syndrome (IBS) is characterized by chronic abdominal pain associated with diarrhea and/or constipation. Visceral hypersensitivity is a biological marker for IBS, but the pathophysiology of this hypersensitivity is unknown. Transient inflammation has been suggested as an inciting agent, and Substance P (SP) is an important neuropeptide in pain processing. Using the pig as an animal model, the major objectives were to 1) develop a model of subacute proctitis; 2) validate a model of visceral discomfort; 3) evaluate the effect of colorectal inflammation on visceral nociceptive threshold; 4) evaluate the effect of colorectal inflammation on SP-immunoreactivity (SP-IR) in the colon, rectum, and lumbar spinal cord.

Nineteen cross-bred male castrated young pigs (initial bodyweight 20-30 kg) were used. Colitis was reliably induced (n = 12) by trinitrobenzene sulfonic acid/ethanol (TNBS/EtOH) enema, and resolved within 5 weeks, as documented endoscopically. Control animals (n = 7) received a saline enema and had no endoscopic abnormalities.

Half of the animals in each group (6 and 3, respectively) were euthanized 5 weeks following enema administration, and the remaining animals were euthanized 5 weeks later. SP-IR in the spinal cord, colon, and rectum was determined via immunohistochemical staining. Colorectal distention (CRD) consisted of sequential one-minute barostat-controlled inflations, interspersed by five-minute deflations, starting at 15 mmHg and increasing in 10mmHg increments until a discomfort response was induced (considered threshold), up to a maximum of 55 mmHg. Mean baseline threshold was 37.1 mmHg and did not differ significantly between TNBS/EtOH and saline groups. However, at weeks 3 and 9 post-enema, the TNBS/EtOH-treated animals had a significantly ($p = 0.045$ and 0.005 respectively) lower CRD threshold, relative to baseline, than the saline-treated animals. SP-IR did not differ statistically between groups in any tissues. But, SP-IR in the dorsal horn of spinal segments L1 and L7 had a significant correlation with visceral sensitivity. This study demonstrates the potential usefulness of the pig as a large animal model for visceral nociception studies. The correlation between spinal SP-IR and visceral sensitivity reinforces a relationship between CNS upregulation of SP and visceral hypersensitivity.

CHAPTER 1
LITERATURE REVIEW

Many factors regulate the sensory function of the gastrointestinal tract. As a result, when alterations of this function occur, a specific etiology can be difficult to ascertain. The experiments described in this dissertation revolve around using the pig as a model for visceral sensitivity studies. This information could potentially be applicable to other species, including humans, which typically suffer from numerous clinical problems with associated gastrointestinal pain.

Visceral Hypersensitivity

Hypersensitivity commonly refers to the development of either hyperalgesia, a significant upregulation of the magnitude of the response to a given peripheral painful stimulus, and/or allodynia, a nociceptive perception of a normally nonpainful stimulus (Willis, 1992). Visceral hypersensitivity (VH) refers to such a response within the abdominal viscera. The Irritable Bowel Syndrome (IBS) is a common functional gastrointestinal disorder with a wide range of clinical presentations, including alterations in bowel habits and enhanced visceral sensitivity (Thompson et al., 1999). VH is a common biologic marker of the Irritable Bowel Syndrome (IBS) and is present in almost all IBS patients (Mertz et al., 1995).

Some IBS patients have also been shown to have altered somatic referral patterns in response to colorectal distention (CRD), which indicates altered spinal processing of visceral sensory information (Kingham and Dawson, 1985; Lembo et al., 1994). Some studies have shown cutaneous allodynia in IBS patients (Verne et al., 2001) whereas

others have shown no alteration in somatic pain tolerance compared to healthy controls (Accarino et al., 1995; Cook et al., 1987; Whitehead et al., 1990). Many IBS patients also report symptoms of extraintestinal functional disorders such as irritable bladder, chronic somatic pain, and sleep disturbances (Whitehead et al., 1982; Whorwell et al., 1986). The crossover of IBS with fibromyalgia, a chronic somatic pain disorder, appears particularly significant (Chang et al., 2000a; Veale et al., 1991; Verne and Price, 2002), and may correlate with the severity of IBS symptoms (Lubrano et al., 2001). Patients with IBS and fibromyalgia were found to have somatic hyperalgesia, whereas patients with IBS alone had somatic hypoalgesia with higher pain threshold and lower pain frequency and severity compared to controls (Chang et al., 2000a). Such alterations may indicate a state of central (with or without a peripheral component) hyperexcitability within the central nervous system (CNS). Hyperexcitability of the CNS has been predominantly characterized through models of cutaneous nociception, but similar mechanisms are now known to play a role in VH as well.

Visceral Afferent Innervation

Visceral spinal afferent nerves function similarly to their somatic counterparts; however they allow only limited localization of an offending stimulus.(Hertz, 1911) Vagal afferents also provide important modulation of nociception (Gebhart and Randich, 1992; Grundy, 1988), and sacral parasympathetic fibers mediate sensory information from the distal colon and rectum (Janig and Morrison, 1986). The receptive fields of upper GI and colonic spinal afferent C fibers normally occur predominantly in the muscularis, serosa, and mesentery, but increase in size and include the mucosa during inflammation (McMahon and Koltzenburg, 1990; Ness and Gebhart, 1990). In the

rectum, sacral Aδ fibers have mucosal receptor fields under non-inflamed conditions (Janig and Koltzenburg, 1991; Sengupta and Gebhart, 1994).

The current concept of visceral pain perception involves a combination of high-threshold nociceptors as well as low-threshold mechanoreceptors (Cervero and Janig, 1992; Willis, 1993). In the colon, both C and Aδ fibers can encode a wide range of stimulus intensity, but C fibers are thought to be primarily slowly-adapting mechanoreceptors, whereas Aδ fibers are predominantly rapidly-adapting mechanoreceptors (Blumberg et al., 1983; Habler et al., 1990; Janig and Koltzenburg, 1991). Additional "silent nociceptors", mechanically insensitive C fibers innervating areas such as the bladder and colon, only respond to distending stimuli after chemical irritation in experimental situations (Janig and Koltzenburg, 1990; Janig and Koltzenburg, 1991). Inputs from these neurons converge on "wide-dynamic range" dorsal horn neurons (Willis and Coggeshall, 1991). Under normal conditions, low-intensity stimuli activate low-threshold afferents, which do not trigger the nociceptive pathway. Transient high intensity stimuli not only increase the intensity of the low-threshold afferents, but also recruit the high-threshold afferents, resulting in nociception (Cervero and Janig, 1992). Inflammation can alter these pathways through persistent stimulation of the peripheral terminals or activation of the "silent nociceptors" (Mayer and Gebhart, 1994).

Spinal and vagal afferents differ significantly in the localization of neuropeptides, in that 85-95% of spinal afferents to the stomach and colon, but only 5% of gastric vagal afferents, contain calcitonin-gene related peptide (CGRP) (Mayer and Raybould, 1990). In the GI tract, Substance P (SP) is found predominantly in the muscular layer and

myenteric plexus, coinciding with the previously stated receptor fields for spinal afferents (Otsuka and Yoshioka, 1993). CGRP is present in numerous splancnic afferents.

SP is a neuropeptide in the tachykinin family, along with neurokinins A and B (NKA and NKB). Of the three tachykinin receptors, NK_1 has the greatest affinity for SP which, in turn, preferentially binds to that receptor (Routh and Helke, 1995). In the spinal cord dorsal horn, NK_1 is heavily concentrated within Laminae I and II, with decreased density in Laminae III and IV, and very little in Lamina V(Charlton and Helke, 1985a; Helke et al., 1986). In the ventral horn, motor neurons throughout the spinal cord contain low to moderate levels of NK_1 binding(Charlton and Helke, 1985b; Charlton and Helke, 1985a). Antibody microprobe studies document the release of SP following noxious peripheral stimuli primarily within the superficial dorsal horn (Laminae I and II), but extending beyond (Duggan et al., 1992; Schaible et al., 1992). CGRP and SP co-exist in many primary afferent neurons and may be co-expressed when these nerves are stimulated (Bueno et al., 2000; Hokfelt et al., 1977b; Wiesenfeld-Hallin et al., 1984). CGRP also inhibits SP endopeptidase (SPE) activity, thus potentiating the biological actions of SP (Le Greves et al., 1985; Woolf and Wiesenfeld-Hallin, 1986).

CGRP and SP clearly play a role in the transmission of nociceptive information within the CNS (Duggan, 1995), but their function in peripheral terminals is less clear. They likely function as neuromodulators or mediators of local tissue responses, which could allow for changes in motility and visceral sensation at a peripheral level.

Mediators of Visceral Hypersensitivity

Inflammation is thought to affect the primary afferents through the action of inflammatory mediators on the peripheral terminals of primary afferents (Cohen and Perl, 1990; Handwerker and Reeh, 2003; Levine et al., 1992; Schaible and Schmidt, 1988) and

the matrix in which peripheral terminals are imbedded (Janig and Morrison, 1986; Mayer and Raybould, 1990). Specifically, prostaglandin E_2 (PGE_2), prostaglandin I_2 (PGI_2), ATP, bradykinin, serotonin, CGRP, SP, and glutamate can directly mediate sensitivity at the primary afferent (Purcell and Atterwill, 1995; Tracey and Walker, 1995). Many interleukins (IL-1, IL-8, IL-6), tumor necrosis factor-α (TNF-α), nerve growth factor (NGF), bradykinin, leukotriene B_4 (LTB_4), SP, complement 5a (C5a), and vasoactive intestinal peptide (VIP) can act indirectly via activation of immunocytes. Hyperalgesia caused by LTB_4 is neutrophil-dependent and extremely potent (Levine et al., 1984; Levine et al., 1992). Noradrenaline, neuropeptide Y (NPY), and NGF act through mediators released from adrenergic nerve varicosities (Bueno et al., 1997; Bueno et al., 2000). While the alteration in responsiveness at the peripheral terminal likely subsides with resolution of the initial inflammatory insult, experimentally documented "memory" persists for hours (Willis, 1993).

Altered afferent input to the dorsal horn results in central sensitization, and the associated release of neuropeptides in the dorsal horn increases the excitability of spinal neurons and leads to an expansion of the receptive fields (Mayer and Gebhart, 1994). The release of CGRP and SP from both central and peripheral terminals of primary afferent neurons in response to a nociceptive stimulus is well recognized, and these peptides are the two most abundant in medium and small dorsal root ganglia (DRG) neurons (Del Bianco et al., 1991; Hokfelt et al., 1977b; Lundberg et al., 1992; Sternini, 1992). The in-vitro application of the SP-antagonist, CP 96345, can prevent capsaicin-mediated sensitization of spinothalamic tract cells (Dougherty et al., 1994). This agent has also been shown to produce mild analgesic effects in thermo- and chemo-sensitive

models and to prevent noxious responses to carageenan injection in the rat paw and thermal skin stimulation in cats (Birch et al., 1992; Lecci et al., 1991; Yamamoto and Yaksh, 1991). Increased spinal cord expression of NK1 and NK2 receptors and SP occurs in experimental models of arthritis (Krause et al., 1995). These findings indicate a role for the neurokinins in central hyperexcitability (Bueno et al., 1997; Bueno et al., 2000).

These local effects can include activation of immune cells which will stimulate the local inflammatory response, leading to central and/or peripheral hyperexcitability. As previously described, mast cells appear to play a major role in the sensitization of primary afferents, and the release of SP is crucial (Bueno et al., 1997). Essentially, a feedback loop is created whereby SP release can trigger mast cell degranulation, and the subsequent histamine release causes further release of SP as well as NGF (Bertrand et al., 1993; Purcell and Atterwill, 1995). The resultant CGRP/SP-immunoreactive network modulates both reflex motor activity and the transmission of sensory information to the CNS.

At a spinal level, SP likely contributes to dorsal horn hyperexcitability via a direct action on the postsynaptic cells or by potentiating the excitatory effects of glutamate (Haley and Wilcox, 1992). Recent studies showing a reduction in nociceptive response to CRD in rats by intrathecal or intravenous administration of the CGRP receptor antagonist, hCGRP8-37, provide direct evidence for the role of CGRP in visceral nociception (Gschossmann et al., 2001; Plourde et al., 1997). Intravenous injection of CGRP results in abdominal cramping and decreased gastric emptying similar to that induced by peritoneal irritation, and the effects are blocked by NK1 antagonists. The

release of SP and CGRP may also result in neurogenic inflammation, although the roles of these peptides in gastrointestinal inflammation are not yet fully understood (Sharkey and Kroese, 2001). In a rat TNBS-colitis model, SP-immunoreactivity was decreased initially throughout the colon, followed by an increase in the circular muscle at 7 days (Miampamba and Sharkey, 1998). A similar pattern was shown in the enteric and primary afferent nerves after intraluminal injection of TNBS in the guinea pig ileum (Miller et al., 1993). Zymosan-induced colitis reduced the number of SP double-labeled (with Fluorogold, to document afferent innervation from the colon) DRG in both the T13-L2 and L6-S2 DRG cells (Traub et al., 1999). Further work suggests that the alteration in substance P in response to inflammation may be regulated through Il-1β (Hurst and Collins, 1993).

Indirect effects of neurotransmitters are also thought to result in enhanced receptor sensitivity. Activation of CGRP, SP, and other non-N-methyl D-aspartate (NMDA) receptors on the post-synaptic terminal of the central terminal can result in increasing depolarization of the postsynaptic membrane, leading to activation of the NMDA receptor by removal of the Mg^{++} block (Mayer et al., 1984; Woolf, 1992). This allows for a further increase in intracellular Ca^{++}, activation of NOS, and availability of NO as an intracellular messenger and diffusible neurotransmitter (Mayer and Gebhart, 1994). Increased intracellular calcium accumulated by NMDA receptor activation may also lead to neuronal cell death, or "excitotoxicity," which may play a role in disinhibition of second-order sensory neurons, associated with the clinical phenomenon of "windup" (Bueno et al., 1997; Mayer and Gebhart, 1994).

The Role of Inflammation

The timing of events is likely very important when considering the role of inflammation in the development of VH. Transient colonic irritation (Al Chaer et al., 2000) or maternal separation (Coutinho et al., 2002) during the neonatal period in rats can lead to provoke chronic visceral hyperalgesia that persists through adulthood despite the absence of histopathological lesions. Neuroplastic changes, including enlargement of dorsal root ganglia cells, have been documented within weeks of chronic partial urinary bladder obstruction and are associated with lowered thresholds for urgency to urinate as well as discomfort (Steers et al., 1990; Steers et al., 1991). However, the associated changes may result in alterations of motility rather than sensation. Repeated rectal distention in humans to noxious pressures (60 mmHg) can result in alteration of reported sensation, and repetitive CRD in rats to noxious (80 mmHg) but not innocuous (20 mmHg) pressures causes an increase in spinal *fos* and *jun* (Traub et al., 1992). These changes are likely a result of alterations in the dorsal horn neurons due to repeated excitability similar to that previously described for inflammation.

Clinical data to support inflammation as a modulator of VH include the fact that IBS patients and patients with Crohn's disease in which inflammation is limited to the small bowel have shown similar patterns of abdominal dermatome referral in response to CRD (Bernstein et al., 1996). Patients with mild active ulcerative colitis have attenuated rectal sensitivity responses, which correlate negatively with UC activity index, implying that either a more severe or more chronic inflammatory insult is needed for the development of hypersensitivity (Chang et al., 2000b). Clinically, most patients suffering from inflammatory-mediated alterations in visceral sensitivity improve in conjunction with resolution of the inflammatory insult, emphasizing the short-term nature of many

changes. The fact that VH associated with inflammation is not limited to the affected site within the GI tract also stresses the role of central hypersensitization in these events.

The alteration of dorsal horn neurons such that previously "subthreshold" stimuli can stimulate a nociceptive response is believed to play a key role in the development of mechanical allodynia. Thus, alterations associated with inflammation cannot only cause short-term VH, but also the alteration of afferent input to the dorsal horn which likely promotes plasticity within the CNS resulting in central hyperexcitability. For this central hyperexcitability to persist, however, additional cofactors, repeated events, or specific timing of the insult such as during the neonatal period likely occurs.

Stress and other psychosocial factors are also thought to contribute to VH and/or CNS hyperexcitability. Clinically, IBS patients often report an exacerbation of symptoms in conjunction with stressful life events, and the length and severity of the associated exacerbation often reflect the severity of the stressful event (Camilleri, 2001; Drossman et al., 1999; Lembo et al., 1999; Sandler, 1990). Psychological stressors can result in many physiologic changes including changes in muscular tone, immune modulation, and mucosal barrier dysfunction, alterations in descending pain modulating systems, and alterations in sleep (Mayer and Gebhart, 1994; Santos and Perdue, 2000; Soderholm and Perdue, 2001). Perhaps the most convincing argument for the role of social stressors in VH is the fact that psychological treatment for anxiety and depression is often an effective tool in the treatment of functional bowel disorders (Mayer and Gebhart, 1994).

Infectious insults to the gastrointestinal tract have also been postulated as contributing events to VH and/or CNS hyperexcitability in the IBS, given that

approximately 33% of patients hospitalized for a bout of infectious diarrhea develop IBS within 3-12 months (Gwee et al., 1996; McKendrick and Read, 1994). The responses to infection are likely mediated through inflammatory changes or immune modulation within the GI tract as a result of the initial insult and are likely to act via similar mechanisms as inflammation alone. In acute *Nippostrongylus brasiliensis* infection, a 2.5-fold increase in nerve content can occur by day 10 post infection, returning to near control values by day 14 (Stead et al., 1991).

In summary, a tremendous range of potential mediators likely contribute to the development of VH. Genetic, environmental, and individual differences compound the situation. In addition, other mediators have been proposed as contributing factors to the development of VH but lie beyond the scope of this review.

Animal Models of Visceral Pain

Balloon distention of a hollow organ, most notably within the gastrointestinal tract, is the most widely used stimulus of visceral pain experimentally (Ness and Gebhart, 1990). CRD in humans produces similar painful sensations, both in intensity, quality, and area of somatic referral, to clinically occurring gastrointestinal-associated pain. CRD has been validated in many animal species to produce brief discomfort with reliable, quantifiable behavioral and physiological responses attenuated by analgesic drugs, thus fulfilling the criteria for a valid model of visceral pain (Ness and Gebhart, 2001).

Animal Models of Inflammatory Bowel Disease

Clearly, the main thrust of this dissertation is to evaluate colitis-mediated visceral hypersensitivity using an animal model. After deciding to further pursue the effect of inflammation in the gastrointestinal tract on nociceptive responses, the next step was to choose an inflammatory model. Numerous naturally occurring and inducible models of

inflammatory bowel disease have been documented. Most involve rodents, and these are most commonly characterized based upon the specific nature of the insult.

Inflammatory Bowel Disease in Humans

Although commonly grouped together under the umbrella of "Inflammatory Bowel Disease" (IBD), Crohn's Disease (CD) and Ulcerative Colitis (UC) have many differences. The pathophysiological mechanisms involved in UC and CD have not been fully elucidated. An infectious cause has long been suspected, especially a role for *Mycobacterium paratuberculosis* in the pathogenesis of CD, but efforts to identify an etiologic agent have thus far been unsuccessful (Ryan et al., 2002; Shafran et al., 2002). UC usually begins with ulceration of the rectal mucosa and progresses orally to include varying portions of the bowel, potentially the entire colon. Clinical signs can begin with constipation and quickly progress to include rectal bleeding urgency, diarrhea, and abdominal discomfort. The course of disease is both acute and chronic, and relapse/remission is often unpredictable. Histologically, UC is predominantly an acute inflammatory process with disease limited to the mucosa and superficial submucosa except in fulminant disease (Fiocchi, 1998). The clinical course of CD is much more variable, but consists of acute and chronic inflammation of the small and large intestine and can include extraintestinal symptoms as well. The most common site of initial involvement is the ileocecal region. Histologically, CD has two different presentations indicative of either acute or chronic disease. Acutely, focal aphthoid ulcerations are noted, often in the epithelium overlying lymphoid aggregates. These ulcerations can undergo cycles of formation and healing, and the focal ulcerations can progress to more cobblestone-like lesions. Severe chronic CD can present with transmural inflammation,

inflammation, and fibrosis, often with granuloma formation. Often, the histological distinction between the two forms can be difficult (Fiocchi, 1998; Riddell, 2000).

Regardless of the initiating event, the chronicity of IBD suggests some form of immune dysregulation. T-helper lymphocytes are primarily responsible for the maintenance of an immune steady-state, and a tremendous amount of data regarding the association between their related cytokines and the IBD syndromes has recently become available. In general, UC demonstrates a predominantly Th2-like (especially IL-5) profile, the increase in cytokine production is limited to the involved mucosa, and eicosanoid production is prominent. In CD, secretion of Th1-type cytokines (IL-12, TNFα, IFNγ,) predominates, cytokine production is increased in involved and uninvolved mucosa, and eicosanoid production is only moderate (Anand and Adya, 1999; MacDonald et al., 2000). In both forms of IBD, increases of proinflammatory cytokines (IL-1, IL-6, IL-8) are evident, although data are inconsistent and TNFα appears to be more important in CD. Although mucosal T cells are activated in both CD and UC, their differential response to IL-2 stimulation represents one of the hallmark immunologic differences between these two syndromes. CD mucosal T cells demonstrate a hyperreactive response to Il-2 stimulation and have high expression of IL-2Rα gene products (Fiocchi, 1998).

