The role of primary afferents from different levels of the neuraxis in colon function

by

Kimberly A. Meerschaert

Bachelor of Arts, Saginaw Valley State University, 2015

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This dissertation was presented

by

Kimberly A. Meerschaert

It was defended on

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and approved by

Kathryn M. Albers, Professor, Neurobiology

Michael S. Gold, Professor, Neurobiology

H. Richard Koerber, Professor, Neurobiology

David J. Levinthal, Assistant Professor, Gastroenterology, Hepatology, and Nutrition

Sarah E. Ross, Associate Professor, Neurobiology

Julie A. Christianson, Professor, Anatomy and Cell Biology

Dissertation Director: Brian M. Davis, Professor, Neurobiology

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Kimberly A. Meerschaert, PhD

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Visceral organs receive sensory innervation from primary afferents arising from multiple levels of the neuroaxis. The reason for these different levels of sensory innervation remains a mystery. The colon receives extrinsic primary afferent neuron (ExPAN) input from thoracolumbar (TL; T10-L2) and lumbosacral spinal levels (LS; L5-S1) and the nodose ganglion (NG). Hypotheses for this dual innervation include: a) that different levels of innervation are involved in different qualitative aspects of pain, b) that different levels are important for the integration of autonomic function, or c) that different levels play complementary roles in immune modulation. The first step in discerning the role of these different afferents is to characterize the molecular identity of afferents from different levels innervating the same organ. This was accomplished by creating a molecular profile of colon afferents from TL, LS, and NG using single cell RT-qPCR. Clustering of colon afferents based on gene expression revealed unique TL, LS, and NG clusters. Next, processes of extrinsic afferents course throughout the myenteric ganglia of the enteric nervous system, where data suggest that they have local effector functions through release of glutamate and/or neuropeptides (e.g. CGRP, substance P) or act indirectly through autonomic reflexes. However, there is little known on how different ExPANs modulate myenteric activity, nor how this may change in inflammatory states. Calcium imaging and optogenetic studies revealed the LS level is largely unaffected by colitis, however, the TL and NG afferents had increased activation after colonic inflammation. Finally, colitis induced changes in colon motility

evoked by autonomic efferent fibers from different levels of the neuraxis was examined. Although TL sympathetic neurons were altered by colitis, the LS parasympathetics were unchanged. Results from these studies indicate that different levels of the neuraxis have unique and overlapping functions during naïve states, and colonic inflammation alters specific levels of the neuraxis.

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Preface

This dissertation would not have been possible without the help and support of several people. First, I must thank my dissertation advisor Dr. Brian Davis. Brian has been a wonderful mentor and has helped me grow as a scientist. Thank you for believing in me and trying your hardest to make me believe in myself. I also want to thank the chair of my dissertation committee, Dr. Kathryn Albers, for always being available for help and support and for trying to keep Brian in check. I was given advice after my first rotation to rotate with Brian or Kathy because they genuinely care about their mentees. After being in their lab for five years, I can say this is true and I am glad I took that advice. Thank you, Brian and Kathy, for caring about me as a scientist but also as a person.

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Finally, I would like to thank my family for their love and support. My parents, Tom and Mary, who never put any limits on what I could become. And of course, my husband Scott.

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1.0 Introduction

1.1 Colon Anatomy

The colon is connected to the small intestine and rectum in the gastrointestinal (GI) tract and its main function is the reabsorption of fluids and processing of waste for removal. In human, the colon consists of the cecum, ascending colon, transverse colon, descending colon, and sigmoid colon (Ellis & Mahadevan, 2014). The cecum is located in the lower right side of the abdomen at the attachment site of the appendix. The main functions of the cecum are to absorb fluids and salts that remain after completion of intestinal digestion and to mix intestinal contents with mucus to facilitate movement. The ascending colon projects superiorly from the cecum to the transverse colon. The juncture between the cecum and transverse colon is called the right colic flexure, or the hepatic flexure. The ascending colon is responsible for water absorption and mixing of fecal content. The transverse colon connects the ascending and descending colon, traveling lengthwise across the abdomen. The transverse colon is also involved in absorption of water and salt and in formation of stool. The descending colon begins at the left colic flexure, also known as the splenic flexure. This portion of the colon lies in the left side of the abdomen, connecting the transverse colon to the sigmoid colon. The main function of the descending colon is to store waste and move stool toward the rectum. The sigmoid colon makes up the last 30-40 centimeters of the colon leading to the rectum and is largely responsible for the storage of stool until defecation occurs.

The colon is a hollow tube organ that consists of multiple layers that include the mucosa, submucosa, muscularis propria, and serosa (Rao & Wang, 2010). The mucosa is the inner most layer interfacing with the lumen and consists of the epithelium, lamina propria, and muscular

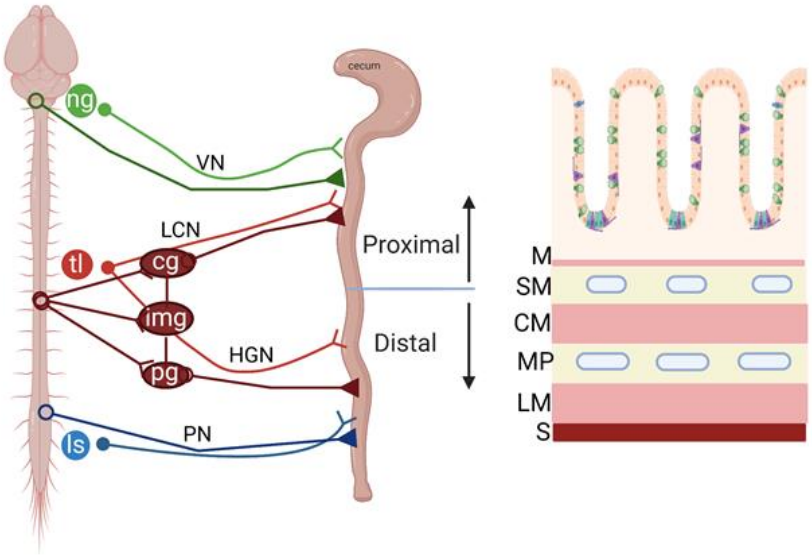

Figure 1 Schematic of neural innervation of the colon.

The vagus nerve (green) contains parasympathetic neurons and afferents originating in the nodose ganglia. The vagus nerve innervates the proximal and distal colon. The TL spinal cord (red) contains preganglionic sympathetic fibers that synapse onto prevertebral ganglia. The postganglionic sympathetic neurons located in the prevertebral ganglia innervate the colon through the hypogastric nerve and the lumbar colonic nerve. Sensory afferents originating in the TL DRG innervates proximal and distal colon. The LS spinal cord (blue) has parasympathetic fibers that innervate the colon through the pelvic nerve. The LS DRG neurons innervate the distal colon. The layered structure of the colon and intrinsic ENS ganglia are also shown. ng-nodose ganglia, tl-thoracolumbar, ls-lumbosacral, VN-vagus nerve, LCN-lumbar colonic nerve, HGN-hypogastric nerve, PN-pelvic nerve, cg-celiac ganglia, img-inferior mesenteric ganglia, pg-pelvic ganglia, M-mucosa, SM-submucosa, CM-circular muscle, MP-myenteric plexus, LS-longitudinal muscle, S-serosa. Created with BioRender.com.

mucosa (**Figure 1**). The first layer facing the intestinal lumen comprises of a single layer of epithelial cells that is attached to a basement membrane overlying the second layer, the lamina propria. The lamina propria consists of subepithelial connective tissue and lymph nodes, underneath which is the third and deepest layer called muscularis mucosa. This is a continuous sheet of smooth muscle cells that lies at the base of the lamina propria. The submucosa contains thick connective tissue that is highly vascularized and supports the mucosa. The submucosa joins

the mucosa to the muscularis propria. The submucosa plexus (or Meissner's plexus) is also found in this layer. The muscularis propria contains the inner circular muscle and outer longitudinal muscle layers. The myenteric plexus (or Auerbach's plexus) lies between the two muscle layers. The serosa is the outer most layer and is a smooth membrane that consists of a thin connective tissue layer and a thin layer of cells that secrete serous fluid.

The colon is innervated by extrinsic sensory and autonomic fibers (**Fig 1.**). The vagus nerve contains afferent neurons (located in the jugular and nodose ganglia) and efferent parasympathetic neurons (located in the dorsal motor nucleus of the vagus and nucleus ambiguus). In humans, the vagus nerve is thought to innervate the ascending and first half of the transverse colon (Berthoud & Neuhuber, 2000). In animal models, vagal innervation has been reported throughout the length of the colon (Altschuler, Escardo, Lynn, & Miselis, 1993; F. Wang & Powley, 2000). An additional source of parasympathetic efferent fibers, which travel through the pelvic nerve, arise from the sacral spinal cord to innervate the descending colon and rectum. Lumbosacral (LS) dorsal root ganglia (DRG) also contain colon afferents that travel through the pelvic nerve to innervate the distal colon. The thoracolumbar (TL) DRG contain an additional source of spinal afferents that innervate the proximal and distal colon. The sympathetic preganglionic efferent fibers are found in the TL spinal cord in the intermediolateral nucleus (IML) and project to postganglionic sympathetic fibers in the prevertebral ganglia. The prevertebral ganglia that innervate the colon consists of the celiac ganglia (CG), inferior mesenteric ganglia (IMG), and pelvic ganglia (PG). The postganglionic sympathetic fibers within these prevertebral ganglia innervate the colon via the lumbar colonic nerves, and the hypogastric nerves.

Mouse and human GI tracts are quite similar in physiology and anatomical structures. However, prominent differences are found that may be influenced by differences in diet, feeding

patterns, body sizes and metabolic requirements (Nguyen, Vieira-Silva, Liston, & Raes, 2015). Both mouse and human have the same distinct layers of the colon and similar cell types. The most obvious anatomical difference is the human colon has clear anatomical divisions and is sub-compartmentalized into pouches (called haustra), whereas the mouse colon does not have clear anatomical divisions and is relatively smooth. In addition, the mouse cecum is large relative to its total GI tract and is an important site for the fermentation of plant materials. By contrast, the human cecum is relatively small, with an anatomical structure similar to that of the colon and does not have a distinct function (Treuting, Arends, & Dintzis, 2018). Overall, the mammalian digestive tract is highly conserved, with major differences between species being likely driven by evolutionary adaptations to species specific diet.

1.2 Sensory Innervation of the Colon

Primary afferents survey the environment via peripheral endings in the target organ. Information is transferred to the central nervous system through central terminals that project into the spinal cord or brainstem. Visceral organs receive sensory innervation from primary sensory neurons arising from multiple levels of the neuraxis. The colon is unique in that it receives sensory neurons from three level of the neuraxis; vagal afferents originating from the nodose ganglia (NG), thoracolumbar (TL) spinal afferents arising from the lower thoracic and upper lumbar dorsal root ganglia (DRG), and lumbosacral spinal afferents originating from the lower lumbar and upper sacral DRG. Sensory neuron afferents travel to the periphery in nerve bundles that also contain efferent neurons from the autonomic nervous system. The vagal and LS spinal neurons travel with parasympathetic fibers whereas the TL spinal afferents travel with sympathetic fibers. It is

important to note that vagal afferents reside in two distinct ganglia from different embryological origins in humans. The jugular or superior vagal ganglia originates from the neural crest whereas, the nodose or inferior vagal ganglia is embryologically derived from the epibranchial placodes (Baker & Schlosser, 2005). In the mouse, these ganglia are fused although staining with markers of neural crest or placodal tissue show there are distinct portions of the fused ganglia that are jugular and nodose in origin (Nassenstein et al., 2010). It is likely that vagal afferents innervating the colon are nodose specific afferents as thoracic viscera are known to receive a much richer jugular afferent innervation than subdiaphragmatic organs (Hayakawa, Kuwahara-Otani, Maeda, Tanaka, & Seki, 2011). Therefore, vagal afferents will be referred to as originating from the nodose ganglia throughout this document, although it is possible a small number of afferents innervating the colon are jugular in origin.

The extent of vagal afferent innervation has been debated throughout history, with most early references suggesting the vagus nerve does not innervate the distal colon. Early reports describing vagal innervation in human colon suggested innervation extended only to the transverse colon (Lannon & Weller, 1947; Woodburne, 1956). However, these studies noted that vagal fibers that pass through the celiac ganglia would be hard to distinguish from sympathetic fibers. Furthermore, Schmidt (Schmidt, 1933) performed vagotomy or pelvic nerve resection in cats and dog and examined nerve degeneration in the large intestine. Vagotomy caused degenerate nerves in the proximal colon but not distal, whereas pelvic nerve resection caused nerve degeneration throughout the colon. Varying effects of vagus nerve stimulation on colon function have also been reported. Vagus nerve stimulation produced no activity in the colon of dog (Bayliss & Starling, 1900; Wells, Mercer, Gray, & Ivy, 1942), a slight but inconsistent contraction of the cecum in pig and monkey (Wells et al., 1942), a strong contraction of the cecum in rabbit (Meltzer & Auer,

1907) and contraction of the ascending colon in cat (Hultén & Jodal, 1969). In contrast, vagus nerve stimulation did cause contraction of the proximal colon and sometimes distal colon in dog (Gray, Hendershot, Whitrock, & Seevers, 1955). Further evidence for vagal innervation of distal colon has been found in ulcerative colitis patients who received vagotomy. Dennis et al. (Dennis, Eddy, Frykman, McCarthy, & Westover, 1948) found patients with vagotomy had a diminished vascular response to a stress reflex in the rectum and stated "The effect of vagotomy on the vascular response of the rectum to stress is as if vagal innervation includes the rectum."

Advances in neural tracing techniques and electrical stimulation studies have provide additional support for vagal innervation throughout the entire colon. Tracing studies from the dorsal motor nucleus of the Vagus (DMN) to the cecum and colon show terminals associated with the myenteric ganglia throughout the colon of the rat, with decreased innervation in the distal colon (Altschuler et al., 1993; Berthoud, Carlson, & Powley, 1991). Vagus nerve stimulation has also been shown to induce contractions in both proximal and distal colon in rat (Berthoud et al., 1991; Tong, Ridolfi, Kosinski, Ludwig, & Takahashi, 2010), nonhuman primates (Dapoigny, Cowles, Zhu, & Condon, 1992), and pig (Larauche et al., 2020).

Colonic primary afferents have been described based on morphology and response properties. To date, the structure and function of most visceral afferent subtypes have not been coordinated in the same experiment although the structure/function relationship has been speculated upon (Grundy, 1988). Several peripheral terminals and functional subtypes have been described in the colon, with different levels of the neuraxis having both overlapping and unique morphology and function.

The electrophysiological properties of vagal afferents innervating the colon have not been well characterized, however, the morphology of vagal nerve endings has been well described.

Three distinct types of vagal afferents occur in the gastrointestinal tract, intraganglionic laminar endings (IGLEs), intramuscular arrays (IMAs) and mucosal nerve endings (Berthoud, Jedrzejewska, & Powley, 1990; Grundy, 2002; Powley & Phillips, 2002). IGLEs terminate in the myenteric plexus of the enteric nervous system (ENS) and form intimate contact with myenteric ganglia. Electrophysiological recordings with dye filling have identified IGLEs as low threshold tension receptors(Tassicker, Hennig, Costa, & Brookes, 1999; Zagorodnyuk, Chen, & Brookes, 2001). IMAs are single afferent axons that branch within the circular muscle layer and run parallel to muscle bundles. These endings form synapse like connections with intramuscular interstitial cells of Cajal (ICCs) and are thought to work together with ICCs and smooth muscle cells to transduce muscle length or stretch information (Powley & Phillips, 2011). Currently, no electrophysiological recordings from vagal afferents have been found to correspond to IMAs. The last classification of vagal nerve terminals are mucosal endings. Several types of mucosal endings have been identified, including endings that terminate in the villi of the small intestines (villus) and within intestinal crypts. Villus afferents project up to the villi and branch extensively along the internal surface of the villus epithelium up to the apical tips. These afferents are ideally positioned to detect substances released from epithelial cells (Collins, Surette, & Bercik, 2012). In contrast, crypt afferents form subepithelial rings below the crypt-villus junction. Single fiber anterograde tracing has found that villus and crypt afferents originate from distinct afferents (Powley, Spaulding, & Haglof, 2011). Although the peripheral terminals of vagal afferents in the colon are not as well characterized gastrointestinal organs, all of the subtypes of nerve endings described above have been reported in the colon (Berthoud et al., 1990; Berthoud, Patterson, Neumann, & Neuhuber, 1997; F. Wang & Powley, 2000).

In contrast to vagal afferents, the electrophysiological properties of spinal afferents have been well characterized. Interestingly, TL and LS colon afferents have both unique and overlapping functional afferent subtypes. Five colon afferent fiber types have been identified (mucosal, muscular, serosal, mesenteric, and muscular/mucosal) (Brierley et al., 2005; Brierley, Jones, Gebhart, & Blackshaw, 2004). These classifications are based on the responses to different types of stimulation to the colon; blunt probing of the colon with a von Frey hair, mucosal stroking with a von Frey hair, and circumferential stretch. Mucosal afferents respond to both probing and mucosal stroking but not stretch. Mucosal afferents are low threshold responders and are thought to play a role in detecting luminal contents as part of defecatory reflexes (Brierley, Hibberd, & Spencer, 2018). Muscular afferents respond to probing and stretch but not mucosal stroking. Muscular afferents include both high threshold fibers with the response properties of dedicated nociceptors (i.e., the ability to encode noxious input via proportional increases in action potential firing, (Sherrington, 1906)), as well as low threshold mechanoreceptors with a slowly adapting response to low intensity stretch (Brierley et al., 2004; Hughes et al., 2009; Malin, Christianson, Bielefeldt, & Davis, 2009). The low threshold fibers likely respond to physiological levels of distention caused by the passage of fecal matter in the distal colon (Brierley et al., 2018). Serosal afferents only respond to blunt probing and are hypothesized to be involved in sharp transient pain associated with spasm or distention (Brierley et al., 2004). However, it should be noted that Spencer et al. (Spencer, Kyloh, & Duffield, 2014) could not identify fibers terminating in this layer of the colon (see below). Mesenteric afferents respond to probing on their receptive fields and mesenteric stretch and has been proposed to detect twisting and torsion of the colon (Lynn & Blackshaw, 1999). Muscular/mucosal afferents respond to all three stimuli and are uniquely situated to relay a diversity of mechanical events to the spinal cord (Brierley et al., 2004). In

addition to these five subtypes, afferents that are initially mechanically insensitive but subsequently respond to mechanical stimuli after exposure to chemicals or inflammatory mediators are termed mechanically insensitive or "silent" afferents. Three subtypes of silent afferents have been described, chemically sensitive afferents that do not become mechanosensitive after chemical stimuli (Brierley et al., 2005), afferents that are not chemically sensitive but gain mechanosensitivity (Feng & Gebhart, 2011), and afferents that are chemically sensitive and mechanically sensitized (Feng & Gebhart, 2011). Both TL and LS have mucosal, muscular, and serosal afferents whereas mesenteric afferents are specific to TL neurons and muscular/mucosal afferents are specific to LS afferents (Brierley et al., 2004). The proportion of subtypes also different between TL and LS afferents. LS afferents are almost evenly distributed between mucosal (23%), muscular (21%), serosal (33%), and muscular/mucosal (23%). In contrast, the majority of TL afferents are mesenteric (50%) and serosal (36%), with a small percentage being mucosal (4%) and muscular (10%) (Brierley et al., 2004).

The morphology of spinal afferents has been investigated using *in vitro* anterograde tracing from mixed nerve trunk as the pelvic or mesenteric nerves enter the gut wall (Song et al., 2009; Spencer, Kerrin, Singer, et al., 2008; Spencer, Kerrin, Zagorodnyuk, et al., 2008). These studies have identified IGLEs in the rectum, intramuscular endings in the smooth muscle, and endings on myenteric ganglia. However, the methods used in the studies can label other extrinsic fibers (e.g. parasympathetic, intestinofugal) so it is possible these nerve endings are not specific to spinal afferents. To address this issue, a recent study directly injected biotinylated dextan biotin into the L6 and S1 DRG to selectively label LS afferents and traced afferent terminals in the colon (Spencer et al., 2014). Spencer et al. found thirteen distinct types of peripheral terminals with most in the submucosa, circular muscle, and myenteric ganglia. Endings were classified as "simple" (few

branches with relatively long axon with unbranching terminals), “branching” (numerous branching axons that lay in the circumferential or longitudinal direction), or “complex” (numerous varicose branches arising from one axon without any preferential directionality in the circumferential or longitudinal orientation). Simple, branching, and complex endings were found in the mucosa and circular muscle. The most common terminals in the myenteric ganglia were intraganglionic varicose endings. Rarely, IGLEs were found in both myenteric and submucosal ganglia which are morphologically similar to IGLEs originating from vagal afferents but tend to have less extensive branching patterns (S. J. Brookes, Spencer, Costa, & Zagorodnyuk, 2013). A small proportion of afferents innervated blood vessels, longitudinal muscle, mucosa, and internodal strands of myenteric ganglia. Interestingly, no fibers were found to terminate in the serosa, and thus there is no anatomical counterpart to fibers functionally described as “serosal”. Similar studies have not been performed for TL afferents and the morphology of these afferents is largely unknown. The differences in morphology and functional properties suggests that afferents arising from different levels of the neuraxis are likely involved in different colon functions.

1.3 Functions of Visceral Afferents

1.3.1 Visceral Pain

Visceral pain is associated with diseases from almost all thoracic and abdominal organs including esophagus, stomach, lungs, small intestines, and colon (Alemi et al., 2013; Cervero & Laird, 1999; Collett, 2013; Edwards & Coghill, 1968; Fass & Navarro-Rodriguez, 2008; Gasbarrini et al., 2007; Kappelman et al., 2007; R. Lee, Hodgson, Jackson, & Adams, 2012).

Visceral pain disorders contribute to a significant burden on healthcare resources and decrease the quality of life of patients (Sikandar & Dickenson, 2012). Classically, it has been hypothesized that spinal afferents, but not vagal afferents, relay nociceptive information (that is perceived by the brain as pain), whereas vagal afferents are thought to be responsible for the affective aspect of visceral pain including depression, anxiety, nausea and fear (Altschuler et al., 1993; Berthoud & Neuhuber, 2000; Grundy, 2002; Sengupta, 2009). However, recent evidence has shown that vagal afferents may have a significant role in nociception and pain.

Vagal afferents that can detect noxious stimuli have been identified in the esophagus (Kollarik, Ru, & Undem, 2007; Yu & Ouyang, 2009; Yu, Ru, Ouyang, & Kollarik, 2008; Yu, Undem, & Kollarik, 2005), lungs (Canning, Mori, & Mazzone, 2006; L. Y. Lee & Pisarri, 2001; L. Y. Lee, Shuei Lin, Gu, Chung, & Ho, 2003; Nassenstein et al., 2008; Taylor-Clark, Ghatta, Bettner, & Undem, 2009), and stomach (Bielefeldt & Davis, 2008; Bielefeldt, Zhong, Koerber, & Davis, 2006; Kang, Bielefeldt, & Gebhart, 2004; McIlwrath, Davis, & Bielefeldt, 2009). Sensitization is an important neuronal mechanism underlying visceral hyperalgesia (Cervero & Laird, 2004); these afferents can be sensitized by inflammatory mediators (Bielefeldt & Davis, 2008; Ho, Gu, Hong, & Lee, 2000; Kang et al., 2004; L. Y. Lee & Morton, 1993; Yu, Kollarik, Ouyang, Myers, & Undem, 2007; Yu & Ouyang, 2009) and have electrophysiological properties similar to their spinal counterparts (Kostreva, Zuperku, Hess, Coon, & Kampine, 1975; Ozaki & Gebhart, 2001; Sengupta, Saha, & Goyal, 1990, 1992).

Further evidence for a role of vagal afferents in detecting the noxious aspect of pain comes from spinal cord injury patients. Patients with complete cervical spinal cord injuries have reported poorly localized crampy abdominal pain (Charney, Juler, & Comarr, 1975; Finnerup, Faaborg, Krogh, & Jensen, 2008; Levinthal & Bielefeldt, 2012; Yung & Groah, 2001), descriptions

commonly associated with visceral pain (Fink, 2000) thought to arise from spinal afferents. The only intact signal to the CNS for abdominal viscera in complete cervical spinal cord injuries are from vagal afferents. This suggests that vagal afferents can contribute to the perception of pain in humans, at least under these conditions. What is not known is whether, and to what extent, vagal afferents contribute to noxious pain sensation under normal conditions and whether a common insult like inflammation changes the role of vagal afferents in the detection of noxious sensations.

The reason for having nociceptors in both spinal and vagal pathways is not known, but current evidence suggests that these pathways might be involved in distinct modalities of nociception. For example, gastric vagal and spinal nociceptors appear to be differentially involved in sensing noxious chemical and mechanical stimuli. Intragastric acid caused c-Fos (a marker for neuronal activation) staining in the NTS but did not induce c-Fos in the spinal cord (Schuligoi et al., 1998). This suggests vagal afferents may be preferentially involved in sensing noxious chemical stimuli. In further support of this idea, vagotomy completely abolished visceromotor response (VMR) to intragastric acid, however, splanchnic nerve resection (which severs connections of spinal afferents) did not significantly alter responses (Lamb, Kang, Gebhart, & Bielefeldt, 2003). In contrast, splanchnic nerve resection significantly reduced VMR to mechanical distention whereas vagotomy did not significantly alter these responses (Lamb et al., 2003). The visceromotor response is a surrogate measure of visceral pain and is mediated through a spinobulbospinal reflex (Kamp, Jones, Tillman, & Gebhart, 2003). Although both afferent types can respond to mechanical and chemical stimuli (Grundy, 1988, 2002, 2004; Kirkup, Brunsden, & Grundy, 2001), it appears vagal afferents may be more important for chemical nociception and spinal afferents more important for mechanical nociception.

Spinal afferents from different levels may have specific roles in visceral pain. Noxious colorectal distention induces c-Fos expression in the LS spinal cord but not the TL spinal cord. However after colonic inflammation, noxious distention induces c-Fos expression in the TL spinal cord suggesting TL afferents play a role in inflammatory pain but not acute pain (Traub, Herdegen, & Gebhart, 1993; Traub, Lim, Sengupta, Meller, & Gebhart, 1994; G. Wang, Tang, & Traub, 2005). In addition, VMR to colorectal distention is almost completely abolished after bilateral dorsal rhizotomy of the L6-S3 dorsal roots in the rat (Traub, 2000). Colonic inflammation causes an exaggerated VMR implying that colonic inflammation induces visceral hypersensitivity (Morteau, Hachet, Caussette, & Bueno, 1994). The VMR was partially recovered after colonic inflammation induced by mustard oil instillation in the colon of rats with dorsal rhizotomy of the L6-S3 root. These results suggest that visceral hypersensitivity seen following inflammation is due in part to the recruitment of TL afferents.

Electrophysiological recordings of TL and LS afferents following colonic inflammation also show functional differences between these levels. Colitis induced by trinitrobenzene sulfonic acid (TNBS) was used to examine the response properties of pelvic and splanchnic nevres in the acute phase and after recovery from colitis in the rat (Hughes et al., 2009). TNBS treatment did not change the percentage of afferent subtypes for TL or LS afferents during the acute phase or after recovery. However, LS afferents had little or no difference in response properties to mechanical stimuli during the acute phase, whereas mesenteric and serosal TL afferents became hypersensitive during the acute phase. Mesenteric and serosal TL afferents exhibited hypersensitivity during the recovery phase and LS serosal afferents also gained hypersensitivity during recovery. These data imply that TL afferents are involved in both the acute and recovery phase of colitis whereas LS afferents are more important for the recovery phase.

The Hughes et al. study directly compared TL and LS afferents in the same experiment, however, there has been some discrepancy in the literature about mechanical hypersensitivity after inflammation in experiments focused on TL or LS afferents. Dextran sodium sulfate (DSS) induced inflammation in the rat did not change the mechanosensitivity of TL afferents, but did increase their responsiveness to serotonin during the acute phase and after recovery (Coldwell, Phillis, Sutherland, Howarth, & Blackshaw, 2007). TNBS induced colitis in mice was associated with changes in the proportion of subtypes of LS afferents in the acute phase and during recovery (Feng et al., 2012a). Furthermore, muscular afferents were sensitized to stretch and serosal afferents showed a decrease response to blunt probing during the acute phase. These results suggest that the species and/or method of inflammation are important factors in hypersensitivity after inflammation.

1.3.2 Autonomic Reflexes

Spinal visceral afferents that innervate colon and bladder have cell bodies in both TL and LS DRG, projecting to organs they innervate along with sympathetic (TL) or parasympathetic (LS) splanchnic nerves and terminate broadly in the spinal cord, including in the region of sympathetic (TL) or parasympathetic (LS) preganglionic neurons (Harrington et al., 2019a; Smith-Edwards et al., 2019). In contrast, vagal afferents that innervate the colon (and possibly bladder (Herrity, Rau, Petruska, Stirling, & Hubscher, 2014)) have cell bodies in the nodose ganglia, run in the vagus nerve with preganglionic parasympathetic fibers and terminate in the brainstem nucleus tractus solitarius (NTS) that in turn projects to nuclei in the brainstem, thalamus and cortex(Norgren, 1978; van der Kooy, Koda, McGinty, Gerfen, & Bloom, 1984).

Vagovagal reflexes refer to reflex circuits involving activation of vagal afferents that in turn activate the dorsal vagal complex (NTS and DMN) in the brainstem and cause release of acetylcholine or nitric oxide from vagal efferent fibers. The main GI functions that are regulated by vagovagal reflexes are esophageal propulsion, gastric volume, contractile activity and acid secretion, contraction of the gallbladder, and secretion of pancreatic enzymes (Furness, Callaghan, Rivera, & Cho, 2014; Travagli, Hermann, Browning, & Rogers, 2003). Certain vagovagal reflexes are more associated with GI disease states including vomiting, nausea, cough, disorder of the lower esophageal sphincter, and early satiety (Andrews & Sanger, 2002).

The NTS has a viscerotopic organization with inputs from the gut terminating in the caudal medial NTS (Altschuler, Bao, Bieger, Hopkins, & Miselis, 1989). The neurons in the NTS not only synapse onto the vagal motor neurons in the DMN and nucleus ambiguus but send projections to a number of brain regions. Specific gut NTS projections have been examined by microinjection of anterograde tracer into the caudal visceral region of NTS (Rinaman, 2010; Rinaman & Schwartz, 2004). Dense labeling was found in the DMN, parabrachial nucleus, paraventricular nucleus of hypothalamus, dorsomedial nucleus of hypothalamus, and bed nucleus of stria terminalis. Moderate labeling was also found in a number of regions including the medullary reticular nucleus, parvicellular reticular nucleus, nucleus ambiguus, paragigantocellular reticular nucleus, pontine reticular nucleus, pontine reticular nucleus, Barrington's nucleus, periaqueductal gray, paraventricular nucleus of thalamus, lateral hypothalamic area, substantia innominate, and the central nucleus of amygdala. Additionally, sparser labeling was found in the locus coeruleus, ventral tegmental area, dorsal raphé nucleus, midbrain reticular nucleus, nucleus accumbens, medial septum, and several hypothalamic subnuclei. No labeled fibers were found in cortical structures known to be involved in visceral sensation suggesting that visceral sensory signals are

relayed to cortex by thalamus and other brain regions. Vagal afferents are thought to convey homeostatic signals to the brain and have a general protective role in maintaining internal homeostasis (Berthoud & Neuhuber, 2000).

Furthermore, the vagus nerve is also important for interoception. Interoception pertains to receiving, encoding, and the representation of internal signals from the body to the brain (Cameron, 2001). Interoception is not only crucial for homeostasis, but also central in a range of cognitive and emotional processes, including memory, decision-making, emotional processing, and social interactions (Critchley & Harrison, 2013; Dunn et al., 2010; Shah, Catmur, & Bird, 2017). As noted in the above paragraph, gut specific NTS neurons project to a number of brain regions associated with cognitive and emotional processes including thalamus, hippocampus, and amygdala. Vagus nerve stimulation has been implicated to influence several higher order processes including memory (Ghacibeh, Shenker, Shenal, Uthman, & Heilman, 2006), emotional regulation (Porges, Doussard-Roosevelt, & Maiti, 1994), and learning (J. Lai & David, 2021). Vagus nerve stimulation is also a FDA approved treatment for depression and epilepsy and is currently being explored as a treatment for a number of autoimmune and inflammatory conditions (Johnson & Wilson, 2018).

In addition to terminating in the dorsal horn where they initiate visceral sensations, LS afferents synapse in the spinal cord on sacral preganglionic parasympathetic neurons that travel in the pelvic nerve to pelvic organs including the colon, bladder, and genitals. Retrograde tracing has demonstrated sacral parasympathetic neurons directly innervate the colon and project to the pelvic ganglia (mixed sympathetic and parasympathetic) which then innervate the colon (S. Brookes, Dinning, & Gladman, 2009). The LS afferents and sacral parasympathetic neurons are involved in the defecation reflex. Distention or irritation of the rectum initiate the defecation reflex and starts

a complex coordination of parasympathetic fibers, enteric neurons, somatic neurons, and the central nervous system (S. Brookes et al., 2009). Healthy individuals have descending central control to maintain fecal continence, however, voluntary control is lost if corticospinal input to the sacral parasympathetic nucleus is absent (Lynch, Antony, Dobbs, & Frizelle, 2001). The defecation reflex can still occur in response to colon distention if the sacral outflow and pelvic nerves are not damaged.

The TL afferents synapse onto preganglionic sympathetic neurons in the spinal cord. Preganglionic sympathetic neurons synapse onto postganglionic sympathetic neurons in the sympathetic chain or prevertebral ganglia, the later innervating the abdominal organs and are canonically associated with inhibiting GI function. Sympathetic efferent fibers signal to myenteric neurons, submucosal neurons, blood vessels, and sphincter muscle (Furness et al., 2014) and inhibit excitatory myenteric neurons, secretomotor neurons, and contract sphincters to slow the passage of content through the GI tract (Lundgren, 2000).

Transneuronal labeling with pseudorabies virus injection into the colon has been used to examine the regions of the central nervous system involved in colon function in the rat (Vizzard, Brisson, & de Groat, 2000). The earliest timepoint (suggesting only one synapse separated the injection site from the labeled neuron) showed labeling in the myenteric ganglia, pelvic ganglia, DRGs, and the spinal cord. The TL and LS spinal cord had the most labeling in the autonomic nuclei, intermediolateral nucleus (IML) and sacral parasympathetic nucleus (SPN), respectively, as well as the dorsal commissure (DMC, an accessory autonomic nuclei (Hancock & Peveto, 1979)) at the 72-hour timepoint. Later timepoints (suggesting these neurons were two or more synapses away from the injection site) saw labeling in the dorsal horn, with superficial laminae showing labeling first and deeper laminae at later timepoints. Central nervous system regions also

had labeling with medullary regions showing labeling at the earlier timepoints. Area postrema, A5, DMN, NTS, lateral reticular nucleus, dorsal raphé nucleus, Barrington's nucleus, paraventricular nucleus, and the medial-frontal lobe had the densest labeling at the latest timepoint. The inferior olive, locus coeruleus, nucleus ambiguus, A7, nucleus subcoeruleus, Edinger-Westphal nucleus, periaqueductal gray, and the red nucleus had moderate labeling. Sparse labeling was found in the parabrachial nucleus, lateral hypothalamic nucleus, and supraoptic nucleus. The colon has wide ranging projection with the CNS suggesting several CNS areas are involved in colon function. To investigate the proportion of labeling due to vagal afferents, a separate cohort of animals received a complete spinal cord transection at T8. There was a significant loss of labeling overall and only the NTS, DMN, A5, area postrema, lateral reticular nucleus, nucleus ambiguus, and paraventricular nucleus still had labeling. An important caveat for this study is only distal colon was injected and a portion of vagal afferents innervating the proximal colon may have been missed. Spinal afferents are generally thought to be involved with higher-threshold sensations including discomfort, bloating, urgency, and pain (Sengupta, 2009).

1.3.3 Neuroimmune Interactions

Afferent/immune interactions are also critical for gut function and homeostasis. Specific neuroimmune interactions are less well described in the colon, although it is assumed similar interactions occur as have been described for the skin (Cohen et al., 2019; Saloman, Cohen, & Kaplan, 2020; Zhang et al., 2021) and other structures (Baral et al., 2018; N. Lai et al., 2020). The vagus nerve has a crucial role in the anti-inflammatory reflex (Tracey, 2002), with vagal afferent being the first step in this reflex. Primary afferents in the vagus nerve detect cytokines, including TNF and IL-1β (Zanos et al., 2018) which in turn activate a brainstem circuit that signals via the

vagal parasympathetic fibers back to the periphery to shut down cytokine production. In this circuit, vagal efferent fibers signal to the celiac ganglia via acetylcholine to α7 nicotinic acetylcholine receptor on sympathetic post-ganglionic neurons (Vida, Peña, Deitch, & Ulloa, 2011). These neurons then signal via the splenic nerve and release norepinephrine in the spleen (Rosas-Ballina et al., 2011). A subpopulation of splenic T cells produce acetylcholine in response to this norepinephrine, relaying the neuronal signals to the splenic α7 nicotinic acetylcholine receptor-expressing macrophages and decrease cytokine release. Vagal afferents have also been shown to modulate immune cells in the lungs during lethal bacterial infections. Baral et al. (Baral et al., 2018) found ablation of trpv1+ vagal afferents in methicillin-resistant S. aureus (MRSA) pneumonia led to improved survival, core body temperature maintenance, and bacterial clearance. Trpv1+ afferents blocked the ability of neutrophils to infiltrate the lungs and suppressed γδ T cells. Blockade of CGRP release with an antagonist significantly increased survival rates and bacterial clearance suggesting vagal afferents suppressed the immune cells through CGRP release. Interestingly, ablation of jugular but not nodose afferents modulated this immune response, implying a specific subset of lung innervating vagal afferents are important for this response.

Spinal afferents have also been shown to modulate the immune system. Spinal afferents innervating the small intestine defend against Salmonella infection by crosstalk with epithelial cells and the intestinal microbiota (N. Lai et al., 2020). Salmonella invades the small intestine through intestinal microfold (M) cells, which are specialized epithelial cells within Peyer's patch follicle-associated epithelium. Spinal afferents signal via CGRP to reduce the numbers of M cells, thereby decreases the entry points of the bacteria. Spinal afferent signaling also maintains levels of segmented filamentous bacteria in the small intestine which is an intestine-resident microbe that provides resistance against Salmonella colonization and invasion. This effect was shown to be

spinal afferent specific, as intrathecal resiniferatoxin (a potent trpv1 agonist that denervates trpv1+ neurons) caused increase bacterial burden whereas direct nodose injection had no effect on bacteria load.

Furthermore, sympathetic efferent neurons as well as enteric neurons have been shown to be involved in neuroimmune interactions. Sympathetic neurons in the small intestine signal to resident muscularis macrophages through the beta-2 adrenergic receptor to polarize the macrophages toward a tissue-protective M2-like phenotype during homeostasis (Gabanyi et al., 2016). Additionally, during GI infection caused by enteric pathogens, these macrophages protect enteric neurons from caspase-11-dependent death through their expression of arginase and protective polyamines (Matheis et al., 2020). During helminth infections (e.g. flatworms including flukes and tapeworms), sympathetic neurons inhibit type 2 innate lymphoid cells responses and helminth clearance through the activation of beta-2 adrenergic receptor in lungs and small intestine (Moriyama et al., 2018). In the small intestine, a subset of enteric neurons express the peptide neuromedin-U (NMU) and make close contact with type 2 innate lymphoid cells that express the receptor NMUR1. NMU induces type 2 innate lymphoid cells proliferation and production of type 2 cytokines, including interleukin (IL)-5, IL-9, and IL-13 (Cardoso et al., 2017; Klose et al., 2017). In contrast, the neuropeptide CGRP restrains type 2 innate lymphoid cell activation and inhibits anti-helminth responses (Nagashima et al., 2019). The origin of CGRP was not investigated and it is possible that CGRP release from extrinsic primary afferents, intrinsic enteric neurons, and/or non-neuronal cells is important for this response.

In addition to neurons signaling to immune cells, primary afferents can be directly activated by ligands released from immune cells allowing bi-directional communication. Neurons express receptors for cytokines and lipid mediators released by immune cells, including canonical

cytokines. Vagal afferents have been confirmed to be activated by IL-1β (Hansen, O'Connor, Goehler, Watkins, & Maier, 2001; Mascarucci, Perego, Terrazzino, & De Simoni, 1998; Watkins, Goehler, Relton, Tartaglia, et al., 1995), tumor necrosis factor alpha (Rogers, Van Meter, & Hermann, 2006; Watkins, Goehler, Relton, Brewer, & Maier, 1995), interferon-gamma (Deng et al., 2018; J. Wang et al., 2017), interferon-alpha (Patil et al., 2020; J. Wang et al., 2017), and IL-8 (J. Wang et al., 2017). Spinal afferents can also be directly activated by IL-1β (Binshtok et al., 2008) and tumor necrosis factor alpha (Jin & Gereau, 2006), as well as IL-6 (Segond von Banchet, Kiehl, & Schaible, 2005) Many immune mediators can functionally alter neuronal signaling, including sensitization of sensory neurons in the context of pain (Bueno & Fioramonti, 2002; Hall & Agrawal, 2014; Samad et al., 2001). Pattern-recognition receptors, canonically expressed by immune cells, are expressed by neurons, allowing simultaneous surveyance of luminal microbial signals by both systems (Barajon et al., 2009; Brun et al., 2013; Veiga-Fernandes & Pachnis, 2017). There has been a renewed interest in neuroimmune interactions, and the field is continually learning more about how crosstalk between neurons and immune cells controls organ homeostasis as well as visceral disease.

1.4 Extrinsic Primary Afferent Neurons and Enteric Nervous System Communication

1.4.1 Intrinsic Innervation of the Colon

The GI tract is unique in that it is innervated by both an intrinsic enteric nervous system (ENS) and extrinsic autonomic and sensory neurons. The ENS consists of the myenteric plexus (Auerbach’s plexus) and the submucosal plexus (Meissner’s plexus). The myenteric plexus is

located between the longitudinal and circular muscle layers of the GI tract and is important for motor functions (Kunze & Furness, 1999). The submucosal plexus is located within the submucosal layer of the GI tract and is thought to be important for secretory functions and regulating blood flow (Kunze & Furness, 1999). The ENS is an autonomous system and is the main driver of GI function. The extrinsic innervation is thought to play a modulatory role in GI function as the GI tract can function without these connections (Costa, Brookes, & Hennig, 2000).

The ENS is composed of multiple subtypes of neurons. Intrinsic primary afferent neurons (IPANs) are similar to the extrinsic primary afferent neurons and sense luminal chemical stimuli, mechanical deformation of the mucosa, and radial stretch and muscle tension (Furness, Jones, Nurgali, & Clerc, 2004). IPANs have circumferential projections and synapse with ascending and descending interneurons to activate intrinsic reflexes that influence motility, secretion, and local blood flow (Furness et al., 2004). Ascending and descending interneurons form chains of connected interneurons that synapse onto the final motor neuron. Excitatory and inhibitory motor neurons are the output neurons and cause contraction or relaxation of the muscle, respectively. The diversity of neurons is required for complex motor patterns e.g., propulsion of luminal content requires coordinated motor patterns along different segments of the GI tract involving both contraction and relaxation of smooth muscle cells (Spencer, Dinning, Brookes, & Costa, 2016).

The ENS also contains non-neuronal cell types that are important for GI function. Interstitial cells of Cajal (ICC) are pacemaker cells in the gut and communicate with myenteric neurons and smooth muscle cells to control motor activity (Sanders, Koh, & Ward, 2006). The epithelium is a single layer of cells that line the luminal border of the GI tract with two major functions: absorbing nutrients and water and forming a protective barrier. Epithelial cells have been shown to play a major role in sensory signaling and the epithelium can directly activate

IPANs and extrinsic primary afferents (Furness et al., 2004; Makadia et al., 2018). There has also been a recent interest in the microbiome as a regulator of gut function. The gut microbiome consists of microorganisms that interact with host cells and has an important role in metabolism, immune defense, and behavior (Dinan & Cryan, 2017). How these different cells communicate to maintain gut homeostasis are ongoing areas of research and have been shown to not only be important for gut function but brain function and behavior.

1.4.2 The Role of ExPANs in ENS Function

As stated above, extrinsic primary afferent neurons (ExPANs) have endings in the multiple layers of the colon including the myenteric and submucosal ganglia and have been hypothesized to communicate with ENS neurons. In addition to previously described indirect autonomic reflexes, ExPANs are hypothesized to have direct local effector functions on myenteric neurons via neuropeptides (eg, substance P and CGRP) and glutamate released from their peripheral terminals in the colon (Holzer, 1988; Maggi, 1995). Previous studies indicate that the majority of colon ExPANs express transient receptor potential vanilloid 1 (TRPV1) (Christianson, McIlwrath, Koerber, & Davis, 2006), a nonselective cation channel activated by capsaicin, and that applied capsaicin causes contraction of the colon in rodents (Matsumoto et al., 2009), large mammals(Hayashi et al., 2010), and humans (Maggi et al., 1990). In addition, CGRP- and TRPV1-immunoreactive ExPAN fibers have been localized to myenteric ganglia, where they form dense networks around myenteric neurons (Anavi-Goffer & Coutts, 2003; Matsumoto et al., 2011; Spencer et al., 2014; Ward, Bayguinov, Won, Grundy, & Berthoud, 2003). Furthermore, a recent calcium imaging study showed capsaicin-evoked transients in ExPAN endings within myenteric ganglia that were temporally correlated with increased activity in myenteric ganglion neurons and

smooth muscle contraction (Spencer, Sorensen, et al., 2016). These findings suggest that peptidergic ExPANs can locally and directly influence myenteric neuron activity to alter colon contractility. A significant and acknowledged caveat for this conclusion is that the peptides thought to be involved in direct communication from ExPANs to myenteric neurons are also expressed in myenteric neurons (Deacon & Conlon, 1987; Mitsui, 2009) and that a small subset of TRPV1-expressing myenteric neurons have been reported (Anavi-Goffer, McKay, Ashford, & Coutts, 2002). Additionally, functional TRPV1 expression has been reported in epithelial cells (De Jong et al., 2014), endothelial cells (Tóth et al., 2014), and immune cells (Bertin et al., 2014; Majhi et al., 2015) in the colon.

To directly test if ExPANs can directly communicate with myenteric neurons, Smith-Edwards et al. (Smith-Edwards et al., 2019) used an *ex vivo* colon nerve spinal cord preparation with calcium imaging to stimulate dorsal or ventral roots at the LS level and image calcium transients in the myenteric neurons of the colon. Surprisingly, dorsal root stimulation without the spinal cord intact did not produce myenteric neuron activity or movement. Ventral root stimulation, however, did cause calcium transients in myenteric neurons and contraction of the colon. If the spinal cord were left intact, dorsal root stimulation could cause myenteric neuron responses and movement, implicating that the ExPANs communicate with myenteric neurons through a spinal cord reflex but not direct communication through effector activity at peripheral terminals. These experiments were all performed in naïve mice and the possibility that colonic inflammation may uncover this direct communication of ExPANS to myenteric neurons due to afferent sensitization remains unexplored. Furthermore, it is possible that a different level of the neuraxis may be the one involved in direct communication of ExPAN to myenteric neurons.

The work in this dissertation was inspired by the intriguing question of "why do viscera receive multiple levels of sensory innervation?" To begin to address this question, I performed single cell RT-qPCR on back-labeled colon and bladder afferents from the different levels of the neuraxis. Next, I examined how colonic inflammation affects LS afferent to myenteric neuron communication. I also investigated how colonic inflammation modulates afferent activation of the second-order neurons at their central terminals in the spinal cord and brainstem. Finally, I assessed how colitis changed the effects of autonomic efferent stimulation on colon motility and alterations in intrinsic ENS circuits. The work in this dissertation suggests that different levels of the neuraxis have both overlapping and unique aspects in colon function and colonic inflammation differentially impacted the different levels of the neuraxis.

2.0 Unique Molecular Characteristics of Visceral Afferents Arising from Different Levels of the Neuraxis

2.1 Introduction

Visceral organs receive sensory innervation from primary sensory neurons arising from multiple levels of the neuraxis. Depending on the organ, innervation can originate from vagal afferents (including afferents in the jugular and nodose (NG) ganglia), thoracolumbar (TL) spinal afferents (arising from lower thoracic and upper lumbar dorsal root ganglia (DRG)) or lumbosacral (LS) spinal afferents (arising from lower lumbar and upper sacral DRG). Our hypotheses to account for why viscera requires monitoring by multiple populations of afferents include: a) that different levels of innervation are involved in different qualitative aspects of stimulus detection, including pain, b) that because the first synapse is on CNS neurons associated with sympathetic or parasympathetic circuits, different afferent populations provide the basis for integration of autonomic/homeostatic functions, or c) that the different levels play complementary roles in immune modulation.

With regard to shaping the nature of the visceral sensory experience, it had been hypothesized that spinal afferents, but not vagal afferents, relay nociceptive sensations (e.g., stabbing, burning, cramping), whereas vagal afferents underlie affective aspects of visceral pain including depression, anxiety, nausea and fear (Altschuler et al., 1993; Berthoud & Neuhuber, 2000; Grundy, 2002; Sengupta, 2009). However, recent evidence has shown that vagal afferents may have a significant role in nociception and pain (Bielefeldt et al., 2006; Canning et al., 2006; Kang et al., 2004; L. Y. Lee et al., 2003; Nassenstein et al., 2008; Taylor-Clark et al., 2009; Yu et

al., 2005). Even spinal afferents from different levels may have specific roles; colonic afferents arising from the TL level have been reported to be important for inflammatory pain but not acute pain, whereas LS colonic afferents are proposed to be involved in both acute and inflammatory pain (Traub, 2000; Traub et al., 1994; G. Wang et al., 2005).

In terms of homeostasis, spinal visceral afferents that innervate colon and bladder have cell bodies in both TL and LS DRG, projecting to organs they innervate along sympathetic (TL) or parasympathetic (LS) splanchnic nerves and terminate broadly in the spinal cord, including in the region of sympathetic (TL) or parasympathetic (LS) preganglionic neurons (Harrington et al., 2019b; Smith-Edwards et al., 2019). In contrast, vagal afferents that innervate the colon (and possibly bladder (Herrity et al., 2014)) have cell bodies in the nodose ganglia, run in the vagus nerve with preganglionic parasympathetic fibers and terminate in the brainstem nucleus tractus solitarius (NTS) that in turn projects to nuclei in the brainstem, thalamus and cortex (Norgren, 1978). Thus, redundant sensory input to the CNS could be informing different components of the autonomic nervous system.

Afferent/immune interactions are also critical for gut function and homeostasis, i.e., the vagus nerve has a crucial role in the anti-inflammatory reflex (Tracey, 2002; van der Kooy et al., 1984), with vagal afferent response to cytokine release as the first step in this reflex. Primary afferents (vagal and spinal) release multiple peptides including calcitonin gene-related peptide (CGRP) and substance P that bind receptors on immune cells, allowing direct afferent and immune communication (Altmayr, Jusek, & Holzmann, 2010; Brain & Williams, 1985; Caceres et al., 2009; Cohen et al., 2019; Riol-Blanco et al., 2014). Ablation or silencing of primary afferents (vagal and spinal) also can modulate the immune response across multiple organs, including the lungs, small intestine and colon (Baral et al., 2018; Engel et al., 2011; N. Lai et al., 2020).

In this study we examined how genes required for sensation, homeostasis, and neuroimmune regulation are divided among different visceral afferents. Single cell RT-qPCR analysis combined with automated hierarchical clustering (AHC) of afferents innervating the bladder and colon were carried out. Results show that location matters; that genes expressed by an afferent and where that afferent terminates (especially for the colon) is determined by the ganglionic location. Furthermore, the clustering of unique genes to afferents from different ganglia suggests that all afferents contribute to homeostatic and immune regulation, as well as signal all aspects of visceral sensation.

2.2 Methods

2.2.1 Animals

Experiments were conducted on male and female adult (6-12 week) C57BL/6 mice from Jackson Laboratory (JAX#000664). Animals were group-housed with a 12-hour light-dark cycle and ad libitum access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and were carried out in accordance with AAALAC-approved practices.