Chemical Models

Many chemicals have been used as mucosal irritants in IBD models (Elson et al., 1995; Wirtz and Neurath, 2000). None of the animal models feature a relapsing/remitting nature of disease characteristic of the human problem, and all chemical models have a relatively short duration (1-8 wks) although those that cause disease for 6-8 weeks allow for the characterization of a chronic inflammatory phase. The extent of colonic

involvement depends upon the method used for chemical instillation, with enema administration resulting in a distal colitis in most cases. Surgical instillation allows delivery to more orad sites within the GI tract such as the ileum. The four main chemical-induced models of IBD include acetic acid, formalin/immune complex, TNBS/ethanol, and indomethacin. Several polymer/microbial-induced models also exist, including carrageenan and dextran sodium sulfate (DSS).

Acetic acid instillation into the colonic lumen produces predominantly mucosal inflammation (rats, mice, guinea pigs, rabbits) with histological similarity to naturally occurring UC (MacPherson and Pfeiffer, 1978; Sharon and Stenson, 1985; Yamada et al., 1992). Depending on the concentration and volume used, the lesion can progress to transmural depth. However, the inflammatory response remains for only days in mice and 2-3 weeks in rats; thus the value of this model lies only in the early phases of inflammation. The mechanism of injury is primarily related to a destruction of epithelial cells and subsequent mucosal and submucosal inflammatory response (Elson et al., 1995).

Trinitrobenzenesulfonic acid/ethanol (TNBS/EtOH) instillation results in acute transmural inflammation, edema, and cryptitis with histological similarity to CD (Torres et al., 1999). TNBS induces a delayed hypersensitivity response to skin contact by haptenating body proteins with trinitrophenyl (TNP) groups, rendering the resultant proteins immunogenic (Neurath et al., 2000). Ethanol works as a mucosal irritant, allowing for easier access for the TNBS molecule. Both T and B lymphocytes and macrophages predominate initially in an IL-12 driven Th1 T-cell-mediated inflammatory response (Neurath et al., 2000). The TNBS model has been used in numerous species,

including rats, mice, and rabbits, with a peak inflammatory response in 2-3 days with an overall duration of 2-3 weeks in mice and up to 8 weeks in rats (Elson et al., 1995). In contrast to acetic acid-induced disease, TNBS/EtOH-induced colitis does appear to have an immunologic component in that susceptibility to disease differs among strains of inbred mice and the dosage of TNBS required to induce lesions varies among species (Beagley et al., 1991; Morris et al., 1989). By eliciting oral tolerance via the administration of a TNBS-protein complex orally simultaneous to TNBS/ethanol enema administration in mice, the predominant Th1 response can be altered to a predominant Th2 response (Neurath et al., 2000; Seder et al., 1998). We have recently documented a TNBS/EtOH-induced ileitis in pigs produced by intraluminal instillation (Merritt et al., 2002a).

An immune complex-induced colitis model involves the instillation of a dilute formalin enema, followed by the intravenous injection of preformed immune complexes (Cominelli et al., 1990; Zipser et al., 1987). The dosage of formalin is critical to ensure that disease is related to the immune complexes rather than the chemical nature of formalin alone. The resultant lesion consists of severe mucosal and submucosal inflammation with crypt distortion, which resolves within 6-8 weeks. This model obviously involves an immunologic component, and IL-1 appears to play an important role (Cominelli et al., 1990). The inflammatory cytokine production in this model mirrors that seen in both syndromes of IBD. Because the immune complexes are formed to ubiquitous antigen, normal intestinal flora may be involved in either initiation or perpetuation of inflammation (Elson et al., 1995; Fedorak and Madsen, 2000).

Indomethacin administration will induce enteritis, primarily in the mid-jejunum. Rat strains vary in their response, with ulceration resolving by 14 days in Fischer rats, lasting at least 14 days in Sprague-Dawley rats, and 77 days in inbred Lewis rats (Sartor et al., 1992; Yamada et al., 1993). Lewis rats develop segmental transmural inflammation throughout the distal jejunum and ileum. Granulomatous inflammation, fibrosis, adhesions, and partial intestinal obstruction may also result; thus, the histological characteristics of this model more closely mimic CD (Elson et al., 1995). Length of disease is related to the dosage of indomethacin used, but inflammation in most models persists for 1-2 weeks. Because indomethacin is a non-selective cyclooxygenase inhibitor, protective mucosal prostaglandins are depleted. Host susceptibility, normal intestinal flora, and bile and/or enterohepatic circulation can also play a role in this model given the species and strain differences in susceptibility, the reduction or absence of disease in germ-free rats (Robert and Asano, 1977), the prevention of lesion formation by bile duct ligation (Yamada et al., 1993), and the lesion attenuation with antibiotic administration (Banerjee and Peters, 1990; Yamada et al., 1993).

The polymer models (DSS and carrageenan) both induce predominantly mucosal and submucosal lesions with histological similarity to UC (Elson et al., 1995). A definitive role of luminal bacteria has been established in the carrageenan model, but the model is not easily reproducible in species other than the guinea pig (Breeling et al., 1988; Onderdonk et al., 1987). DSS can be administered easily in drinking water, and results in chronic lesions (Cooper et al., 1993). The colitis is similar to UC; however lymphoid aggregates, fissuring ulceration, and focal inflammation seen in the chronic phases more closely resemble CD (Elson et al., 1995).

Mouse Knock-out Models

Genetic manipulation, primarily in mice, has yielded both transgenic (dominant or dominant-negative expression of a gene product) and knockout (targeted gene deletion) animals that develop intestinal inflammation (Elson et al., 1995; Wirtz and Neurath, 2000). In general, once lesions develop in these animals, they will persist until the animal's death or, in some cases, replacement of the deleted molecule (cytokine, etc.), but a relapsing/remitting course of disease has not yet been duplicated. The number of different knockouts (KO) and transgenic animals that develop IBD-like disease supports the notion that IBD is a multifactorial syndrome (Elson et al., 1995; Wirtz and Neurath, 2000).

One of the most important findings among the many genetically manipulated animals was that IL-2 and IL-10 knockout mice and HLA-B27 transgenic rats do not develop intestinal inflammation in the absence of luminal bacteria (Kuhn et al., 1993; Kundig et al., 1993; Sadlack et al., 1993). The IL-2 KO mice surviving beyond the 10^{th} week of life develop continuous mucosal/submucosal colitis without small intestinal or major internal organ involvement. Immunological abnormalities include increased numbers of activated T and B cells, a potential Th2-like shift, increased IgG1, and anti-colon antibodies (Kundig et al., 1993; Sadlack et al., 1993). Thus, these mice develop histological and immunological abnormalities similar to those seen in UC. The IL-10 KO mice develop chronic transmural duodenitis, jejunitis, and proximal colitis with an enhanced Th1 response due to the lack of IL-10 downregulation (Kuhn et al., 1993). This model therefore more closely mimics CD, although the early lesions are histologically more consistent with UC. Rats transgenic for human HLA-B27 and β_2-microglobulin develop multiorgan disease (including colitis, arthritis, orchitis, and a

psoriasis-like condition), but the resultant colitis lacks the acute neutrophilic component of IBD (Hammer et al., 1990). T-cell receptor KO and $G\alpha_{i2}$ KO mice also develop colitis, as do SCID mice to which $CD4^+$ T cells expressing high levels of CD45RB have been transferred (Elson et al., 1995; Fedorak and Madsen, 2000; Wirtz and Neurath, 2000).

The Pig as a Model

The pig was chosen as an animal model for these studies for a number of reasons. Because the majority of previous work has been performed in rodent models, a large animal model would be useful to represent larger mammalian species. A larger animal allows for colonic endoscopic examination; thus the inflammatory status can be documented without sacrificing additional groups of animals. From a practical perspective, experimental use of pigs is more economical than other commonly used larger mammals such as dogs and cats.

Study Objectives

Using the pig as a large animal model, the major objectives of this dissertation were to 1) develop a model of subacute proctitis; 2) validate an objective evaluation of visceral discomfort; 3) evaluate the effect of colorectal inflammation on visceral nociceptive threshold; 4) evaluate the effect of colorectal inflammation on immunoreactivity of Substance P in the colon, rectum, and lumbar spinal cord.

CHAPTER 2
METHODS

IACUC Approval

All procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Pilot animals were approved under Protocol # A579 "Evaluation of visceral discomfort in a chronic proctitis model in the pig" and animals for the major study were approved under Protocol # B167 "Porcine Model of Inflammatory-Mediated Visceral Hypersensitivity."

Model Development

The primary focus of pilot studies in this project was to establish a reliable method for visceral sensitivity testing, and to determine the appropriate methodology for TNBS/EtOH enema administration.

Animals

Three animals were used for the initial phases of the pilot study. These animals were used mostly to develop the visceral sensitivity testing protocol. An additional two animals were used to generate preliminary data regarding differences in visceral sensitivity, if present, between saline and TNBS-EtOH enema treatment.

Training

The first objective of the pilot studies was to find an acceptable method of pig restraint which would allow for their comfort and easy manipulation, and observation by research personnel. A standard hog transport crate was used, with modifications so that the animal could not escape through a large top opening (Fig.2-1). Otherwise, the

animals were able to move freely in the crate and turn back and forth. They were slowly acclimated to the crate, using corn treats as a training tool. Once acclimated, the pigs were easily transported from their normal housing to and from the laboratory and would remain in the crate without incident during the studies.

Figure 2-1. Pig in crate used for all procedures. View from top.

Once an acceptable transport device was obtained, the pigs were easily accustomed to all laboratory procedures. Food, most notably whole corn, was used as a tool for positive reinforcement for all training procedures.

Procedures

The next phase of the pilot studies was to develop a standard protocol for CRD. A standard rectal distention balloon and catheter designed for use in humans (Medtronics, Shoreview, MN) were initially used. While the balloon volume was appropriate, the

catheter system was too flexible. With a small amount of abdominal strain, the pigs would, in essence, defecate out the balloon. A design modification of the catheter was accomplished such that the catheter would remain in place without causing additional discomfort to the animal or any local damage to the rectal mucosa. The resultant system is described in detail below.

Based on the potentially subjective nature of clinical signs of abdominal discomfort, we chose to use a ramp protocol of CRD in which the threshold of discomfort was used as the response variable of interest. This also allowed for a minimal amount of discomfort to the animals.

Main Study Design

The main study was designed based upon pilot data.

Animals

The study was designed such that eighteen two to three month-old mixed breed swine would be used. Initial bodyweight for all animals was between 20-30 kg. The pigs were divided into four groups (two groups of six and two groups of three) using a random number chart. Animals in groups 1 and 2 were sacrificed at week 9 and those in groups 3 and 4 were sacrificed at week 14. Due to unforeseen complications, one additional animal was added to the 14 week saline control group, for a total of 19. Except as described below, pigs were meal fed a commercial swine grower diet (LabDiet) at a rate of 0.2-0.25 kg/kg bodyweight daily.

For the first 3 weeks of the study, pigs were handled 1-2 times daily in order to acclimatize them to human contact. As the animals became increasingly tractable, they were trained to load into and out of a standard transport crate and to remain in the crate

for 30-45 minute time periods, followed by acclimation to rectal thermometer and finally to intra-rectal balloon insertion.

Table 2-1. Animal grouping

Group #	Enema	Euthanasia Week
1	Saline	9
2	Saline	14
3	TNBS/EtOH	9
4	TNBS/EtOH	14

Study Timeline

The timeline of events did not vary between animals and is described below in Table 2-2.

Table 2-2. Timeline of study events

Week	Description	Distention studies	Other events	Animals sacrificed
1	Training	None		
2	Training	None		
3	Training	One	Endoscopy	
4	Enema	None	Monitoring	
5	Post-enema	None	Monitoring	
6	Post-enema	None	Monitoring, Endoscopy	
7	Post-enema	One		
8	Post-enema	One		
9	Post-enema	One	Endoscopy	Groups 1 & 2
10	Post-enema	One		
11	Post-enema	One		
12	Post-enema	One		
13	Post-enema	One		
14	Post-enema	One	Endoscopy	Groups 3 & 4

Procedures

Enema Administration

Enema administration occurred during week 4 of the study. Prior to this procedure, feed was withheld for 12 hours although water was available on a free-choice basis. Each animal was anesthetized with a butorphanol (0.15-0.30 mg/kg)/xylazine (4-8 mg/kg)/ketamine (4-8 mg/kg) combination administered intramuscularly. Pigs in Groups 1 and 3 received 40 ml of 100% EtOH mixed with 5 grams of TNBS diluted in 10 ml of water. The enema was retained in a 10-cm portion of the distal colon and proximal rectum for 12 minutes by use of two Foley catheters with 60-ml balloons. For control animals (groups 2 and 4), the retention enema consisted of 50 ml of 0.9% saline. Each pig was observed until fully recovered from anesthesia. They were then monitored 2-3 times daily until vital signs remained within normal ranges for 3 consecutive days and daily thereafter. Clinical evaluation included measurement of rectal temperature, observation of general attitude and fecal output, and monitoring for signs of GI distress such as vomiting, constipation, diarrhea, and anorexia. Three pigs became excitable in response to the initial anesthetic protocol and were subsequently anesthetized with a combination of xylazine (2.2 mg/kg) and tiletamine/zolazepam (2.2 mg/kg) intramuscularly. Due to a delay in approval of the revised anesthetic protocol, the first of these pigs was re-allocated and became a non-treated control animal for the duration of the study.

Endoscopic Evaluation

Videoendoscopic examination of the rectum and distal colon was performed (Pentax EFG 1-meter videoendoscope) and recorded during weeks 3, 6, 9, and 14 of the study. Prior to the procedure, the rectum was evacuated using 1-2 liters of warm soapy

water as an enema, and the endoscopy was performed with the animals standing in the transport crate previously described. When possible, each endoscopic examination was documented with a series of still images. For each endoscopic procedure, the appearance of the rectum and colon was scored on a 0-2 scale for each of the following: erythema, edema, granularity, friability, and erosions, with an overall range of 0-10 (Table 2-3) (D'Argenio et al., 2001).

Table 2-3. Endoscopic lesion scoring

Lesion	None	Mild	Moderate	Marked	Severe
Erythema	0	0.5	1	1.5	2
Edema	0	0.5	1	1.5	2
Granularity	0	0.5	1	1.5	2
Friability	0	0.5	1	1.5	2
Erosions	0	0.5	1	1.5	2

Visceral Sensitivity Evaluation

Visceral sensitivity was assessed by means of colorectal distention (CRD). Animals of all groups underwent CRD procedures once during week 3, then once weekly from week 7 until the end of their respective protocol.

Rectal catheter

Visceral discomfort was stimulated by CRD using a barostat (IsoBar 3, G&J Electronics Corp., Willowdale, Ont.). This involved a commercially available 500-ml polyethylene bag (Medtronics, Shoreview, MN) attached to a rectal catheter with dental floss (Fig. 2-2). The catheter had separate channels dedicated for volume control and pressure transduction, respectively. A thin metal rod was placed within the lumen of the catheter and secured with silicone. The distal end of this rod was smoothed, and the silicone filling completely covered the end of the rod such that it would not interfere with

the pressure measurements or volume alteration functions of the catheter system. The proximal end of the rod extended approximately 15 cm from the catheter tip. The purpose of this rod was to provide stiffened support for the catheter such that, when secured in place, it would not be expelled by the abdominal strain demonstrated during an animal's discomfort response.

Figure 2-2. Polyethylene rectal distention balloon attached to catheter.

Prior to insertion, the catheter was lubricated and a roll of 1" white tape was attached 10 cm from the catheter tip. After insertion, the tape was used to secure the apparatus to the animal's tail such that the balloon would remain at a standardized distance from the external anal sphincter. (Fig. 2-3)

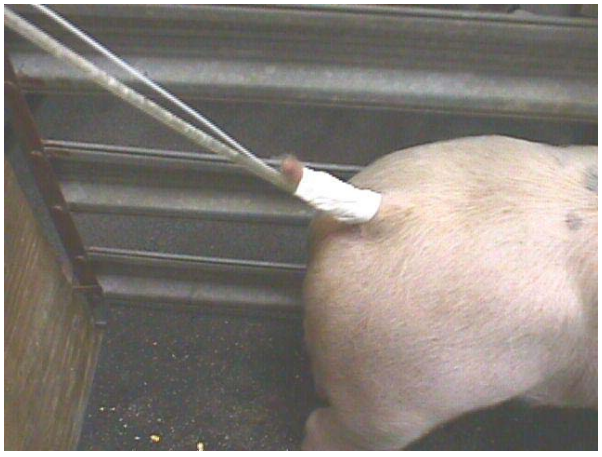

Figure 2-3. Close-up of rectal catheter attached to pig's tail.

Ramp Protocol

For nociceptive response testing, a stepwise pattern of inflations from 15 to 55 mmHg (60-second inflation with a 5-minute deflation period between inflations) was used. The inflation pattern continued until the animal displayed a response of discomfort or, if no response was obtained, a maximum pressure of 55 mmHg.

Assessment of Response

Three observers, blinded as to treatment group, monitored each pig throughout each distention protocol. At the conclusion of each inflation period, each observer independently displayed a "yes" or "no" response to a fourth investigator responsible for control of the barostat. When at least two of three observers declared a "yes" response, the fourth investigator discontinued the inflation protocol and recorded the pressure at which the response occurred. Based upon pilot study observations, a discomfort response was considered to include at least three of the following behaviors occurring during one inflation period: abrupt change in behavior (i.e. the animal discontinues previous behavior), arching of the back, abdominal strain, shifting of the hindlimbs. A still image of a typical response is displayed in Figure 2-4.

Each animal was observed for at least 5-minutes following the final balloon deflation.

Figure 2-4. Typical discomfort response. Note the arched back and wide-based stance of the hindlimbs.

Tissue Collection, Processing, and Analysis

Animals were euthanized during the final week of the study after the conclusion of that week's other events (CRD, endoscopy). Animals were first anesthetized with the xylazine/butorphanol/ketamine combination described previously, followed by a barbiturate overdose (Beauthanasia D, Shering Plough, 0.22ml/kg) administered intravenously. Those pigs requiring telazol/xylazine anesthesia for enema administration, received that anesthetic combination prior to Beauthanasia administration. Necropsy examinations were performed immediately after euthanasia. Tissue from all animals was processed in similar fashion.

Necropsy

Complete necropsy examinations were performed on each animal. Any gross abnormalities, if present, were recorded. The gastrointestinal tract was examined in its entirety. Samples taken for later histological analysis were cut, rinsed with 0.9% NaCl, and immersed immediately in 10% buffered formalin. Sections of the rectum were taken

at points 10 and 12 cm orad to the external anal sphincter and, respectively, labeled R1 and R2. Sections of the colon were taken at points 15 and 20 cm orad to the orad-most rectal section and labeled C1 and C2, respectively. A section of ileum was taken 2 cm orad to the ileocecal band (labeled I), a section of cecum was taken along the medial cecal band (CE), and a section of jejunum was taken at a random location within the mid-jejunum (J). The remaining portions of the gastrointestinal tract were opened and examined for gross lesions. The thoracic and remaining abdominal contents were also examined for the presence of lesions.

After completion of this portion of the examination, the spinal cord was removed and fixed. The vertebral column was isolated from the cranial to mid-thoracic level caudal to its termination and stripped of all excess tissue. A dorsal hemilaminectomy was performed via the use of a Stryker saw along the length of the column. The spinal cord and associated spinal nerve roots were extricated and immersed in formalin as described above.

Tissue Preparation

After formalin immersion for a period of 18-24 hours, sections were cut, placed in cassettes, and then dehydrated in ethanol and embedded in paraffin in routine fashion. Sections were cut onto Superfrost Plus slides for staining at a later date. All slides and blocks were stored at room temperature.

Histological Analysis

For routine histological analysis, slides were heat-fixed and deparaffinized in routine fashion then stained with hematoxylin and eosin. Sections of the colon and rectum of each animal were initially evaluated by the author and a pathologist (EWU) in order to gauge the range of lesions present in the study population. Based upon the initial

analyses, cellular infiltrates in the tissues were confined to lympocytes, predominantly in mucosal and submucosal aggregates. In addition, the tissues had a varying degree of edema. Thus, the degree of edema was scored from 1 (normal) to 4 (severe) and the total number of lymphoid aggregates per slide were counted. (Table 2-3). Spinal sections were evaluated for detectable abnormalities, but not scored.

Table 2-4. Histologic scoring system for gastrointestinal tissues.

Criterion	Category	Score
Edema	Normal	1
	Mild	2
	Moderate	3
	Severe	4
Lymphoid aggregates/section	0	1
	1	2
	2-3	3
	4-6	4

Immunohistochemical Analysis

Immunostaining for Substance P antigen was performed on the formalin-fixed, paraffin-embedded tissue using a rabbit anti-human Substance P polyclonal antibody (BYA1145-1, Accurate Chemical and Scientific Corp., Westbury, NY). This antibody has been validated in rat, monkey, feline, porcine, and bovine tissues. Slides were processed in duplicate (antibody and negative control). All slides were heat fixed and deparaffinized by immersion in xylene (2 x 5min) followed by decreasing concentrations of ethanol (2 x 3 min at 100%; 2 x 3 min at 95%) and then a rinse in deionized water. Slides were then stained using the DAKO EnVision Peroxidase staining system (DakoCytomation, Inc., Carpinteria, CA). All procedures were performed at room temperature. Briefly, slides were carefully dried and the tissue section was outlined with a hydrophobic pen (PAP pen), leaving at least a 3 mm margin. Next, they were incubated

with DAKO EnVision Peroxidase Blocking Reagent for 5 min, rinsed with PBS and placed in a PBS bath for 5 min. Slides were then incubated with primary antibody or control, respectively, for 30 minutes. Based on serial dilutions of 1:50 to 1:2500, the apparent optimal dilution of anti-substance P antibody was 1:500. This dilution was used for all subsequent staining.