2.2.2 Back-labeling of Neurons

Mice were anesthetized with isoflurane (2%) and a laparotomy was performed to access the pelvic viscera. Fluorescent retrograde dyes (cholera toxin subunit beta (CTB) or wheat germ

agglutinin (WGA); Invitrogen Carlsbad, CA) were injected into colon or bladder as previously described (Christianson et al., 2007). Briefly, using an insulin syringe (31-gauge needle), 5-10μL of Alexa Fluor 555-conjugated CTB was injected in 2-3μL aliquots beneath the serosal layer of the distal colon or at the base of the bladder. Alexa Fluor 488-conjugated CTB was injected in the proximal colon just distal to the cecum. To ensure dye did not spread between areas, colons were removed and visualized under a fluorescence microscope. In all cases, there was at least a 20mm area of middle colon that had no visible fluorescence. In a separate cohort of mice, 5-10μL of Alexa Fluor 488-conjugated CTB or WGA was injected in 2-3μL aliquots beneath the serosal layer in the urinary bladder body. WGA was used to examine whether CTB was labeling a specific subset of afferents (n=3, 3 female). We found that the number of cells expressing a given gene was virtually the same (except for some of the rarely detected genes like MrgprD and Trpm8) regardless of which tracer was used (**Table 1**). Therefore, CTB was used in subsequent experiments. In a subset of these animals, 555-conjugated CTB was injected into both proximal and distal colon, combined with 488-CTB injection into the bladder to label convergent bladder/colon afferents. All incisions were then sutured, and animals were allowed to recover before returning to their home cage.

2.2.3 Dissociation of Ganglia and Single Neuron Isolation

Three to five days after back-labeling, mice were anesthetized with isoflurane and transcardially perfused with cold Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS). The NG, TL dorsal root ganglia (DRGs; T10-L1), and LS DRGs (L5-S1) were dissected into cold HBSS and enzymatically treated with cysteine, papain, collagenase type II, and dispase type II to facilitate isolation by mechanical trituration. Cells were plated on poly-d-lysine/laminin-coated coverslips

Table 1 Comparison of the percentage of genes detected in CTB or WGA backlabeled neurons.

The number of bladder backlabeled neurons expressing a given gene was virtually the same (except for some of the rarely detected genes like MrgprD and Trpm8) regardless of retrograde tracer used. Genes are listed from highest to lowest expression for CTB. The number of cells analyzed is in parentheses.

Genes	CTB (43)	WGA (48)
Asic2	100.00%	97.92%
Calcα	100.00%	97.92%
Nefh	100.00%	100.00%
RET	100.00%	89.58%
Scn10a	100.00%	97.92%
Scn11a	100.00%	97.92%
Scn9a	100.00%	97.92%
TrkA	100.00%	95.83%
Th	95.35%	93.75%
Tac1	86.05%	85.42%
TrpV1	86.05%	79.17%
CD274	81.40%	87.50%
P2rX3	69.77%	70.83%
TrpM3	65.12%	56.25%
Gfrα1	60.47%	62.50%
OPRK1	60.47%	60.42%
Gfrα3	58.14%	50.00%
Piezo2	53.49%	47.92%
TrkB	53.49%	50.00%
Asic1	39.53%	41.67%
TrpA1	39.53%	27.08%
Asic3	20.93%	33.33%
Gfrα2	20.93%	18.75%
P2rY2	18.60%	18.75%
MrgprD	11.63%	2.08%
MrgprA3	6.98%	2.08%
OPRM1	4.65%	10.42%
OPRD1	2.33%	0.00%
P2rY1	2.33%	6.25%
Sst	2.33%	0.00%
TrpM8	2.33%	18.75%
TrkC	0.00%	8.33%

in 35mmx10mm petri dishes. Coverslips were flooded with Dulbecco modified Eagle medium F12 (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin 2 hours later. Cells were picked up from each culture using glass capillaries (World Precision Instruments) held by a 3-axis micromanipulator. Typically, there were 10-20 back-labeled cells on each coverslip. To avoid selection bias based on cell size, only isolated, single neurons (those not adjacent to another labeled cell) were used. Six to twelve coverslips were plated for each mouse and cells were sampled from multiple coverslips. To monitor for size selection bias, the relative size of each cell was recorded at time of collection. The great majority of back-labeled cells were small (10-20 μm in diameter) or medium (20-30μm). Occasionally, large cells were seen and collected in a manner proportional to their relative abundance. Collected cells were transferred to tubes containing 3uL of lysis buffer (Epicentre, MessageBOOSTER kit), and stored at - 80^{o}.

2.2.4 Single Cell Amplification and qPCR

The methods for single cell RT-qPCR and clustering and validation of these techniques have been previously described (Adelman et al., 2019). Briefly, the RNA collected from each cell was reverse transcribed and amplified using T7 linear amplification (Epicentre, MessageBOOSTER kit for cell lysate), cleaned with RNA Cleaner & Concentrator-5 columns (Zymo Research), and assessed using qPCR with optimized primers and SsoAdvanced SYBR Green Master Mix (BIO-RAD). Cycle time (Ct) values were determined via regression. Quantification threshold for PCR was defined as the point at which there was a 95% replication rate (35 Ct) (Reiter et al., 2011). GAPDH threshold was thus defined as 25 Ct to ensure detection of transcripts at least a thousand-fold less common than GAPDH.

2.2.5 Primer Design and Validation

Unique forward and reverse primer sequences were chosen for each gene within 500 bases of the 3' end. Stock solutions of cDNA were generated by extracting RNA from the whole DRG and both 10 and 160pg aliquots of the RNA were amplified using the same procedure described above for single cells. Serial dilutions of these aliquots were used to calculate primer efficiencies over the range of RNA concentrations observed in single cells. Expression level was determined relative to GAPDH and corrected for these primer efficiencies (Pfaffl, 2001).

2.2.6 Automated Hierarchical Clustering

All back-labeled neurons were clustered utilizing the unweighted pair group method with averaging (UPGMA) using expression information obtained from single cell PCR in MATLAB (MathWorks, Natick, MA). The preprocessing for this data analysis consists of taking the ΔCt values and replacing the samples that failed to generate a value for a given gene with the limit of detection for that gene.

2.2.7 Tissue Preparation and Estimation of the Number of Back-labeled Neurons

Three to five days after back-labeling, mice were isoflurane-anesthetized and transcardially perfused with cold isotonic saline. The NG, TL, and LS DRGs were removed and fixed with 4% paraformaldehyde solution for 30 minutes. For a negative control, the L3 DRG was also removed. After post-fixation, the ganglia were moved to 25% sucrose for overnight cryoprotection. After cryoprotection, ganglia were embedded in optimal cutting temperature compound (OCT; Fisher)

and cryosectioned at 14 µm, allowed to dry, and immediately stored at -80°C. Each ganglion was placed on six slides with serial sections alternating between slides. Ganglia sections were analyzed using fluorescent microscopy at 20x. Only two slides from the six were analyzed with 42µm between slides; visual confirmation was used to avoid analyzing the same cells more than once. At least 6 sections from each ganglion were analyzed per animal. For each animal, the number of 555-conjugated CTB positive, 488-conjugated CTB positive cells, and double labeled cells were counted. The percentage of CTB positive cells at each level of the neuraxis was found for each mouse and averaged within groups.

2.2.8 Calcium Imaging Protocol

Colon neurons were back-labeled and prepared for culture as described above except proximal and distal colon were both labeled with CTB-555. Cells were incubated at 37°C and imaged within 36 hours as previously described (Malin et al., 2006). Prior to imaging, cells were incubated for 30 minutes at 37°C with 1µl fura-2AM (2µM, Molecular Probes) and 2µl of 20% Pluronic F-127 dissolved in DMSO (AnaSpec) and diluted in HBSS containing 10mg/ml BSA (Sigma). Coverslips were mounted on an inverted Olympus IX71 (Thornwood, NY) microscope stage with HBSS buffer flowing at 5ml/min, controlled by a fast step system (AutoMate Scientific). Perfusate temperature was maintained at 30°C using a heated stage and an in-line heating system (Warner Instruments). Chemicals were delivered with a rapid-switching local perfusion system. Firmly attached, CTB-positive neurons were identified using a 555nm filter and chosen as regions of interest using Simple HCI software (Compix Imaging Systems, Sewickley, PA). Unlabeled, adjacent cells were also identified and imaged. All fields were first tested with a brief application (4s) of 50mM K+ (high K+) to ensure that cells were responsive. Following a

five-minute recovery period, agonists were applied in a randomized order with at least a five-minute recovery period between agonists. Stock solutions of capsaicin (Sigma) and mustard oil (Sigma) were made in 1-methyl-2-pyrrolidinone and α,β-methylene ATP (Sigma) was made in water. The stock solution of mouse interferon-α A (PBL Assay Science, Piscataway, NJ), recombinant mouse interleukin-4 (PeproTech, Rocky Hill, NJ), recombinant mouse interferon-γ and recombinant human interkeukin-8 (R&D Systems, Minneapolis, MN) was prepared in PBS containing 0.1% BSA, as previously described (Wang et al., 2017). Final concentrations applied to cells were 1μM capsaicin, 30μM α,β-methylene ATP , and 50μM mustard oil for four seconds and 500ng/ml for interleukin-4, interleukin-8 and interferon-γ and 1000iu/ml interferon-α for 90 seconds. Absorbance data at 340nm and 380nm were collected at one frame per second. Responses were measured as the ratio of 340/380nM excitation and 510nM emission over baseline (ΔF/F0; DG4, Sutter Instruments, Novato, CA). Peak responses were included in the analysis if the response was 5 standard deviations above baseline. The prevalence of responsive colon afferents was determined as a percentage of total healthy (high K+-responsive) CTB-positive cells. Any cell with significantly diminished Fura-2 signal over the duration of the experiment or that did not recover to baseline prior to the second agonist application was not included in the analysis.

2.2.9 Experimental Design and Statistical Analysis

For back-labeling experiments, the mean values of the number of back-labeled cells were compared between level (NG, TL, LS) and target (Proximal, Distal, Dual) using a two-way analysis of variance (ANOVA) with Tukey's post hoc analysis. A two-tailed paired t-test was used to test for significance of the difference in the extent of spread (length) of CTB injections in the proximal and distal colon. For calcium imaging experiments, mean peak values of the calcium

transients were compared between level (NG, TL, LS) and target (Proximal, Distal, Dual) using a mixed effects model with Tukey's post hoc analysis. Statistical tests were performed in Excel (Microsoft, Redmond, WA) and GraphPad Prism (GraphPad Software, San Diego, CA). Data are expressed as mean ± standard error of the mean, where n represents mice used, unless indicated otherwise. The number of animals and statistical values are reported in the results section; significance is defined as $p < 0.05$.

2.3 Results

2.3.1 Distribution of back-labeled neurons from injection sites in distal and proximal colon

In mouse, the spinal innervation of the distal colon and bladder (including dually projecting colon and bladder neurons) have been described (Robinson et al., 2004; Christianson et al., 2007), whereas innervation of the proximal colon has not been fully characterized. To fill this knowledge gap, the spinal and nodose sensory innervation of the proximal colon was investigated and compared to innervation of the distal colon.

Injection of CTB into the proximal and distal colon of individual mice labeled, on average, a total of 659 ± 75 CTB-positive neurons within the nodose and spinal ganglia (n=6, 4 males, 2 females; **Table 2**, **Figure 2A-C**). There were nearly equal percentages of neurons arising from nodose (32.29% ± 3.73, $p > 0.05$), TL (30.29% ± 4.02) and LS (37.43% ± 5.77) ganglia. Injections into the distal colon produced more CTB-labeled cells (451 ± 84) compared to the proximal colon (181 ± 54, $F_{(3,15)} = 22.19$, $p = 0.0256$; **Table 2**), resulting in 65.42% ± 8.12 of CTB-positive afferents originating from distal colon, 27.87% ± 7.55 from proximal colon and 6.70% ± 1.02

Table 2 Number of NG (nodose), TL (thoracolumbar) and LS (lumbosacral) CTB-positive afferents projecting to proximal and distal colon.

The total number of CTB back-labeled neurons from different levels of the neuraxis innervated proximal, distal or both proximal and distal colon (dual) almost equally. The NG and TL have approximately equal numbers of neurons that innervate proximal and distal colon, whereas LS cells almost exclusively innervate the distal colon.

	NG	TL	LS	All
Proximal	96 ± 26	78 ± 30	6 ± 3	181 ± 54
Distal	97 ± 24	108 ± 23	246 ± 58	451 ± 84
Dual	20 ± 4	18 ± 2	6 ± 1	44 ± 6
Total	214 ± 26	204 ± 30	258 ± 56	659 ± 75

being dual-labeled (i.e., innervating both proximal and distal colon). This difference in the number of cells innervating the proximal and distal colon occurs because the majority of LS afferents project to the distal colon; of the 258 ± 56 back-labeled cells in LS ganglia, 252 ± 59 were labeled by injections into the distal colon (this included 6 ± 1 dual-labeled afferents; **Table 2**). Thus, LS colon afferents almost exclusively innervate the distal colon. For the proximal colon, the largest afferent contribution came from nodose and TL ganglia (58.29% ± 7.20 (NG), 38.22% ± 6.00 (TL), and 3.49% ± 1.63 (LS), $F_{(4,20)} = 25.21$, $p < 0.0001$ for NG vs. LS, $p = 0.0002$ for TL vs. LS; **Figure 2D**). The size of the proximal and distal injections was not a factor as the distance over which fluorescence could be detected in the proximal colon (13.0mm ± 1.9) and distal colon (14.0mm ± 1.1, $t_{(7)} = 0.5739$, $p = 0.58$) were equivalent (n=8, 5 males, 3 females; **Figure 2E**). In addition, there was a 20-30mm gap between the leading edge of each injection. That TL afferents projected equally to proximal and distal colon, while LS afferents were more restricted to the distal

colon is consistent with the idea that neural components that regulate colon function change along the length of the colon (Li et al., 2019).

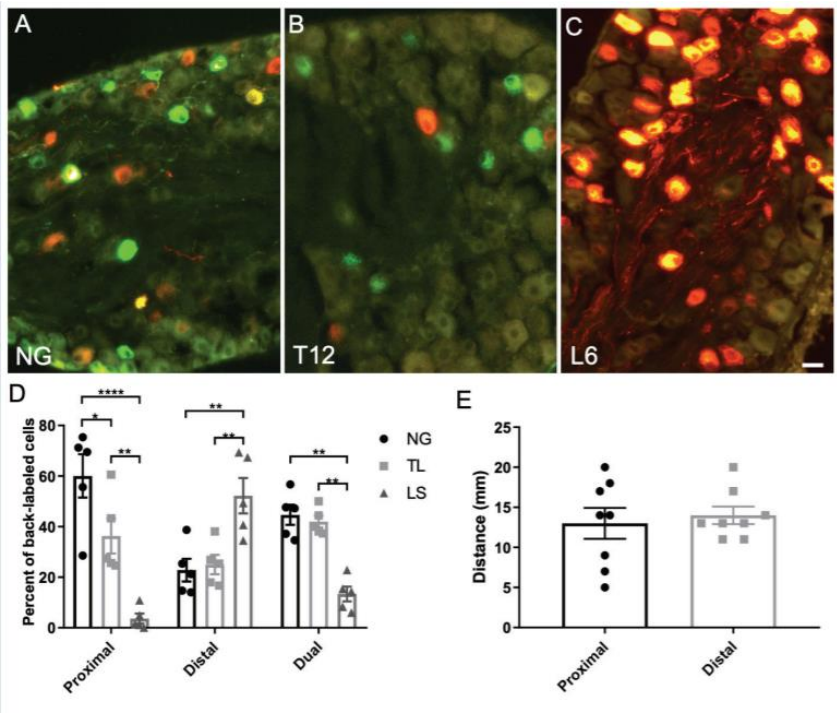

Figure 2 Proximal and distal colon have distinct patterns of innervation from different levels of the neuraxis

Retrograde labeled cell bodies in the NG (A), T12 DRG (B) and L6 DRG (C) following injection of CTB-488 (green) into the proximal colon and CTB-555 (red) into the distal colon. In all ganglia, the majority of cells contained one color of CTB. D) Quantification of the percentage of CTB positive cells across the neuraxis. NG and TL neurons project to proximal and distal colon, whereas LS neurons project primarily to distal colon. Afferents that project to both proximal and distal colon (dual) were most common in the NG and TL. E) Distance of fluorescence spread after CTB injection into the proximal and distal colon. The size of the injection ranged from 5 to 20 mm with 20-30mm between injection sites. Calibration bar A-C = 20μm. * p<.05, **p<.01, *** p=.001, ****p<.001, using a two-way analysis of variance (ANOVA) (D) or paired two tailed t-test (E).

2.3.2 Automated Hierarchical Clustering (AHC) Based on mRNA Expression and Afferent Location Reveal Distinct Neuronal Clusters.

To classify afferent subtypes, we used single-cell RT-qPCR to analyze transcripts of 28 genes previously used in a study for classification of identified cutaneous afferents (Adelman et al., 2019). These genes were shown to identify clusters that largely replicated the clusters produced by bulk, single cell RNAseq of DRG neurons unidentified with respect to innervation target or functional phenotype (Usoskin et al., 2015; Zeisel et al., 2018). Importantly, Adelman et al. combined AHC of these 28 genes with functional identification of neuron subtype with use of an ex vivo skin-nerve physiological preparation. This approach allowed correlation of molecular phenotype of individual cells with physiological response properties of intact afferents (e.g., conduction velocity, response to mechanical and thermal stimuli). Using this approach, these 28 genes produced clusters that corresponded to functional afferent subtypes (e.g., CMH (C-mechanoheat), CH (C-heat), and HTMR (high threshold mechanoreceptors)). For analysis of colon and bladder afferents, 4 additional genes encoding opioid receptors and the immune checkpoint inhibitor programmed cell death 1 ligand 1 (Cd274; AKA PDL-1) were added. For colon afferents only, 16 genes associated with immune function were also examined. Based on previous RNAseq analysis, these immune genes are highly expressed in both the nodose (Wang et al., 2017) and lumbar DRG (Usoskin et al., 2015).

The protocols used here were previously shown to produce a linear correlation between starting mRNA transcript level and the amount of mRNA detected following reverse transcription and amplification (Adelman et al., 2019). Thus, for each gene we can determine the relative level of expression for each cell analyzed (but cannot compare the level of expression between mRNA transcript species because of differential primer efficiency). Expression level information was

converted into the heat maps shown in **Figs. 3** and **4**. In addition, descriptors of expression level in each cluster are reported as “undetected” (every cell in a group had no detectable level of transcript), “low” (when the average expression level for cells in a cluster was in the lowest 30th percentile), “moderate” (between 31-70th percentile) or “high” (71-100th percentile).

Clusters were named by combining the approaches of Zeisel et al. (Zeisel et al., 2018) and Hockley et al. (Hockley et al., 2018). Abbreviations used for clusters are “PEP” for peptidergic (clusters containing cells with expression of mRNA coding for substance P (Tac1) and calcitonin gene related peptide (Calca)), “NP” for nonpeptidergic (clusters of cells not expressing neuropeptide related mRNAs), “NF” for neurofilament (clusters of cells with expression of the high molecular weight neurofilament gene (Nefh), a marker of myelinated afferents (Lawson and Waddell, 1991; Adelman et al., 2019)), “tl” for clusters containing cells originating predominately (over 85%) from thoracolumbar ganglia, “ls” for clusters of cells from lumbosacral ganglia, “ng” for clusters of cells from nodose ganglia, and “m” for mixed clusters containing cells from TL and LS ganglia. Clusters are named based on whether they are ng, ls, tl or m plus whether they are PEP, NP, NF or some combination (e.g., NF-PEP). Because it is not possible to interrogate the unbiased algorithms used in this study, the contribution of each gene to the decision-making process is unknown. Therefore, genes that are highlighted in discussion of each cluster were chosen based on our inspection of the heatmaps and identification of ones most highly expressed in a cluster and ones that are either absent or expressed at low levels.

2.3.2.1 Colon Afferents

Using the previously validated AHC methods of Adelman et al., (Adelman et al., 2019), a total of 132 colon afferents from NG (n=43), TL (n=48) and LS (n=41) separated into 13 distinct

clusters based on mRNA level (n=7 animals, 4 males, 3 females; **Figure 3**, **Figure 5A**). 16 cells did not fit into any of these clusters and are not represented on the heatmap in **Figure 3.**

As the AHC is unbiased, it is interesting that the first branch point in the cell dendrogram separates nodose afferents from spinal afferents (**Figure 3**, **Figure 5A**). Major distinguishing characteristics of nodose afferents were the high levels of purinergic receptors (*P2rx2*, *P2rx3*) and *Cd274*, and low expression of calcitonin gene related polypeptide a (*Calca)* and preprotachykinin-1 (*Tac1)*. Nodose afferents were further divided into four clusters based on genes involved in growth factor receptor signaling, chemokine receptors and GPCR expression.

For AHC of spinal afferents, the first branch point separated cells based on levels of the heavy neurofilament (*Nefh*), a marker of myelination. The *Nefh* branch point gave rise to 5 *Nefh*-low and 4 *Nefh*-high final clusters. In the *Nefh*-low group, two clusters came almost exclusively from TL ganglia, one from LS ganglia, whereas two other clusters contained a mixture of TL and LS neurons. For the *Nefh*-high clusters, two contained only LS afferents and the other two contained a mix of TL and LS neurons. Thus, even at the initial level of analysis, the AHC revealed multiple mRNA-based clusters that were unique to afferents arising from anatomically distinct neuron populations.

Nodose afferent clusters

Four nodose clusters with nearly equal number of cells (8-11 in each cluster) were identified (**Figure 3, Figure 5A**). Each cluster had both proximal colon and distal colon projecting cells. The **ngNPa** cluster has high levels of interleukin-8 receptor type 2 (*Cxcr2*), prostaglandin I2 receptor (*Ptgir*), and interferon gamma receptor 2 (*Ifngr2*). The **ngNPb** cluster also had high *Cxrc2*, interleukin-1 receptor type 1 (*Il1r1*) and cholinergic receptor nicotinic alpha 3 subunit (*Chrna3*). The **ngNF** cluster was a distinct nodose cluster with high expression of *Nefh*, glial cell

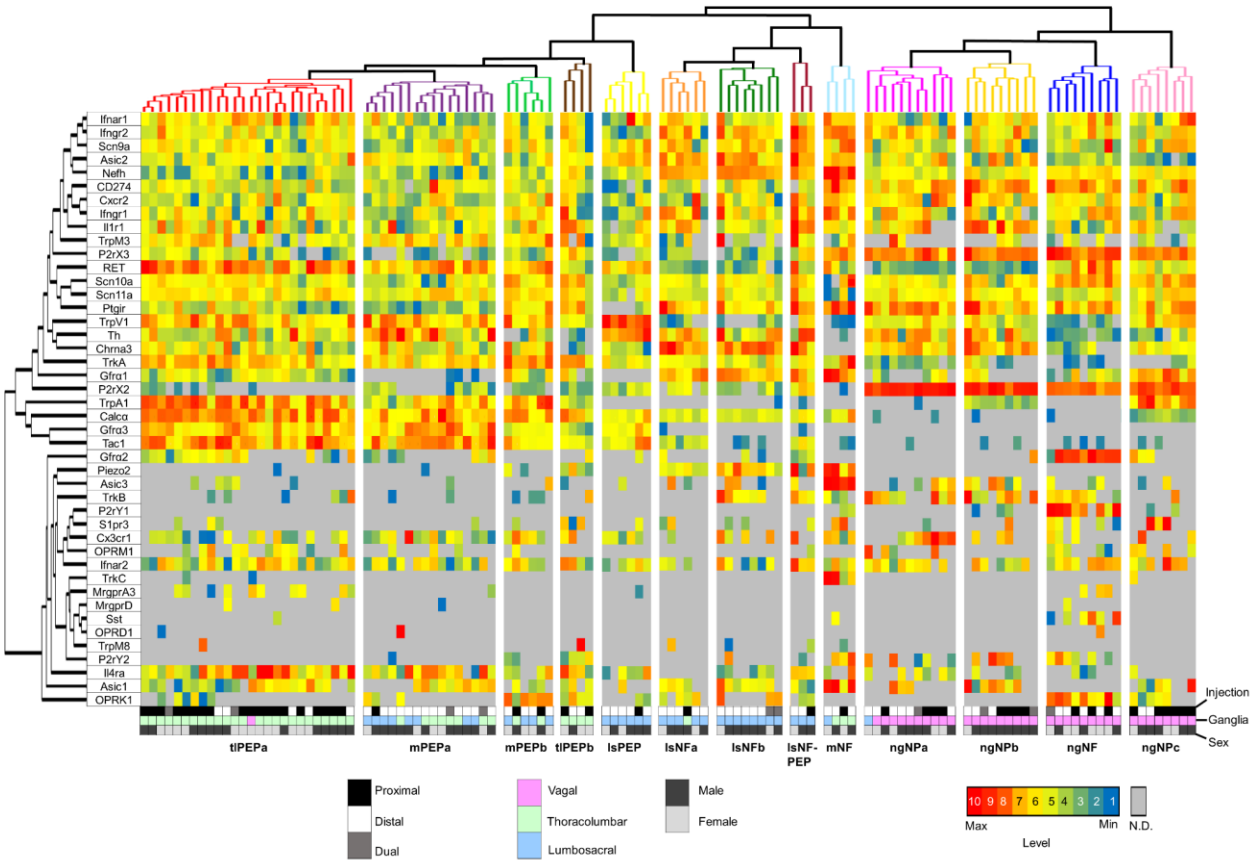

Figure 3 Colon afferents cluster into 13 distinct molecular clusters

Heatmap of mRNA expression across 116 colon afferents. Neurons and transcripts were clustered using the UPGMA Matlab algorithm. Red indicates high level of transcripts and blue the lowest. Gray bars indicate not detected (N.D.). The numbers in the color bar (bottom right) designate the colors that correspond to "low" (1-3), "moderate" (4-7) and "high" expression (7-10) used in the text. The bottom three rows of the heatmap indicate whether individual cells were back-labeled from injections into the proximal colon (black), distal colon (white) or proximal and distal (gray), whether the cell body was in the NG (pink), TL (green) or LS (blue) ganglia, and whether cells were taken from male (dark gray) or female (light gray) mice. Cells grouped into clusters designated tlPEPa (# of cells=26), mPEPa (# of cells =16), mPEPb (# of cells =6), tlPEPb (# of cells 4), lsPEP (# of cells =6), lsNFa (# of cells =6), lsNFb (# of cells =8), lsNF-PEP (# of cells =3), mNF(# of cells =4), ngNPa (# of cells =11), ngNPb (# of cells =9), ngNF (# of cells =9), and ngNPc (# of cells =8).

line-derived neurotrophic factor family receptor alpha 2 (*Gfra2;* one of three co-receptors for *Re*t), sodium voltage-gated channel alpha subunit 10 (*Scn10a*), purinergic receptor *P2ry1* (a Gq coupled GPCR detected only with uniform, moderate expression in this one cluster), and opioid receptor kappa 1 (*Oprk1*). The nodose **ngNF** group was also unique in the uniform and low level of transient receptor potential cation channel family v member 1 (*Trpv1)* and *Chrna3*. The final nodose cluster (**ngNPc**) had high expression of *Scn10a* and glial cell line-derived neurotrophic factor family receptor alpha 1 (*Gfra1,* along with moderate expression of its co-receptor *Ret*) and was the only nodose cluster with a moderate level of transient receptor potential cation channel family a member 1 (*Trpa1*).