Following antibody incubation, slides were placed in a PBS bath (2 x 5 min). They were then incubated with DAKO Envision Peroxidase labeled polymer for 30 min, followed by a PBS rinse and placement in a PBS bath for 5 min. DAKO Envision DAB substrate was applied for 5 min, and then slides were rinsed in deionized water for 1 min. Next, they were counterstained with 50% Gill's Hematoxylin for 2 min, and then placed in a running deionized water bath until the water ran clear. Finally, they were then placed in a bluing solution (deionized water with ammonium hydroxide, 10 quick dunks), followed by a deionized water bath (5 min), and then dehydrated in increasing concentrations of ethanol (2 min at 95%, 3 x 2 min at 100%). Slides were kept in xylene until coverslips were permanently affixed with Permount.

Ventral Horn Immunohistochemical Analysis

Substance P-immunoreactive neurons within the ventral horn (total of both right and left hemisections) of each spinal section were counted and recorded. Examples of neurons considered immunoreactive and non-immunoreactive are identified in Figure 2-5.

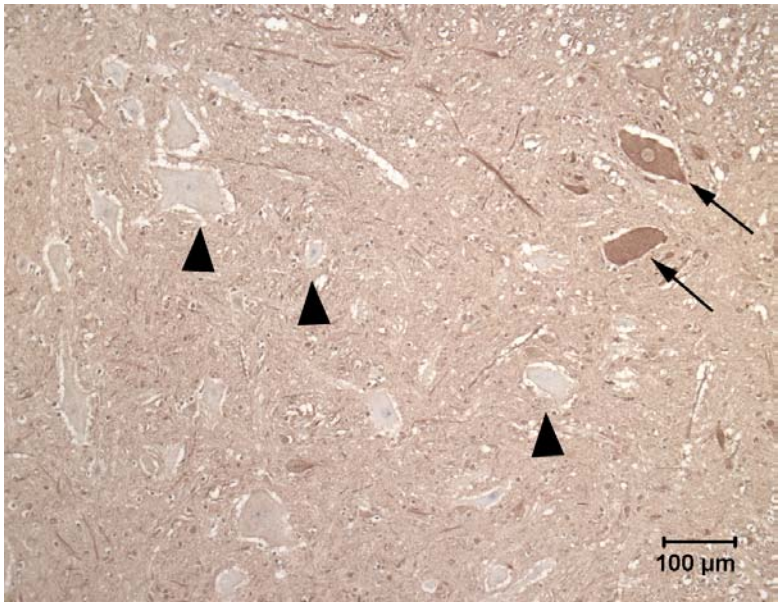

Figure 2-5. Ventral horn neurons. Examples of SP-immunoreactive (arrows) and non-immunoreactive (arrowheads) neurons within the ventral horn.

Quantitative Immunohistochemical Analysis

Substance P immunoreactivity was evaluated quantitatively using an algorithm described and validated by Matkowskyj (Matkowskyj et al., 2000). Essentially, the information within a control slide is subtracted from the information within an antibody-treated slide in order to quantitatively identify antibody-generated chromagen content of the slides in question. In order to for this to occur effectively, slides were read then and images saved in tagged-image file format (TIFF), which allows for compression without loss of data.

The slides were read using a Zeiss Axioplan 2 Microscope. Corresponding images were captured for antibody-stained and control images for each tissue section using SPOT image capture software. These images were saved in TIFF format and reopened with Adobe Photoshop (Version 6.0, Adobe Systems, Inc., San Jose, CA), where 3 100x100 pixel areas of interest for each slide were captured and saved as new jpg files. These files were opened with Matlab (Version 6.5, The MathWorks, Inc., Natick, MA),

which calculated the energy contained within each image. The net energy differential (energy within control images subtracted from energy within antibody images) was used to represent the net chromagen content within the region of interest as arbitrary units.

For spinal sections, image one was taken at the apex of the dorsal horn, and image two was taken along the dorsal margin, approximately half-way to midline. The 3 100-pixel squares were captured as shown. (Fig. 2-6, 2-7, and 2-8) For colon and rectal sections, both original images were taken at arbitrary points along the junction between the circular and longitudinal muscle layers. The 100-pixel squares were taken at consecutive points along this junction, careful not to include blood vessels. (Fig. 2-9)

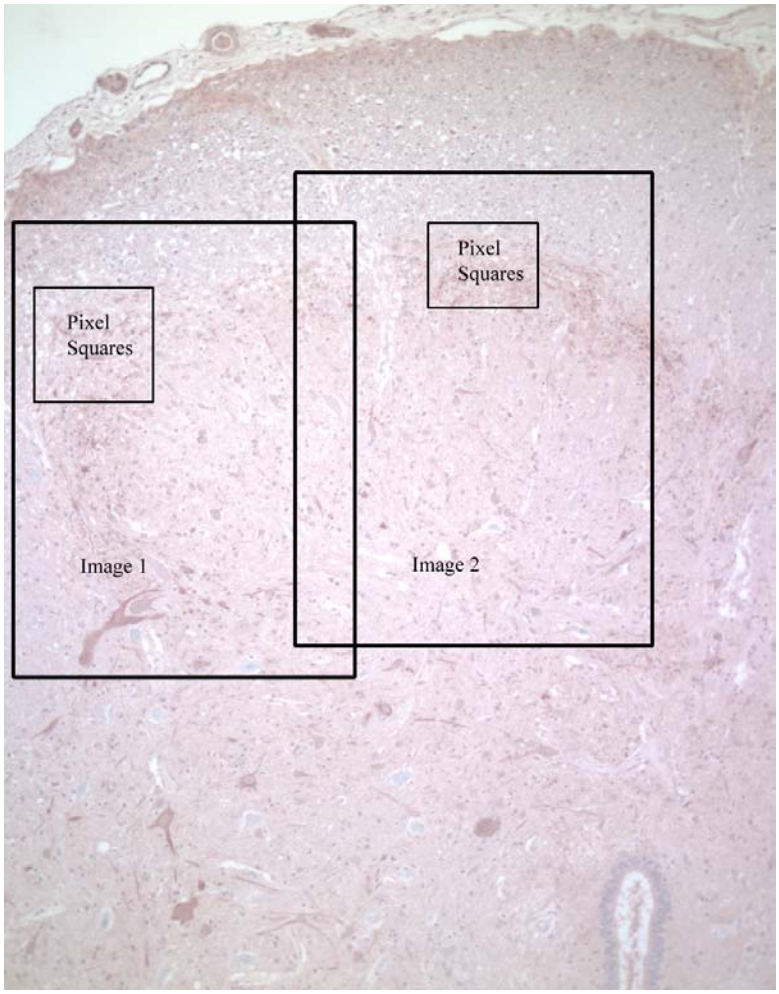

Figure 2-6. Image selection for spinal sections. Spinal cord, 10x magnification. Note the tip of dorsal horn at upper left of image, central canal at lower right.

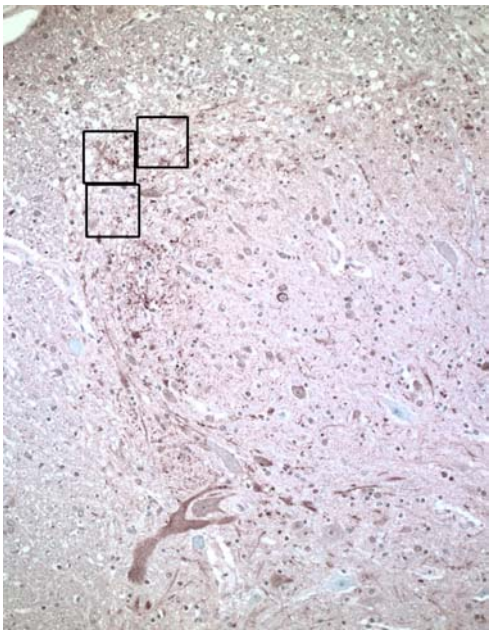

Figure 2-7. Pixel square selections for Cord Image 1. Spinal cord, 20x magnification. See figure 2-6 for orientation.

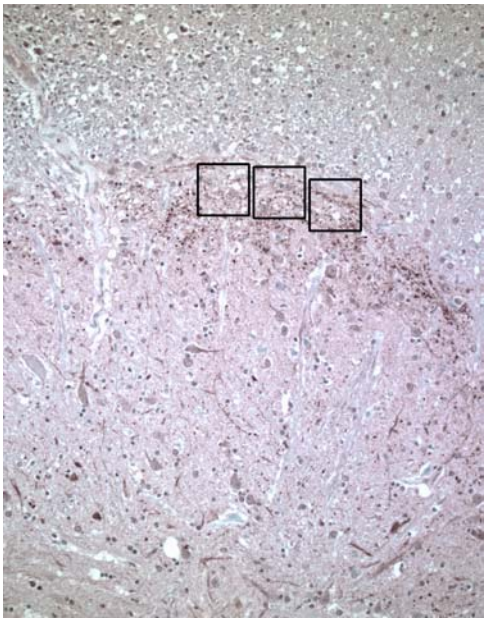

Figure 2-8. Pixel square selections for Cord Image 2. Spinal cord, 20x magnification. See figure 2-6 for orientation.

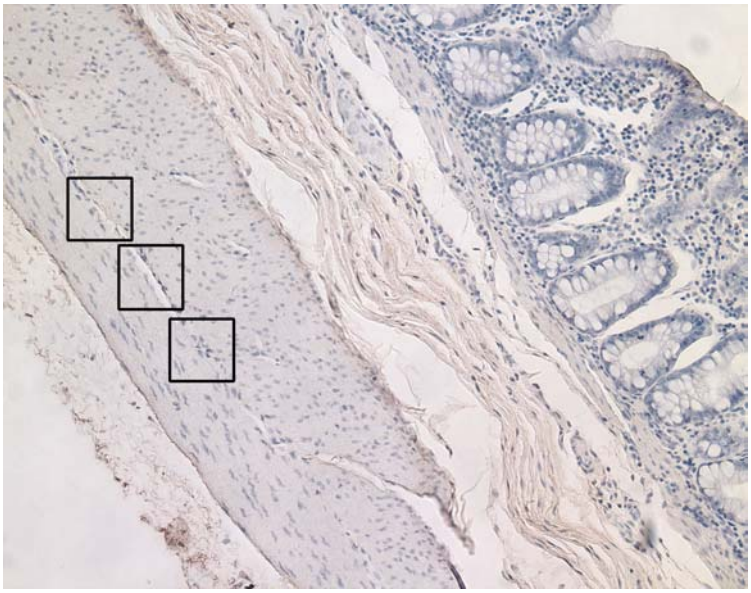

Figure 2-9. Pixel square selections for colonic and rectal myenteric plexus. Colon, 20x magnification.

Statistical Analysis

All statistical analyses were performed using SPSS 11.0 for Windows (SPSS, Inc., Chicago, IL). Significance was placed at $p \leq 0.05$.

Colorectal Distention

Week 3 threshold pressures were compared between saline and TNBS groups (combined groups 1 and 3 vs. 2 and 4) using an independent samples t-test. For all subsequent weeks, threshold pressures were compared between saline and TNBS groups (combined groups 1 and 3 vs. 2 and 4) using a one-way analysis of variance (ANOVA).

In an effort to gauge a change in each individual animal's threshold relative to baseline, an alteration from baseline threshold pressure (ABTP) for each study from weeks 7-14 by subtracting the baseline threshold pressure from the threshold pressure for that study. Thus, if the pressure for a particular week was lower than that animal's threshold pressure at week 3, the alteration number for that week would be negative.

ABTP were compared between saline and TNBS groups (combined groups 1 and 3 vs. 2 and 4) using a one-way ANOVA for weeks 7-14.

Endoscopy

Week 3 endoscopy scores were compared between saline and TNBS groups (combined groups 1 and 3 vs. 2 and 4) using an independent samples t-test. Scores for weeks 6, 9, and 14 were compared between saline and TNBS groups (combined groups 1 and 3 vs. 2 and 4) using a one-way ANOVA.

Histology and Immunohistochemistry

Inflammatory scores were compared between the four groups using a one-way ANOVA, followed by Tukey's HSD multiple comparison procedure. Scores for each colonic and rectal section were evaluated independently.

Chromagen content (SP-IR as represented by arbitrary energy units/pixel (EU/pixel)) in each section (rectum 1&2, colon 1&2, spinal sections L1, L2, L6, and L7) was compared between the four groups using a one-way ANOVA, followed by Tukey's HSD multiple comparison procedure. For each spinal section, the number of SP-immunoreactive neurons in the ventral horn was similarly compared between groups.

Baseline threshold pressures and chromagen content satisfied the Shapiro-Wilk test for normality, thus a parametric approach was justified. A one-way ANOVA was chosen for sequential analyses due to the disparate times of euthanasia for groups 1 and 2 vs. 3 and 4. Essentially, half of the animals were discontinued mid-way through the study, so a one-way analysis was used, though a two-way ANOVA for repeated measures would have been most appropriate had all animals continued through until week 14 of the study.

Correlations

The relationship between an individual animal's visceral sensitivity, as measured by the weekly alteration from CRD sensitivity threshold, and each of the following were also evaluated using linear regression and Pearson's correlation: 1) ventral horn SP-immunoreactive neuron count for each evaluated spinal cord section; 2) dorsal horn SP-immunoreactivity (EU/pixel) for each evaluated image of each spinal cord section; 3) gastrointestinal pathology for each evaluated section (edema and lymphoid aggregate counts evaluated independently). In addition, correlation analysis was similarly performed between the gastrointestinal edema and lymphoid aggregate counts and the spinal SP-immunoreactivity. Linear regression analysis was subsequently performed on correlations identified as significant in this fashion.

CHAPTER 3
RESULTS – ANIMAL STUDIES

Animals were well trained and tolerated the distention procedures very well. Pilot studies allowed for refinement of all procedures and subsequent design of the main study.

Development of colitis

After TNBS/EtOH enema administration, most animals developed mild bloody diarrhea for approximately 24 hours and all became febrile (103-104^{0}F) for 2-5 days. One animal developed signs of sepsis and died despite therapy within 5 hours following its TNBS/EtOH enema. Necropsy examination revealed a perforated rectum. This animal was replaced in the study. None of the other pigs displayed any evidence of clinical illness, other than the diarrhea noted previously and occasional mild depression for 12-24 hours. All animals maintained an excellent appetite throughout the study.

Endoscopic Evaluation

Endoscopic evaluations were easily performed using the previously described procedure. Most animals' rectum and distal colon could be easily and fully observed after the first 1-L enema. If further evacuation was needed, the enema was repeated. No animal required more than 2 enemas and all animals tolerated the procedure well.

Raw data from the endoscopic evaluations are presented in Appendix A. Mean baseline endoscopy score (week 3) was 0.33 for the TNBS animals and 0.50 for the saline animals. These values did not differ significantly. Endoscopy scores for subsequent weeks (TNBS animals combined as Group 1 and saline animals combined as Group 2) are presented in Figure 3-1.

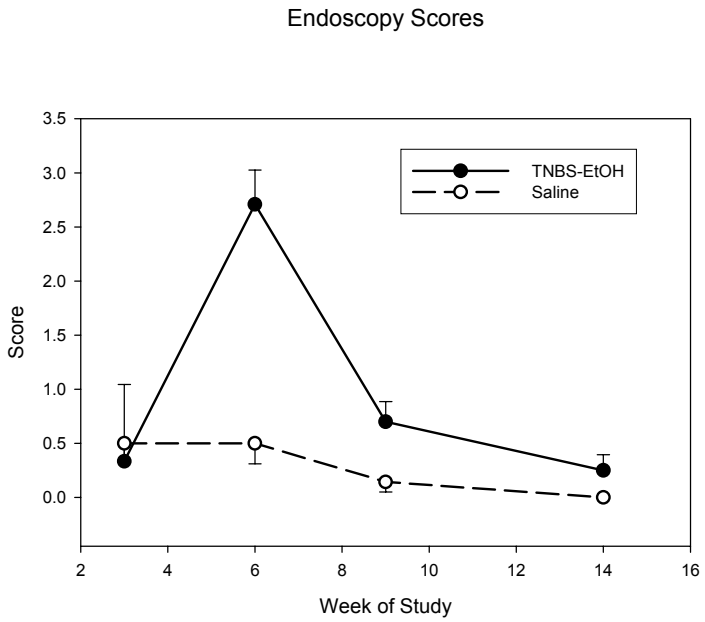

Figure 3-1. Endoscopy scores. Data expressed as mean ±SEM.

TNBS and saline means differed significantly at weeks 6 (p=0.000) and 9 (p=0.033) but not week 14 (p=0.134). Examples of normal and abnormal endoscopic evaluations are presented in Figures 3-2 and 3-3, respectively.

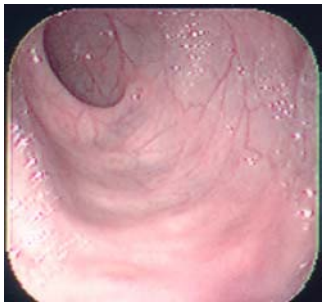

Figure 3-2. Normal endoscopy (Grade 0).

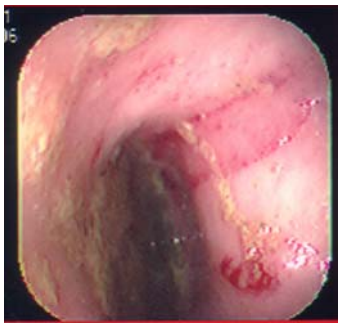

Figure 3-3. Abnormal endoscopy (Grade 3).

Visceral Sensitivity

CRD studies were completed for all animals, all weeks, with the exception of one pig (#3179) that developed a small amount of rectal bleeding during balloon insertion for its week 9 (final) trial. The procedure was not repeated for this animal. All other trials were completed successfully. For 116/125 completed trials, the animal displayed a discomfort response as judged by at least 2/3 blinded observers. For the remaining 9 trials, a majority opinion discomfort response was not observed (represented by 55* in the raw data). For statistical analyses, these trials were considered to have a threshold pressure of 55 mmHg. For all trials, 55% of the decisions were unanimous, whereas 45% involved a "yes" response from 2/3 observers.

Raw data from the visceral sensitivity studies are presented in Appendix A. Mean baseline threshold pressure (week 3) was 40.8 mmHg for the TNBS/EtOH animals and 30.7 mmHg for the saline animals. These values did not differ significantly. For subsequent weeks, mean values $\pm$SEM for TNBS/EtOH (combined as Group 1) and saline (combined as Group 2) animals are presented in Figure 3-4.

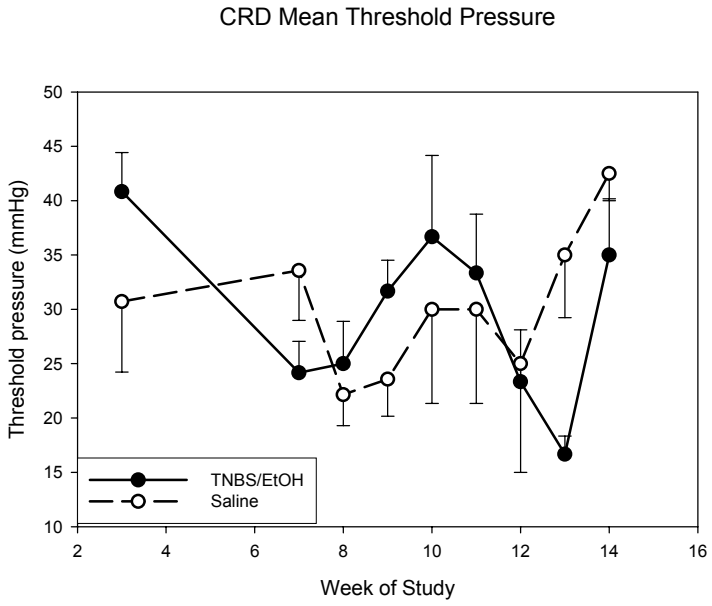

Figure 3-4. Mean threshold pressures. Data expressed as mean ±SEM. TNBS/EtOH and saline groups differed significantly ($p<0.05$) at week 13.

Mean TNBS/EtOH pressures differed significantly from the mean saline pressure for week 13 ($p=0.006$). There was a trend towards difference for weeks 7 ($p=0.084$) and 9 (0.093). Complete ANOVA tables for all analyses are presented in Appendix B.

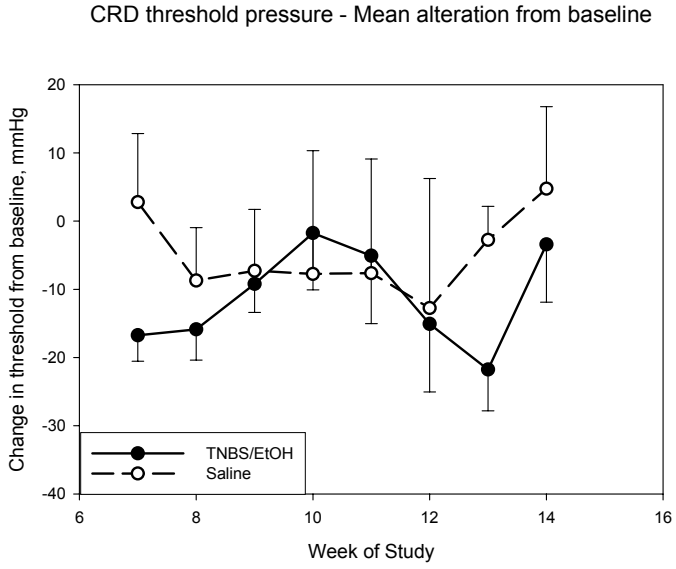

Figure 3-5. Mean ABTP. Data expressed as mean ±SEM. TNBS/EtOH and saline groups differed significantly ($p<0.05$) at week 7.