Spinal Afferents - Thoracolumbar clusters

Of the two TL clusters (**tlPEPa** and **tlPEPb**), **tlPEPa**, overall the largest cluster, had high levels of *Calcα* and *Ret*. This cluster also had moderate expression of glial cell line-derived neurotrophic factor family receptor alpha 3 (*Gfra3*; which was absent in nodose clusters), *Tac1,* tropomyosin receptor kinase a (*Trka*), *Trpa1*, *Trpv1* and interleukin-4 receptor alpha (*Il4ra*) (**Figure 3, Figure 5A**). The **tlPEPa** cluster neurons project primarily to the proximal colon. As anatomical origin (proximal vs. distal) was not a variable in the AHC algorithm, this clustering indicates that transcripts of proximal colon afferents are unique from those of the distal colon. The **tlPEPb** cluster, the second smallest of the spinal groups, had moderate *Il4ra,* low *Trpa1* and high *Trka,* relative to the **tlPEPa** cluster. This cluster also had high interleukin-1 receptor type 1 (*Il1r1*) and moderate transient receptor potential cation channel family m member 8 (*Trpm8*), purinergic receptor *P2ry2* and *Oprk1*.

Spinal Afferents - Lumbosacral clusters

Within the four identified LS clusters, 20/23 of the back-labeled afferents were from the distal colon (two were dually labeled) indicating that the distal colon receives functionally distinct afferent input relative to the proximal colon. All but the **lsPEP** cluster had high or moderate *Nefh,* piezo type mechanosensitive ion channel component 2 (*Piezo2*), interferon gamma receptor 2 (*Ifngr2*) and *Gfra1*. The sodium voltage-gated channel alpha subunit 9 (*Scn9a*) was expressed in all LS neurons (including the **lsPEP** cluster) as was the acid sensing ion channel 2 (*Asic2*). The **lsNF-PEP** cluster was distinguished by high levels of the immune genes interferon gamma receptor 1 (*Ifngr1*), *Ifngr2*, *Cxcr2*, *Il1rl*, *Ptgir,* and interferon alpha receptor 2 (*Ifnar2*). This cluster also had high *Chrna3* and moderate *Th, P2rx2, Tac1*, and *Calca*. The **lsNFa** and **lsNFb** clusters were the most similar of the LS clusters, distinguished by relatively high levels of *Ifngr1* in the **lsNFa** cluster and *Ifngr2* in the **lsNFb** cluster. The **lsNFb** cluster also had moderate tropomyosin receptor kinase b (*Trkb*), which was absent from other spinal clusters except the **lsNF-PEP** cluster. As mentioned above, the **lsPEP** cluster had transcripts not detected in other LS clusters and was unique in having the high levels of *Trpv1* and tyrosine hydroxylase (*Th*).

Spinal afferents - Mixed clusters

There were 3 mixed clusters of spinal afferents that were defined by neurons within both TL and LS ganglia (**Figure 3**, **Figure 5A**). The **mPEPa** and **mPEPb** clusters had high or moderate *Calca, Gfra3, Ret,* and *Trpv1* transcripts. The **mPEPa** cluster also had moderate levels of the immune genes *Il4ra, Ifngr2,* and fractalkine receptor (*Cx3cr1*). The **mPEPb** cluster was the only cluster with high transient receptor potential cation channel family m member 3 (*Trpm3*) and *Oprk1,* a gene with minimal expression in other spinal clusters (although present at high levels in one nodose cluster).

The other mixed cluster (**mNF**) was similar to the LS clusters with respect to the relative level of *Nefh*, Piezo2, Scn9a, *Gfra1*, and *Asic2*. This cluster also contained the immune related genes *Ifnar1*, *Ifngr2*, *Cd274*, *Cxcr2*, *Ifngr1*, *Ptgir*, and *Cx3cr1* as well as *P2rx3*, acid sensing ion channel 1 (*Asic1*), acid sensing ion channel 3 (*Asic3*), and tropomyosin receptor kinase c (*Trkc*). This was the only cluster (spinal or NG) that had no detectable level of *Chrna3*.

Comparison of gene clustering of vagal and spinal colon afferents

A significant finding of the current study is the nearly exclusive manner in which colon NG neurons segregate from colon spinal afferents. Only one colon DRG neuron appeared in the 4 nodose clusters and only one nodose neuron sorted with neurons in the spinal clusters. The major contributor to this segregated pattern was the uniformly high levels of *P2rx2* and *P2rx3* in NG neurons. Similarly high levels of the prostacyclin receptor (*Ptgir*) and *Cd274* (AKA PDL-1 (programmed cell death ligand 1)) were in the NG. *Ptgir* and *Cd274* have important roles in immune responses, consistent with the role of nodose afferents in neuroimmune regulation (Baral et al., 2018; Sharkey & Mawe, 2002; Tracey, 2002). The individual NG clusters were also unique. No NG cluster had characteristics similar to the peptidergic clusters at TL or LS levels. There was a conspicuous absence or low level of *Trka, Calca, Gfra3* and *Tac1* in NG neurons, consistent with previous findings (Mazzone et al., 2020; Trancikova et al., 2018; J. Wang et al., 2017).

2.3.2.2 Bladder Afferents

For the bladder, 32 genes were analyzed from 128 back-labeled afferents from LS (80) and TL (48) levels (n=11 animals, 3 males, 8 females; **Figure 4**, **Figure 5B**). Neurons sorted into 7 distinct clusters with 9 cells not fitting into any cluster (not shown in the heat map). Because some afferents can project to both bladder and colon (Christianson et al., 2007; Keast & De Groat, 1992), in some experiments CTB conjugated to different fluorophores was injected into bladder and

colon. From these animals (n=5, 2 males, 3 females) 14 convergent bladder/colon afferents were analyzed. Interestingly, these convergent afferents did not cluster into a separated group but were spread across the different clusters.

Unlike colon afferents, the first branchpoint in clustering of bladder afferents was defined by *Gfra3* and *Gfra1*. Neurons in these groups had either high levels of *Gfra1* or *Gfra3*, but never

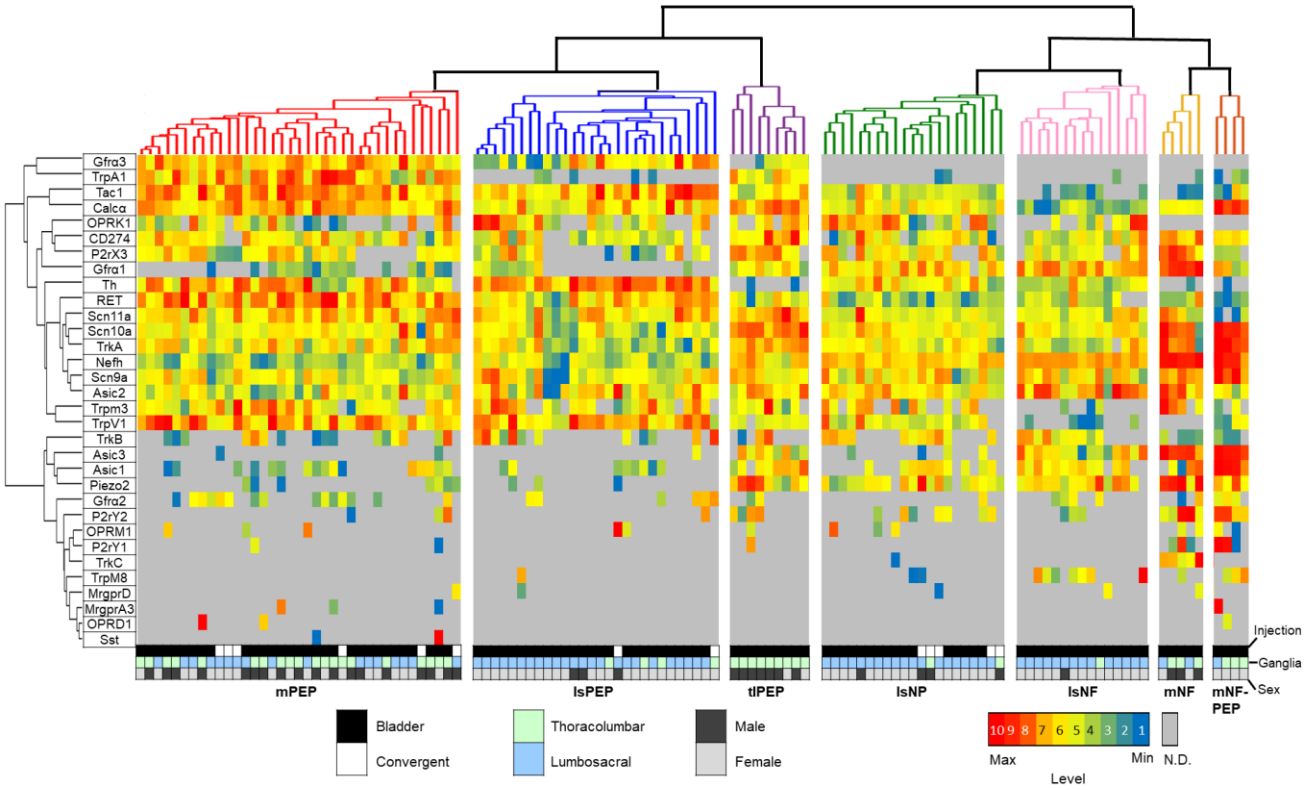

Figure 4 Bladder afferents cluster into 7 distinct molecular clusters.

Heatmap of mRNA expression across 119 bladder afferents. Color coding for bladder afferents used the same methodology and parameters used for colon afferents. "Convergent" designates afferents that were back-labeled from retrograde makers placed in both colon and bladder. Bladder afferent groups were clustered into **mPEP** (# of cells 37), **lsPEP** (# of cells 28), **tlPEP** (# of cells 9), **lsNP** (# of cells 21), **lsNF** (# of cells 15), **mNF** (# of cells 5), and **mNF-PEP** (# of cells 4). As in the colon, some clusters arose from afferents located primarily at either TL or LS levels.

both. *Nefh,* the gene associated with the first branchpoint for colon afferents, was also highly segregated, being expressed at moderate to high levels in all *Gfra1* clusters, but moderately in only one *Gfra3* cluster. Similar to colon afferents, there were distinct TL and LS clusters as well as mixed clusters.

Thoracolumbar cluster

Only one bladder afferent cluster was comprised exclusively of TL afferents (**tlPEP**) (**Figure 4**, **Fig 5B**). The **tlPEP** cluster had high *Calcα, Cd274*, *Scn10a*, *Scn11a*, and *Trka* and moderate levels of *Nefh*, *P2rx3*, and *Piezo2*. This cluster was also distinctive in having both *Gfra1* and *Gfra3*, although at low to moderate levels. The low co-expression of *Gfra1* and *Gfra3* is likely the reason for positioning of this cluster at the border of the major branchpoint.

Lumbosacral clusters

Bladder afferents formed three LS exclusive clusters. The **lsPEP** cluster had low *Gfra3* and almost no *GFRa1* (undetectable in 19/28 and moderate to low in the rest), high *Th* and moderate *Calca, Tac1,* Ret and *Tprv1*. The **lsNP** and **lsNF** clusters had no cells with detectable *Gfra3,* but moderate *Piezo2*. The **lsNP** and **lsNF** clusters can be separated by the high levels of *Asic2*, *Gfra1,* and *Nefh* in the **lsNF** cluster. The **lsNP** cluster also had high *Trka* (moderate in the **NP** vs. low in the **NF**).

Mixed clusters

Bladder afferents formed three mixed clusters, **mPEP**, **mNF**, and **mNF-PEP**. The **mPEP** cluster was the largest and had the highest level of *Gfra3* of any bladder cluster. **mPEP** also had high *Tac1* and *Ret* and moderate *Calca, Trpa1, Trpv1, Th,* and *Scn11a*. The mixed **mNF** and **mNF-PEP** clusters were small and similar to the LS clusters based on *Gfra1* and *Nefh*, but also had high levels of several other genes including *Scn10a, Scn9a, Asic3,* and *Piezo2*. The **mNF** cluster can be

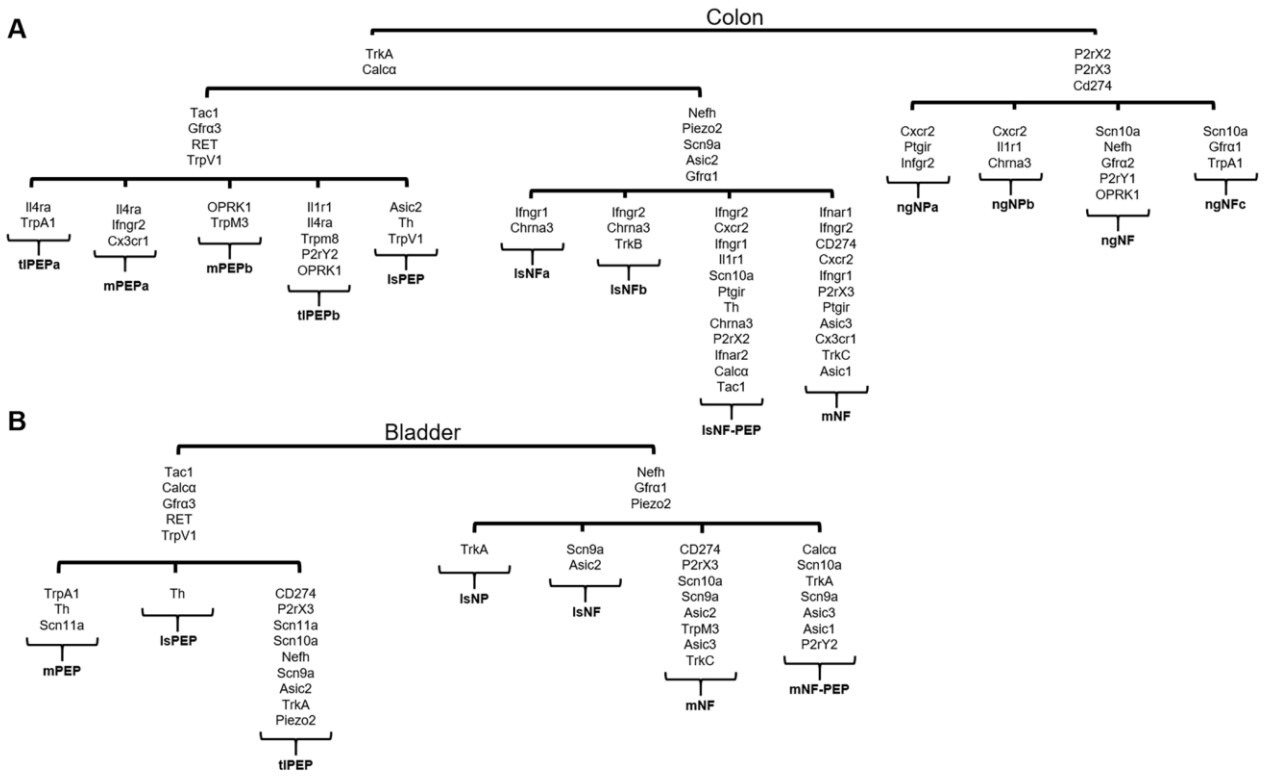

Figure 5 Colon and bladder afferent clusters have similar genes expressed but in different combinations.

A) Schematic of major branch points from colon afferent clustering. Transcripts that differentiate each branch point are shown. B) Schematic of major branch points for bladder afferent clustering and the distinct genes in each cluster.

differentiated from the **mNF-PEP** cluster by high *Cd274*, *P2rx3*, *Asic2*, *Trpm3*, and *Trkc*. In contrast, the **mNF-PEP** cluster had high *Calca*, *Trka*, *Asic1*, and *P2ry2*.

2.3.2.3 Comparison of Gene Clustering for Bladder and Colon Afferents

Comparison of high level transcripts across colon and bladder clusters shows many are present in afferents that innervate the two organs. However, different combinations of transcripts

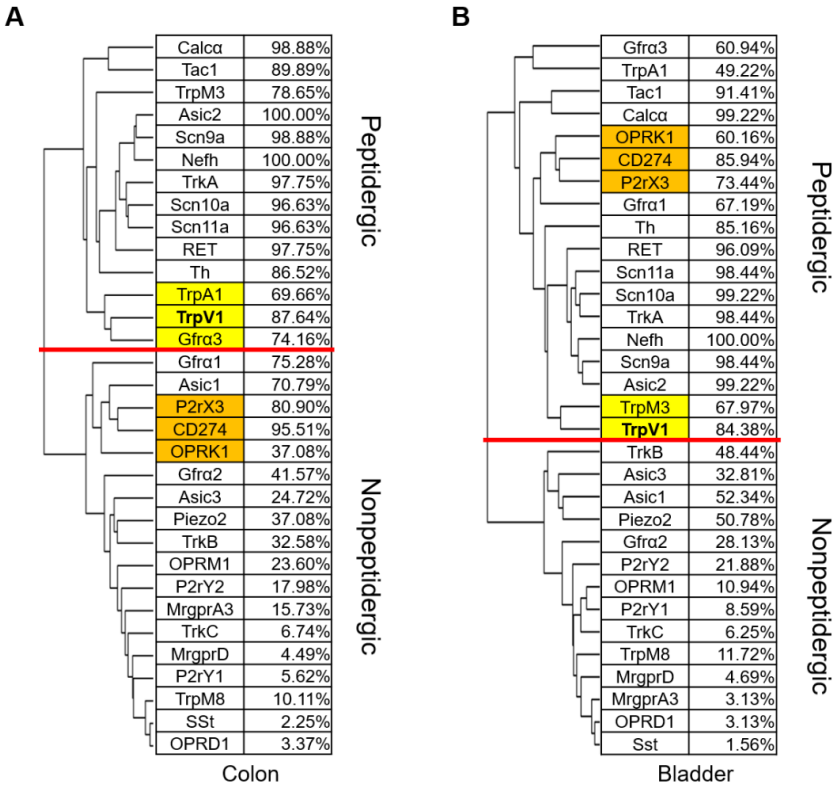

Figure 6 Spinal afferents of colon and bladder cluster into peptidergic and nonpeptidergic groups.

A) Dendrogram of gene clustering for colon spinal afferents and percentage of cells with detectable transcripts. B) Dendrogram of gene clustering for bladder spinal afferents and the percentage of cells with detectable transcripts. Red line indicates the first major branch point in the dendrogram and separates peptidergic- from nonpeptidergic-related groups. Highlighted cells indicate differences between colon and bladder gene clustering.

define the clusters (**Figure 5A-B**). Dendrograms that illustrate the segregation of the 32 genes examined in spinal afferents for both organs (**Figure 6A-B**) show some significant differences; the first major branch point for colon is associated with a peptidergic phenotype (e.g., *Calca*, *Tac1*, *Trka*, *Trpa1*, *Trpv1*, *Gfra3*) vs. a non-peptidergic phenotype (e.g., *Asic1, Trkb*, *Gfra2*, *Mrgprd, Mrgpra3, Trpm8, Piezo2*). The clustering in bladder shows a similar first branchpoint, however three high level and closely clustered genes *(Oprk1*, *Cd274* and *P2rx3)*, are shifted from the nonpeptidergic to the peptidergic group. In addition, in colon, *Trpv1* nearest neighbors are

peptidergic-associated genes (*Gfra3* and *Trpa1*), whereas in the bladder *Trpv1* was separated from these genes by 3 branchpoints and clustered with *Trpm3*. Given that the overall percentage of cells expressing all 32 genes was not dramatically different for bladder and colon afferents, these differences suggest that at least some bladder afferents have novel properties not present in colon afferents.

2.3.3 Calcium Imaging and Validation of Gene Function in Colon Afferents

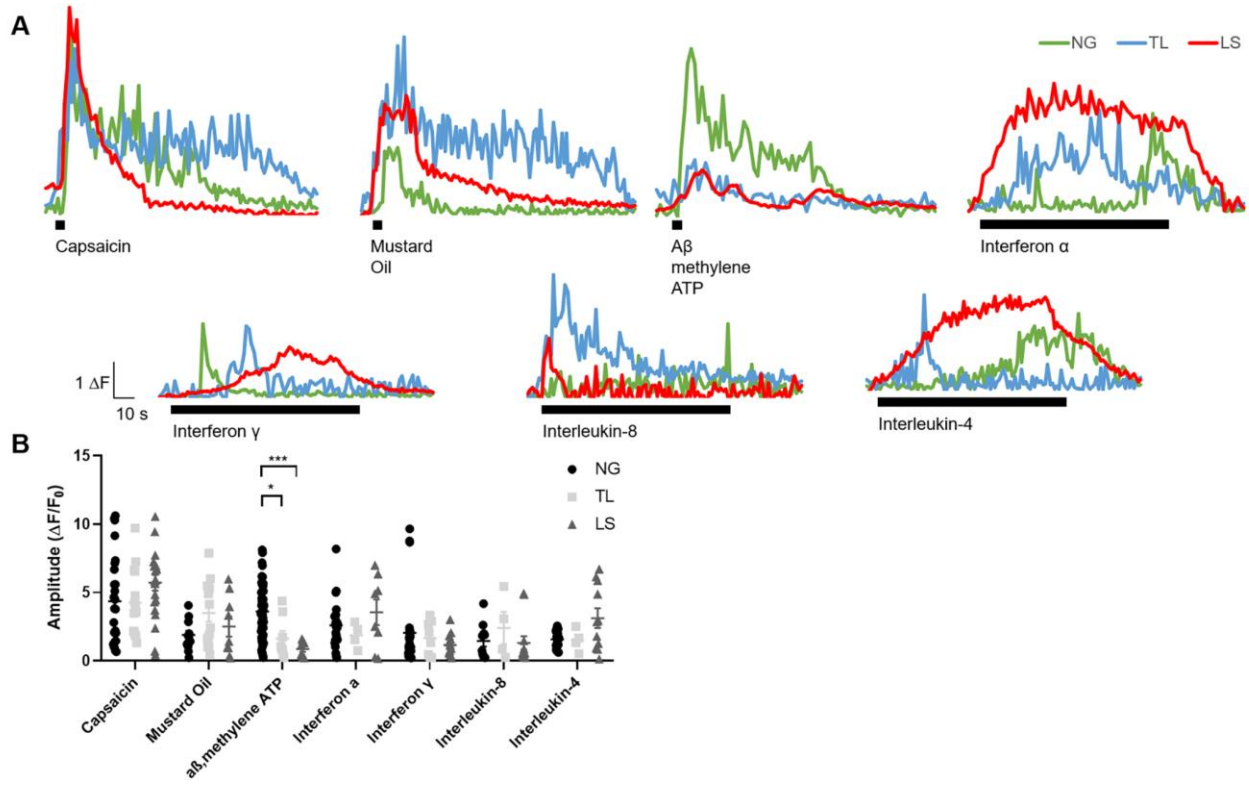

Figure 7 Colon afferents have functional receptors identified by single cell RT-qPCR.

A) Sample traces of calcium transients from the NG, TL, and LS afferents to agonists. Black bar represents the length of agonist presentation and corresponds to 4 seconds (short bars) or 90 seconds (long bars). B) Amplitude of calcium transients from applied agonists from the different levels of the neuraxis. Amplitudes were similar for all agonists at all levels except for responses for α,β methyl ATP, which was significantly larger in NG afferents. *p<.05, ***p<.001, using a mixed effects model.

Because RNA expression may not correlate with synthesis of functional protein (Adelman et al., 2019), we performed *in vitro* calcium imaging of back-labeled colon afferents to examine the relationship between mRNA expression and function. Afferents from different levels of the neuraxis were analyzed. Three agonists (capsaicin, mustard oil and α,β-methylene ATP) known to activate colon afferents (Christianson, Bielefeldt, Malin, & Davis, 2010; Shinoda, La, Bielefeldt, & Gebhart, 2010a) via membrane bound receptors that were included in this analysis were used. We also examined the response properties of identified afferents to ligands of immune-related receptors (Interferon α, Interferon γ, Interleukin-4, and Interleukin-8) previously shown to produce calcium transients in primary afferents (Oetjen et al., 2017; J. Wang et al., 2017). The percent of responders was compared to the percent of cells that exhibited detectable (low, medium, or high) levels of gene transcripts (**Table 3**).

Calcium imaging was performed on 131 colon afferents (56 NG (n=6, 4 males, 2 females), 35 TL afferents (n=8, 2 males, 6 females), and 40 LS afferents (n=8, 4 males, 4 females); **Figure 7A**). Many TL afferents were positive for *Trpa1* (91.67% vs. 48.84% for NG afferents and 43.90% for LS afferents) and this correlated with a high percentage of mustard oil responsive neurons (63.64%, compared to 36.58%, NG and 47.37%, LS). Capsaicin activated 74.28% of NG neurons, 77.78% of TL neurons, and 95.45% of LS neurons, consistent with the relatively broad *Trpv1* expression across all levels (**Table 3, Figure 4**). Adelman et al. (Adelman et al., 2019) had shown for cutaneous afferents that neurons responsive to capsaicin had a ΔCt of 9 or lower for *Trpv1* (i.e., a ΔCt of 9 was the expression threshold). In this study, this level of *Trpv1* was met in approximately 80% of the colon afferents, roughly matching the percentage of neurons with capsaicin responses (**Table 3**).

Table 3 Calcium imaging of back-labeled colon afferents reveals relationship between mRNA level and receptor function.

The percentage of cells that respond to selected agonists was compared to the percentage with detectable levels of the corresponding receptor gene(s). Capsaicin and mustard oil showed good agreement between responders and *Trpv1* or *Trpa1*, respectively. For α,β-methylene ATP, this was also true for NG afferents. TL and LS neurons exhibited many fewer responders, which is likely due to the reduced level of *P2rx2/3* in these cells. For immune-related receptors (*Ifnar1/2*, *Ifngr1/2*, *Cxcr2* and *Il4ra*), almost all afferents had detectable transcripts, but in most cases, fewer than half of the cells exhibited calcium transients in response to ligands. The number of cells analyzed is in parentheses.

	NG		TL		LS	
Agonist (Gene)	Percentage of Responsive Cells	Percentage of RNA expression	Percentage of Responsive Cells	Percentage of RNA expression	Percentage of Responsive Cells	Percentage of RNA expression
Capsaicin (Trpv1)	74.28% (35)	93.02%	77.78% (18)	95.83%	95.45% (22)	78.05%
Mustard Oil (Trpa1)	36.58% (41)	48.84%	63.64% (22)	91.67%	47.37% (19)	43.90%
αβ,methylene ATP (P2rx2/P2rx3)	96.15% (52)	97.67% / 97.67%	34.78% (23)	41.67% / 91.67%	34.48% (29)	75.61%/ 68.29%
Interferon α (Ifnar1/Ifnar2)	43.14% (51)	95.35% / 76.74%	18.18% (22)	97.92% / 95.83%	38.09% (21)	100%/ 80.4%
Interferon γ (Ifngr1/Ifngr2)	58.33% (48)	95.35% / 97.67%	34.78% (23)	100% / 97.92%	24.24% (33)	100% / 100%
Interleukin-8 (Cxcr2)	37.04% (27)	97.67%	15.38% (26)	100%	46.15% (26)	100%
Interleukin-4 (Il4ra)	41.18% (51)	34.88%	23.53% (17)	91.67%	47.83% (23)	65.85%

The P2rX2/3 and P2rX3 selective agonist α,β-methylene ATP activated 96.15% of NG neurons, 34.78% of TL neurons, and 34.48% of LS neurons. For the NG, the percentage of α,β-methylene ATP responders (96.15%) matched the uniformly high level of *P2rx3* and *P2rx2*

detected in NG afferents. In contrast, for TL and LS neurons, there were fewer responders to α,β-methylene ATP (34.78% and 34.48%, respectively) relative to the percentage of neurons expressing detectable levels of *P2rx3* and *P2rx2* (see **Table 3**). Adelman et al. found that for cutaneous afferents expressing the *P2rx3* receptor (*P2rx2* was not examined), a ΔCt of 6 was the threshold for presence of functional receptors. This value was measured in only 20.45% of TL neurons and 14.28% of LS neurons, consistent with the low percentage of afferents responding to α,β-methylene ATP. In terms of the amplitude of the calcium transients in response to α,β-methylene ATP, for neurons that did respond, the peak responses of NG afferents was significantly higher than for LS and TL afferents ($F_{(12,210)}$= 3.065, p = 0.0204 for TL, p < 0.0001 for LS; **Figure 7B**). This suggests that for P2X receptors, there is a direct relationship between the transcript level and response to ligand.

With the exception of *Il4ra* in NG and LS neurons, all immune-related receptors assayed were detected in nearly all cells. Unlike other ligands examined, the percentage of neurons activated by the immune-receptor ligands was significantly lower than the percentage of cells positive for these receptor genes (except for *l4ra* in the NG). In NG neurons, calcium responses to interferon-α, interferon-γ, interleukin-8, and interleukin-4 occurred in approximately half (37-58%) of the cells expressing the corresponding receptor mRNA. A lower percentage of TL and LS neurons exhibited calcium transients in response to these ligands (24-47%, LS and 15-34%, TL neurons). These results indicate that mRNA alone cannot be used as an indicator of functional activity for these receptors. This may be because these immune receptors are not ionotropic receptors, and often act as receptor complexes that activate complex intracellular signaling cascades (Bezbradica & Medzhitov, 2009). For cells where immune ligands did cause a calcium

transient, the amplitude of these transients were similar to those for capsaicin, mustard oil, and α,β-methylene ATP (**Figure 7B**).

2.4 Discussion

Identification of unique protein or mRNA markers that can be used as surrogates for functional phenotyping has long been a goal of primary afferent biology. Here we employed single cell mRNA analyses of up to 42 genes to determine if a unique molecular signature for colon and bladder primary afferents mapped to their origin, i.e, NG, TL, or LS ganglia. Using unbiased, AHC, we found that for both colon and bladder afferents, most clusters had neurons primarily from a single level of the neuraxis (>85% of cells from NG, TL or LS) than from mixed levels (**Figure 8**). For the 13 colon clusters, 4 originated in the NG (**ngNPa-c, ngNF**), 2 from TL (**tlPEPa&b**) and 4 from LS (**lsPEP, lsNFa&b** and **lsNF-PEP**). Of the 7 bladder clusters, 1 came from TL (**tlPEP**) and 3 from LS (**lsPEP, lsNP**, and **lsNF**) ganglia (**Figure 8**). For colon, there were also clusters that primarily innervated the proximal (**tlPEPa**) or distal (**mPEPa, lsPEP, lsNFa&b**) colon. Genes in our panel were chosen based on a validated role in stimulus detection (e.g., TRP channels, piezo2, P_2X receptors, ASIC channels, cytokine receptors) or responsiveness (e.g., opioid receptors, neuropeptides, sodium channels), suggesting that function-specific components of sensory information arriving in the CNS is segregated by sensory ganglia at different levels.

The mRNA analysis from the current study revealed some key differences between colon and bladder afferents compared to studies examining unidentified (i.e., not back-labeled from a

specific target) DRG afferents (Usoskin et al., 2015; Zeisel et al., 2018). Almost all spinal afferents expressed some level of *Calcα* whereas previous studies of unidentified afferents reported

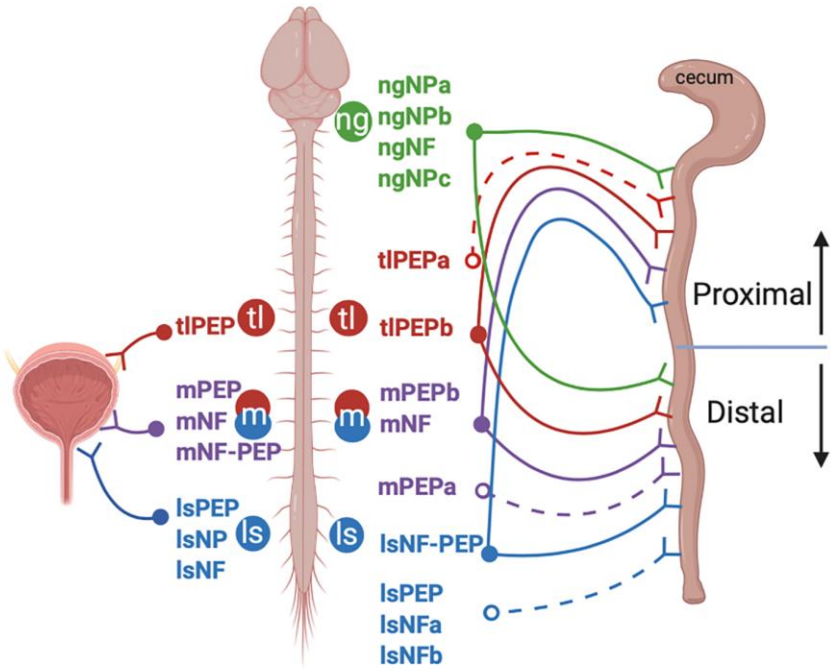

Figure 8 Automated hierarchical clustering shows afferent clusters for colon and bladder arise from distinct sensory ganglia.

For both bladder and colon, the majority of the identified clusters are from one anatomical level; i.e., not from mixed (m) ganglia (11/13 for colon and 4/7 for the bladder). For the colon, there are also clusters that project selectively to either the proximal or distal colon (dotted lines). Illustration created with BioRender.com.

clusters with no *Calcα* expression. In our study > 80% of colon and bladder afferents express *Th* whereas in previous studies of unidentified afferents, Th expression was restricted to putative C-LTMRs. However, studies of unidentified sacral DRG afferents did replicate the findings here with both showing high levels of *Trpv1*, *Chrna3*, and *Tac1* (Smith-Anttila et al., 2020).

Unlike RNAseq analysis, our study of visceral afferents focused on a small number of curated genes. The clusters identified are similar to those detected in a previous RNAseq analysis of back-labeled distal colon afferents from both TL and LS levels (Hockley et al., 2018). Using single-cell consensus clustering (Kiselev et al., 2017) of 314 cells in the work by Hockley and

coworkers, 7 clusters were identified based on the presence of peptidergic (*Calcα, Tac1, Trpa1*) or nonpeptidergic (*Mrgprd, P2rx3, Gfrα2*) markers, and the expression of *Nefh* (which was coexpressed with *Piezo2*). Hockley et al., also identified mixed clusters arising from a combination of TL and LS afferents and clusters that were exclusively from LS afferents (defined as "pelvic nerve" afferents). The present study identified clusters that arise exclusively from TL afferents that were not identified by Hockely et al. However, these TL clusters have a majority of afferents originating from the proximal colon, which were not included in Hockley et al.

The NG clusters were unique in that unlike TL or LS clusters, NG clusters projected virtually equally to proximal and distal colon (45.13% proximal vs. 45.44% distal, compared to a 38.25%/52.94% (proximal/distal) for TL ganglia and 2.54%/95.35% for LS ganglia). This suggests that unlike clusters arising from DRG afferents, innervation of different NG clusters is required throughout the length of the colon. This could reflect a role for NG afferents in neuroimmune monitoring, which would be in line with the high percentage of NG neurons that exhibited calcium transients in response to cytokine application.

RNAseq studies have been performed on NG afferents and compared to jugular afferents (Kupari, Häring, Agirre, Castelo-Branco, & Ernfors, 2019; Mazzone et al., 2020; J. Wang et al., 2017). In mouse, jugular afferent somata are located in a ganglion fused to the placode-derived NG and like DRG neurons, are neural crest derived (Baker & Schlosser, 2005). These studies found that like the DRG neurons examined here, jugular neurons are molecularly distinct from NG afferents. However, cluster analysis found several more unique NG clusters then identified in the present study (18 nodose clusters with a major breakdown between "mechanosensitive" groups (1-11) and "nociceptive" clusters (12-18); (Kupari et al., 2019). A likely reason for this difference is

that we used back-labeled neurons from colon whereas in Kupari et al. clusters from all the thoracic and abdominal organs innervated by the NG were represented.

A major theory to explain the need for overlapping sensory innervation of the viscera, especially for those organs that receive nodose and spinal input like the colon, is that different pathways relay unique aspects of the sensory experience. For example, in the case of pain arising from abdominal organs (including the colon), spinal afferents have been proposed as the transducer of noxious pain (e.g., sharp, burning) whereas nodose afferents transmit affective aspects of visceral pain (e.g., fear, anxiety, nausea) (Berthoud & Neuhuber, 2000; Grundy, 2002; Sengupta, 2009). These distinctions are supported by patient reports of sensations produced by whole vagal nerve stimulation (Ben-Menachem, 2002; Sackeim et al., 2001), but contradicted by the similarities in firing properties of nodose and spinal afferents innervating the gut (Bielefeldt et al., 2006; Ozaki & Gebhart, 2001; Sengupta et al., 1990; Yu et al., 2005) and the anecdotal reports of noxious gut pain in patients with spinal transections, i.e., patients that lack ascending input from spinal afferents (Finnerup et al., 2008; Levinthal & Bielefeldt, 2012; Yung & Groah, 2001)).

Given that in most cases activation of visceral afferents does not produce conscious sensations (Critchley & Harrison, 2013), the data presented here could be used to argue that the driving force behind the evolution of the complex pattern of sensory innervation arises out of the requirements of homeostatic regulation and not because the different afferent populations are "labeled-lines" carrying segregated qualities of visceral sensation. First, the projections of NG and TL clusters overlap anatomically in a manner that suggests these molecularly distinct afferent populations are making unique contributions to homeostatic function; both NG and TL afferents innervate the entire colon, with approximately equal numbers of afferents, providing direct input to their associated parasympathetic (NG) and sympathetic (TL) CNS circuits in the spinal cord and

brainstem. Second, while it is clear that neurons at NG, TL and LS levels have unique transcriptomic profiles with respect to receptor expression (e.g., TRP and purinergic channels, cytokine receptors) that should "tune" them to different aspects of the internal milieu, all clusters, regardless of origin, express receptors required for monitoring of homeostasis-relevant stimuli. Third, many organs, in particular the small and large bowel, contain large numbers of innate immune cells and their interactions with their local microbiome affect the entire body (Cryan & O'mahony, 2011; Maynard, Elson, Hatton, & Weaver, 2012). The near ubiquitous expression of immune-related genes in all clusters is consistent with the idea that these afferents play a role in integration of information required for immunological homeostasis. Moreover, the efferent function of visceral afferents, i.e., their ability to release molecules (e.g., peptides likes CGRP and substance P) that can modulate immune cells has recently been shown to play a central role in somatic and visceral tissue responses to pathogenic insult (Baral et al., 2018; N. Lai et al., 2020; Saloman et al., 2020).

The corollary to this hypothesis is that different aspects of conscious visceral sensation (e.g., both noxious and affective aspects of visceral pain) is the result of integration of information gathered from all sensory ganglia and then processed by different (but likely overlapping) CNS circuits. Five colon afferent fiber types have been identified (mucosal, muscular, serosal, mesenteric, and muscular/mucosal) (Brierley et al., 2005; Brierley et al., 2004), as well as four classes of bladder afferents (serosal, muscular, muscular/urothelial, and urothelial) (Xu & Gebhart, 2008; Zagorodnyuk, Costa, & Brookes, 2006). For colon, both TL and LS have mucosal, muscular, and serosal afferents whereas mesenteric afferents are specific to TL neurons and muscular/mucosal afferents are specific to LS afferents. For the bladder, the TL level is comprised of primarily serosal and muscular afferents whereas the LS afferents contain all four subtypes.

This suggests that for both bladder and colon, some TL and LS afferents detect similar mechanical stimuli, whereas others code for unique aspects of these stimuli. That neurons from all levels of the neuraxis (including the NG) have a role in conscious sensation including pain is supported by the data presented here; all of the colon clusters express proteins shown to play important roles and/or are "required" for nociception in both somatic and visceral afferents (Bielefeldt & Davis, 2008; Delafoy et al., 2006; Kiyatkin, Feng, Schwartz, & Gebhart, 2013; McIlwrath et al., 2009). It seems unlikely that noxious pain would be detected by only a subset of these afferents from one or two levels with the remainder transmitting signals specific to non-noxious qualities of pain (i.e., affective aspects). The more parsimonious explanation is that afferents in all clusters, at all levels, are collaborating to provide a comprehensive report of peripheral conditions and occasionally this information reaches qualitative and quantitative thresholds required for conscious visceral sensation, including pain.

3.0 Changes in Extrinsic Primary Afferent Neuron to Myenteric Neuron Communication Following Inflammation

3.1 Introduction

It has previously been hypothesized that ExPANs have a direct local effector function on myenteric neurons due to the dense network of ExPAN endings around myenteric neurons (Anavi-Goffer & Coutts, 2003; Matsumoto et al., 2011; Spencer et al., 2014; Ward et al., 2003) and that capsaicin (thought to active ExPANs and not myenterics) produces smooth muscle contraction and myenteric neuron activity (Hayashi et al., 2010; Maggi, 1995; Matsumoto et al., 2009; Spencer, Sorensen, et al., 2016). However, recent work from our lab has shown that LS ExPANs do not directly activate myenteric neurons or produce movement. Afferent activation is able to induce colon contractions via spinal cord circuits as evidenced by calcium transients in myenteric neurons and muscle movement following LS afferents in an ex vivo preparation that included ExPANs in continuity with the colon via the spinal cord (Smith-Edwards et al., 2019). These results indicated that ExPANs modulate myenteric activity through a spinal reflex. However, these experiments were all performed in naïve mice, leaving the possibility that inflammation could induce direct communication of ExPANS to myenteric neurons, possibly in a manner similar to how inflammation converts “silent” colon afferents to active nociceptors in models of colitis (Feng & Gebhart, 2011; Feng, Joyce, & Gebhart, 2016; Feng et al., 2012a). Furthermore, it is possible that the three levels of the neuraxis that innervate the colon exhibit different levels of plasticity in response to inflammation in terms of activation of myenteric circuits.

One of the most common diseases characterized by colonic inflammation is inflammatory bowel disease (IBD). IBD consists of Crohn‘s Disease and ulcerative colitis and is characterized by chronic inflammation with unknown etiology (Loftus Jr, 2004). Common symptoms of IBD include abdominal pain, bloating, diarrhea, and fatigue. The observed changes in motility are assumed to regulated at least in part by changes in myenteric activity. Animal models of colitis cause visceral hypersensitivity (Ciesielczyk, Furgała, Dobrek, Juszczak, & Thor, 2017; Feng et al., 2012a; Miranda et al., 2007) and have been shown to sensitize spinal afferents innervating distal colon, including converting up to 25% of "silent" afferents (aka mechanically insensitive afferents (MIA)) to being able to code noxious mechanical sensation (Feng et al., 2012a; Jones, Xu, & Gebhart, 2005; Kiyatkin et al., 2013; Miranda et al., 2007; Shinoda, La, Bielefeldt, & Gebhart, 2010b). Although vagal afferents innervating the colon have not been well studied, evidence of afferent sensitization due to visceral inflammation have been seen in esophagus, lungs, and stomach (Holzer, 2003; Kollarik, Ru, & Brozmanova, 2010; Kollarik et al., 2007; Lamb et al., 2003) suggesting colon inflammation is likely to sensitive vagal afferents as well. Furthermore, peptides released by ExPANs (e.g. CGRP and SP) have been found to be upregulated in IBD (Holzer, 1998), although it is important to note these peptides can also be released by intrinsic ENS neurons. Therefore, it is possible after ExPANs have been sensitized by colitis, a direct local effector function on myenteric neurons may develop.

The colon receives extrinsic primary afferent neuron (ExPAN) input from TL and LS spinal afferents as well as vagal NG afferents (Chapter 2). It has been proposed that afferents arising from different levels of the neuraxis are differentially involved pain processing. LS afferents are thought to be involved in both acute and inflammatory pain, whereas TL afferents are only involved in inflammatory pain (Traub, 2000; Traub et al., 1993; Traub et al., 1994; Traub, Sengupta, &

Gebhart, 1996; G. Wang et al., 2005). In addition, electrophysiological recordings from TL and LS afferents in a model of colitis suggest TL afferents were sensitized during both the acute inflammatory phase and during recovery. In contrast, LS afferents were not sensitized during the acute inflammatory phase but became sensitized during the recovery phase (Hughes et al., 2009). These findings suggest that afferents arising from different levels of the neuraxis are differentially affected by colonic inflammation and likely play different roles during colitis.

In the periphery, ExPAN processes course throughout the myenteric ganglia of the enteric nervous system (ENS), where data suggests that they may have local effector functions through the release of neuropeptides (e.g. CGRP, substance P) at their peripheral terminals or act indirectly through spinal or vagovagal reflexes that is unique for each population. However, we have no idea how different ExPAN's modulate myenteric activity, nor how this may change in inflammatory states. The goal of this series of experiments is to determine if ExPAN regulation of myenteric activity changes in a model of inflammatory colon disease. For these studies, an *ex vivo* colon nerve preparation was used to investigate the ability of LS ExPANs to directly communicate with myenteric neurons during a model of colitis. Even after colonic inflammation, LS ExPAN stimulation did not cause a change in myenteric neuron activity or invoked movement. Furthermore, colitis did not change the ability of parasympathetic efferent fibers to evoke myenteric activity or movement. This suggested that another level of innervation may be more involved in regulating myenteric neuron activity after inflammation. *In vivo* optogenetic stimulation of colon afferents was used to stimulate all colon afferents and c-Fos staining in the areas where these afferents terminate (spinal cord and brainstem) was used as a surrogate for activation of second-order neurons. Interestingly, the TL level had little to no c-Fos staining in vehicle-treated animals in response to optogenetic activation of colon afferents, however,

following induction of inflammation, activation at the TL level resulted in dramatic increase in c-Fos staining in the dorsal horn and intermediolateral cell column. LS spinal cord had c-Fos staining in vehicle animals in response to optogenetic activation, but only the dorsal horn showed increased activation after colitis. The NTS (nucleus tractus solitarius, site of vagal afferent termination) also had c-Fos staining in vehicle mice and exhibited increased c-Fos staining after inflammation. These findings suggest that TL and NG afferents have increased activation at their central terminals in this model of colitis and are not only sensitized but may play an important role in activating brainstem and spinal circuits that could modulate colon function in inflammatory states.

3.2 Methods

3.2.1 Animals

Male and female mice aged 8–12 weeks were analyzed in these studies. E2a-Cre mice (RRID:IMSR_JAX:003724) were crossed with mice containing a floxed-STOP-GCaMP6s sequence in the Rosa26 locus (Ai96 mice, RRID:IMSR_JAX:028866). TRPV1-Cre mice (RRID:IMSR_JAX:017769) were crossed with mice containing a floxed-STOP-ChR2 sequence (Ai32 mice, RRID:IMSR_JAX:012569). Animals were housed in a facility approved by Assessment and Accreditation of Laboratory Animal Care (AAALAC), with a 12-hour light/dark cycle and free access to water and standard chow. Animal use protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

3.2.2 *Ex vivo* Colon Nerve Preparation

E2a-GCaMP6 mice were killed with isoflurane and transcardially perfused with cold carbogenated ACSF. Using a protocol modified from Brierley et al (Brierley et al., 2004), the distal colon (4 cm) was isolated along with the intact pelvic nerve, L6 DRG, and L6 dorsal and ventral roots; DRG and roots remained in the spinal column. The spinal column was pinned to a Sylgard-lined dish, and the attached colon was opened longitudinally along the mesenteric border and pinned out with the serosal side up. The preparation was transferred to the fluorescent microscope stage and superfused with ACSF maintained at 35°C–37°C. Concentric electrodes were placed on the dorsal and ventral roots, and GCaMP signals in myenteric neurons were imaged.

3.2.3 GCaMP Imaging of Myenteric Neurons

After mice were killed by isoflurane, entire colons were removed from E2a-GCaMP6 mice and placed in carbogenated ACSF containing (in mmol/L) 117.9 NaCl, 4.7 KCL, 25 NaHCO3, 1.3 NaH2PO4, 1.2 MgSO4-7H2O, 2.5 CaCl2, 11.1 D-glucose, 2 sodium butyrate, 20 sodium acetate, and 0.004 nifedipine. Colons were opened longitudinally along the mesenteric border and pinned out with the serosal side up in a glass dish lined with Sylgard 182 (Dow Corning, Midland, MI). The colon was stretched roughly 140% and 200% along the longitudinal and circular muscle axes, respectively. The dish was transferred to the stage of a Leica STP 8000 upright fluorescent microscope, placed on a heated platform (Okolab, Burlingame, CA) and superfused with ACSF maintained at 35°C–37°C, except in experiments testing temperature effects on interstitial cells of Cajal (ICC) activity. Fluorescence in the myenteric plexus was imaged with a Prime 95B Scientific Complementary Metal-Oxide Semiconductor (CMOS) camera (Photometrics; Roper Scientific,

Tucson, AZ) and signals were collected with Metamorph software (Molecular Devices, San Jose, CA). Image stacks were collected at a 40-Hz sampling rate with 25ms exposure time. Electrical pulses (20 Hz at 100 μs for 1 second) were delivered to the dorsal or ventral root during image acquisition of myenteric neurons. A pulse duration of 100μs was chosen because it is too short to elicit muscle fiber contractions but reliably induces neuronal action potential firing.

3.2.4 Optogenetic Stimulation of Colon Afferents

VMR to laser stimulation of the colon was measured using previously described protocols (Makadia et al., 2018). Mice were fasted overnight to help reduce fecal content for fiber insertion. Mice were kept under isoflurane anesthesia during all procedures. Initially, mice were maintained in a nose cone administering 2% isoflurane (vaporized with 95% O2/5% CO2). A 400-mm optical fiber (Thor Labs, Newton, NJ) enclosed by polyethylene tubing (1.4-mm diameter) was inserted approximately 20mm into the colorectum. The optical fiber was connected to a 473-nm laser power source (Laserglow Technologies, Toronto, Ontario), enabling blue-laser stimulation of the colon. After fiberoptic insertion, the level of isoflurane was lowered to 1% and then lowered by 0.125% every 10 minutes, down to 0.6%. When a steady level of anesthesia was reached, mice were responsive to toe pinch reflex testing but were not ambulating. The laser stimulus (10 Hz, 15 mW) was delivered 4 times, at 30 seconds each, with a 5-minute break in between. Visual confirmation of abdominal contraction was used to confirm response to laser stimulation.

3.2.5 Immunohistochemistry of Spinal Cord and Brainstem

Ninety minutes after laser stimulation, mice were deeply anesthetized and perfused with 4% paraformaldehyde. Spinal cord from the T12-L1 and L5-S1 regions and the brainstem were dissected and post-fixed for 1 hour with 4% paraformaldehyde. Gelatin-embedded transverse spinal cord sections (35 μm) and brainstem sections (50μm) were washed 3 times for 5 minutes in 0.1 mol/L phosphate-buffered saline, blocked (5% normal donkey serum and 0.1% Triton X-100 in 0.1 mol/L PB) for 2 hours at room temperature and incubated overnight with the rabbit anti-cFos antibody (1:1000; 226 003; Synaptic Systems Goettingen, Germany). Sections were washed, incubated for 2 hours at room temperature in Cy3-conjugated anti-rabbit IgG secondary antibody (1:500), washed, mounted, and cover-slipped. The total numbers of c-Fos–positive cells were counted in 7–10 sections per mouse. Every fourth section was mounted on a slide to allow 140μm or 200μm between sections to avoid recounting cells. Cells were counted as positive if the fluorescence intensity was 5 standard deviations above background. The average number of c-Fos positive cells per section for each mouse was used for statistical analysis. For the NTS, the obex was found and sections 1mm rostal and 1mm caudal were counted for a total of 11 sections.