Mean ABTP for TNBS/EtOH (combined as Group 1) and saline (combined as Group 2) animals for all weeks are presented in Figure 3-5. Mean ABTP for TNBS/EtOH animals differed significantly from the mean for saline animals for week 7 ($p=0.045$), although there was a trend for week 13 ($p=0.056$)

When examining data from individual animals, rather than the groups as whole, only 2/7 saline animals had >2 weeks with threshold pressures below baseline, whereas 11/12 TNBS/EtOH animals had >2 weeks as such.

Correlations

Correlations were made between endoscopic scores on weeks 6, 9, and 14and weekly ABTP. Complete Pearson's correlation data are presented in Appendix C. Of these, endoscopy scores on weeks 6 and 9 had a significant negative correlation with week 13 sensitivity, and the linear regression between these factors are presented in Figure 3-8. No other comparisons had significant Pearson's correlations, thus linear regression was not performed.

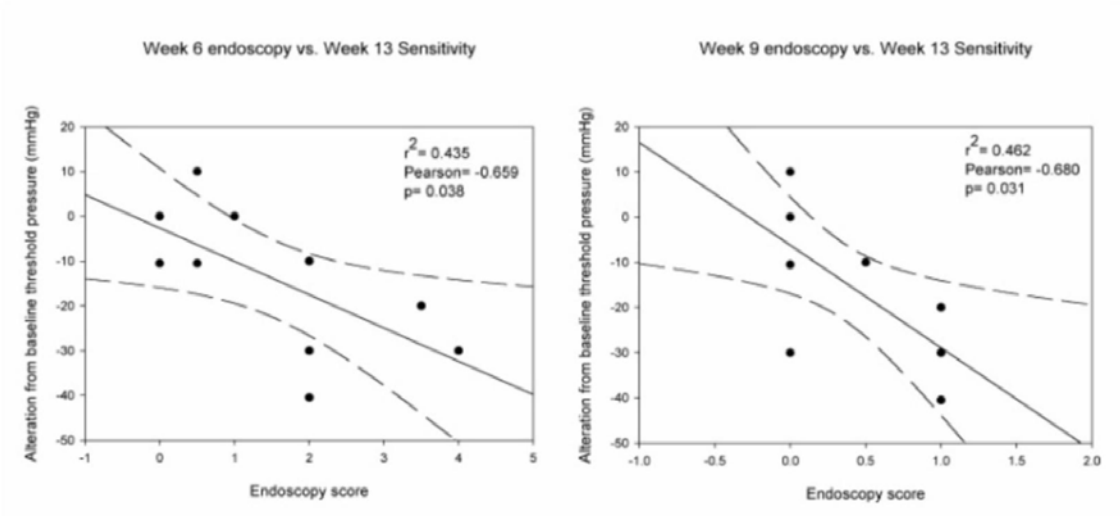

Figure 3-6. Correlation between endoscopy scores on weeks 6 and 9 and week 13 ABTP. Dashed lines represent 95% confidence interval.

CHAPTER 4
RESULTS – TISSUE ANALYSIS

Histological Analysis

An initial review of the gastrointestinal sections revealed that the predominant abnormality seen was submucosal edema. Also, the number of lymphoid aggregates seen in the mucosal and submucosal areas appeared to differ between sections. Thus, these factors were used in order to quantitatively evaluate the gastrointestinal sections as described in the materials and methods section. Examples of each histologic edema score are presented below in Figures 4-1 through 4-4.

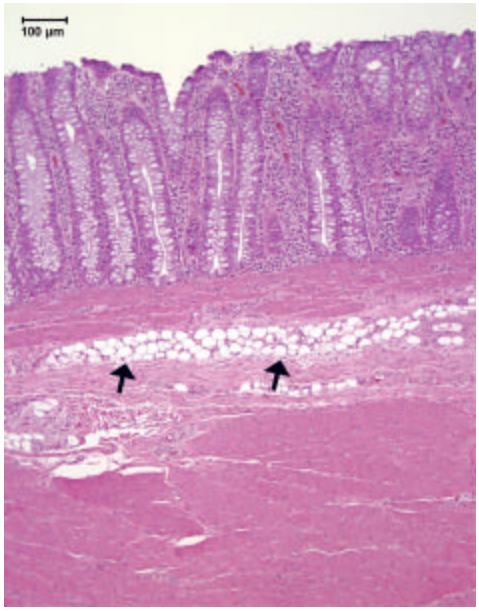

Figure 4-1. Example of edema grade 1. Arrows denote submucosal edema. Porcine rectum, 10x magnification, bar denotes 100 µm.

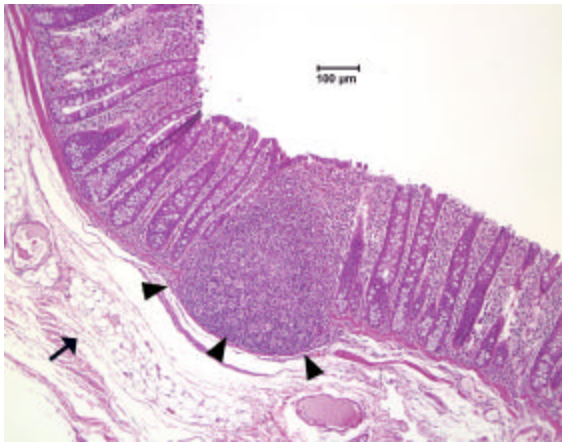

Figure 4-2 Example of edema grade 2. Arrow denotes submucosal edema. Arrowheads denote a mucosal lymphoid aggregate. A dilated lymphatic is also present just below the lymphoid aggregate. Porcine rectum, 10x magnification, bar denotes 100 µm.

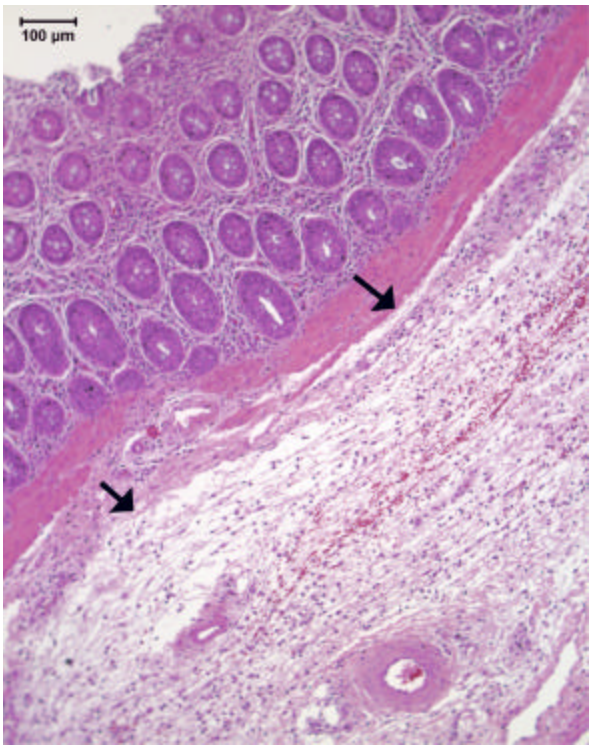

Figure 4-3. Example of edema grade 3. Arrows denote submucosal edema. Mucosal edema is also evident, as noted by an increased space between mucosal glands. Porcine rectum, 10x magnification, bar denotes 100 µm.

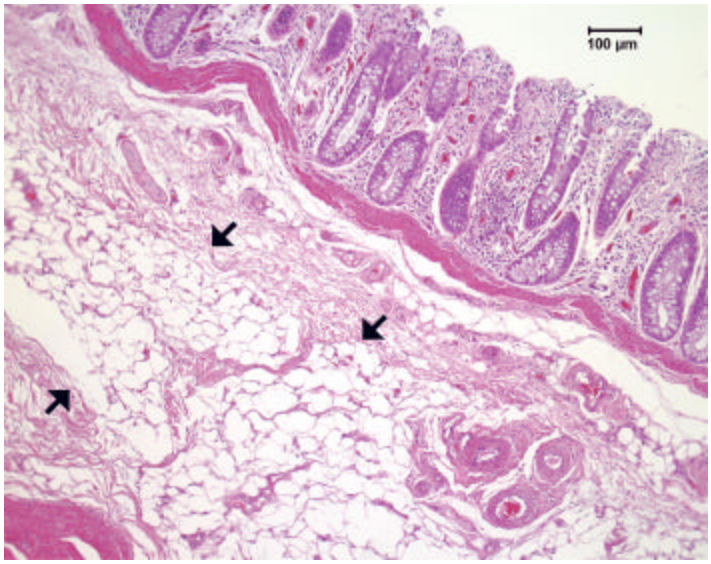

Figure 4-4. Example of edema grade 4. Arrows denote submucosal edema. Porcine colon, 10x magnification, bar denotes 100 µm.

Mean edema and lymphoid aggregate scores for all sections are presented in Tables 4-1 and 4-2, respectively. Statistical analysis did not reveal any significant differences between groups for any of the colonic or rectal sections. Complete ANOVA tables and Tukey's multiple comparison analyses are provided in Appendix B.

Table 4-1. Mean lymphoid aggregate scores.

Section	Group	Mean	SD	SEM
C1	1	2.17	0.75	0.31
	2	2.33	0.58	0.33
	3	1.67	1.03	0.42
	4	2.25	1.50	0.75
C2	1	2.17	0.75	0.31
	2	2.00	1.00	0.58
	3	2.00	0.89	0.37
	4	2.25	0.50	0.25
R1	1	2.17	0.98	0.40
	2	2.67	1.53	0.88
	3	1.67	1.03	0.42
	4	2.00	1.41	0.71
R2	1	2.17	0.98	0.40
	2	1.67	1.15	0.67
	3	2.17	0.98	0.40
	4	2.50	1.00	0.50

Table 4-2. Mean edema scores.

Section	Group	Mean	SD	SEM
C1	1	1.50	0.55	0.22
	2	1.67	0.58	0.33
	3	2.33	0.82	0.33
	4	2.00	1.41	0.71
C2	1	1.50	0.55	0.22
	2	1.33	0.58	0.33
	3	2.00	1.10	0.45
	4	2.25	1.26	0.63
R1	1	2.33	0.52	0.21
	2	2.67	0.58	0.33
	3	1.83	1.17	0.48
	4	3.25	0.96	0.48
R2	1	2.33	0.82	0.33
	2	2.33	1.15	0.67
	3	2.17	1.47	0.60
	4	3.00	1.15	0.58

Spinal cord sections were also evaluated. Some sections contained mild gliosis, but this was considered within normal limits, thus the spinal cords were not scored.

Immunohistochemical Analysis

Substance P-immunoreactivity (SP-IR) was seen in all tissues. The immunoreactivity appeared specific, and very little background staining was evident in the spinal cord sections. Gastrointestinal sections had a higher degree of non-specific chromagen uptake, but specific immunoreactivity was also present. Negative control slides had little to no chromagen uptake for all sections.

Spinal cord

Substance P-immunoreactivity was most prominent in the superficial dorsal horn (lamina I and II) (Figure 4-5). Some large immunoreactive neurons were also detected in the ventral horn of some sections.

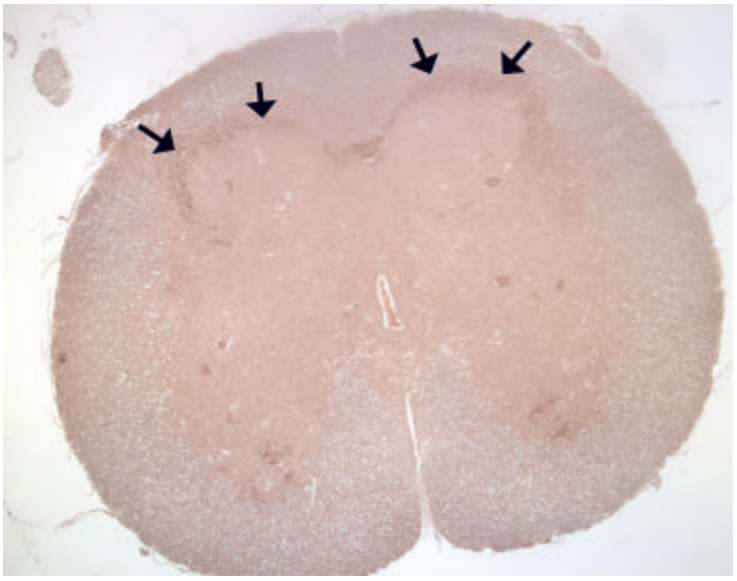

Figure 4-5. SP-IR in spinal cord. Arrows denote margin of immunoreactivity in dorsal horn. Porcine spinal cord, L7 segment, 2.5x magnification. For details of site selection and immunoreactivity in those regions, see Figures 2-6 through 2-8.

Quantitative data describing the Substance P-immunoreactivity (EU/pixel) within the dorsal horn are presented in Table 4-3. Statistical analysis did not reveal any significant differences between experimental groups for any spinal segment. Initially, data from images 1 and 2 were analyzed individually. But, as this did not change any analyses, data were pooled as collective data for the dorsal horn. Complete ANOVA tables and multiple comparison procedural results are presented in Appendix B.

Numbers of Substance P-immunoreactive neurons within the ventral horn are presented in Table 4-4. Statistical analysis did not reveal any significant differences between experimental groups for any spinal segment. Complete ANOVA tables and multiple comparison procedural results are presented in Appendix B.

Table 4-3. Mean dorsal horn Substance P-immunoreactivity (EU/pixel)

Section	Group	Mean	SD	SEM
L1	1	150.64	43.67	17.83
	2	104.14	10.42	6.02
	3	152.38	61.16	24.97
	4	110.74	22.53	11.26
L2	1	145.01	32.53	13.28
	2	131.65	28.48	16.44
	3	158.66	37.05	15.13
	4	146.44	64.27	32.13
L6	1	183.18	58.26	23.79
	2	180.43	45.04	26.00
	3	179.13	48.98	19.99
	4	143.30	36.66	18.33
L7	1	138.91	41.26	16.85
	2	157.64	32.08	18.52
	3	141.95	76.53	31.24
	4	202.21	17.41	10.05

Table 4-4. Mean ventral horn Substance P-immunoreactive neurons

Section	Group	Mean	SD	SEM
L1	1	1.50	3.67	1.50
	2	0.00	0.00	0.00
	3	6.83	7.49	3.06
	4	4.00	8.00	4.00
L2	1	0.83	1.60	0.65
	2	0.00	0.00	0.00
	3	8.00	8.67	3.54
	4	7.75	8.73	4.37
L6	1	10.50	9.20	3.76
	2	6.33	3.79	2.19
	3	14.67	16.60	6.78
	4	5.50	6.81	3.40
L7	1	3.33	4.18	1.71
	2	2.33	4.04	2.33
	3	3.83	3.92	1.60
	4	2.67	2.52	1.45

Gastrointestinal Tract

In the gastrointestinal tissues, the most intense regions of SP-IR were in the submucosal region between circular and longitudinal muscle layers, corresponding to the location of the myenteric plexus (Fig. 4-6). Some peripheral chromagen uptake was present in all tissues, likely related to staining artifact. Quantitative-IHC data for the gastrointestinal tissues are presented in Table 4-5. Values did not differ significantly between experimental groups. Complete ANOVA tables are presented in Appendix B.

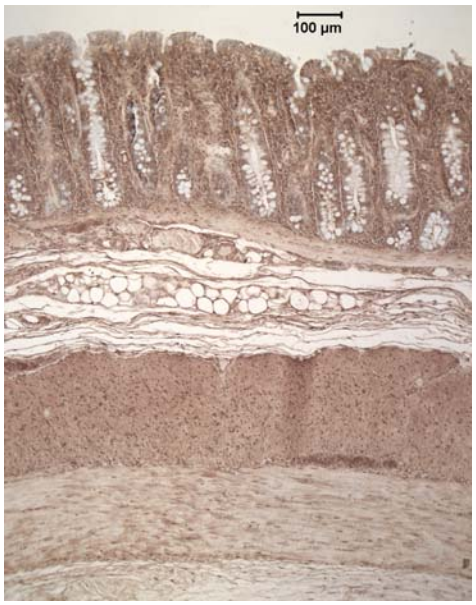

Figure 4-6. SP-IR in porcine colon. 10x magnification. For pixel square location, see Figure 2-9.

Table 4-5. Quantitative IHC data for gastrointestinal tissues.

Section	Group	Mean	SD	SEM
R1	1	92.96	34.20	13.96
	2	86.72	46.11	26.62
	3	100.13	40.16	17.96
	4	140.90	65.47	32.74
R2	1	117.04	65.24	26.63
	2	61.06	33.01	19.06
	3	98.29	50.79	20.74
	4	93.14	52.82	30.49
C1	1	135.69	29.87	13.36
	2	94.96	16.00	9.24
	3	150.16	89.22	36.42
	4	156.23	76.90	38.45
C2	1	137.68	71.19	35.59
	2	67.92	45.83	26.46
	3	100.83	84.43	37.76
	4	203.68	36.68	21.18

Correlations

Complete Pearson's correlation data are presented in Appendix C. The following comparisons had a significant *negative* correlation: R2 lymphoid aggregate score and

Week 10 and 12 ABTP (Figure 4-7); R1 histological scores and Ventral horn SP-IR neurons in the L1 and L2 segment (Figure 4-8); Dorsal horn SP-IR in the L1 segment and Week 9 ABTP (Figure 4-9) ; Dorsal horn SP-IR in the L7 segment and Week 11, 12, and 14 ABTP (Figure 4-9); Ventral horn SP-IR neurons in the L2 segment and Week 11, 12, 13, and 14 ABTP (Figure 4-10); Ventral horn SP-IR neurons in the L6 segment and Week 13 and 14 ABTP (Figure 4-11); Ventral horn SP-IR neurons in the L1 segment and Week 8 ABTP (Figure 4-12); Dorsal horn SP-IR in the L6 segment and R1 lymphoid aggregate score(Figure 4-13).

In addition, the following comparisons had a significant *positive* correlation: SP-IR in the C1 section and R2 lymphoid aggregate score; SP-IR in the R1 section and C1 lymphoid aggregate score; SP-IR in the R1 section and C2 lymphoid aggregate score (Figure 4-13). All other comparisons did not have significant Pearson's correlations, thus linear regression was not performed.

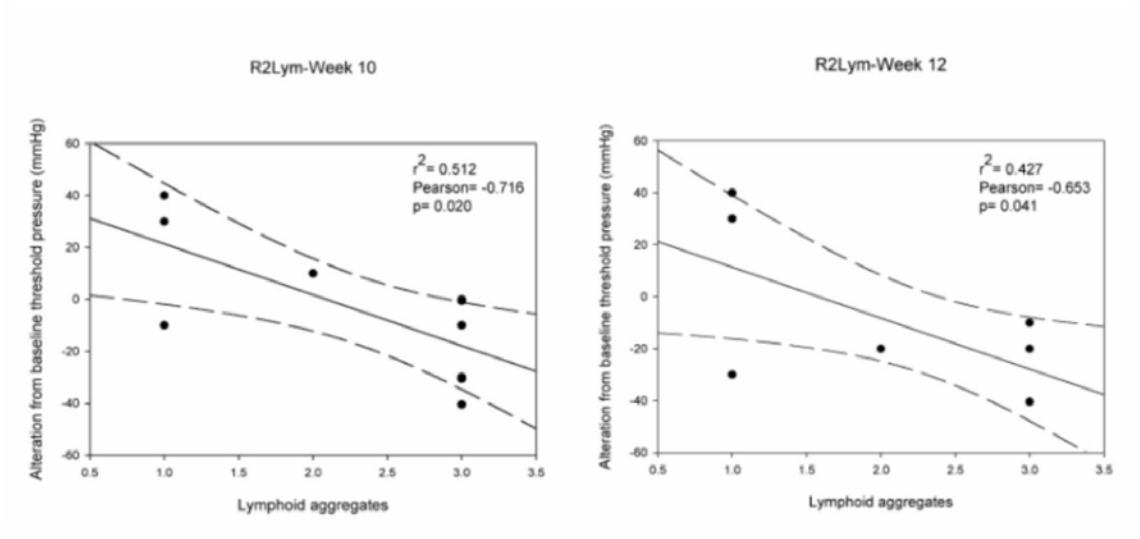

Figure 4-7. Correlation between lymphoid aggregates in the rectal section R2 and ABTP. Dashed lines represent 95% confidence interval.

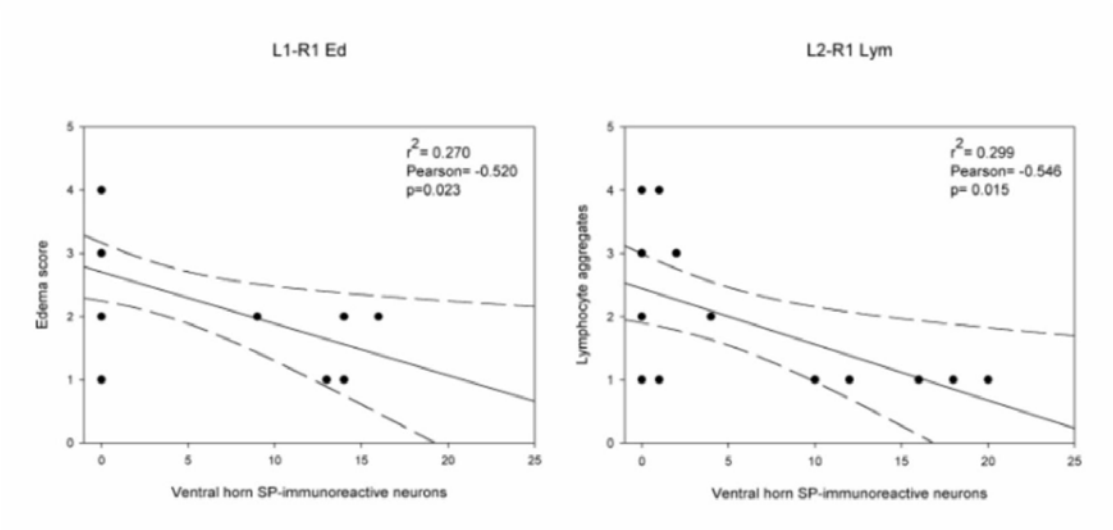

Figure 4-8. Correlation between histological scores in the rectal section R1 and ventral horn SP-immnuoreactive neurons in spinal sections L1 and L2. Dashed lines represent 95% confidence interval.