3.2.6 Dextran Sodium Sulfate Induced Colitis

Dextran sodium sulfate (DSS) was administered in drinking water. A 3% DSS solution was prepared by dissolving DSS (36,000-50,000 MW; MP Biomedicals, Santa Ana, CA) into sterile water that was provided ad libitum via water bottle for 5 days. Vehicle control mice were provided sterile water without DSS.

3.2.7 Experimental Design and Statistical Analysis

Image files collected in Metamorph were exported to ImageJ software. In each time-series image stack, circular regions of interest were drawn around neuron cell bodies. We quantified response amplitude by calculating ΔF/F0 as % [(F - F0)/F0] 100, where F is the peak fluorescence signal and F0 is the mean fluorescence signal at baseline, the average of 20 frames immediately before stimulation. Regions of interest were drawn in the areas surrounding the analyzed cells, and fluorescence signals from cells of interest were normalized to signals from nonresponding cells. Tissue movement in response to stimuli was determined using a Template Matching plugin (Align Slices in Stack) in ImageJ, which quantifies movement along the x- and y-axes, representing the circular and longitudinal muscles, respectively. Statistical tests were performed in Excel and GraphPad Prism. Data points represent the average of 5–10 visual fields per mouse. Data are expressed as mean ± standard error of the mean, where n represents the number of mice used, unless indicated otherwise. Statistical analyses were performed using t-test, Mann Whitney test, or two-way analysis of variance (ANOVA) with tests specified in the result section. Significance was defined as $P < .05$.

3.3 Results

3.3.1 The Effects of Colitis on LS ExPAN to Myenteric Neuron Communication

Our lab has previously shown that LS ExPANs do not directly communicate with myenteric neurons in naïve mice (Smith-Edwards et al., 2019). However, there is still the

possibility that when LS ExPANs become sensitized after colitis, a local effector function may be uncovered. Therefore, I tested this hypothesis by using an *ex vivo* LS colon nerve preparation to separately stimulate LS ExPANs and preganglionic parasympathetic fibers and use calcium imaging in the ENS to examine myenteric neuron activity.

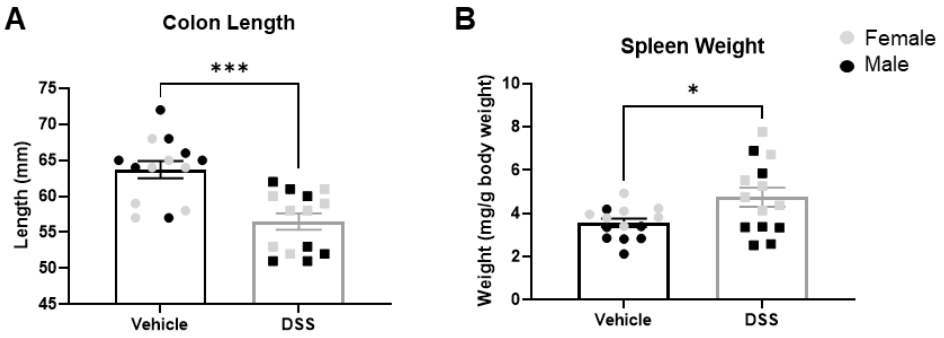

Figure 9 Dextran sodium sulfate (DSS) treatment induces colonic inflammation.

A) Mice treated with DSS had significantly shorter colon length. B) DSS treated mice had a significant increase in spleen weight. These changes have been associated with colonic inflammation. *p<.05, ***p<.001 Colon length was analyzed with a Mann Whitney test, Spleen weight was analyzed by a Welch's t test.

The DSS model of colitis was used in these experiments due to the simplicity of administration and ease of changing administration parameters to control the onset, severity, and duration of inflammation. Furthermore, DSS has been shown to mimic clinical characteristics of ulcerative colitis and studies that validated DSS model by using different therapeutic agents for human IBD show that DSS-induced colitis can be used as a relevant model for the translation of mice data to human disease (Melgar et al., 2008). Using a model of DSS colitis that we have previously shown to cause visceral hypersensitivity (Najjar et al., 2021), I confirmed that DSS caused increased spleen weight (3.55 ± 0.19 vs. 4.75 ± 0.44, $t_{(18)}$ = 2.46, p = 0.0244, **Figure 9B**)

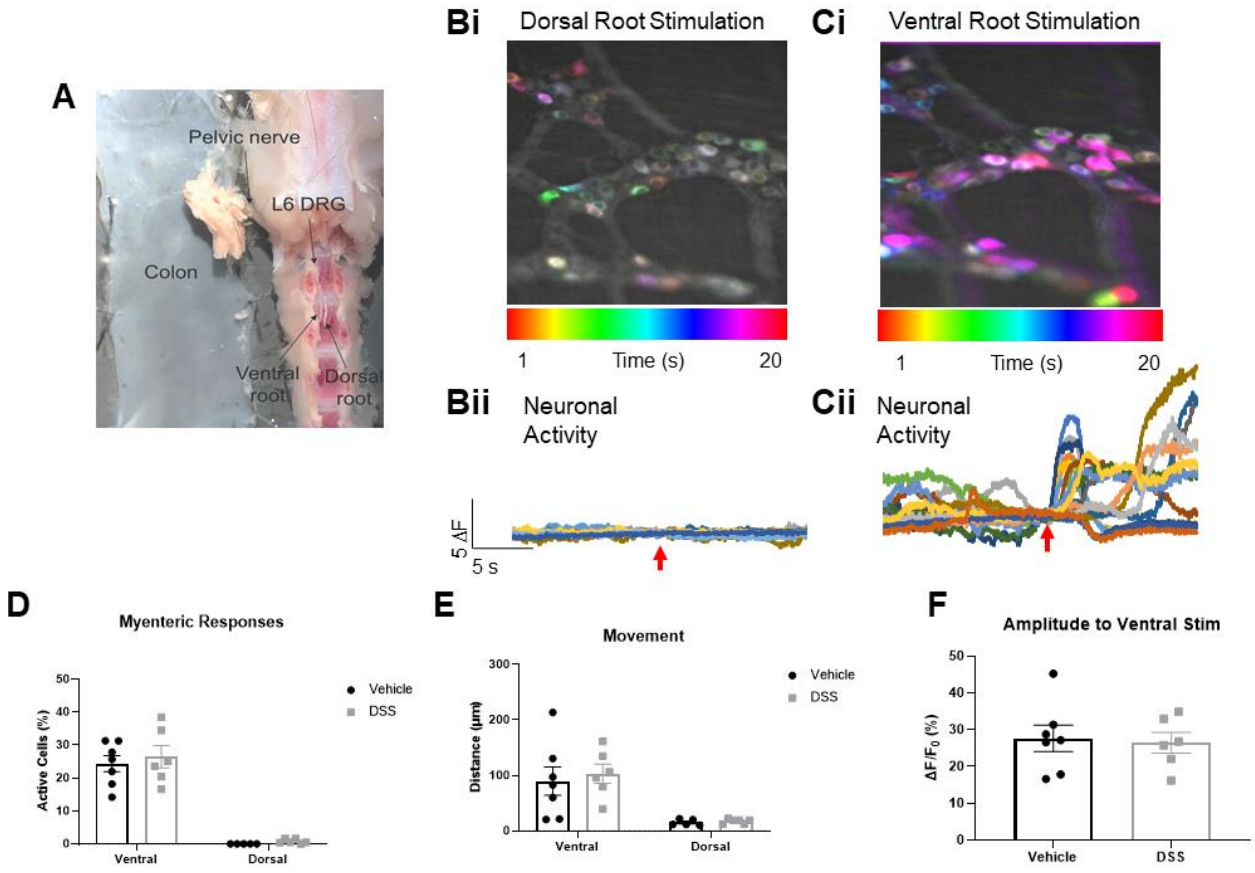

Figure 10 DSS does not alter responses to LS dorsal or ventral root stimulation.

A) Image of ex vivo colon nerve preparation with cut dorsal and ventral roots. B) Myenteric neuron responses to dorsal root stimulation, time-lapse color-coded image of myenteric neuron activity (Bi) and traces of calcium transients (Bii). C) Myenteric neuron responses to ventral root stimulation, time-lapse color-coded image of myenteric neuron activity (Ci) and traces of calcium transients (Cii). D) Percentage of responsive myenteric neurons to dorsal and ventral root stimulation in vehicle and DSS treated mice. E) Evoked movement from dorsal and ventral root stimulation in vehicle and DSS treated mice. F) Amplitude of calcium transients in myenteric neurons to ventral root stimulation in vehicle and DSS treated mice. Ventral root stimulation activated myenteric neurons and evoked muscle movement. Dorsal root stimulation did not activate myenteric neurons or evoke colon movement. DSS treatment had no significant effect on the percentage of responsive myenteric neurons or the amplitude of responses and did not change the evoked movement. D and E were analyzed with a repeated measures two-way ANOVA, there were no significant interactions for D or E therefore post-hoc analysis was not perofmred. F was analyzed with an unpaired t test.

and decreased colon length (63.71 ± 1.19 vs. 56.50 ± 1.13, U = 28.50, p = 0.0008, **Figure 9A**), two characteristics indicative of DSS-induced colitis (Chassaing, Aitken, Malleshappa, & Vijay-Kumar, 2014) (n = 14, 7 males, 7 females). There have been reports of sex differences in the DSS model (Bábíčková et al., 2015). However, we saw no obvious difference in the spleen weight or colon length of male and female mice (**Figure 9**), although male mice tended to have lower spleens weights than female. Therefore, subsequent experiments combined male and female mice.

To test if colonic inflammation induces the ability of LS ExPANs to directly activate myenteric neurons, our *ex vivo* preparation (**Figure 10A**) was used to electrically stimulate the L6 dorsal root in E2a-GCaMP mice and myenteric activity was measured by calcium imaging. The L6 ventral root was also stimulated as a control as parasympathetic preganglionic stimulation has been shown to induce calcium transients in myenteric neurons (Smith-Edwards et al., 2019). We confirmed our previous results in the vehicle mice, dorsal root stimulation did not activate myenteric neurons or induce muscle movement (15.35 ± 2.45μm, n= 5, **Figure 10B**) whereas ventral root stimulation activated 24.35 ± 2.47% of myenteric neurons and induced colon movement (89.61 ± 25.56μm, n = 7, **Figure 10C**). Dorsal root stimulation in DSS treated mice also did not activate myenteric neurons (0.77 ± 0.28%, n = 6, **Figure 10D**) or induce movement (17.59 ± 1.66μm, n = 6, **Figure 10E**). In addition, DSS did not modulate the percentage of responsive myenteric neurons to ventral root stimulation (26.44 ± 3.42%, F-main$_{(1, 20)}$ = 0.08, p = 0.7756, n = 6, **Figure 10D**) or induced movement (102.95 ± 17.41μm; F-main$_{(1, 20)}$ = 0.09, p = 0.7559, n = 6, **Figure 10E**). The amplitude of calcium transients induced by ventral root stimulation was also not significantly different between vehicle (27.66 ± 3.60%, n=7, **Figure 10F**) and DSS mice (26.45 ± 2.827%; $t_{(11)}$ = 0.26, p = 0.8006). Based on these results, we concluded that colonic inflammation did not change communication between LS ExPANs and efferent fibers

to myenteric neurons implying that LS ExPANs do not have a local effector function to myenteric neurons, even after visceral hypersensitivity.

3.3.2 The Effects of DSS on *in vivo* Stimulation of Colon ExPANs Activation of Central Terminals

The response of myenteric neurons or colon contractility to activation of afferents at the LS level was not changed after DSS. However, there may still be DSS induced changes at other level of the neuraxis. Although *ex vivo* preparations for the TL and NG levels have not been developed, we started to address this question by using *in vivo* optogenetic stimulation of TRPV1-expressing colon afferents (which accounts for 78.05% of LS, 95.83% of TL, and 93.02% of NG afferents; Chapter 2) and looking at activation of central targets as determined by c-Fos expression. LS ExPANs can indirectly activate myenteric neurons through a spinal reflex loop and optogenetic activation of colon afferents induced c-Fos staining in parasympathetic nuclei of the LS spinal cord (Smith-Edwards et al., 2019). Using the same optogenetic protocol, we investigated if the TL and NG also had activation in their respective central terminals and how colonic inflammation may modulate this activation. Mice groups included TRPV1-ChR2 vehicle (n = 6), TRPV1-ChR2 DSS (n = 7), control vehicle (n = 6), and control DSS (n = 6).

3.3.2.1 LS Spinal Cord

For the LS level, c-Fos staining was quantified in the LS dorsal horn (DH), sacral parasympathetic nucleus (SPN), and dorsal commissure (DCM). Activation of colon afferents in TRPV1-ChR2 vehicle mice induced c-Fos expression in the SPN (5.54 ± 0.47, **Figure 11Ci**) and DCM (10.98 ± 1.64, **Figure 11Ai**) that was not seen in littermate wildtype (non-ChR2 expressing)

controls (SPN 0.40 ± 0.10, p <0.0001, **Figure 11D**; DCM 0.87 ± 0.23, p = 0.0007, **Figure 11B**). DSS treated TRPV1-ChR2 and control mice had no significant differences from vehicle treated TRPV1-ChR2 and control mice at the SPN (DSS control 0.57 ± 0.26, p > 0.9999, **Figure 11D**; DSS TRPV1-ChR2 5.86 ±0.90, $F_{(1, 21)}$ = 0.33, p = 0.8945, **Figure 11Cii**) or DCM (DSS control 0.96 ± 0.32, p > 0.9999, **Figure 11B**; DSS TRPV1-ChR2 12.30 ± 2.51, $F_{(1, 21)}$ = 0.34, p = 0.9452, **Figure 11Aii**). Neurons in autonomic nuclei were activated by laser stimulation of ChR2-expressing TRPV1 colon afferent as indicated by increases in the number of neurons expressing c-Fos. Surprisingly, DSS treatment did not change the extent of this activation. This implies that colonic inflammation does not change the LS ExPAN spinal reflex circuit, confirming the results of the GCaMP experiments above. In contrast, there was a significant interaction between genotype and treatment for the LS DH ($F_{(1, 21)}$ = 6.07, p = 0.0225). The LS DH had a significant increase of c-Fos staining following blue light stimulation in TRPV1-ChR2 DSS mice (9.86 ± 2.36, **Figure 11Eii**) compared to vehicle treated TRPV1-ChR2 mice (3.84 ± 0.97, p = 0.0107, **Figure 11Ei**) or WT controls (p = 0.0005 DSS; p = 0.0004 Vehicle, **Fig, 11F**). These data provide evidence that although inflammation produces sensitization of the primary afferent input to the dorsal horn, the more direct connection with the deeper layers of the spinal cord involved in homeostatic autonomic reflexes (i.e., the SPN and DCM) do not change with inflammation. The increase in LS DH activation after inflammation supports the idea that LS afferents become sensitized after inflammation which leads to visceral hyperalgesia (Feng et al., 2012b).

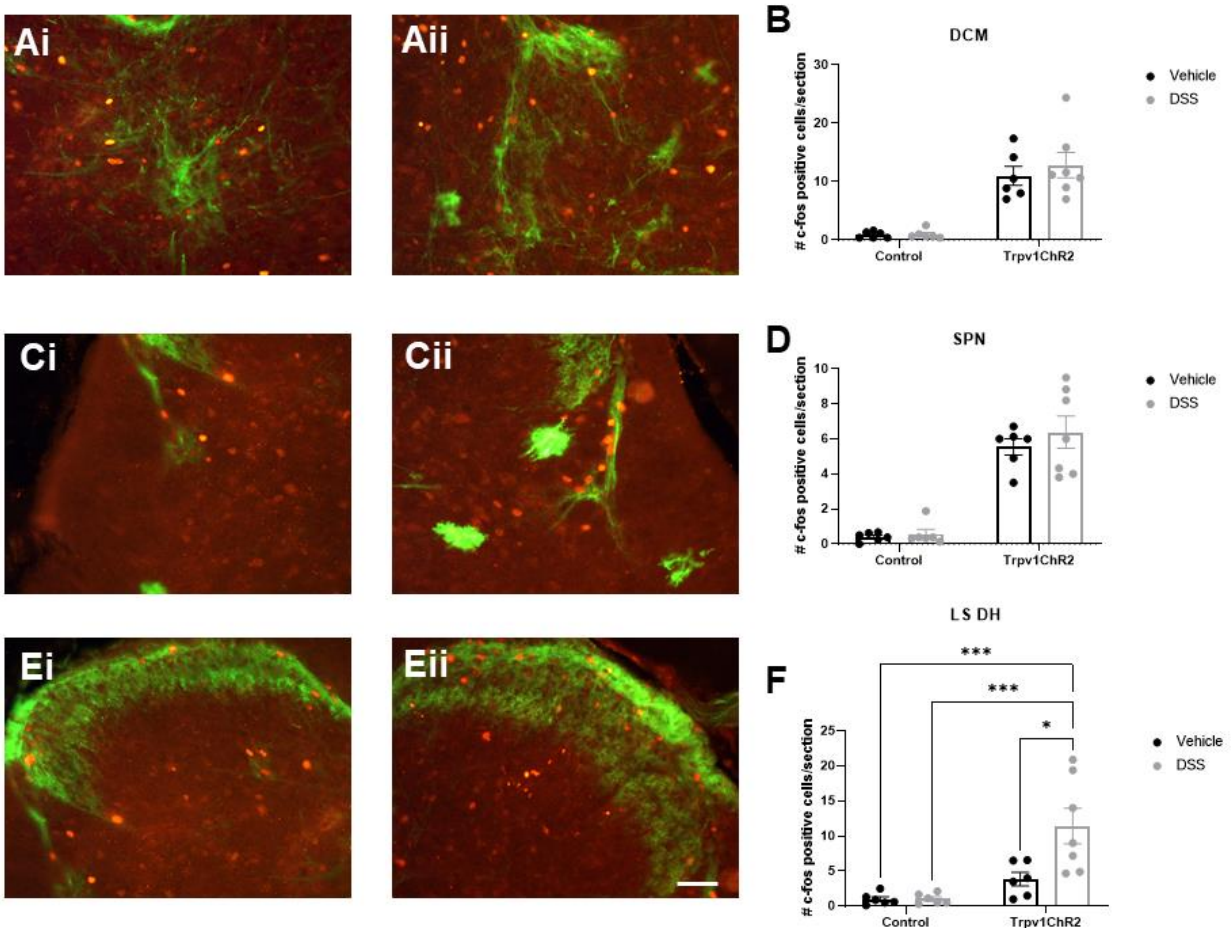

Figure 11 Optogenetic stimulation of ExPANs activates neurons in the DCM, SPN, and LS DH.

A) LS spinal cord sections of DCM from TRPV1-ChR2 mice have yellow fluorescent protein (YFP) in sensory afferents and c-Fos expression in activated cells from blue light stimulation in vehicle (Ai) and DSS mice (Aii). B) Quantification of c-Fos positive cells in the DCM. TRPV1-ChR2 mice had significantly more c-Fos activation compared to control littermates. DSS did not the level of c-Fos positive cells in control or TRPV1-ChR2 mice. C) LS spinal cord sections of SPN from TRPV1-ChR2 mice show c-Fos activation after optogenetic activation in both vehicle (Ci) and DSS mice (Cii). D) Quantification of c-Fos positive cells in the SPN after blue light activation of colon afferents. TRPV1-ChR2 mice had significantly higher number of c-Fos positive cells compared to controls. DSS did not change the number of positive cells in control or TRPV1-ChR2 mice. E) LS spinal cord sections of DH show c-Fos positive cells in TRPV1-ChR2 vehicle mice (Ei). TRPV1-ChR2 mice treated with DSS have c-Fos positive cells throughout the DH (Eii). F) Quantification of LS DH c-Fos positive cells. TRPV1-ChR2 DSS mice had significantly higher number of c-Fos positive cells compared to control littermates and TRPV1-ChR2 vehicle

mice. *p<.05, ****p=.0001. All locations were analyzed with a two-way ANOVA. Post-hoc analysis was only performed for F because there was a significant interaction. DCM-dorsal commissure, SPN-sacral parasympathetic nucleus, DH-dorsal horn. Scale bar 50μm.

3.3.2.2 TL Spinal Cord

To investigate DSS changes of TL afferents inputs to second-order neurons, the TL DH, intermediolateral nucleus (IML), and intercalated nucleus (ICI) were examined for c-Fos labeling. For all regions examined, there were few cFos positive cells in vehicle wildtype or ChR2-expressing mice after laser activation. However, a significant increase in the number of c-Fos cells were observed in the TL DH after laser stimulation of TRPV1-ChR2 DSS mice (6.41 ± 1.19, F-interaction$_{(1, 21)}$ = 15.81, **Figure 12Eii**) compared to TRPV1-ChR2 vehicle (1.29 ± 0.39, p < 0.0001, **Figure 12Ei**), control DSS (1.19 ± 0.35, p < 0.0001), and control vehicle mice (1.19 ± 0.44, p < 0.0001, **Figure 12F**). The increase in c-Fos positive cells in the TL DH only after inflammation (and laser activation) supports previous research that suggest TL afferents are not involved in acute pain but inflammatory pain (Traub, 2000; G. Wang et al., 2005). c-Fos expression in TL autonomic spinal regions was also only seen after DSS treatment and laser application in TRPV1-ChR2 mice. The IML (7.89 ± 0.76, F-interaction$_{(1, 21)}$ = 55.49, n = 7, **Figure 12Aii**) and ICI (22.99 ± 1.60, F-interaction$_{(1, 21)}$ = 72.31, , **Figure 12Cii**) had significantly more c-Fos positive cells in TRPV1-ChR2 DSS mice after laser activation compared to TRPV1-ChR2 vehicle (IML 1.05 ± 0.22, , p < 0.0001, **Figure 12Ai**; ICI 4.31 ± 0.99, , p < 0.0001, n = 6, **Figure 12Ci**), control DSS (IML 0.92 ± 0.33, , p < 0.0001, **Figure 12B**; ICI 1.98 ± 0.76, , p < 0.0001, **Figure 12D**), and control vehicle (IML 0.37 ± 0.12, , p < 0.0001; ICI 1.41 ± 0.42, , p < 0.0001). The activation of

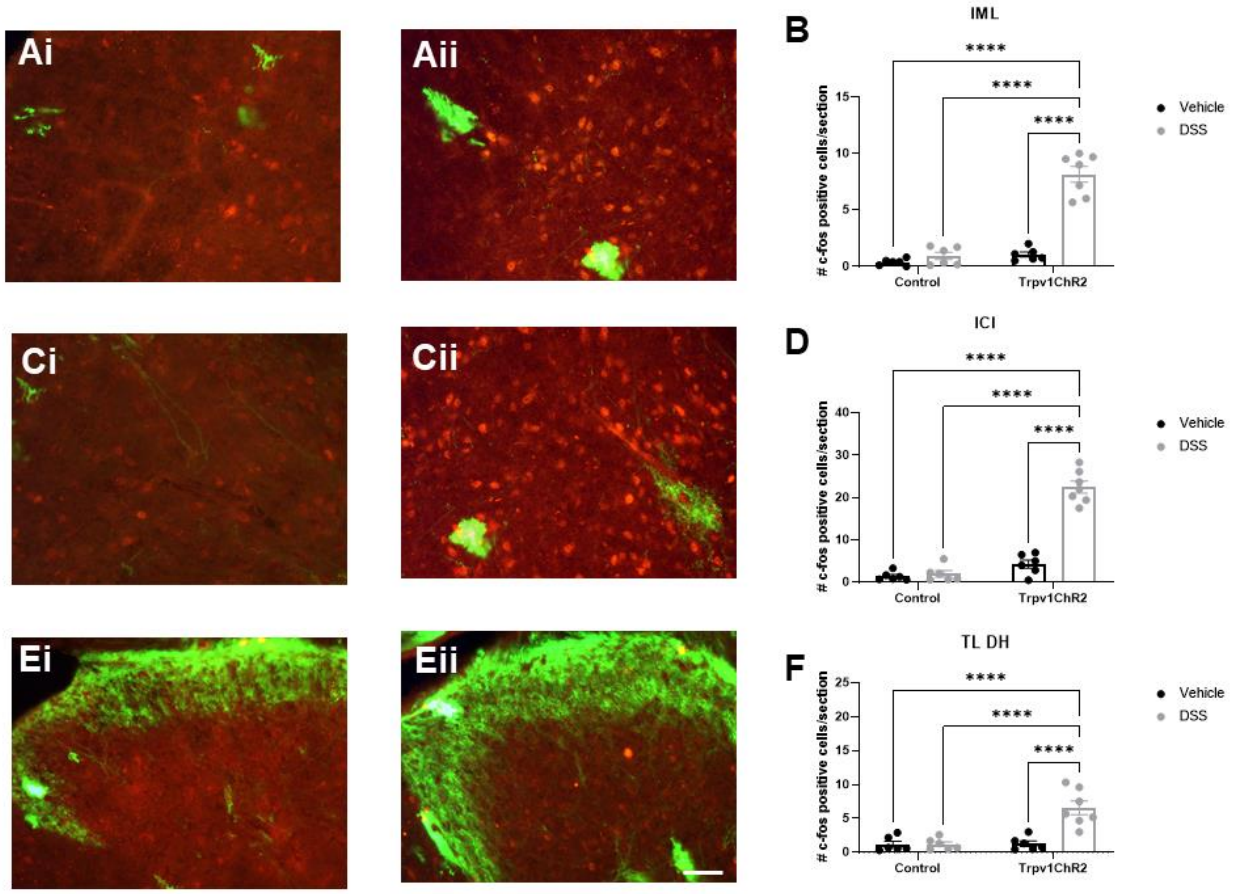

Figure 12 Optogenetic stimulation of ExPANs activates neurons in the IML, ICI, and TL DH after inflammation.

A) TL spinal cord sections of IML from TRPV1-ChR2 mice have yellow fluorescent protein (YFP) in sensory afferents and c-Fos expression in activated cells from blue light stimulation in DSS mice (Aii) but not vehicle treated mice (Ai). B) Quantification of c-Fos positive cells in the IML. TRPV1-ChR2 mice treated with DSS had significantly higher numbers of c-Fos positive cells compared to all other groups. C) TL spinal cord sections of ICI from TRPV1-ChR2 mice do not show c-Fos activation after optogenetic activation in vehicle mice (Ci) but DSS mice (Cii) have c-Fos staining. D) Quantification of c-Fos positive cells in the ICI after blue light activation of colon afferents reveals only TRPV1-ChR2 mice treated with DSS have a significant number of c-Fos positive cells. E) TL spinal cord sections of DH show little to no c-Fos positive cells in TRPV1-ChR2 vehicle mice (Ei). TRPV1-ChR2 mice treated with DSS have c-Fos positive cells throughout the DH (Eii). F) Quantification of LS DH c-Fos positive cells. TRPV1-ChR2 DSS mice had significantly higher number of c-Fos positive cells compared to control littermates and TRPV1-ChR2 vehicle mice. ****p=.0001. All locations were analyzed with a two-way ANOVA and

had significant interactions therefore post hoc analyses were performed. IML- intermediolateral nucleus, ICI- intercalated nucleus, DH-dorsal horn. Scale bar 50μm.

autonomic nuclei only after DSS suggests sensory-induced sympathetic outflow is likely changed after colonic inflammation.

3.3.2.3 Nucleus Tractus Solitarius

c-Fos positive cells in the NTS were quantified to examine whether NG afferents inputs to NTS was affected by DSS. Laser activation in TRPV1-ChR2 mice produced significantly more c-Fos positive cells in the NTS (222.17 ± 10.96, F-interaction$_{(1, 22)}$ = 6.670, **Figure 13Ai**) compared to WT control vehicle (123.51 ± 7.73, p = 0.0194, **Figure 13B**) and WT control DSS (145.83 ± 16.06, p = 0.0260, **Figure 13B**). In addition, laser activation in TRPV1-ChR2 DSS mice produced significantly more c-Fos staining than TRPV1-ChR2 vehicle mice (335.33 ± 32.63, p = 0.0039, **Figure 13Aii**) and both control groups ($p < 0.0001$). When examining the rostral caudal spread of the NTS, the highest density of c-Fos positive cells were found from -400μm to +200μm from the obex (**Figure 13C**). Laser treatment in TRPV1-ChR2 vehicle mice produced the greatest increase in c-Fos activation compared to vehicle controls at -1000μm (p = 0.0061), -800μm (p = 0.0091), and +600μm (p = 0.0049) from the obex. There was a significant increase in the number of c-Fos positive cells from -400μm (p = 0.0011), -200μm (p = 0.0306), and at the level of the obex (p = 0.0164) in the TRPV1-ChR2 DSS mice compared to TRPV1-ChR2 vehicle mice following laser activation. These data suggest that like the TL regions of the spinal cord involved in autonomic regulation and unlike the corresponding LS regions, the connection between vagal afferents and their second-order counterparts are sensitized by inflammation.

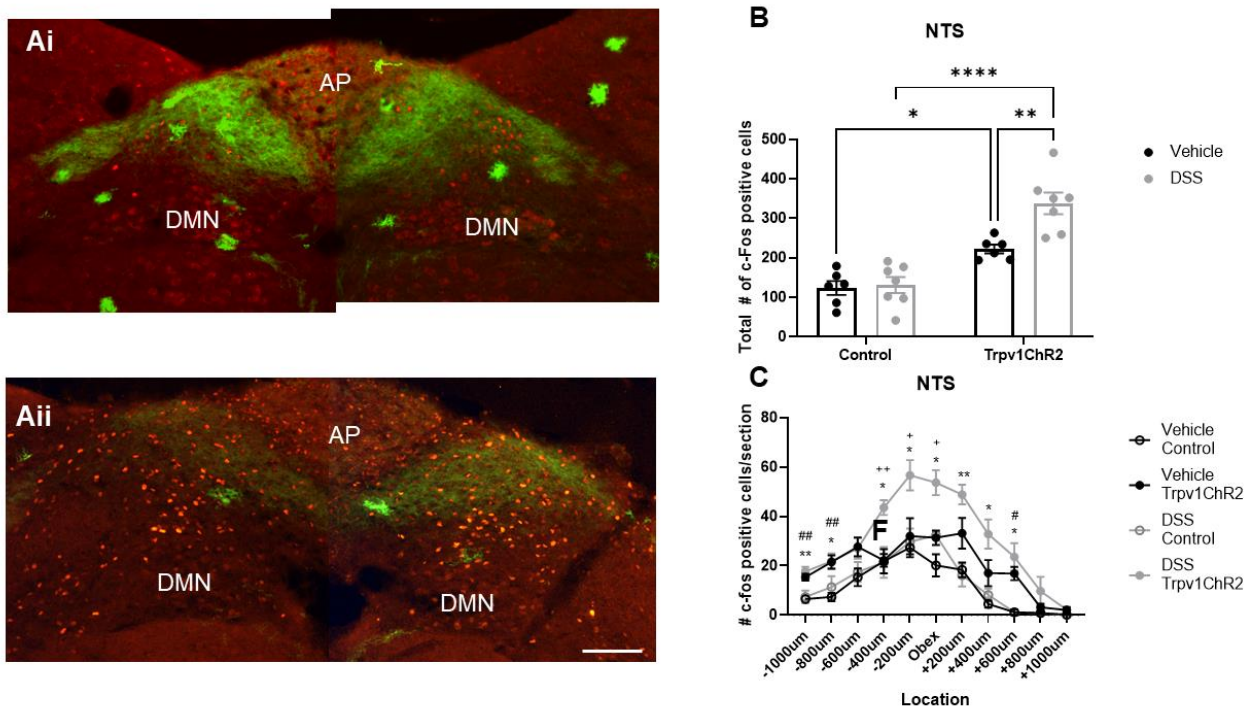

Figure 13 Optogenetic stimulation of ExPANs activates neurons in the NTS and DSS inflammation increases the number of c-Fos positive cells.

A) Brainstem sections of NTS at -200µm from obex from TRPV1-ChR2 treated with vehicle (Ai) and DSS (Aii) showed c-Fos positive cells after optogenetic stimulation. B) Quantification of the total number of c-Fos positive cells throughout the NTS. TRPV1-ChR2 mice showed a significant increase in c-Fos positive cells compared to control littermates after vehicle and DSS treatment. TRPV1-ChR2 DSS mice also showed a significant increase in the total number of c-Fos positive cells compared to TRPV1-ChR2 vehicle mice. C) Quantification of c-Fos positive cells along the rostrocadual extent of the NTS. TRPV1-ChR2 DSS mice had a significant increase in c-Fos positive cells compared to both control littermate groups throughout the NTS with the exception of the most rostral sections and -600µm. TRPV1-ChR2 DSS mice also showed a significant increase in c-Fos staining at -400µm-obex compared to TRPV1-ChR2 vehicle mice. TRPV1-ChR2 vehicle mice showed a significant increase in c-Fos positive cells compared to littermate controls at -1000µm, -800µm, and +600µm from obex after blue light stimulation. *p<.05, **p<.01, ****p=.0001 for C, *p<.05 TRPV1-ChR2 DSS vs. controls, **p<.01 TRPV1-ChR2 DSS vs. controls, #p<.05 TRPV1-ChR2 vehicle vs. controls, ##p<.01 TRPV1-ChR2 vehicle vs. controls, +p<.05 TRPV1-ChR2 DSS vs. TRPV1-ChR2 vehicle, ++p<.01 TRPV1-ChR2 DSS vs. TRPV1-ChR2 vehicle for D. C was analyzed

with a two-way ANOVA and D was analyzed with a repeated measures two-way ANOVA and post hoc analyses were performed due to significant interactions. NTS-nucleus tractus solitarius, DMN-dorsal motor nucleus of the vagus, AP-area postrema. Scale bar 200μm.

3.4 Discussion

The ability of ExPAN neurons to communicate with myenteric neurons, either directly or indirectly through autonomic reflexes, and how this may change with visceral disease is currently not fully understood. It had been previously hypothesized that spinal ExPANs have a local effector function at their peripheral terminals, although we have shown this is not the case at the LS level in naïve animals (Smith-Edwards et al., 2019). We hypothesized that after colonic inflammation, there may be plastic changes that induce direct ExPAN to myenteric neuron communication related to the observed afferent sensitization and hypersensitivity. However, using a DSS-model of colitis that we have previously shown does cause visceral hypersensitivity (Najjar et al., 2021), we found no evidence of inflammation-induced direct connections between L6 dorsal root afferents and myenteric neurons, although afferent input to the DH appears to be sensitized. Moreover, myenteric activity and muscle movement invoked by L6 ventral root stimulation did not change. These data suggest that both the sensory and autonomic circuits arising from this portion of the spinal cord that regulate ENS function are not affected in this model of colon inflammation.

The next question was whether this was also true at other levels of the neuraxis that regulate colon function. To begin to answer this question, optogenetic activation of colon afferents in vehicle and DSS treated mice was utilized. Spinal cord and brainstem, areas where the central terminals of ExPANs synapse on other portions of the autonomic nervous system, were stained

with c-Fos as an indirect measure of neuron activation. In vehicle treated TRPV1-ChR2 mice, optogenetic activation of colon afferents induced c-Fos immunoreactivity in LS spinal cord and brainstem autonomic nuclei, however, the TL spinal cord exhibited little to no c-Fos staining. Interestingly after colonic inflammation, activation of colon afferent induced cFos staining in the TL spinal cord and the NTS showed increased staining. In contrast, no change was seen autonomic regions of the LS spinal cord despite increases in c-Fos expression in the dorsal horn. This supports previous research in the rat that TL afferents are selectively recruited during inflammation, whereas LS afferents are involved in both acute and inflammatory pain (Traub, 2000; Traub et al., 1993; Traub et al., 1994). The TL spinal cord had increased c-Fos expression in both dorsal horn neurons, which project to the CNS and are involved in higher order functions including pain (Sikandar & Dickenson, 2012), and autonomic nuclei (IML) that are involved in spinal reflexes. Similar to what has been reported for LS afferents (Smith-Edwards et al., 2019), TL afferents provide input to areas involved in visceral hypersensitivity and sympathetic autonomic nuclei that directly affect colon motility (Smith-Edwards et al., 2021), suggesting that TL afferents are directly involved in both visceral pain and dysmotility following colitis. These results suggest that the TL afferent input to the sympathetic nervous system may be more affected by inflammation than the parasympathetic fibers at the LS level.

An important caveat for these optogenetic experiments is the laser is only inserted as far as the distal colon and although the laser power is several times that needed to excite colon afferents, it is unlikely that afferents innervating the proximal colon are activated to the same extent as distal colon afferents. This is further complicated by the presence of fecal pellets that varies between animals further reducing optogenetic activation. Mice are fasted overnight to reduce this problem; however, it was clear that some animals had fecal pellets closer to the last 25mm of the colon than

others. I previously showed that distinct levels of the neuraxis innervated proximal and distal colon differently (Meerschaert et al., 2020); the LS afferents almost exclusively innervated the distal colon, whereas TL and NG afferents projected almost equally to proximal and distal colon. Thus, for the optogenetic experiments, it is likely all LS afferents are being activated whereas a majority of the TL and NG that innervate distal and middle colon are activated, with proximal afferents activation dependent on fecal content and colon position within the abdomen. Proximal colon distention has been examined in the rat (Martinez, Wang, Mayer, & Tache, 1998; Martinez, Wang, & Tache, 2006) and c-Fos staining was found in the LS spinal cord and NTS, although these studies did not investigate the TL level of the spinal cord.

Although these experiments are beginning to tease apart the ability of ExPANs to indirectly communicate with the ENS through autonomic reflexes, activity in the ENS was not directly measured for the TL and NG levels. Furthermore, the ability of TL or NG to directly communicate with myenteric neurons was not investigated. Similar *ex vivo* colon nerve preparation for the TL and NG have not been described, and although we began to try different preparations for the TL and NG level, we never found a preparation that reliability activated these levels and did not find it practical to continue to work on these preparations. An alternative approach to *ex vivo* preparations could be a mix of optogenetic and viral RCaMP in myenteric neurons to selectively activate nerves from the different levels and examine myenteric neuron responses. Future studies further investigating the TL and NG levels are needed to fully understand how these different levels are involved in communication to the ENS in homeostatic conditions and after visceral disease.

4.0 The Effects of Colon Inflammation on Extrinsic Autonomic Efferent Fibers and Intrinsic ENS Circuits

4.1 Introduction

IBD is associated with both visceral hypersensitivity as well as dysmotility. Manometry and scintigraphy studies in patients with IBD, specifically ulcerative colitis, reveal a loss of muscle contractility but an increase in propulsive contractive waves (Bassotti, Antonelli, Villanacci, Baldoni, & Dore, 2014; Bassotti et al., 2020). This change in motility can cause diarrhea, and may be linked to increased intraluminal fluid and of inflamed contents causing colon distention (Bassotti, Gaburri, Imbimbo, Morelli, & Whitehead, 1994). Altered motility in IBD involves changes in ENS neuronal circuits (Galeazzi, Haapala, van Rooijen, Vallance, & Collins, 2000; Jacobson, McHugh, & Collins, 1995; Main, Blennerhassett, & Collins, 1993), disruption of ICC networks (Lu et al., 1997; Suzuki et al., 2004), and decreased contractility of smooth muscle cells (Al-Saffar & Hellström, 2001; Depoortere, Thijs, Van Assche, Keith, & Peeters, 2000; Zhao, Bossone, Piñeiro-Carrero, & Shea-Donohue, 2001).

Intrinsic ENS neurons are found in the myenteric plexus and submucosal plexus. The myenteric plexus is located between the longitudinal and circular muscle layers of the GI tract and is important for motor functions (Kunze & Furness, 1999). The submucosal plexus is located within the submucosal layer of the GI tract and is thought to be important for secretory functions and regulating blood flow (Kunze & Furness, 1999). Modulation of structural morphology as well as functional properties are found in both myenteric and submucosal neurons after colonic inflammation (Lakhan & Kirchgessner, 2010; Moynes, Lucas, Beyak, & Lomax, 2014). Enteric

reflexes are initiated by the activation of IPANs (intrinsic primary afferent neurons). IPANs are located in both plexuses and respond to changes in the chemical and contractile state of the organ. IPANs innervate one another, interneurons, and motor neurons to regulate gut functions (Furness et al., 2004). Ascending and descending interneurons form connections to neurons within the myenteric or submucosal plexus of neighboring ganglia and are innervated by IPANs and other interneurons. Motor neurons are innervated by interneurons or IPANs and in turn release neurotransmitters onto effector cells within the smooth muscle, the vasculature, or the mucosal epithelium. The myenteric plexus has further heterogeneity along the proximal/distal axis (Costa et al., 2020; Drokhlyansky et al., 2020; Z. Li et al., 2019); the proximal colon has more complex circuits corresponding to more diverse motor patterns associated with mixing luminal contents, absorption of water, and fecal pellet formation. In contrast, the distal colon has simpler circuits with the major motor output being peristalsis associated with fecal pellet expulsion via the anus.

Motor patterns are driven by both ENS neurons as well as ICC (interstitial cells of Cajal) pacemaker cells. There are multiple layers of ICC networks in the colon; ICC-SM are found between the submucosa and circular muscle, ICC-CM are found in the circular muscle, ICC-LM are located in the longitudinal muscle, ICC-MY are associated with the myenteric plexus, and ICC-SS are found in the subserosal connective tissue (Komuro, 2006). In the colon, the ICC-SM and ICC-MY appear to have the intrinsic pacemaker activity. Electrical repetitive oscillations (or slow waves) that produce non-propulsive ‘ripple’ contractions are present in the colon smooth muscle and the pacemakers for this activity has been identified as the ICC-SM (Huizinga et al., 2011). ICC-MY, normally under tonic inhibition, become disinhibited and generate membrane potential oscillations during large contractions (Huizinga et al., 2011). Colonic migrating motor complexes (CMMCs) are spontaneous rhythmic waves of contractions that propagate down the length of the

colon. Our lab has proposed a model for the generation of CMMCs that begins with the myogenic ripples generated by ICC-SM oscillations. IPANs become activated by the sensory input generated by the ICC-SM and begin the neural program involved in the generation of a CMMC and disinhibit the ICC-MY. Once ICC-MY and longitudinal muscle become excited, more IPANs become activated, thus recruiting activity in more myenteric neurons until the large contractions of the CMC occur.

Extrinsic autonomic fibers have also been shown to influence motility. For example, electrical stimulation of LS parasympathetic fibers causes contraction in the distal colon (Smith-Edwards et al., 2019), and distention or irritation of the rectum initiate the defecation reflex via complex coordination of parasympathetic fibers, enteric neurons, somatic neurons, and the central nervous system (S. Brookes et al., 2009). In contrast, sympathetic activation is usually associated with inhibition of GI tract function and motility. In the colon, stimulation of sympathetic postganglionic neurons decreases propagating contractions in the distal colon, but initiate non-propagating contractions involved in the mixing and processing of fecal content in the proximal colon (Smith-Edwards et al., 2021). The role of the vagus nerve in colon motility is largely unknown. Stimulation of the vagus nerve has been shown to cause contractions in both proximal and distal regions of the colon (Dapoigny et al., 1992; Larauche et al., 2020; Tong et al., 2010).

Visceral diseases are often associated with both visceral hypersensitivity as well as motility changes. Motor patterns in the colon require multiple different cell types, neuronal and non-neuronal and involves both intrinsic and extrinsic innervation. The goal of the present studies was to investigate the role of extrinsic autonomic fibers in dysmotility caused by colon inflammation. In addition, modulation of intrinsic circuits by colitis was also examined. DSS-induced colitis altered the ability of sympathetic fibers to modulate colon motility but had no effect on LS

parasympathetic fibers. Ascending intrinsic circuits and ICC-SM were also altered by DSS inflammation. The results from these experiments suggest that not only are afferents from different levels of the neuraxis differentially effected by DSS but also autonomic efferent fibers associated with the sympathetic and parasympathetic branches of the ANS are differentially modulated.

4.2 Methods

4.2.1 Animals

Male and female mice aged 8–12 weeks were analyzed in these studies. E2a-Cre mice (RRID:IMSR_JAX:003724) were crossed with mice containing a floxed-STOP-GCaMP6s sequence in the Rosa26 locus (Ai96 mice, RRID:IMSR_JAX:028866). Animals were housed in a facility approved by Assessment and Accreditation of Laboratory Animal Care (AAALAC), with a 12-hour light/dark cycle and free access to water and standard chow. Animal use protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

4.2.2 *In vivo* Transit Assay

Carmine red (Sigma-Aldrich) was prepared as a 6% solution dissolved in DI water with 0.5% methylcellulose (Sigma-Aldrich). Mice were gavaged with 150 µl of the carmine solution and returned to a clean cage in with free access to water and standard chow. Animals were not fasted beforehand. Feces were collected every 30 min (up to 7 hr from time of gavage) and streaked across a sterile white napkin detect the presence of the red carmine dye. A cutoff time of 7 hours

was used, and mice were transferred from home cage to a white transfer bucket and feces were collected for 30 minutes after. All mice had carmine red presence in fecal pellets by 30 minutes after transfer. The time from gavage to initial appearance of carmine in the feces was recorded as the total intestinal transit time for that animal. Animals were tested before and after treatment with DSS or vehicle and transit time was calculated as Δtime (post-treatment time - pre-treatment time).

4.2.3 Fecal Water Content

After transit experiments or before sacrificing mice for motility studies, a subset of mice were used to measure fecal water content after vehicle and DSS treatment. Mice were placed in a white transfer bucket, and a fresh fecal sample was collected after natural defecation. Fecal water content was calculated after drying the fecal samples overnight in a 60°C dry oven according to the equation: (fecal weight before drying - fecal weight after drying)/feces weight before drying × 100.

4.2.4 Whole Colon Motility Recordings

After mice were killed by isoflurane, entire colons were removed from mice and placed in carbogenated ACSF maintained at 35°C–37°C. Colons were allowed to equilibrate for 10-20 minutes before motility recordings. Whole colon motility video recordings (15-20 minutes; HDR-CX440; Sony) were used to measure colonic migrating motor complexes (CMMCs) and any non-specific contraction (includes both CMMCs and non-propagating contractions). CMMCs had to migrate at least 1cm to be included in analysis and only anterograde (oral to anal migration)

CMMCs were counted. Video recordings were transferred to ImageJ and the Manual Tracking plugin was used to analyze non-specific contractions along the colon.

4.2.5 Extrinsic *ex vivo* Colon Preparation

In a separate cohort of mice, the extrinsic autonomic nerves were kept intact during motility recordings. Using a modified version of the Smith-Edwards et al. (Smith-Edwards et al., 2021) preparation, mice were euthanized with isoflurane and transcardially perfused with carbogenated (95% O2 and 5% CO2) ACSF, containing: 117.9 mM NaCl, 4.7 mM KCL, 25 mM NaHCO3, 1.3 mM NaH2PO4, 1.2 mM MgSO4-7H2O, 2.5 mM CaCl2, 11.1 mM D-glucose, 2 mM sodium butyrate, and 20 mM sodium acetate. The colon, lumbar colonic nerves, sympathetic postganglionic neurons, and pelvic nerves were isolated, removed, and pinned to a Sylgard-lined dish superfused with ACSF at 35°C. Fecal contents were removed by gently flushing the lumen with ACSF. Whole colon motility was recorded during spontaneous activity (no nerve stimulation), with pelvic nerve stimulation, and with sympathetic nerve stimulation (lumbar colonic or hypogastric nerve). Nerves were stimulated with electrical pulses (20 Hz at 100 μs for 10 seconds) every 2 minutes. After whole colon motility recordings, the colon was cut adjacent to the mesentery, taking caution not to sever nerves innervating the colon, and pinned flat, with sympathetic prevertebral ganglia (and underlying aorta) positioned to the side. For changes in local contractions, colon tissue movement was recorded before, during, and after nerve stimulation (pelvic and sympathetic) using a CMOS camera (Prime 95B Photometrics) under a 10x objective on an upright DM6000FS Leica fluorescent microscope. Images were collected with Metamorph software at 6.67-Hz sampling rate and 150-ms exposure.

4.2.6 GCaMP Imaging of Myenteric Neurons

After mice were killed by isoflurane, entire colons were removed from E2a-GCaMP6 mice and placed in carbogenated ACSF. Colons were opened longitudinally along the mesenteric border and pinned out with the serosal side up in a glass dish lined with Sylgard 182 (Dow Corning, Midland, MI). The colon was stretched roughly 140% and 200% along the longitudinal and circular muscle axes, respectively. The dish was transferred to the stage of a Leica STP 8000 upright fluorescent microscope, placed on a heated platform (Okolab, Burlingame, CA) and superfused with ACSF maintained at 35°C–37°C. Fluorescence in the myenteric plexus was imaged with a Prime 95B Scientific Complementary Metal-Oxide Semiconductor (CMOS) camera (Photometrics; Roper Scientific, Tucson, AZ) and signals were collected with Metamorph software (Molecular Devices, San Jose, CA). Image stacks were collected at a 20-Hz sampling rate with 50ms exposure time. To examine evoked activity, concentric electrodes were placed on the colon 5–10 mm oral and anal to the imaging field. Electrical pulses (20 Hz at 100 μs for 1 second) were delivered to the colon during image acquisition of myenteric neurons or ICC. A pulse duration of 100μs was chosen because it is too short to elicit muscle fiber contractions but reliably induces neuronal action potential firing.

4.2.7 Experimental Design and Statistical Analysis

Image files collected in Metamorph were exported to ImageJ software. In each time-series image stack, circular regions of interest were drawn around neuron cell bodies. We quantified response amplitude by calculating ΔF/F0 as % [(F -F0)/F0]*100, where F is the peak fluorescence signal and F0 is the mean fluorescence signal at baseline, the average of 20 frames immediately

before stimulation. Regions of interest were drawn in the areas surrounding the analyzed cells, and fluorescence signals from cells of interest were normalized to signals from nonresponding cells. Tissue movement in response to stimuli was determined using a Template Matching plugin (Align Slices in Stack) in ImageJ, which quantifies movement along the x- and y-axes, representing the circular and longitudinal muscles, respectively. Statistical tests were performed in Excel and GraphPad Prism. Data points represent the average of 3-5 visual fields per mouse. Data are expressed as mean ± standard error of the mean, where n represents the number of mice used, unless indicated otherwise. Statistical analyses were performed using t-test, one-way ANOVA, or two-way ANOVA and the test used per experiment is specified in the result section. Significance was defined as $P < .05$.

4.3 Results

4.3.1 *In vivo* Changes in Transit Induced by DSS

The first experiments tested was how *in vivo* GI transit was changed after DSS. We performed a transit assay by gavage of a non-absorbable red dye in mice before and immediately after the 5 day treatment with DSS or vehicle (n = 6). Transit times were not significantly different between vehicle and DSS treated mice ($t_{(10)}$=0.4173, p = 0.6853, **Figure 14A**). DSS colitis has previously been shown to only affect transit times during the recovery period, but not in the acute phase (Kodani et al., 2018; Yeom & Kim, 2015). To assess an *in vivo* change in absorption, fecal water content was measured in vehicle and DSS mice (n = 6 vehicle, 7 DSS). Water content was significantly increased in DSS mice (65.88 ± 4.60%, t(11)=2.964, p = 0.0129, **Figure 14B**)

compared to vehicle (49.39 ± 2.65%) indicating that while overall speed of fecal content is not changed by DSS, the processing of fecal material is. One of the main GI symptoms of IBD is diarrhea that can be caused by increased luminal osmolality, increased secretion, decreased fluid absorption, or altered motility (Surawicz, 2010). Diarrhea is usually caused by multiple mechanisms all which are affected by inflammation. However, for the purpose of these studies, I will be focusing on motility to test the hypothesis that DSS-induced inflammation causes diarrhea-like symptoms via increased motility leading to decreased fluid absorption (Surawicz, 2010).

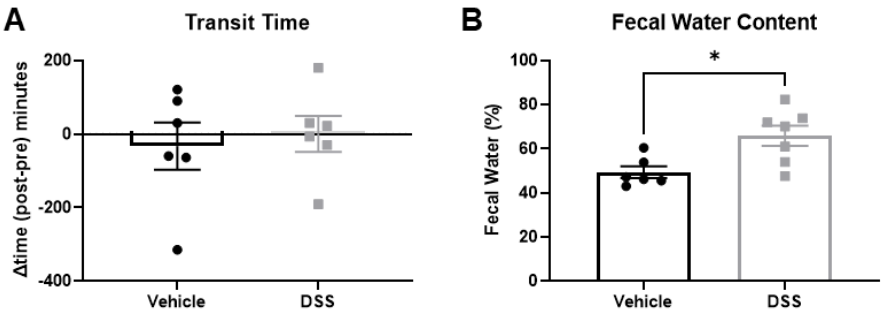

Figure 14 Dextran sodium sulfate (DSS) alters the processing of fecal material in vivo.