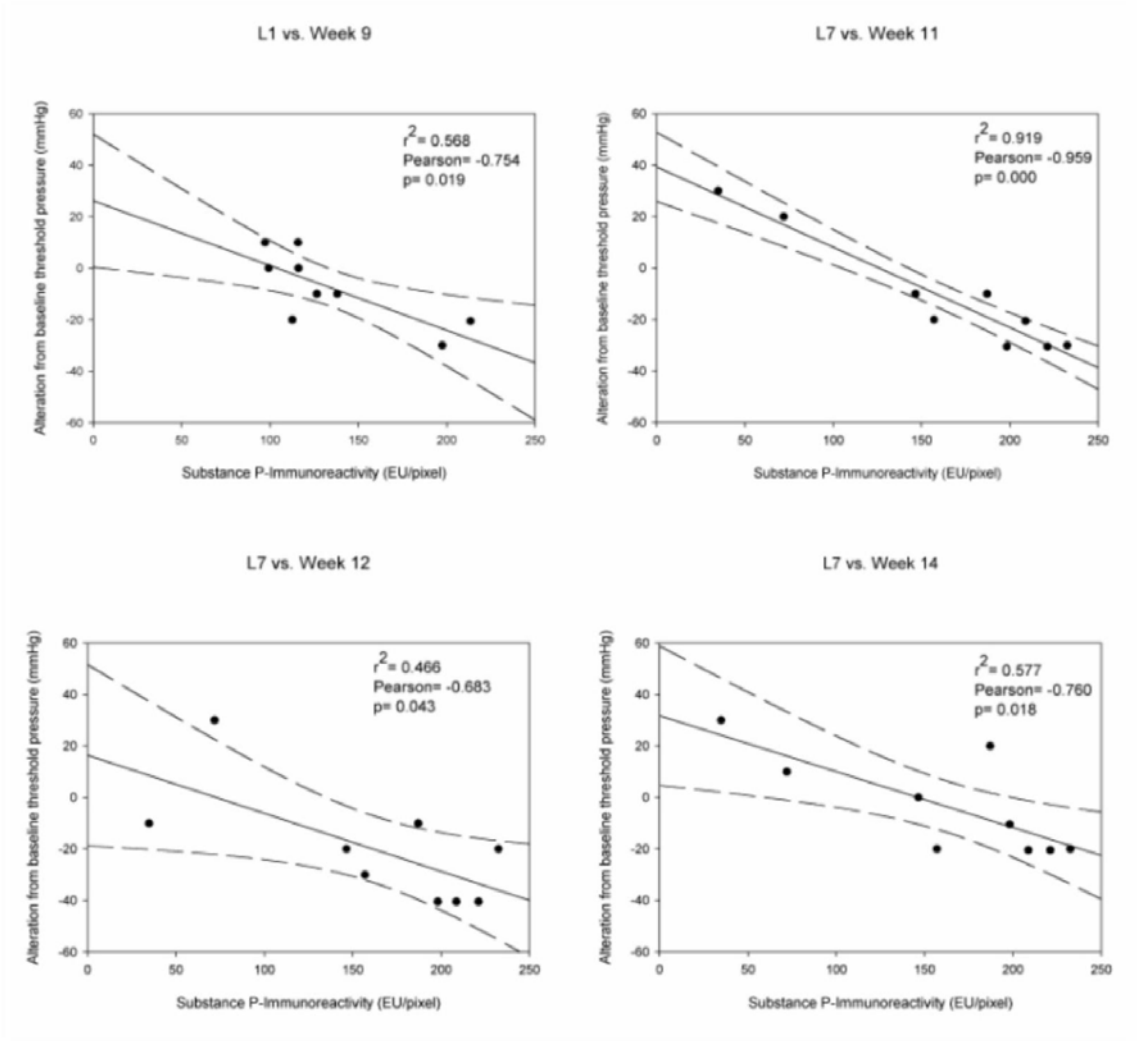

Figure 4-9. Dorsal horn SP-IR correlation with ABTP. Clockwise from the top left, plots represent linear regression analysis for segment L1 vs. Week 9 and segment L7 vs. Weeks11, 14, and 12. Dashed lines represent 95% confidence intervals.

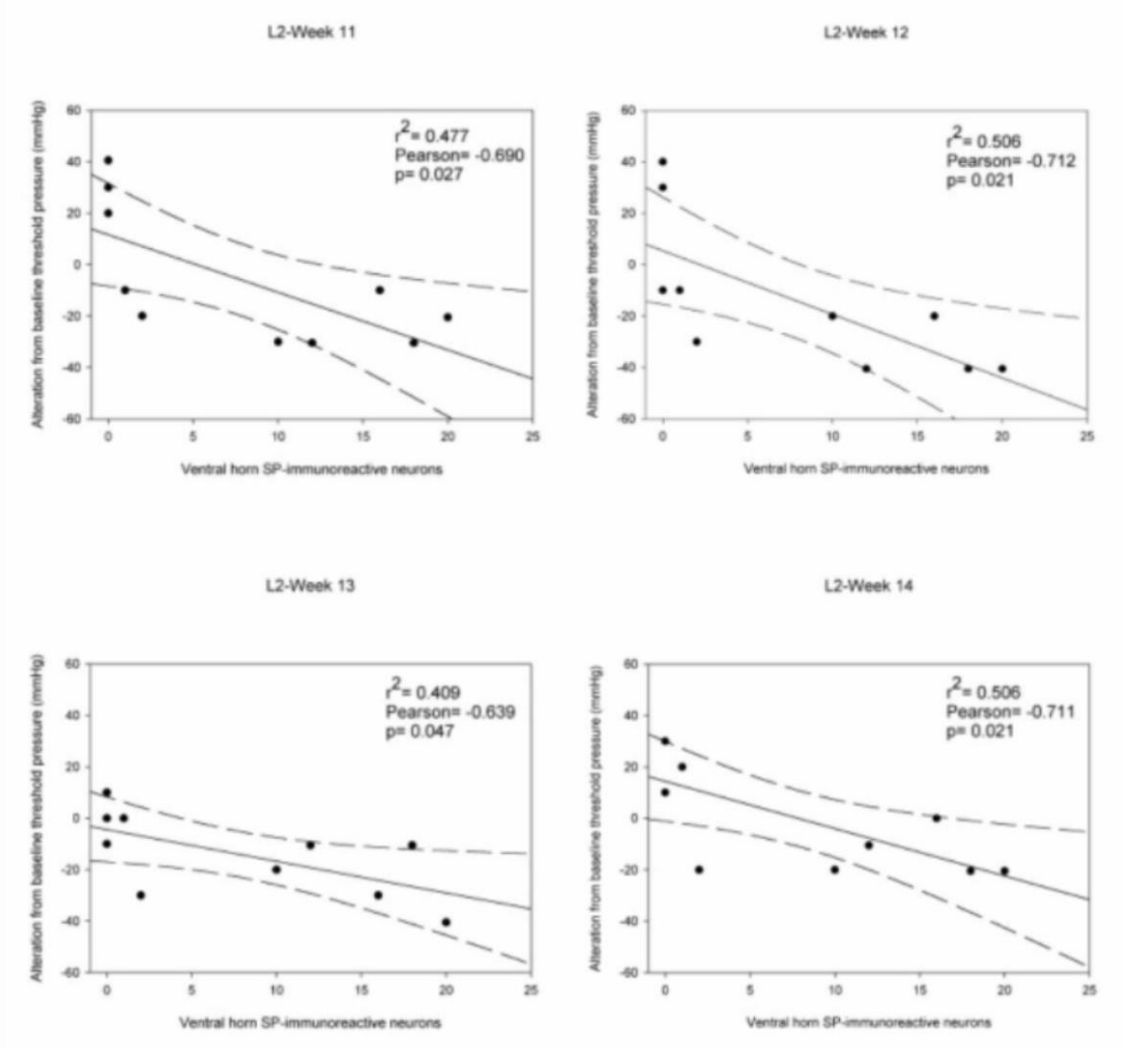

Figure 4-10. Ventral horn correlation with ABTP for the L2 spinal segment. Clockwise from the top left, plots represent linear regression analysis for weeks 11, 12, 14, and 13. Dashed lines represent 95% confidence intervals.

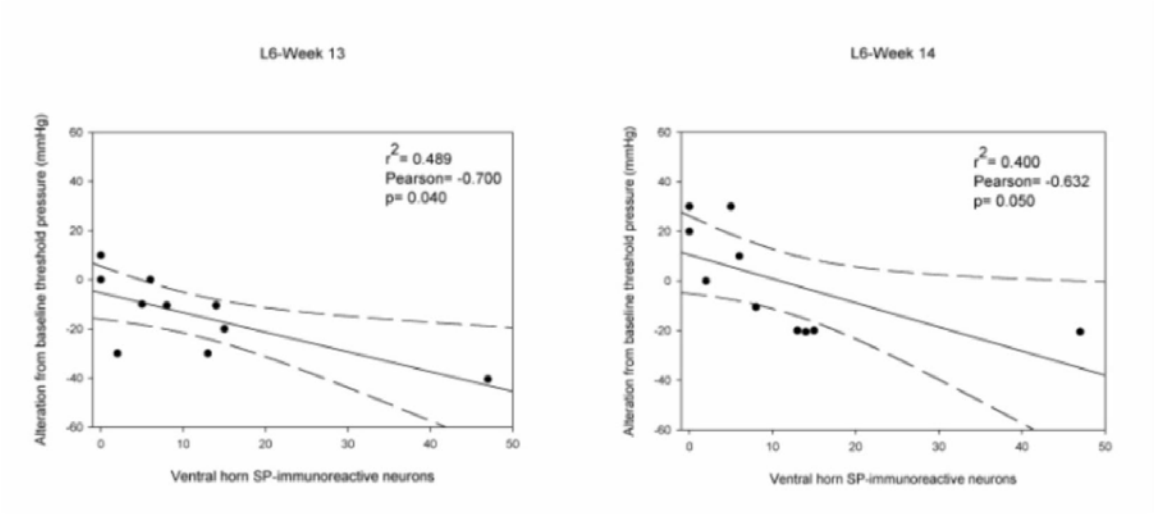

Figure 4-11. Ventral horn correlation with ABTP for the L6 spinal segment. Plots on the left and right represent linear regression analysis for weeks 13 and 14, respectively. Dashed lines represent 95% confidence intervals.

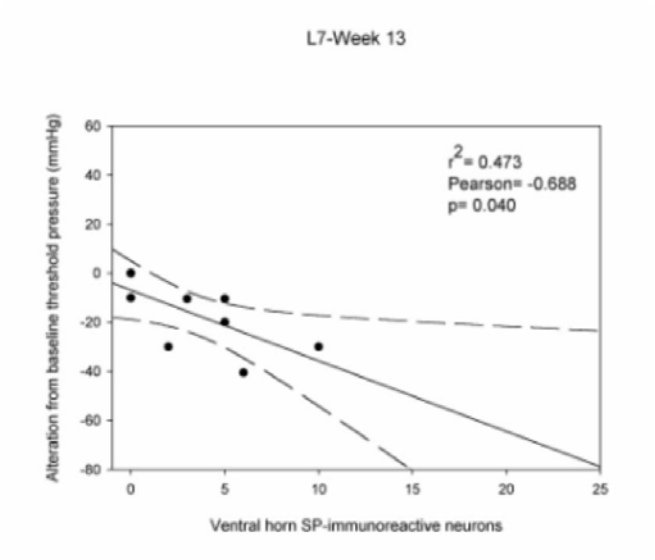

Figure 4-12. Ventral horn correlation with ABTP for the L7 spinal segment. Dashed lines represent 95% confidence intervals.

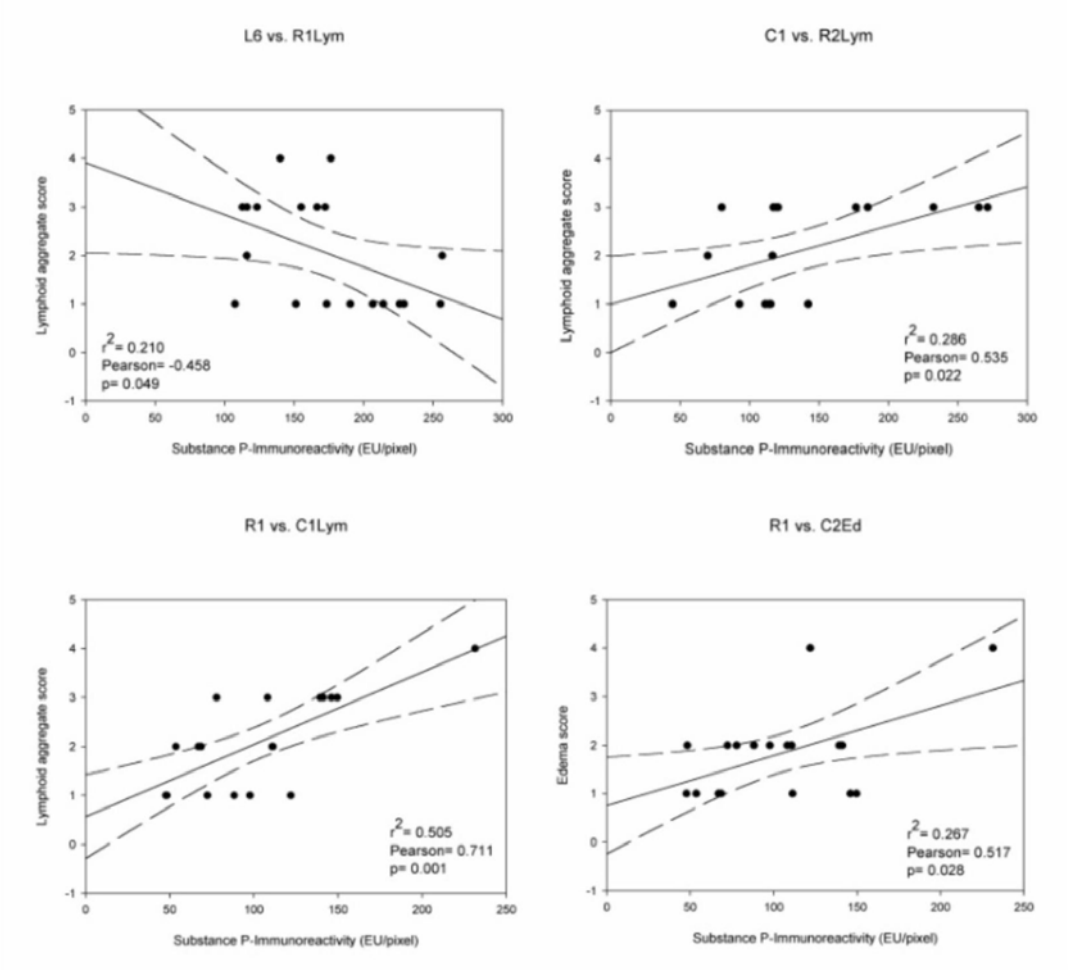

Figure 4-13. SP-IR correlation with histological scores. Clockwise from the top left, plots represent linear regression analysis for spinal segment L6 dorsal horn vs. R1 Lymphoid aggregate score; colon section C1 vs. R2 lymphoid aggregate score; rectal section R1 vs.C2 edema score; rectal section R1 vs. C1 lymphoid aggregate score. Dashed lines represent 95% confidence intervals.

CHAPTER 5
DISCUSSION

Model Development

The first two major objectives outlined for this study were clearly met. Animals developed a subacute proctitis after TNBS/EtOH enema administration and showed clinical signs of abdominal discomfort in response to colorectal balloon distention.

Subacute Proctitis

A subacute proctitis developed in the TNBS/EtOH groups. The degree of inflammation was such that the animals became febrile and most briefly developed bloody diarrhea. These signs were transient and did not affect the animals' appetite at any point. Some animals appeared slightly depressed for 12-24 hours, but this did not persist. Unfortunately, one pig suffered a perforated rectum shortly after TNBS/EtOH instillation, resulting in spontaneous death. The timing of this event was somewhat unusual in that the peak inflammatory response following TNBS/EtOH instillation is usually 2-3 days (Elson et al., 1995) but the animal died within 8 hours after the procedure. Due to the transmural nature of the resultant colitis, the effective dose of TNBS is close to the lethal dose in mice (Beagley et al., 1991). However, the currently used dose of TNBS/EtOH did not cause systemic illness in any other pig used in the pilot or main portions of the current study or those treated with ileal instillation in another study in this laboratory (Merritt et al., 2002a). One possible confounding factor was the use of inflated Foley catheters for enema retention. Because these catheters were inflated to a set volume, rather than pressure, colonic contractions over an inflated balloon,

especially in the presence of a chemical irritant, could have resulted in rupture. The timing of such an event more closely coincides with the animal's clinical picture than rupture due to the TNBS alone. In future studies, use of a pressure transducer with a "pop-off" valve attached to the Foley valve could avoid similar problems.

Endoscopy was an extremely useful tool for evaluation of ongoing distal colonic and rectal inflammation and allowed scoring the severity of inflammation without animal sacrifice. The duration of inflammation was similar to previous reports in mice (2-3 weeks) and dogs and rats (up to 8 weeks) (Elson et al., 1995; Shibata et al., 1993). Because animals were not evaluated until one week after TNBS/EtOH instillation, we likely did not capture the peak inflammatory response endoscopically. Individual animals varied in both severity and duration of the lesion, typical of TNBS/EtOH colitis (Elson et al., 1995). The development of lesions in all animals was more consistent than that reported in dogs, but endoscopic lesion appearance was similar (Shibata et al., 1993).

Histological analysis at weeks 9 and 14 (5 and 10 weeks post-enema) did not reveal significant differences between groups. Overall, colonic and rectal tissues had little to no cellular infiltrate, and mucosal and submucosal edema was the only prominent abnormality. If the colonic or rectal edema was related to TNBS/EtOH instillation, one would expect to see the highest scores in the rectum of animals in that experimental group sacrificed at 9 weeks. Because edema scores did not differ significantly between groups, other contributing factors must be considered. Since tissue from all animals was handled similarly, a processing artifact is unlikely. Repeated CRD studies could have resulted in rectal irritation and edema. But, one would therefore expect a difference between colonic and rectal edema and a difference between the 9 and 14 week animals as

a result of additional CRD procedures. These differences were not evident; thus, a specific cause of the rectal edema could not be identified.

The presence of edema did not appear to have an impact on remaining study variables. A significant correlation between edema and sensitivity among individual animals was not identified, indicating that VH was not related to an ongoing inflammatory process.

The other indicator of histological differences, number of lymphoid aggregates, could be misleading. This number is dependent upon section location and angle as well as normal variability throughout the gastrointestinal tract, and cellular infiltrate is more commonly used as an indicator of disease (Kruschewski et al., 2001). But, because very little cellular infiltrate was present in any of the sections, and the number of lymphoid aggregates appeared to vary between tissues based on a cursory examination, this count was used. Similar to the edema scoring, the number of lymphoid aggregates did not differ significantly between groups nor did it appear to have a major impact on other aspects of the study.

Visceral Discomfort

Pigs were easily trained to allow balloon insertion, and the ramp protocol for balloon distention reliably induced a discomfort response in most trials. One of the important factors in developing a pig model of visceral discomfort was the determination of "discomfort". Previous animal models have used both subjective methods such as an abdominal writhing response in rodents (Reichert et al., 2001) or more objective measurements such quantification of abdominal muscle contractions (Al Chaer et al., 2000; Coutinho et al., 2002; Ness and Gebhart, 1988) or a particular behavior (Merritt et al., 2002b). A conscious passive avoidance behavior, such as pushing a lever to

discontinue the painful stimulus, has also been described in rats (Messaoudi et al., 1999; Ness et al., 1991).

For CRD studies, we chose to evaluate discomfort subjectively, thus utilizing a threshold of discomfort as our primary response variable. Most quantitative measures involve a comparison of a particular variable between distention pressures rather than determination of a threshold response. Such a method would have required repeated painful colorectal distentions over the course of many weeks (Gschossmann et al., 2001; Ness and Gebhart, 1990). We did not want to cause discomfort above threshold in our subject animals for several reasons, including concern for their well-being. From a physiologic standpoint, repeated painful CRD can increase spinal *fos* and *jun* proto-oncogenes which, in turn, have been associated with central hyperexcitability (Traub et al., 1992). Thus, we wanted to minimize the effect of repeated CRD procedures themselves on visceral sensitivity by subjecting the animals to the fewest possible number of painful CRD. Similarly, we were concerned that the pigs could develop aversive behavior to the laboratory, crate, personnel, or testing procedure in general if we used repeated painful distentions. Because stress alone can influence visceral nociception (Bradesi et al., 2002; Coutinho et al., 2002), we wanted to minimize the physical and psychological stress imposed on our animal subjects.

We considered using real-time abdominal myoelectrical data to contribute to the threshold response determination. But, due to the nature of the pigs, we felt that movement artifact would make interpretation difficult in a time-dependent situation. Thus, in an attempt to limit the subjective nature of the behavioral observation technique, strict criteria were used to define a discomfort response, and observers were blinded as to

treatment. Most pigs had an obvious discomfort response with agreement of all three observers in 55% and 2/3 in 45% of trials. Some pigs were difficult to judge, especially for the baseline trials when they were more nervous and less accustomed to the testing procedure. In hindsight, obtaining an average baseline result from at least two, if not three trials, may have allowed a more thorough evaluation of the animals' true baseline responses.

Also in hindsight, equal sample sizes amongst all groups would have been preferable to the current design. The pilot animals produced very consistent CRD data. Because the main study was designed based upon their results, we presumed that saline-treated groups would react similarly, thus allowing for a smaller sample size. Because saline-treated animals exhibited more variation in CRD threshold response than expected, larger control groups likely would have diminished the impact of individual variability and increased the power of the study.

Effect of Inflammation on Nociceptive Threshold

Once a consistent model was established, a secondary goal of this study was to investigate the effect of subacute inflammation on nociceptive threshold.

Weekly Nociceptive Thresholds

The two methods used to express visceral sensitivity, threshold pressure and ABTP, describe the same data in slightly different ways. ABTP expresses the weekly thresholds as a variation of the animal's own baseline threshold pressure, accounting for individual variability in sensitivity. Similar comparisons have been used previously in human visceral sensitivity testing (Sabate et al., 2002). For this reason, the ABTP results were used for all correlations, and will be stressed during discussions of sensitivity.

The only significant difference in ABTP between experimental groups was at week 7, although the difference at week 13 approached significance ($p=0.056$). The mean pressures for week 13 were also significantly different between groups. These results suggest an interesting biphasic response. The initial period of hypersensitivity, manifested by a decrease in threshold pressure relative to baseline, following TNBS/EtOH instillation is likely related to colonic inflammation and its associated mediators. This response is predictable based on previously documented effects of gastrointestinal inflammation (Messaoudi et al., 1999; Ness and Gebhart, 2000; Sharkey and Kroese, 2001). The second period of hypersensitivity occurred after the endoscopic resolution of inflammation in all animals and following histologic resolution of inflammation in experimental group 1. A post-inflammatory period of visceral hypersensitivity supports the concept of plasticity within the afferent arm of the visceral nociceptive pathway.

Some degree of individual variation in nociceptive threshold is expected (Elmer et al., 1998), and such variability was observed in this study. When examining individual responses, only 2/7 saline animals had >2 weeks with threshold pressures below baseline, whereas 11/12 TNBS/EtOH animals had >2 weeks as such. Within the TNBS/EtOH-treated groups, variability in the resultant inflammatory response may have contributed to this individual variation. Week 6 and 9 endoscopy scores had a significant negative correlation with week 13 ABTP ($p=0.038$ and 0.031, respectively; $r^2= 0.435$ and 0.462, respectively), indicating that those animals with the highest endoscopy scores required less pressure to induce a nociceptive response. Because the r^2 value was less than 0.5 for each, this correlation should be considered a strong trend, rather than a significant

determination. Because a similar correlation did not exist between ABTP and histological scoring, any effect of inflammation on visceral sensitivity appears related more to the degree of inflammation initially present in each animal rather than the remaining level of inflammation present at the time of testing (or in this case one week later). This further supports the notion of neuronal plasticity rather than the direct action of inflammatory mediators for the later period of hypersensitivity seen at week 13.

The CRD protocol used in this study evaluated threshold pressure, rather than quantifying a particular pain response to a given stimulus. Thus, a reduction in sensory threshold truly indicates allodynia, rather than hypersensitivity. Based on information in other species, animals with allodynia were likely also hypersensitive, but this cannot be proven given the constraints of the testing system used for this study (Mayer and Gebhart, 1994).

The two saline-treated animals which consistently demonstrated negative ABTP likely contributed to the lack of a group effect in many weekly sensitivity thresholds. The sensitivity profiles of these two animals appeared to differ from the remaining saline-treated animals. These animals had threshold pressures approximately 20 mmHg below their initial baseline threshold at week 7 (the first CRD post enema) and then remained at those thresholds (within 10 mmHg) for all subsequent weeks. Given the consistency of this response, one potential explanation is that the reported baseline for those two animals was erroneously high and the remaining weeks represented their normal threshold. Alternatively, these animals became hypersensitive; possible causes include the saline retention enema, soapy water enemas used prior to endoscopy, repeated endoscopic examinations, or repeated CRD procedures. To the author's knowledge, none of these

have been reported as causes of visceral or somatic hypersensitivity, nor do they represent "noxious" procedures. As stated previously, CRD to noxious pressures has been reported to cause hypersensitivity, but not non-noxious distentions (Traub et al., 1992). Normal individual variability in visceral sensory threshold is another possible explanation. This is the more plausible explanation, and would likely coincide with an erroneously high baseline reported in these animals.

Animal selection

Other investigators have recently described models involving various insults during the neonatal period that resulted in visceral hypersensitivity during adulthood (Al Chaer et al., 2000; Coutinho et al., 2002; Ruda et al., 2000). Similar findings have not, to the author's knowledge, been extensively evaluated using a slightly older population of animals. At the time of TNBS/EtOH enema administration, pigs in this study were approximately 8-10 weeks old, corresponding to early adolescence in humans. This stage of development may be important for the development of IBS (Sandler, 1990; Van Ginkel et al., 2001), a concept further supported by the results of the present study.

Castrated male pigs were chosen for this study to avoid the any potential effect of hormonal variation during the estrous cycle on nociceptive threshold. Gender differences in both the perception and modulation of pain have been documented in humans (Gear et al., 1996), and women are at a higher risk for the development of IBS (Mayer et al., 1999). IBS-related pain can also vary with phase of the menstrual cycle (Heitkemper and Jarrett, 1992). In animal models of pain, female rodents display lower nociceptive thresholds to both shock and thermal stimuli (Marks and Hobbs, 1972; Pare, 1969; Romero and Bodnar, 1986).

Furthermore, gonadectomy has been reported to decrease both nociceptive threshold and response to analgesia in rodents (Marks and Hobbs, 1972; Romero et al., 1988). Thus, one cannot assume that the results obtained from our castrated animals would be the same as those obtained in intact male pigs or in females. However, since they were castrated at a similar age, any potential effect of gonadectomy should have been uniform across all animals.

Substance P

When attempting to quantify SP in various tissues, immunohistochemical analysis offers several advantages over molecular analyses. Most importantly, immunohistochemistry offers localization of immunoreactivity rather than protein quantification in a particular tissue. For porcine tissue, specific anti-pig SP polyclonal antibody has been raised (Balemba et al., 2001; Balemba et al., 2002), but it is not commercially available. The present study used a rabbit polyclonal SP antibody that had been validated previously at a similar dilution in porcine gastrointestinal tissue (Kulkarni-Narla et al., 1999). Early antibodies to SP showed some cross-reactivity to NK-A or NK-B, however more recently purified antibodies have apparently overcome this problem (Duggan, 1995; Hoyle, 1998).

In the gastrointestinal tissue, the location chosen for Q-IHC sampling was based upon the location of myenteric plexus (Goyal and Hirano, 1996). SP has previously been identified in the mucosa and submucosal plexus in the porcine gastrointestinal tract (Balemba et al., 2001; Balemba et al., 2002). During initial microscopic review of the gastrointestinal tissues in this study, SP-IR was consistently seen in the myenteric plexus, but not consistently in the areas of the other plexes. Some non-specific immunoreactivity was seen in the mucosa, most prominently surrounding the tissue periphery. Thus, the

quantitative analysis described in this report focused on the region of the myenteric plexus. For precise neuroanatomical detail in the enteric nervous system, whole mount preparations of the gastrointestinal tissue are preferred (Balemba et al., 2001; Miampamba and Sharkey, 1998). The transverse sections used in this study provide less specific detail in that most neurons in the plexus are captured only in part or in cross-section. However, as the purpose of the study was to evaluate the amount of SP-IR present, rather than to map its distribution, this method met study requirements.

The spinal cord segments were chosen as a representative sample of those receiving afferent input from the colon and rectum. The distal colon and rectum have dual sacral and lumbar afferent innervation (Ness and Gebhart, 1990). Based on Fluorogold labeling of the descending colon, DRG in the T13-L2 and L6-S2 regions received afferent input, but the number of positive neurons in the T13-L2 region was greater following colonic inflammation (Traub et al., 1999). Thus, spinal segments L1 and L2 were chosen to represent the proximal extent of innervation, while L6 and L7 were chosen to represent the distal portion.

The algorithm used to quantify SP-IR in the gastrointestinal tissues and spinal cord dorsal horn has been previously validated for use with DAB-based immunohistochemistry (Matkowskyj et al., 2000). This technique allows for precise documentation of the chromagen content in a specific image by subtracting the cumulative strength of the negative control image from that contained in the corresponding immunostained image. Thus it allowed for a quantitative evaluation of the SP-IR within specific locations in the spinal cord, colon, and rectum. Because the chromagen content is expressed in arbitrary units, these data cannot be reasonably

compared to other studies. But, this provided a more objective comparison between groups than a subjective scoring system.

Direct Relationship between Histopathology and Sensitivity

Based on an initial review of the gastrointestinal sections, very little cellular infiltrate was noted. The only apparent abnormalities were mucosal and submucosal edema. Also, the number of lymphoid aggregates appeared to vary between sections. One explanation for this variation is the inherent variability due to section location and angle of the cut. But, an analysis of the lymphoid aggregate counts was performed to investigate the possibility of a treatment effect.

Although the correlation between the R2 lymphoid aggregate score and ABTP in weeks 10 and 12 had significant Pearson's correlations, only the week 10 linear regression had a r^2 value >0.5. None of the correlations between histological scoring and SP-IR in either the spinal cord or gastrointestinal tract had an r^2 value >0.5, thus these should be considered trends at best. Visual inspection of these plots does not give the impression of a strong linear relationship (Fig. 4-8 and 4-13). Based on these facts and the lack of correlation between ABTP and any edema scores, the histopathologic changes seen within the gastrointestinal tract at the time of euthanasia did not appear to play a significant role in visceral sensitivity.

Central versus Peripheral Sensitization

The lack of a difference in SP-IR in either the colon or rectum between any of the experimental groups was somewhat unexpected. Other investigators have shown an initial decrease and subsequent increase in SP-IR throughout the colon following TNBS/EtOH instillation in rats (Miampamba and Sharkey, 1998) and in the primary afferent nerves in a guinea pig TNBS ileitis model (Miller et al., 1993). These reported

changes occurred within 2 weeks following the inflammatory insult, and the long-term effects of inflammation upon SP-IR have not been fully elucidated. Thus, while this study did not evaluate the early effects of inflammation upon SP-IR, the information presented for the periods 5 and 10 weeks after TNBS/EtOH instillation provide new insight into the pathophysiology of inflammatory-mediated VH. Due to the relatively small sample size and individual variation in inflammatory response, a small difference between groups may have gone undetected. In addition to a lack of detectable difference between groups, SP-IR in the gastrointestinal tract did not correlate significantly with ABTP for any week. These results, in conjunction with the significant correlation between dorsal horn SP-IR and ABTB, support a central rather than peripheral mechanism of sensitization in these animals.

No significant difference was detected between experimental groups in either dorsal horn SP-IR or ventral horn SP-IR neurons. Similar to the reasoning described for gastrointestinal tissues, individual variability and sample size may have prevented detection of a small difference between groups. Alternatively, groups truly did not differ in immunoreactivity. Because VH did not develop in all of the TNBS/EtOH animals, the correlation between weekly ABTP and SP-IR may provide more insight into the relationship between SP in the spinal cord and the level of visceral sensitivity in a given animal.

The significant correlation between dorsal horn SP-IR at the L1 and L7 segments with multiple weekly ABTP supports a central mechanism of hypersensitivity. This correlation was strongest between L7 and week 11, but also significant between L7 and week 14 and between L1 and week 9. The argument for a role of SP in the development

of hypersensitivity in these animals would be stronger had SP-IR correlated significantly with other weeks, especially when the TNBS/EtOH and saline groups differed significantly. But, the correlation between SP-IR in the dorsal horn and ABTP across all experimental groups further validates the importance of SP within the dorsal horn in the development of central sensitization, regardless of the inciting cause.

SP has been associated with afferent transmission of nociception for years (Hokfelt et al., 1977a; Mayer and Raybould, 1990; Otsuka and Yoshioka, 1993). Not surprisingly, SP is thought to play a role in central sensitization and inflammatory-mediated VH (Kishimoto, 1994; Miampamba et al., 1992; Persson et al., 1995; Schneider et al., 2001; Swain et al., 1992). Zymosan-induced colitis reduced the number of SP-labeled cells in both the T13-L2 and L6-S2 afferent DRG in rats (Traub et al., 1999). Also, TNBS ileitis has been shown to induce hyperexcitability in nociceptive DRG neurons.(Moore et al., 2002) In the present study, SP-IR in the dorsal horn was greatest in the superficial laminae (I and II) which receive afferent input, consistent with previous reports (Duggan, 1995; Kawata et al., 1989; Routh and Helke, 1995). The strongest correlation with ABTP in the lumbar spinal cord was at L7, corresponding to lumbosacral afferent input from the distal colon and rectum.

Given the previously suggested importance of SP in the development of central sensitization and the changes previously associated with gastrointestinal inflammation, alterations in SP-IR were expected in this study. However, a specific correlation between visceral sensitivity in individual animals and SP-immunoreactivity in the spinal cord dorsal horn has not, to our knowledge, been previously documented. This association between SP in the dorsal horn and the lack of an association with SP-IR in the colon or

rectum highlights the role of SP in central sensitization and the importance of this process in post-inflammatory visceral hypersensitivity.

In the ventral horn, the correlation between SP-IR neurons and ABTP, especially at the L2 segment implies an alteration in motor pathways. However, these interpretations should be considered cautiously because the r^2 for all linear regressions were less than 0.51, and visual inspection of the correlation does not imply a consistent linear relationship. SP-IR has been documented in large motoneurons of the ventral horn, but an alteration in ventral horn SP-IR has not previously been attributed to rectal or colonic inflammation (Charlton and Helke, 1985b; Charlton and Helke, 1985a). However, given the appearance of the linear regression plots and the function of the large ventral horn motoneurons, the true relevance of the correlation between ventral horn SP-IR neurons and visceral sensitivity requires further investigation.

Conclusions

Current Study

The main accomplishment of this study was to integrate models of colonic inflammation and visceral pain in the pig. The use of a large animal subject, such as the pig, allowed for endoscopic scoring of gross mucosal changes within the distal colon and rectum. This provided an evaluation of each animal's individual response to TNBS/EtOH instillation, rather than relying on a group average based on histopathologic changes seen in animals sacrificed at various time points. This information allowed for a more thorough characterization of individual responses while also decreasing the required number of animals. Because of the variability in response to TNBS/EtOH, the endoscopic information proved useful, in that the resultant colitis severity scores following instillation correlated negatively with ABTP.

TNBS/EtOH instillation resulted in a biphasic pattern of visceral hypersensitivity. The first period occurred in the presence of ongoing inflammation, but the second period occurred after the gross resolution of inflammation in the subject animals and the histological resolution of inflammation in cohorts euthanized at week 9. These results, combined with a lack of consistent correlation between ABTP and histological scoring, suggest that either central and/or peripheral mechanisms of hypersensitization, rather than inflammatory mediators, appear responsible for observed alterations in visceral sensitivity.

Although TNBS/EtOH instillation did not have a significant effect on spinal or colonic SP-immunoreactivity, a strong correlation between dorsal horn SP-IR at the L1 and L7 levels and ABTP was evident. Due to the small sample size and variation within each group, this correlation more likely represents the true involvement of spinal SP in a visceral hypersensitivity response, consistent with central sensitization. The inclusion of both saline- and TNBS/EtOH-treated animals in these correlations suggests the importance of SP in VH regardless of the inciting cause, an unexpected but intriguing result.

Future Studies

Based upon results of this study, the pig may serve as an excellent large animal model for future studies concerning the pathophysiology and treatment of inflammatory-mediated IBS. One of the first options would be to determine the immunoreactivity of other mediators of central sensitization, such as CGRP, NMDA, NGF, and others, within the spinal cords of the pigs in this study. These studies could be easily accomplished as paraffin-embedded tissue from all animals has been retained. Combined with the ABTP, endoscopic scores, histologic scores, and SP-IR, further immunohistochemical

characterization of this model could contribute to a more thorough understanding of the post-inflammatory neuroplastic changes described in this model.

The porcine CRD model could be used to evaluate the effect of potential therapeutic agents on visceral sensitivity thresholds, either with or without a previous inflammatory insult. In such studies, endoscopic evaluation would allow for classification of the subjects based on lesion severity.

Due to the chronic nature of IBS and the temporal separation between insult and documentation of VH in neonatal rodent models, studies of longer duration are also warranted. For example, a porcine study similar to that described in this report, but that extended to adulthood, would more fully characterize the duration and nature of alterations in visceral sensitivity. If the bi-phasic pattern of VH seen in this study persisted in a relapsing/remitting fashion, the model would offer a similar clinical picture to that seen in IBS. Furthermore, one could evaluate the effect of repeated versus single-dose TNBS/EtOH instillations on the visceral sensitivity pattern to determine whether or not a repetitive inflammatory insult further increases the severity or duration of VH.

APPENDIX A
INDIVIDUAL ANIMAL DATA

Table A-1. Raw data from CRD studies.

An #	Group	Wk 3	Wk 7	Wk 8	Wk 9	Wk 10	Wk 11	Wk 12	Wk 13	Wk 14
3161	3	45	15	25	25	15	15	25	25	25
3171	1	55	25	15	25					
3162	4	25	45	25	25	25	15	15	25	45
3185	1	35	35	25	45					
3197	1	55*	45	45	35					
3184	4	15	55*	35	35	55	55*	55	25	45
3191	3	45	25	15	25	35	25	15	15	25
John	1	45	25	15	25					
Red	1	35	35	15	25					
3166	1	35	15	15	25					
3198	2	25	25	15	35					
3179	2	25	25	25	xx					
3182	4	55*	25	25	15	25	25	15	45	45
3189	2	15	35	15	15					
3190	3	55*	15	55*	55*	55	35	15	15	35
3193	4	55*	25	15	15	15	25	15	45	35
3192	3	45	25	25	35	55	35	25	15	45
3130	3	25	15	15	25	15	55*	15	15	55*
3126	3	15	15	35	35	45	35	45	15	25

Table A-2. Raw data from endoscopic examinations.

Animal #	Group	Wk 3	Wk 6	Wk 9	Wk 14
3171	1	0.5	3.0	0.5	
3185	1	0.5	5.0	2.0	
3197	1	0.5	2.0	0.5	
John	1	0.5	3.0		
Red	1	0.0	2.0		
3166	1	0.0	3.0	0.5	
3198	2	0.5	0.5	0.5	
3179	2	1.0	0.5	0.0	
3189	2	0.0	1.5	0.5	
3161	3	0.0	3.5	1.0	
3191	3	0.5	4.0	1.0	
3190	3	0.5	2.0	1.0	0.0
3192	3	0.0	2.0	0.0	0.5
3130	3	0.5	2.0	0.5	0.0
3126	3	0.5	1.0	0.0	0.5
3162	4	0.5	0.0	0.0	
3184	4	1.0	0.5	0.0	
3182	4	0.5	0.0	0.0	0.0
3193	4		0.5	0.0	0.0

APPENDIX B
ANOVA TABLES

Table B-1. One-way ANOVA analysis for threshold pressure.

Week	Groups	Sum of Squares	df	Mean Square	F	Sig.
3	Between	452.6942	1	452.6942	2.222232	0.154358
	Within	3463.095	17	203.7115		
	Total	3915.789	18			
7	Between	391.0401	1	391.0401	3.361862	0.084292
	Within	1977.381	17	116.3165		
	Total	2368.421	18			
8	Between	36.09023	1	36.09023	0.261874	0.615419
	Within	2342.857	17	137.8151		
	Total	2378.947	18			
9	Between	289.7243	1	289.7243	3.172748	0.09275
	Within	1552.381	17	91.31653		
	Total	1842.105	18			
10	Between	106.6667	1	106.6667	0.330323	0.581267
	Within	2583.333	8	322.9167		
	Total	2690	9			
11	Between	26.66667	1	26.66667	0.119626	0.73836
	Within	1783.333	8	222.9167		
	Total	1810	9			
12	Between	6.666667	1	6.666667	0.028319	0.870539
	Within	1883.333	8	235.4167		
	Total	1890	9			
13	Between	806.6667	1	806.6667	13.35172	0.006457
	Within	483.3333	8	60.41667		
	Total	1290	9			
14	Between	135	1	135	1.234286	0.298847
	Within	875	8	109.375		
	Total	1010	9			

Table B-2. One-way ANOVA analysis for ABTP.

Week	Groups	Sum of Squares	df	Mean Square	F	Sig.
7	Between	1687.269	1	1687.269	4.679076	0.04507
	Within	6130.179	17	360.5987		
	Total	7817.447	18			
8	Between	226.6931	1	226.6931	0.737567	0.402389
	Within	5224.991	17	307.3524		
	Total	5451.684	18			
9	Between	16.34226	1	16.34226	0.048641	0.828073
	Within	5711.658	17	335.9799		
	Total	5728	18			
10	Between	86.4	1	86.4	0.114968	0.743287
	Within	6012.125	8	751.5156		
	Total	6098.525	9			
11	Between	15.50417	1	15.50417	0.019584	0.892164
	Within	6333.396	8	791.6745		
	Total	6348.9	9			
12	Between	13.06667	1	13.06667	0.014309	0.907734
	Within	7305.458	8	913.1823		
	Total	7318.525	9			
13	Between	866.4	1	866.4	4.978863	0.05618
	Within	1392.125	8	174.0156		
	Total	2258.525	9			
14	Between	160.0667	1	160.0667	0.329147	0.581927
	Within	3890.458	8	486.3073		

Table B-3. One-way ANOVA analysis for endoscopy scores.

Week	Groups	Sum of Squares	df	Mean Square	F	Sig.
3	Between	0.122807	1	0.122807	1.252632	0.278616
	Within	1.666667	17	0.098039		
	Total	1.789474	18			
6	Between	21.56031	1	21.56031	24.88431	0.000112
	Within	14.72917	17	0.866422		
	Total	36.28947	18			
9	Between	1.278151	1	1.278151	5.545698	0.032564
	Within	3.457143	15	0.230476		
	Total	4.735294	16			
14	Between	0.125	1	0.125	3	0.133975
	Within	0.25	6	0.041667		
	Total	0.375	7			

Table B-4. One-way ANOVA analysis for gastrointestinal histologic scores.

Section	Group	Sum of Squares	df	Mean Square	F	Sig.
C1LYM	Between	1.3640	3	0.4547	0.4377	0.7293
	Within	15.5833	15	1.0389		
	Total	16.9474	18			
C1ED	Between	2.2895	3	0.7632	0.9954	0.4218
	Within	11.5000	15	0.7667		
	Total	13.7895	18			
C1TOT	Between	0.8640	3	0.2880	0.1347	0.9379
	Within	32.0833	15	2.1389		
	Total	32.9474	18			
C2LYM	Between	0.2061	3	0.0687	0.1076	0.9544
	Within	9.5833	15	0.6389		
	Total	9.7895	18			
C2ED	Between	2.2412	3	0.7471	0.8676	0.4795
	Within	12.9167	15	0.8611		
	Total	15.1579	18			
C2TOT	Between	2.7895	3	0.9298	0.8204	0.5026
	Within	17.0000	15	1.1333		
	Total	19.7895	18			
R1LYM	Between	2.1140	3	0.7047	0.5074	0.6831
	Within	20.8333	15	1.3889		
	Total	22.9474	18			
R1ED	Between	5.0482	3	1.6827	2.1791	0.1330
	Within	11.5833	15	0.7722		
	Total	16.6316	18			
R1TOT	Between	10.3202	3	3.4401	1.2767	0.3182
	Within	40.4167	15	2.6944		
	Total	50.7368	18			
R2LYM	Between	1.1930	3	0.3977	0.3890	0.7626
	Within	15.3333	15	1.0222		
	Total	16.5263	18			
R2ED	Between	1.7982	3	0.5994	0.4316	0.7334
	Within	20.8333	15	1.3889		
	Total	22.6316	18			
R2TOT	Between	4.7982	3	1.5994	0.6695	0.5838
	Within	35.8333	15	2.3889		
	Total	40.6316	18			

Table B-5. Tukey's HSD analysis for gastrointestinal histologic scores.

Section	(I) Grp	(J) Grp	Mean Diff (I-J)	Std. Error	Sig.	95% CI-Lower	95% CI-Upper
C1LYM	1	2	-0.1667	0.7207	0.9955	-2.2439	1.9106
		3	0.5000	0.5885	0.8300	-1.1961	2.1961
		4	-0.0833	0.6579	0.9992	-1.9796	1.8129
	2	1	0.1667	0.7207	0.9955	-1.9106	2.2439
		3	0.6667	0.7207	0.7921	-1.4106	2.7439
		4	0.0833	0.7785	0.9995	-2.1603	2.3270
	3	1	-0.5000	0.5885	0.8300	-2.1961	1.1961
		2	-0.6667	0.7207	0.7921	-2.7439	1.4106
		4	-0.5833	0.6579	0.8118	-2.4796	1.3129
	4	1	0.0833	0.6579	0.9992	-1.8129	1.9796
		2	-0.0833	0.7785	0.9995	-2.3270	2.1603
		3	0.5833	0.6579	0.8118	-1.3129	2.4796
C1ED	1	2	-0.1667	0.6191	0.9929	-1.9511	1.6178
		3	-0.8333	0.5055	0.3831	-2.2903	0.6237
		4	-0.5000	0.5652	0.8128	-2.1290	1.1290
	2	1	0.1667	0.6191	0.9929	-1.6178	1.9511
		3	-0.6667	0.6191	0.7084	-2.4511	1.1178
		4	-0.3333	0.6687	0.9582	-2.2608	1.5941
	3	1	0.8333	0.5055	0.3831	-0.6237	2.2903
		2	0.6667	0.6191	0.7084	-1.1178	2.4511
		4	0.3333	0.5652	0.9336	-1.2956	1.9623
	4	1	0.5000	0.5652	0.8128	-1.1290	2.1290
		2	0.3333	0.6687	0.9582	-1.5941	2.2608
		3	-0.3333	0.5652	0.9336	-1.9623	1.2956
C1TOT	1	2	-0.3333	1.0341	0.9880	-3.3139	2.6472
		3	-0.3333	0.8444	0.9784	-2.7669	2.1003
		4	-0.5833	0.9440	0.9248	-3.3042	2.1375
	2	1	0.3333	1.0341	0.9880	-2.6472	3.3139
		3	0.0000	1.0341	1.0000	-2.9805	2.9805
		4	-0.2500	1.1170	0.9959	-3.4694	2.9694
	3	1	0.3333	0.8444	0.9784	-2.1003	2.7669
		2	0.0000	1.0341	1.0000	-2.9805	2.9805
		4	-0.2500	0.9440	0.9932	-2.9709	2.4709
	4	1	0.5833	0.9440	0.9248	-2.1375	3.3042
		2	0.2500	1.1170	0.9959	-2.9694	3.4694
		3	0.2500	0.9440	0.9932	-2.4709	2.9709
C2LYM	1	2	0.1667	0.5652	0.9907	-1.4623	1.7956
		3	0.1667	0.4615	0.9833	-1.1634	1.4967
		4	-0.0833	0.5159	0.9984	-1.5704	1.4037
	2	1	-0.1667	0.5652	0.9907	-1.7956	1.4623
		3	0.0000	0.5652	1.0000	-1.6290	1.6290
		4	-0.2500	0.6105	0.9760	-2.0095	1.5095
	3	1	-0.1667	0.4615	0.9833	-1.4967	1.1634
		2	0.0000	0.5652	1.0000	-1.6290	1.6290
		4	-0.2500	0.5159	0.9614	-1.7370	1.2370
	4	1	0.0833	0.5159	0.9984	-1.4037	1.5704
		2	0.2500	0.6105	0.9760	-1.5095	2.0095
		3	0.2500	0.5159	0.9614	-1.2370	1.7370

Table B-5. Continued

Section	(I) Grp	(J) Grp	Mean Diff (I-J)	Std. Error	Sig.	95% CI-Lower	95% CI-Upper
C2ED	1	2	0.1667	0.6562	0.9940	-1.7245	2.0578
		3	-0.5000	0.5358	0.7878	-2.0441	1.0441
		4	-0.7500	0.5990	0.6052	-2.4764	0.9764
	2	1	-0.1667	0.6562	0.9940	-2.0578	1.7245
		3	-0.6667	0.6562	0.7428	-2.5578	1.2245
		4	-0.9167	0.7087	0.5807	-2.9594	1.1260
	3	1	0.5000	0.5358	0.7878	-1.0441	2.0441
		2	0.6667	0.6562	0.7428	-1.2245	2.5578
		4	-0.2500	0.5990	0.9746	-1.9764	1.4764
	4	1	0.7500	0.5990	0.6052	-0.9764	2.4764
		2	0.9167	0.7087	0.5807	-1.1260	2.9594
		3	0.2500	0.5990	0.9746	-1.4764	1.9764
C2TOT	1	2	0.3333	0.7528	0.9700	-1.8363	2.5029
		3	-0.3333	0.6146	0.9472	-2.1048	1.4381
		4	-0.8333	0.6872	0.6286	-2.8139	1.1472
	2	1	-0.3333	0.7528	0.9700	-2.5029	1.8363
		3	-0.6667	0.7528	0.8123	-2.8363	1.5029
		4	-1.1667	0.8131	0.4983	-3.5101	1.1768
	3	1	0.3333	0.6146	0.9472	-1.4381	2.1048
		2	0.6667	0.7528	0.8123	-1.5029	2.8363
		4	-0.5000	0.6872	0.8845	-2.4806	1.4806
	4	1	0.8333	0.6872	0.6286	-1.1472	2.8139
		2	1.1667	0.8131	0.4983	-1.1768	3.5101
		3	0.5000	0.6872	0.8845	-1.4806	2.4806
R1LYM	1	2	-0.5000	0.8333	0.9305	-2.9018	1.9018
		3	0.5000	0.6804	0.8816	-1.4611	2.4611
		4	0.1667	0.7607	0.9961	-2.0259	2.3592
	2	1	0.5000	0.8333	0.9305	-1.9018	2.9018
		3	1.0000	0.8333	0.6361	-1.4018	3.4018
		4	0.6667	0.9001	0.8792	-1.9276	3.2609
	3	1	-0.5000	0.6804	0.8816	-2.4611	1.4611
		2	-1.0000	0.8333	0.6361	-3.4018	1.4018
		4	-0.3333	0.7607	0.9709	-2.5259	1.8592
	4	1	-0.1667	0.7607	0.9961	-2.3592	2.0259
		2	-0.6667	0.9001	0.8792	-3.2609	1.9276
		3	0.3333	0.7607	0.9709	-1.8592	2.5259
R1ED	1	2	-0.3333	0.6214	0.9488	-2.1242	1.4576
		3	0.5000	0.5074	0.7597	-0.9623	1.9623
		4	-0.9167	0.5672	0.3996	-2.5515	0.7182
	2	1	0.3333	0.6214	0.9488	-1.4576	2.1242
		3	0.8333	0.6214	0.5526	-0.9576	2.6242
		4	-0.5833	0.6712	0.8205	-2.5177	1.3511
	3	1	-0.5000	0.5074	0.7597	-1.9623	0.9623
		2	-0.8333	0.6214	0.5526	-2.6242	0.9576
		4	-1.4167	0.5672	0.1010	-3.0515	0.2182
	4	1	0.9167	0.5672	0.3996	-0.7182	2.5515
		2	0.5833	0.6712	0.8205	-1.3511	2.5177
		3	1.4167	0.5672	0.1010	-0.2182	3.0515

Table B-5. Continued

Section	(I) Grp	(J) Grp	Mean Diff (I-J)	Std. Error	Sig.	95% CI-Lower	95% CI-Upper
R1TOT	1	2	-0.8333	1.1607	0.8884	-4.1786	2.5120
		3	1.0000	0.9477	0.7207	-1.7314	3.7314
		4	-0.7500	1.0596	0.8924	-3.8038	2.3038
	2	1	0.8333	1.1607	0.8884	-2.5120	4.1786
		3	1.8333	1.1607	0.4187	-1.5120	5.1786
		4	0.0833	1.2537	0.9999	-3.5300	3.6967
	3	1	-1.0000	0.9477	0.7207	-3.7314	1.7314
		2	-1.8333	1.1607	0.4187	-5.1786	1.5120
		4	-1.7500	1.0596	0.3815	-4.8038	1.3038
	4	1	0.7500	1.0596	0.8924	-2.3038	3.8038
		2	-0.0833	1.2537	0.9999	-3.6967	3.5300
		3	1.7500	1.0596	0.3815	-1.3038	4.8038
R2LYM	1	2	0.5000	0.7149	0.8957	-1.5605	2.5605
		3	0.0000	0.5837	1.0000	-1.6824	1.6824
		4	-0.3333	0.6526	0.9553	-2.2143	1.5476
	2	1	-0.5000	0.7149	0.8957	-2.5605	1.5605
		3	-0.5000	0.7149	0.8957	-2.5605	1.5605
		4	-0.8333	0.7722	0.7070	-3.0589	1.3923
	3	1	0.0000	0.5837	1.0000	-1.6824	1.6824
		2	0.5000	0.7149	0.8957	-1.5605	2.5605
		4	-0.3333	0.6526	0.9553	-2.2143	1.5476
	4	1	0.3333	0.6526	0.9553	-1.5476	2.2143
		2	0.8333	0.7722	0.7070	-1.3923	3.0589
		3	0.3333	0.6526	0.9553	-1.5476	2.2143
R2ED	1	2	0.0000	0.8333	1.0000	-2.4018	2.4018
		3	0.1667	0.6804	0.9946	-1.7944	2.1277
		4	-0.6667	0.7607	0.8169	-2.8592	1.5259
	2	1	0.0000	0.8333	1.0000	-2.4018	2.4018
		3	0.1667	0.8333	0.9970	-2.2351	2.5685
		4	-0.6667	0.9001	0.8792	-3.2609	1.9276
	3	1	-0.1667	0.6804	0.9946	-2.1277	1.7944
		2	-0.1667	0.8333	0.9970	-2.5685	2.2351
		4	-0.8333	0.7607	0.6976	-3.0259	1.3592
	4	1	0.6667	0.7607	0.8169	-1.5259	2.8592
		2	0.6667	0.9001	0.8792	-1.9276	3.2609
		3	0.8333	0.7607	0.6976	-1.3592	3.0259
R2TOT	1	2	0.5000	1.0929	0.9671	-2.6499	3.6499
		3	0.1667	0.8924	0.9976	-2.4052	2.7386
		4	-1.0000	0.9977	0.7504	-3.8755	1.8755
	2	1	-0.5000	1.0929	0.9671	-3.6499	2.6499
		3	-0.3333	1.0929	0.9897	-3.4833	2.8166
		4	-1.5000	1.1805	0.5942	-4.9023	1.9023
	3	1	-0.1667	0.8924	0.9976	-2.7386	2.4052
		2	0.3333	1.0929	0.9897	-2.8166	3.4833
		4	-1.1667	0.9977	0.6543	-4.0421	1.7088
	4	1	1.0000	0.9977	0.7504	-1.8755	3.8755
		2	1.5000	1.1805	0.5942	-1.9023	4.9023
		3	1.1667	0.9977	0.6543	-1.7088	4.0421

Table B-6. One-way ANOVA for ventral horn SP-immunoreactive neurons.

Section	Groups	Sum of Squares	df	Mean Square	F	Sig.
L1	Between	128.4035	3.0000	42.8012	1.1882	0.3477
	Within	540.3333	15.0000	36.0222		
	Total	668.7368	18.0000			
L2	Between	257.0482	3.0000	85.6827	2.0811	0.1457
	Within	617.5833	15.0000	41.1722		
	Total	874.6316	18.0000			
L6	Between	253.2895	3.0000	84.4298	0.6434	0.5990
	Within	1968.5000	15.0000	131.2333		
	Total	2221.7895	18.0000			
L7	Between	5.6111	3.0000	1.8704	0.1250	0.9438
	Within	209.5000	14.0000	14.9643		
	Total	215.1111	17.0000			

Table B-7. Tukey's HSD analysis for ventral horn SP-immunoreactive neurons.

Section	(I) Grp	(J) Grp	Mean Diff(I-J)	SEM	Sig.	95% CI-Lower	95% CI-Upper
L1	1	2	1.5000	4.2439	0.9843	-10.7317	13.7317
		3	-5.3333	3.4652	0.4403	-15.3205	4.6538
		4	-2.5000	3.8742	0.9156	-13.6660	8.6660
	2	1	-1.5000	4.2439	0.9843	-13.7317	10.7317
		3	-6.8333	4.2439	0.4026	-19.0650	5.3984
		4	-4.0000	4.5840	0.8188	-17.2117	9.2117
	3	1	5.3333	3.4652	0.4403	-4.6538	15.3205
		2	6.8333	4.2439	0.4026	-5.3984	19.0650
		4	2.8333	3.8742	0.8830	-8.3326	13.9993
	4	1	2.5000	3.8742	0.9156	-8.6660	13.6660
		2	4.0000	4.5840	0.8188	-9.2117	17.2117
		3	-2.8333	3.8742	0.8830	-13.9993	8.3326
L2	1	2	0.8333	4.5372	0.9977	-12.2435	13.9102
		3	-7.1667	3.7046	0.2557	-17.8439	3.5105
		4	-6.9167	4.1419	0.3724	-18.8542	5.0208
	2	1	-0.8333	4.5372	0.9977	-13.9102	12.2435
		3	-8.0000	4.5372	0.3279	-21.0769	5.0769
		4	-7.7500	4.9007	0.4177	-21.8746	6.3746
	3	1	7.1667	3.7046	0.2557	-3.5105	17.8439
		2	8.0000	4.5372	0.3279	-5.0769	21.0769
		4	0.2500	4.1419	0.9999	-11.6875	12.1875
	4	1	6.9167	4.1419	0.3724	-5.0208	18.8542
		2	7.7500	4.9007	0.4177	-6.3746	21.8746
		3	-0.2500	4.1419	0.9999	-12.1875	11.6875
L6	1	2	4.1667	8.1004	0.9544	-19.1799	27.5133
		3	-4.1667	6.6140	0.9208	-23.2291	14.8957
		4	5.0000	7.3946	0.9045	-16.3124	26.3124
	2	1	-4.1667	8.1004	0.9544	-27.5133	19.1799
		3	-8.3333	8.1004	0.7357	-31.6799	15.0133
		4	0.8333	8.7494	0.9997	-24.3839	26.0505
	3	1	4.1667	6.6140	0.9208	-14.8957	23.2291
		2	8.3333	8.1004	0.7357	-15.0133	31.6799
		4	9.1667	7.3946	0.6126	-12.1458	30.4791
	4	1	-5.0000	7.3946	0.9045	-26.3124	16.3124
		2	-0.8333	8.7494	0.9997	-26.0505	24.3839
		3	-9.1667	7.3946	0.6126	-30.4791	12.1458
L7	1	2	1.0000	2.7354	0.9826	-6.9505	8.9505
		3	-0.5000	2.2334	0.9959	-6.9915	5.9915
		4	0.6667	2.7354	0.9947	-7.2838	8.6171
	2	1	-1.0000	2.7354	0.9826	-8.9505	6.9505
		3	-1.5000	2.7354	0.9455	-9.4505	6.4505
		4	-0.3333	3.1585	0.9996	-9.5138	8.8471
	3	1	0.5000	2.2334	0.9959	-5.9915	6.9915
		2	1.5000	2.7354	0.9455	-6.4505	9.4505
		4	1.1667	2.7354	0.9730	-6.7838	9.1171
	4	1	-0.6667	2.7354	0.9947	-8.6171	7.2838
		2	0.3333	3.1585	0.9996	-8.8471	9.5138
		3	-1.1667	2.7354	0.9730	-9.1171	6.7838

Table B-8. One-way ANOVA for SP-immunoreactivity (QIHC – EU/pixel)

Section	Groups	Sum of Squares	df	Mean Square	F	Sig.
L1	Between	8487.5138	3	2829.1713	1.4157	0.2772
	Within	29975.3478	15	1998.3565		
	Total	38462.8616	18			
L2	Between	1542.5863	3	514.1954	0.2947	0.8286
	Within	26170.6220	15	1744.7081		
	Total	27713.2084	18			
L6	Between	4541.2806	3	1513.7602	0.6128	0.6172
	Within	37056.2933	15	2470.4196		
	Total	41597.5739	18			
L7	Between	9247.0372	3	3082.3457	1.0665	0.3947
	Within	40460.8227	14	2890.0588		
	Total	49707.8599	17			
R1	Between	7141.7124	3	2380.5708	1.1332	0.3695
	Within	29411.3091	14	2100.8078		
	Total	36553.0216	17			
R2	Between	6336.3136	3	2112.1045	0.7050	0.5647
	Within	41940.1497	14	2995.7250		
	Total	48276.4633	17			
C1	Between	7798.2417	3	2599.4139	0.5905	0.6313
	Within	61626.3441	14	4401.8817		
	Total	69424.5858	17			
C2	Between	31907.1178	3	10635.7059	2.3118	0.1326
	Within	50607.6520	11	4600.6956		
	Total	82514.7698	14			

Table B-9. Tukey's HSD analysis for spinal SP-Immunoreactivity (QIHC – EU/pixel)

Section	(I) Grp	(J) Grp	Mean Diff(I-J)	SEM	Sig.	95% CI-Lower	95% CI-Upper
L1	1	2	46.5000	31.6098	0.4778	-44.6041	137.6041
		3	-1.7317	25.8093	0.9999	-76.1179	72.6545
		4	39.9089	28.8557	0.5281	-43.2574	123.0752
	2	1	-46.5000	31.6098	0.4778	-137.6041	44.6041
		3	-48.2317	31.6098	0.4475	-139.3358	42.8724
		4	-6.5911	34.1425	0.9973	-104.9948	91.8126
	3	1	1.7317	25.8093	0.9999	-72.6545	76.1179
		2	48.2317	31.6098	0.4475	-42.8724	139.3358
		4	41.6406	28.8557	0.4936	-41.5257	124.8068
	4	1	-39.9089	28.8557	0.5281	-123.0752	43.2574
		2	6.5911	34.1425	0.9973	-91.8126	104.9948
		3	-41.6406	28.8557	0.4936	-124.8068	41.5257
L2	1	2	13.3519	29.5356	0.9682	-71.7742	98.4780
		3	-13.6559	24.1157	0.9406	-83.1610	55.8493
		4	-1.4372	26.9622	0.9999	-79.1464	76.2719
	2	1	-13.3519	29.5356	0.9682	-98.4780	71.7742
		3	-27.0078	29.5356	0.7976	-112.1339	58.1183
		4	-14.7892	31.9021	0.9659	-106.7359	77.1575
	3	1	13.6559	24.1157	0.9406	-55.8493	83.1610
		2	27.0078	29.5356	0.7976	-58.1183	112.1339
		4	12.2186	26.9622	0.9680	-65.4905	89.9278
	4	1	1.4372	26.9622	0.9999	-76.2719	79.1464
		2	14.7892	31.9021	0.9659	-77.1575	106.7359
		3	-12.2186	26.9622	0.9680	-89.9278	65.4905
L6	1	2	2.7550	35.1456	0.9998	-98.5397	104.0497
		3	4.0541	28.6962	0.9989	-78.6527	86.7609
		4	39.8828	32.0834	0.6106	-52.5862	132.3518
	2	1	-2.7550	35.1456	0.9998	-104.0497	98.5397
		3	1.2991	35.1456	1.0000	-99.9956	102.5938
		4	37.1278	37.9615	0.7638	-72.2830	146.5386
	3	1	-4.0541	28.6962	0.9989	-86.7609	78.6527
		2	-1.2991	35.1456	1.0000	-102.5938	99.9956
		4	35.8287	32.0834	0.6852	-56.6403	128.2977
	4	1	-39.8828	32.0834	0.6106	-132.3518	52.5862
		2	-37.1278	37.9615	0.7638	-146.5386	72.2830
		3	-35.8287	32.0834	0.6852	-128.2977	56.6403
L7	1	2	-18.7320	38.0135	0.9594	-129.2209	91.7570
		3	-3.0398	31.0379	0.9996	-93.2536	87.1741
		4	-63.3003	38.0135	0.3768	-173.7892	47.1886
	2	1	18.7320	38.0135	0.9594	-91.7570	129.2209
		3	15.6922	38.0135	0.9754	-94.7967	126.1811
		4	-44.5683	43.8943	0.7434	-172.1499	83.0133
	3	1	3.0398	31.0379	0.9996	-87.1741	93.2536
		2	-15.6922	38.0135	0.9754	-126.1811	94.7967
		4	-60.2605	38.0135	0.4175	-170.7494	50.2284
	4	1	63.3003	38.0135	0.3768	-47.1886	173.7892
		2	44.5683	43.8943	0.7434	-83.0133	172.1499
		3	60.2605	38.0135	0.4175	-50.2284	170.7494

Table B-10. Tukey's HSD for gastrointestinal SP-Immunoreactivity (QIHC – EU/pixel)

Section	(I) Grp	(J) Grp	Mean Diff(I-J)	SEM	Sig.	95% CI-Lower	95% CI-Upper
R1	1	2	6.2406	32.4099	0.9973	-87.9611	100.4422
		3	-7.1699	27.7542	0.9937	-87.8394	73.4995
		4	-47.9365	29.5861	0.3994	-133.9305	38.0574
	2	1	-6.2406	32.4099	0.9973	-100.4422	87.9611
		3	-13.4105	33.4728	0.9774	-110.7015	83.8806
		4	-54.1771	35.0067	0.4374	-155.9265	47.5723
	3	1	7.1699	27.7542	0.9937	-73.4995	87.8394
		2	13.4105	33.4728	0.9774	-83.8806	110.7015
		4	-40.7666	30.7468	0.5626	-130.1341	48.6009
	4	1	47.9365	29.5861	0.3994	-38.0574	133.9305
		2	54.1771	35.0067	0.4374	-47.5723	155.9265
		3	40.7666	30.7468	0.5626	-48.6009	130.1341
R2	1	2	55.9847	38.7022	0.4930	-56.5059	168.4754
		3	18.7542	31.6002	0.9324	-73.0940	110.6024
		4	23.9025	38.7022	0.9248	-88.5881	136.3932
	2	1	-55.9847	38.7022	0.4930	-168.4754	56.5059
		3	-37.2306	38.7022	0.7726	-149.7212	75.2601
		4	-32.0822	44.6895	0.8883	-161.9752	97.8108
	3	1	-18.7542	31.6002	0.9324	-110.6024	73.0940
		2	37.2306	38.7022	0.7726	-75.2601	149.7212
		4	5.1484	38.7022	0.9991	-107.3423	117.6390
	4	1	-23.9025	38.7022	0.9248	-136.3932	88.5881
		2	32.0822	44.6895	0.8883	-97.8108	161.9752
		3	-5.1484	38.7022	0.9991	-117.6390	107.3423
C1	1	2	40.7222	48.4528	0.8344	-100.1090	181.5534
		3	-14.4775	40.1749	0.9833	-131.2486	102.2936
		4	-20.5413	44.5067	0.9662	-149.9030	108.8205
	2	1	-40.7222	48.4528	0.8344	-181.5534	100.1090
		3	-55.1997	46.9142	0.6506	-191.5589	81.1595
		4	-61.2635	50.6731	0.6315	-208.5483	86.0213
	3	1	14.4775	40.1749	0.9833	-102.2936	131.2486
		2	55.1997	46.9142	0.6506	-81.1595	191.5589
		4	-6.0638	42.8266	0.9989	-130.5421	118.4146
	4	1	20.5413	44.5067	0.9662	-108.8205	149.9030
		2	61.2635	50.6731	0.6315	-86.0213	208.5483
		3	6.0638	42.8266	0.9989	-118.4146	130.5421
C2	1	2	69.7556	51.8048	0.5548	-86.1535	225.6647
		3	36.8474	45.5007	0.8486	-100.0891	173.7839
		4	-66.0022	51.8048	0.5964	-221.9113	89.9069
	2	1	-69.7556	51.8048	0.5548	-225.6647	86.1535
		3	-32.9082	49.5349	0.9083	-181.9858	116.1694
		4	-135.7578	55.3817	0.1241	-302.4316	30.9160
	3	1	-36.8474	45.5007	0.8486	-173.7839	100.0891
		2	32.9082	49.5349	0.9083	-116.1694	181.9858
		4	-102.8496	49.5349	0.2199	-251.9272	46.2280
	4	1	66.0022	51.8048	0.5964	-89.9069	221.9113
		2	135.7578	55.3817	0.1241	-30.9160	302.4316
		3	102.8496	49.5349	0.2199	-46.2280	251.9272

APPENDIX C
CORRELATIONS

Table C-1. Correlation between weekly ABTP and histological scores.

		R1Ed	R2Ed	C1Ed	C2Ed	R1Lym	R2Lym	C1Lym	C2Lym
WK7	Pearson	0.5748	0.0016	0.5309	-0.2071	-0.2221	-0.3288	-0.0819	-0.1467
	Sig.	0.1055	0.9967	0.1414	0.5929	0.5656	0.3876	0.8342	0.7064
	N	9	9	9	9	9	9	9	9
WK8	Pearson	0.3911	-0.0343	0.5000	-0.3129	-0.2786	-0.4649	-0.1237	-0.1789
	Sig.	0.2980	0.9301	0.1705	0.4124	0.4678	0.2073	0.7513	0.6451
	N	9	9	9	9	9	9	9	9
WK9	Pearson	0.1835	-0.3763	0.4941	0.0226	-0.1318	-0.3527	-0.0580	-0.3731
	Sig.	0.6365	0.3182	0.1764	0.9540	0.7354	0.3519	0.8821	0.3227
	N	9	9	9	9	9	9	9	9
WK10	Pearson	-0.1127	-0.0591	-0.2116	-0.0964	0.1248	-0.7156	-0.2305	-0.0367
	Sig.	0.7566	0.8712	0.5573	0.7911	0.7312	0.0200	0.5217	0.9198
	N	10	10	10	10	10	10	10	10
WK11	Pearson	0.0179	0.0062	-0.3151	-0.0490	0.2922	-0.5014	-0.4092	-0.1950
	Sig.	0.9609	0.9865	0.3751	0.8930	0.4126	0.1398	0.2403	0.5892
	N	10	10	10	10	10	10	10	10
WK12	Pearson	0.2052	0.1763	-0.1177	0.1953	0.1483	-0.6532	-0.4057	-0.4032
	Sig.	0.5696	0.6262	0.7461	0.5888	0.6827	0.0405	0.2448	0.2480
	N	10	10	10	10	10	10	10	10
WK13	Pearson	0.5357	0.3173	-0.0761	0.3515	0.2669	-0.2148	-0.2030	-0.3455
	Sig.	0.1105	0.3717	0.8346	0.3193	0.4560	0.5513	0.5738	0.3281
	N	10	10	10	10	10	10	10	10
WK14	Pearson	0.2509	0.1209	-0.0467	0.1958	0.5130	-0.2266	-0.2653	-0.0735
	Sig.	0.4844	0.7394	0.8982	0.5877	0.1294	0.5289	0.4588	0.8402
	N	10	10	10	10	10	10	10	10

Table C-2. Correlation between endoscopic histological scores.

		ENDO6	ENDO9	ENDO14
R1ED	Pearson	-0.2507	-0.0909	-0.6325
	Sig.	0.3006	0.7286	0.1778
	N	19	17	6
R2ED	Pearson	0.0119	-0.1300	-0.7071
	Sig.	0.9613	0.6189	0.1161
	N	19	17	6
C1ED	Pearson	0.0576	0.1240	0.3162
	Sig.	0.8147	0.6354	0.5415
	N	19	17	6
C2ED	Pearson	-0.0819	-0.0731	0.0000
	Sig.	0.7389	0.7803	1.0000
	N	19	17	6
R1LYM	Pearson	-0.0310	-0.0473	-0.3162
	Sig.	0.8997	0.8571	0.5415
	N	19	17	6
R2LYM	Pearson	0.0946	0.0711	-0.9258
	Sig.	0.7002	0.7864	0.0080
	N	19	17	6
C1LYM	Pearson	-0.1570	-0.0574	-0.5000
	Sig.	0.5208	0.8267	0.3125
	N	19	17	6
C2LYM	Pearson	0.0908	0.1718	-0.6124
	Sig.	0.7118	0.5096	0.1963
	N	19	17	6

Table C-3. Correlation between weekly ABTP and endoscopic scores.

		ENDO6	ENDO9	ENDO14
WK7ALT	Pearson	-0.3344	-0.2093	0.6171
	Sig.	0.1617	0.4201	0.1918
	N	19	17	6
WK8ALT	Pearson	-0.3366	-0.1315	0.4796
	Sig.	0.1588	0.6148	0.3358
	N	19	17	6
WK9ALT	Pearson	-0.0883	0.1023	0.5377
	Sig.	0.7193	0.6961	0.2712
	N	19	17	6
WK10ALT	Pearson	-0.1579	-0.2787	0.8015
	Sig.	0.6630	0.4355	0.0552
	N	10	10	6
WK11ALT	Pearson	-0.1981	-0.3012	0.3543
	Sig.	0.5833	0.3977	0.4908
	N	10	10	6
WK12ALT	Pearson	-0.2275	-0.3777	0.7039
	Sig.	0.5274	0.2820	0.1185
	N	10	10	6
WK13ALT	Pearson	-0.6594	-0.6798	0.0982
	Sig.	0.0381	0.0306	0.8532
	N	10	10	6
WK14ALT	Pearson	-0.4252	-0.4966	0.2728
	Sig.	0.2206	0.1443	0.6010
	N	10	10	6

Table C-4. Correlation between weekly ABTP and ventral horn SP-immunoreactive neurons.

		L1	L2	L6	L7
WK7	Pearson	-0.5814	-0.0524	0.1507	0.2590
	Sig.	0.1006	0.8935	0.6987	0.5009
	N	9	9	9	9
WK8	Pearson	-0.6872	0.1131	0.4095	0.0036
	Sig.	0.0408	0.7720	0.2738	0.9927
	N	9	9	9	9
WK9	Pearson	-0.5938	-0.3538	0.3460	0.4836
	Sig.	0.0919	0.3503	0.3617	0.1872
	N	9	9	9	9
WK10	Pearson	-0.2473	-0.4879	-0.2496	-0.1101
	Sig.	0.4909	0.1525	0.4867	0.7781
	N	10	10	10	9
WK11	Pearson	-0.0777	-0.6905	-0.4310	-0.5102
	Sig.	0.8311	0.0271	0.2136	0.1605
	N	10	10	10	9
WK12	Pearson	-0.4260	-0.7116	-0.5183	-0.4932
	Sig.	0.2196	0.0210	0.1248	0.1773
	N	10	10	10	9
WK13	Pearson	-0.4349	-0.6392	-0.6996	-0.6881
	Sig.	0.2091	0.0466	0.0243	0.0405
	N	10	10	10	9
WK14	Pearson	-0.1321	-0.7111	-0.6323	-0.5071
	Sig.	0.7161	0.0211	0.0498	0.1636
	N	10	10	10	9

Table C-5. Correlation between weekly ABTP and dorsal horn SP-Immunoreactivity (EU/pixel).

		L1	L2	L6	L7
WK7	Pearson	-0.5990	-0.1063	0.4221	0.6345
	Sig.	0.0883	0.7854	0.2578	0.0664
	N	9	9	9	9
WK8	Pearson	-0.4369	0.0404	0.6071	0.2733
	Sig.	0.2396	0.9177	0.0829	0.4767
	N	9	9	9	9
WK9	Pearson	-0.7536	-0.4110	0.2366	0.4278
	Sig.	0.0190	0.2718	0.5400	0.2507
	N	9	9	9	9
WK10	Pearson	-0.2153	-0.1344	-0.0697	-0.5936
	Sig.	0.5502	0.7112	0.8483	0.0920
	N	10	10	10	9
WK11	Pearson	-0.3204	-0.1908	-0.3231	-0.9589
	Sig.	0.3667	0.5974	0.3625	0.0000
	N	10	10	10	9
WK12	Pearson	-0.3894	-0.3100	-0.3341	-0.6829
	Sig.	0.2660	0.3834	0.3455	0.0426
	N	10	10	10	9
WK13	Pearson	-0.5911	-0.3393	-0.5613	-0.3126
	Sig.	0.0719	0.3375	0.0914	0.4128
	N	10	10	10	9
WK14	Pearson	-0.4596	-0.1198	-0.2605	-0.7598
	Sig.	0.1815	0.7417	0.4672	0.0175
	N	10	10	10	9

Table C-6. Correlation between histological scores and ventral horn SP-Immunoreactive neurons.

		L1	L2	L6	L7
R1ED	Pearson	-0.5195	-0.2684	-0.3009	-0.0790
	Sig.	0.0226	0.2666	0.2106	0.7555
	N	19	19	19	18
R2ED	Pearson	-0.4454	-0.3154	-0.3382	-0.3710
	Sig.	0.0560	0.1885	0.1567	0.1296
	N	19	19	19	18
C1ED	Pearson	-0.1880	0.0805	0.0469	0.0042
	Sig.	0.4409	0.7432	0.8488	0.9868
	N	19	19	19	18
C2ED	Pearson	-0.3785	-0.1591	-0.2429	-0.0721
	Sig.	0.1101	0.5154	0.3163	0.7762
	N	19	19	19	18
R1LYM	Pearson	-0.1895	-0.5465	-0.4168	-0.4302
	Sig.	0.4372	0.0155	0.0759	0.0748
	N	19	19	19	18
R2LYM	Pearson	0.4050	0.3388	0.0349	-0.1559
	Sig.	0.0854	0.1559	0.8873	0.5367
	N	19	19	19	18
C1LYM	Pearson	-0.0044	0.0048	0.1901	-0.1278
	Sig.	0.9856	0.9846	0.4356	0.6133
	N	19	19	19	18
C2LYM	Pearson	0.1984	0.0774	0.1477	-0.2713
	Sig.	0.4155	0.7529	0.5461	0.2761
	N	19	19	19	18

Table C-7. Correlation between histological scores and dorsal horn SP-immunoreactivity (EU/pixel).

		L1	L2	L6	L7
R1ED	Pearson	-0.2147	0.0891	-0.0186	0.4321
	Sig.	0.3773	0.7168	0.9398	0.0734
	N	19	19	19	18
R2ED	Pearson	-0.3134	-0.1273	-0.1942	0.2624
	Sig.	0.1913	0.6035	0.4256	0.2929
	N	19	19	19	18
C1ED	Pearson	-0.0161	0.1654	0.3380	0.2725
	Sig.	0.9479	0.4985	0.1569	0.2740
	N	19	19	19	18
C2ED	Pearson	-0.1937	0.0451	-0.1003	0.2545
	Sig.	0.4269	0.8544	0.6829	0.3081
	N	19	19	19	18
R1LYM	Pearson	-0.3227	-0.0766	-0.4582	-0.2263
	Sig.	0.1778	0.7554	0.0485	0.3666
	N	19	19	19	18
R2LYM	Pearson	0.2614	0.3601	-0.1268	0.1989
	Sig.	0.2797	0.1299	0.6049	0.4289
	N	19	19	19	18
C1LYM	Pearson	-0.0575	-0.2722	-0.0193	0.3424
	Sig.	0.8152	0.2596	0.9375	0.1643
	N	19	19	19	18
C2LYM	Pearson	0.1942	0.3416	-0.1088	0.0140
	Sig.	0.4257	0.1523	0.6574	0.9561
	N	19	19	19	18

Table C-8. Correlation between histological scores and gastrointestinal SP-immunoreactivity (EU/pixel).

		R1	R2	C1	C2
R1ED	Pearson	0.2629	0.2498	-0.0916	0.1728
	Sig.	0.2919	0.3174	0.7179	0.5380
	N	18	18	18	15
R2ED	Pearson	0.2812	0.2003	-0.0275	0.2655
	Sig.	0.2584	0.4254	0.9137	0.3389
	N	18	18	18	15
C1ED	Pearson	0.3411	0.3058	-0.0544	0.0361
	Sig.	0.1660	0.2172	0.8302	0.8985
	N	18	18	18	15
C2ED	Pearson	0.5171	0.4543	0.0105	0.0215
	Sig.	0.0280	0.0582	0.9669	0.9394
	N	18	18	18	15
R1LYM	Pearson	0.4296	0.0837	-0.0916	-0.3919
	Sig.	0.0752	0.7412	0.7177	0.1485
	N	18	18	18	15
R2LYM	Pearson	0.3943	0.3397	0.5347	0.4025
	Sig.	0.1054	0.1678	0.0222	0.1370
	N	18	18	18	15
C1LYM	Pearson	0.7106	0.1241	0.2472	0.2224
	Sig.	0.0010	0.6236	0.3227	0.4257
	N	18	18	18	15
C2LYM	Pearson	0.3322	-0.1034	0.0914	0.1333
	Sig.	0.1780	0.6832	0.7185	0.6359
	N	18	18	18	15

Table C-9. Correlation between weekly ABTP and gastrointestinal SP-immunoreactivity (EU/pixel).

		R1	R2	C1	C2
WK7	Pearson	-0.5907	-0.2644	-0.5939	-0.5692
	Sig.	0.0939	0.4918	0.1206	0.1823
	N	9	9	8	7
WK8	Pearson	-0.5229	-0.2624	-0.6841	-0.3253
	Sig.	0.1486	0.4951	0.0613	0.4765
	N	9	9	8	7
WK9	Pearson	-0.2196	-0.4166	-0.4460	-0.6606
	Sig.	0.5702	0.2647	0.2680	0.1062
	N	9	9	8	7
WK10	Pearson	-0.2325	-0.5033	-0.5639	-0.4152
	Sig.	0.5472	0.1672	0.0895	0.3064
	N	9	9	10	8
WK11	Pearson	-0.4332	-0.1295	-0.2594	-0.6313
	Sig.	0.2442	0.7398	0.4693	0.0932
	N	9	9	10	8
WK12	Pearson	-0.3518	-0.1994	-0.5293	-0.4983
	Sig.	0.3531	0.6071	0.1156	0.2088
	N	9	9	10	8
WK13	Pearson	-0.1143	0.1269	-0.3005	-0.2102
	Sig.	0.7697	0.7449	0.3989	0.6173
	N	9	9	10	8
WK14	Pearson	-0.1242	-0.0231	-0.2996	-0.5600
	Sig.	0.7502	0.9529	0.4003	0.1489
	N	9	9	10	8

Table C-10. Correlation between dorsal horn SP-immunoreactivity (EU/pixel) and gastrointestinal SP-immunoreactivity (EU/pixel).

		R1	R2	C1	C2
L1	Pearson	0.0226	0.3396	0.4028	0.4250
	Sig.	0.9291	0.1679	0.0975	0.1143
	N	18	18	18	15
L2	Pearson	-0.0317	0.3874	0.1117	0.3825
	Sig.	0.9007	0.1122	0.6591	0.1594
	N	18	18	18	15
L6	Pearson	-0.1789	0.1665	-0.0111	0.1565
	Sig.	0.4776	0.5089	0.9650	0.5774
	N	18	18	18	15
L7	Pearson	0.4127	-0.0619	0.2964	0.5232
	Sig.	0.0997	0.8133	0.2480	0.0549
	N	17	17	17	14

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BIOGRAPHICAL SKETCH

Linda Christine (Chris) Sanchez was born in Clearwater, Florida in 1970. After attending public schools in Pinellas County, she enrolled at the University of Florida in 1988. Throughout her undergraduate studies, Chris played tenor saxophone in the University of Florida marching and jazz bands. She earned her Doctor of Veterinary Medicine degree from the University of Florida in 1995, and completed in internship in Equine Medicine and Surgery at a private clinic, Equine Medical Associates in Edmond, Oklahoma in 1996.

Chris returned to the UF College of Veterinary Medicine for a residency program in Large Animal Internal Medicine and was awarded Diplomate status in the American College of Veterinary Internal Medicine, specialty of Internal Medicine in 1999. She was awarded an Alumni Fellowship to pursue her graduate education in the Island Whirl Equine Colic Research Laboratory following the completion of her residency.

Chris has been appointed to the Faculty of the UF College of Veterinary Medicine and will join the Large Animal Medicine service of the Department of Large Animal Clinical Sciences following completion of this degree. Her outside interests include running, cycling, scuba diving, and snow skiing.