A) Mice treated with DSS did not have a significant change in whole GI transit time compared to vehicle treated mice. B) DSS treated mice showed an increase in fecal water content compared to vehicle treated mice. The overall speed of transit is not changed after DSS treatment, but the processing of fecal content is altered after DSS. *p<.05, Colon length was analyzed with a Mann Whitney test, Both data sets were analyzed with an unpaired t test.

4.3.2 DSS Alterations of Whole Colon Motility

Transit studies involving the entire GI tract in the setting of inflammation primarily restricted to the colon (i.e., the DSS model) may not be sensitive enough to detect transit changes restricted to distal portion of the GI tract. Therefore, we next tested how colon motility was altered

after DSS with an *ex vivo* colon nerve preparation (**Figure 15A**). Colons with intact pelvic and sympathetic nerves were used to measure how DSS alters motility (n = 7). DSS did not significantly alter the frequency ($F_{(2, 24)}$ = 0.3547, p = 0.7050, **Figure 15B**) or amplitude ($F_{(2, 24)}$ = 2.516, p = 0.1018, **Figure 15C**) of contractions with extrinsic nerves attached. This was surprising, as DSS has been shown to alter colon motility (Van Crombruggen et al., 2008).

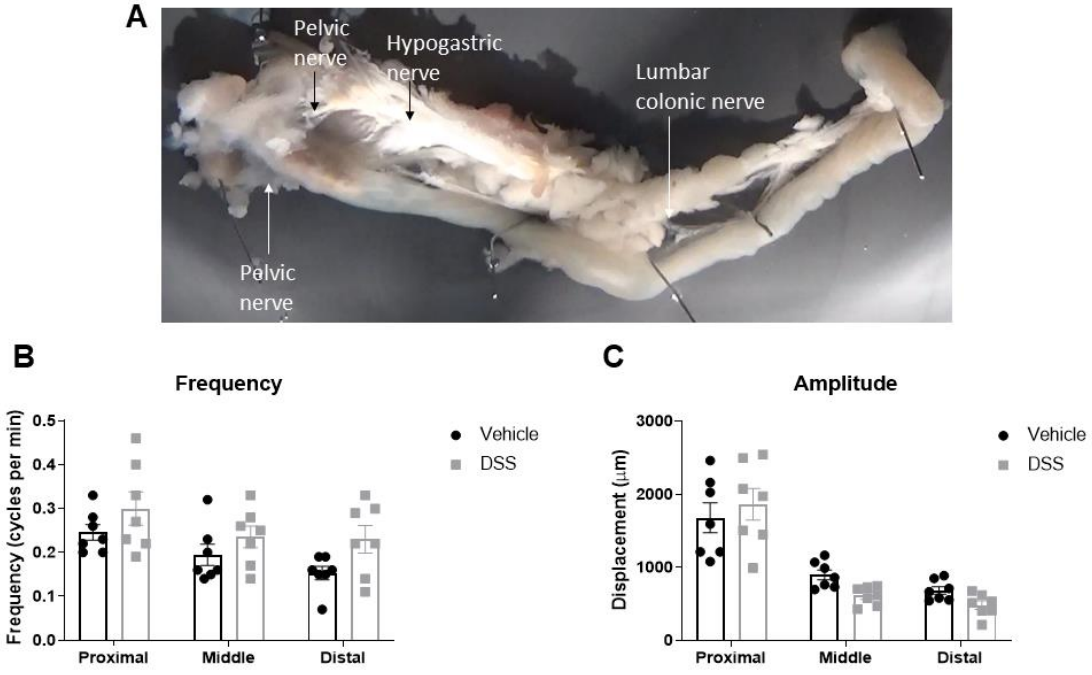

Figure 15 DSS did not alter the frequency or amplitude of spontaneous contractions in the colon.

A) Image of ex vivo preparation with intact pelvic and sympathetic nerves. B) Quantification of the frequency of spontaneous contractions in the proximal, middle, and distal colon. C) Quantification of the amplitude of spontaneous contractions in the proximal, middle, and distal colon. Data was analyzed with repeated measures two-way ANOVA.

Next, we tested if isolated colons (nerves cut) would show dysmotility after DSS. We have shown previously that just having the sympathetic nerves intact changes spontaneous whole colon

motility (Smith-Edwards et al., 2021) likely due to intestinofugal afferent neurons communicating with the sympathetic postganglionic neurons. In isolated colon, DSS did cause an increase in the frequency of contractions specifically in the distal colon (F-interaction$_{(2, 62)}$ = 7.427, p = 0.0244, **Figure 16A**) and was trending toward a decrease in the middle colon (p = 0.0994). There was a significant decrease in the amplitude of contraction in the middle colon (F-interaction$_{(2, 62)}$ = 7.085, p = 0.0100, **Figure 16B**). These experiments imply that DSS treatment induces changes in myenteric/ICC circuits that regulate motility but that extrinsic nerve fibers are able to compensate for these changes. Of course, it is recognized that our *ex vivo* preparations are incomplete, and the normalization of contractions seen when the intestinofugal system is present does not mean that *in vivo*, DSS-induced changes in other extrinsic input (e.g., ExPANs or parasympathetic) do not contribute to pathological symptoms.

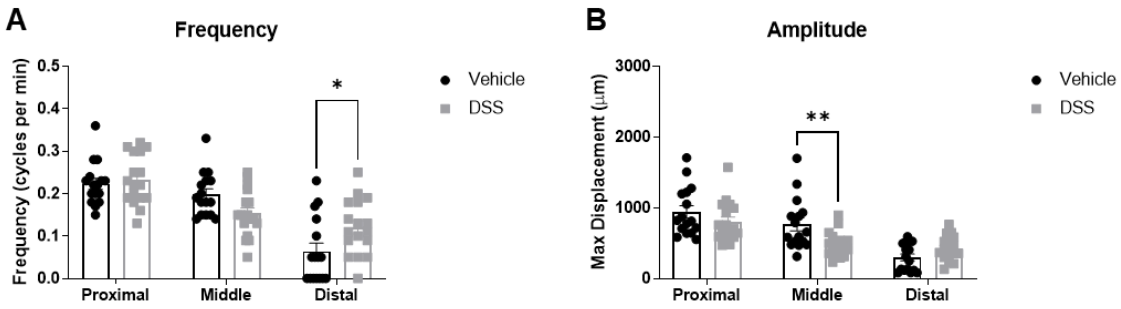

Figure 16 DSS alters whole colon motility in isolated colons.

A) Quantification of the frequency of spontaneous contractions in the proximal, middle, and distal colon. DSS treatment increased the frequency of contractions in the distal colon. B) Quantification of the amplitude of spontaneous contractions in the proximal, middle, and distal colon. DSS treatment significantly decreased the amplitude of contractions in the middle colon. *p<.05, **p<.01. Data were analyzed with a repeated measures two-way ANOVA.

4.3.3 DSS Modulation of Evoked Colon Motility Induced by Extrinsic Autonomic Nerve Stimulation

To further test how extrinsic autonomic fibers are influencing colon motility after DSS, the pelvic nerve and sympathetic nerve (lumbar colonic or hypogastric) were stimulated and changes in whole colon motility were observed. Nerves were stimulated every 2 minutes because this is a short enough time to interrupt normal CMMC frequency but long enough for a motor program to propagate down the colon.

In vehicle treated mice, pelvic nerve stimulation caused a time-locked CMMC that increase the frequency of contractions in the proximal (F-main$_{(1, 12)}$ = 19.18, p = 0.0009, **Figure 17Ai**), middle (F-main$_{(1, 12)}$ = 8.966, p = 0.0112, **Fig, 17Aii**), and distal colon (F-main$_{(1, 12)}$ = 15.31, p = 0.0021, **Fig, 17Aiii**). Pelvic nerve stimulation did not change the amplitude of contractions (**Fig, 17B**). DSS treatment did not alter the responses to pelvic nerve stimulation, which was to be expected based on the LS *ex vivo* preparation experiments in Chapter 3 (i.e., where there was no change in myenteric activity in response to either dorsal or ventral root stimulation). The latency of local contractions induced by pelvic nerve stimulation was also measured, as there is a clear time delay between the start of stimulation and proximal contraction. In vehicle mice, pelvic nerve stimulation caused delayed contractions in the middle (5.46 ± 0.94s) and proximal colon (14.16 ±1.38s, F-main$_{(2, 24)}$ = 103.8, p < 0.0001, **Figure 17C**) compared to distal colon (1.85 ± 0.19s). DSS treated mice had significantly longer respond latency in the proximal colon (18.75 ± 1.76s, p = 0.0317, **Fig, 17C**) compared to vehicle mice. The pelvic nerve innervates the distal colon almost exclusively (Meerschaert et al., 2020) implying that contractions in the proximal colon are likely mediated through intrinsic ascending circuits. Therefore, pelvic nerve stimulation induces local

contractions in the distal colon that propagates in the oral direction toward the proximal colon though ascending circuits and initiates a CMMC.

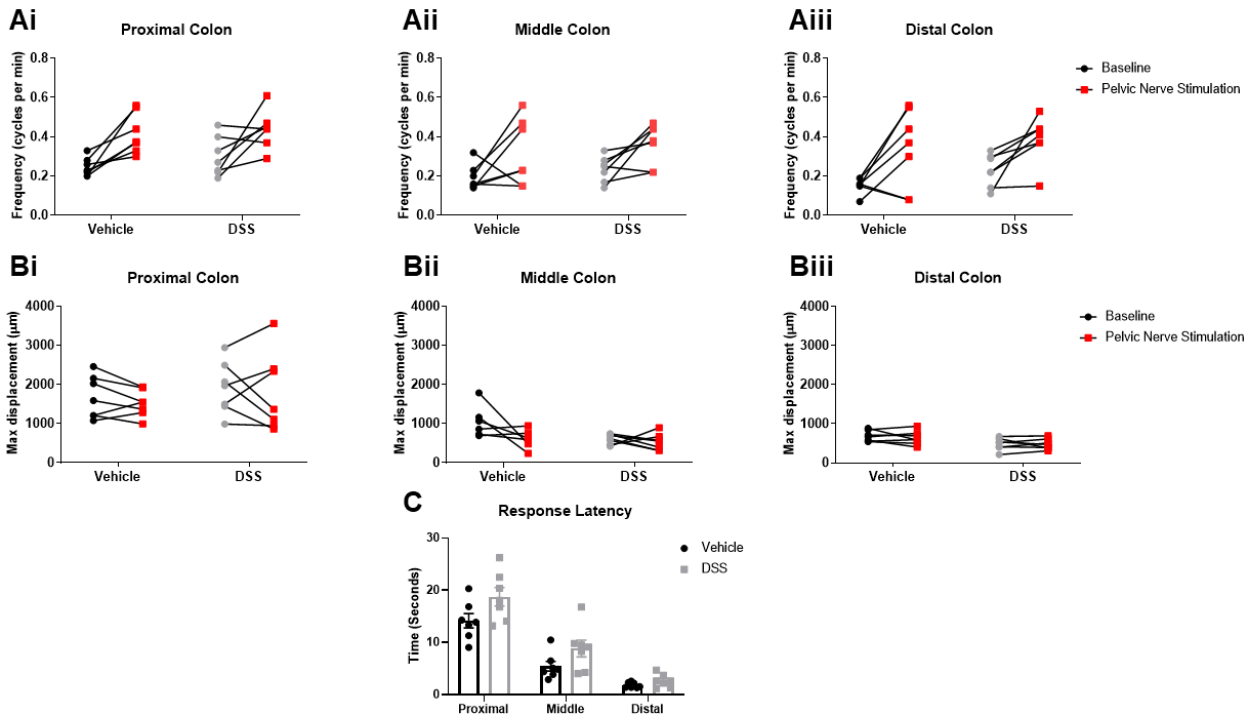

Figure 17 Pelvic nerve stimulation increases the frequency of colon contractions.

A) Quantification of the frequency of contractions with pelvic nerve stimulation in the proximal colon (Ai), middle colon (Aii), and distal colon (Aiii). Pelvic nerve stimulation increases the frequency of contractions compared to baseline. DSS mice were not significantly different from vehicle treated mice. B) Quantification of the amplitude of contractions during pelvic nerve stimulation in the proximal (Bi), middle (Bii), and distal colon (Biii). C) Quantification of the response latency to pelvic nerve stimulation showed an increase in the response latency in the proximal colon after DSS. Data were analyzed with a repeated measures two-way ANOVA. The analyses did not have significant interactions thus post hoc analyses were not performed.

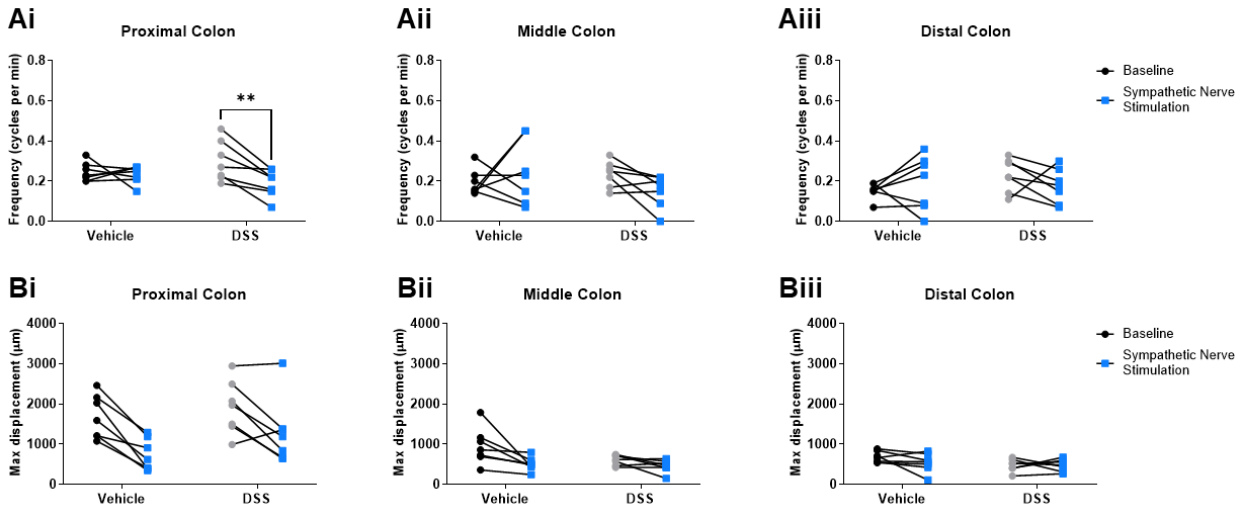

Figure 18 Sympathetic nerve stimulation decreases the frequency of colon contractions after DSS colitis.

A) Quantification of the frequency of contractions with sympathetic nerve stimulation in the proximal colon (Ai), middle colon (Aii), and distal colon (Aiii). Sympathetic nerve stimulation decreased the frequency of contractions in the proximal colon after DSS. B) Quantification of the amplitude of contractions during sympathetic nerve stimulation in the proximal (Bi), middle (Bii), and distal colon (Biii). Sympathetic nerve stimulation decreased the amplitude of contractions in vehicle and DSS treated mice. ***p*<.01. Data were analyzed with a repeated measures two-way ANOVA and post hoc analyses were performed if the ANOVA had a significant interaction.

Sympathetic nerve stimulation did not change the frequency of overall contractions in vehicle treated mice (**Figure 18A**). We had previously shown that sympathetic nerve stimulation decreases the frequency of CMMCs but increases non-propagating contractions (Smith-Edwards et al., 2021) resulting in no change overall. Interestingly, DSS treatment did decrease the frequency of contractions in the proximal colon (F-interaction$_{(1, 12)}$ = 5.198, p = 0.0066, **Figure 18Ai**), which we have previously shown is important for non-propagating contractions for mixing fecal content (Smith-Edwards et al., 2021). In response to sympathetic nerve stimulation, the amplitude of contraction was decreased in both vehicle and DSS mice at the proximal (F-main$_{(1, 12)}$ = 31.15, p = 0.0001, **Figure 18Bi**) and middle colon (p = 0.0073, **Figure 18Bii**). These experiments show

that the pelvic nerve does not seem to be affected by DSS, in contrast, the sympathetic postganglionic fibers are modulated after DSS and may contribute to dysmotility.

4.3.4 Intrinsic Modulation of ENS Circuits Induced by DSS

Motility is largely mediated by the intrinsic ENS including the myenteric neurons and ICC. The latency responses to pelvic nerve stimulation suggest that intrinsic ascending circuits may be impaired in our model of colitis. To further investigate changes induced by DSS in the intrinsic ENS, E2a-GCaMP mice were treated with DSS (n = 9) or vehicle (n = 8) and isolated colon calcium imaging was performed. For myenteric neurons, spontaneous activity and evoked responses were measured. Evoked responses were electrically stimulated in the colon either oral or anal to the imaging field to activation descending or ascending circuits, respectively. Analysis of all myenteric neurons found that DSS did not alter the percentage of spontaneous active neurons (F-main $_{(2, 30)}$ = 0.9901, p = 0.3834, **Figure 19A**) or the percentage of responders to anal (F-main $_{(2, 45)}$ = 1.538, p = 0.2260, **Figure 19Di**) or oral (F-main $_{(2, 30)}$ = 1.175, p = 0.3226, **Figure 19Ci**) stimulation. The evoked movement from anal (p = 0.1862, **Figure 19Dii**) and oral (p = 0.9471, **Figure 19Cii**) stimulation was also not significantly modified after DSS. These results were surprising since colonic inflammation is known to modulate intrinsic neurons (Brierley & Linden, 2014; Mawe, 2015). One possible reason we did not detect changes is that myenteric neurons are a heterogeneous population and specific functional subtypes may be affected by inflammation. To begin to further examine this possibility, preliminary analysis on the three most affected DSS (defined by changes in spleen weight and colon length (for DSS-treated mice) and three vehicle-treated mice was performed to separate out cells that responded specifically to oral stimulation, specifically to anal stimulation, or responded to both. Examining the responses using these criteria,

we found a significant decrease in the myenteric neurons that responded specifically to anal stimulation in the proximal and distal colon after DSS ($p < .05$, **Figure 20**). These data support the idea that ascending circuits are impaired after DSS inflammation.

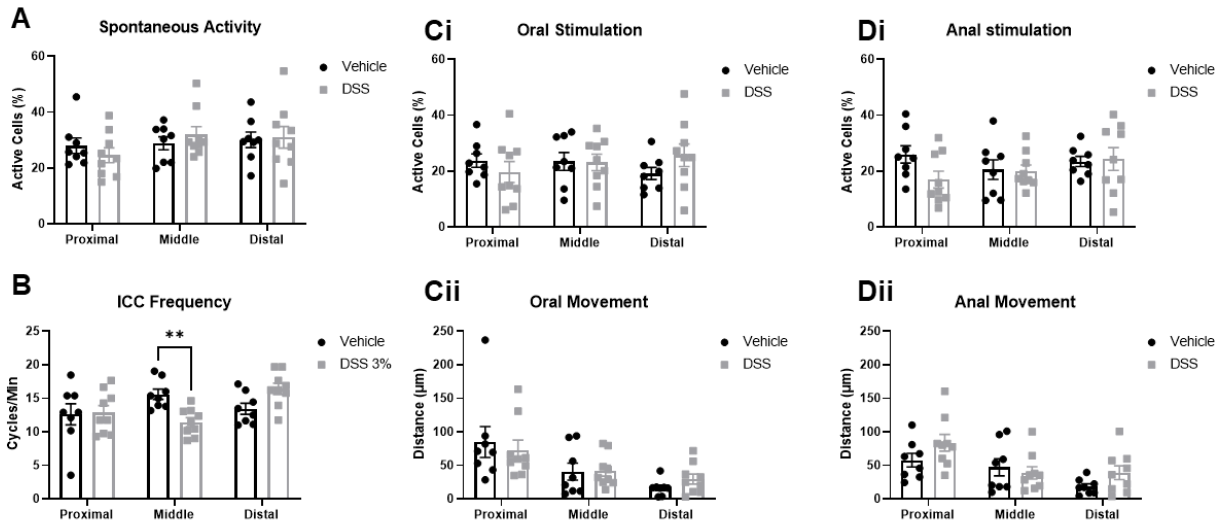

Figure 19 DSS decreased ICC frequency but did not alter myenteric responses.

A) Percentage of myenteric neurons with spontaneous activity. DSS did not later spontaneous activity. B) Quantification of ICC frequency in vehicle and DSS treated mice. DSS decreased the frequency of ICC in the middle colon. C) Stimulation of the colon oral to the imaging field causes myenteric neuron activity (Ci) and muscle movement (Cii). DSS did not change the percentage of active cells or colon movement to oral stimulation. D) Stimulation of the colon anal to the imaging field causes myenteric neuron activity (Di) and colon movement (Dii). DSS did not later the responses to anal stimulation. $^{**}p<.01$. Data were analyzed with a repeated measures two-way ANOVA.

The ICC-SM are known to be involved in motor patterns in the colon and can be modulated by colonic inflammation (Der et al., 2000). ICC-SM are slow wave generators, and the frequency of these calcium waves was assessed. The frequency of ICC-SM was significantly decreased only in the middle colon in DSS mice (F-interaction$_{(2, 45)} = 6.645$, $p = 0.0140$, **Figure 19B**). ICC-SM

has a gradient of frequencies along the colon that moves the calcium waves in the anal to oral direction. The decrease in middle colon frequency will shift this gradient and may contribute to the dysmotility seen with DSS.

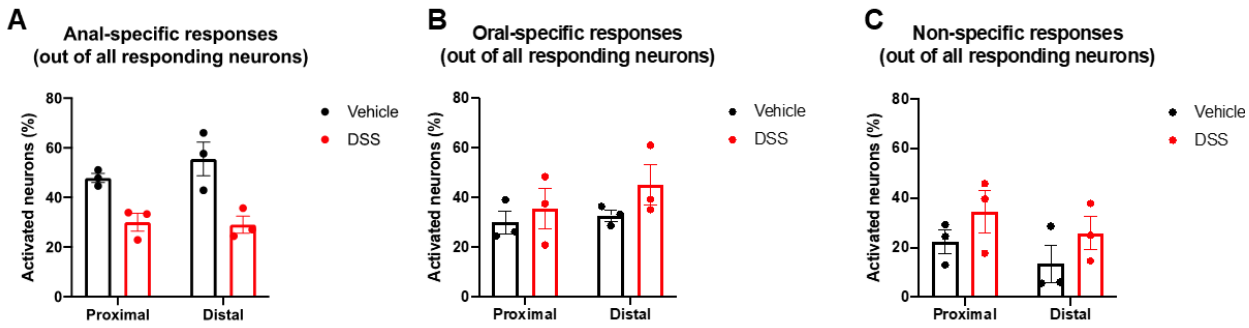

Figure 20 DSS decreased anal-specific myenteric neuron responses.

A) The percentage of myenteric neurons that only responded to anal stimulation. DSS significantly decreased anal-specific responses in proximal and distal colon. B) The percentage of activated myenteric neurons that only responded to oral stimulation. C) The percentage of activated neurons that responded to both oral and anal colon stimulation. All data were analyzed with repeated measures two-way ANOVA. There were no significant interactions between variables therefore post hoc analyses were not performed.

4.4 Discussion

The experiments in this chapter were designed to test how motility circuits are changing after colitis and if extrinsic autonomic efferent fibers from different levels of the neuraxis are distinctly affected. Sympathetic nerve stimulation evoked motility did show a change after DSS, whereas pelvic nerve stimulation was not affected. We also saw changes in the intrinsic ENS that may contribute to dysmotility associated with DSS. Ascending ENS circuits and ICC-SM specifically in the middle colon had impairments due to DSS. The data from these experiments

indicates changes in the extrinsic sympathetics, intrinsic ascending circuits, and ICC all contribute to dysmotility caused by DSS inflammation.

Our data showed a specific impairment of ascending circuits in the intrinsic ENS that was not detected until neurons specifically responding to anal stimulation were separated out from neurons specifically responding to oral stimulation and non-specific responders. This has previously been shown in a TNBS model of colitis; the changes in electrical properties of myenteric interneurons and motoneurons after TNBS colitis are subtle, and no significant changes are detected when considered as a single population (Linden, Sharkey, & Mawe, 2003). However, when the neurons are divided into two subpopulations — those with ascending projections and those with descending projections — the neurons with ascending projections exhibit a slight increase in excitability. In contrast, we found a decrease in the neurons that responded specifically to anal stimulation and an increased latency to pelvic nerve stimulation in the proximal colon (mediated through ascending circuits) implicating decreased responses in ascending circuits. The difference between our results and the previous results may be due to the different inflammation model used or due to species differences as the previous study used guinea pig.

In addition to myenteric alterations with DSS, the ICC-SM also showed a decrease in frequency, specifically in the middle colon. ICC-SM exhibit a gradient of frequencies along the colon that moves in the anal to oral direction. The decrease in middle colon frequency will shift this gradient and may contribute to the dysmotility seen with DSS. Ulcerative colitis patients have a decrease in the number of ICC-SM cells suggesting this population is impaired by colitis (Villanacci et al., 2008). Furthermore, ICC-MY are increased in ulcerative colitis patients and has been shown to be altered by inflammation (Der et al., 2000). This population of ICC were not

examined in these experiments; however, future experiments will examine this population as it is likely affected by inflammation and important for motility.

The main goal of these experiments was to examine whether extrinsic autonomic fibers modulation of colon motility was affected by DSS inflammation. Parasympathetic stimulation has been shown to induce contractions in the distal colon (Smith-Edwards et al., 2019). We showed the pelvic nerve stimulation can travel up the colon through ascending circuits to initiate a CMMC in the proximal colon. DSS inflammation did not change the frequency or amplitude of contractions following pelvic nerve stimulation further supporting the idea that the LS level may not be involved in DSS changes in colon function. In contrast, sympathetic nerve stimulation evoked motility following DSS treatment did exhibit a decrease in the frequency of contractions that was not seen in vehicle mice. This may be due to a decrease in non-propagating contractions that are normally increased in healthy animals following sympathetic stimulation. Sympathetic neurons have previously been shown to be altered in the DNBS model of colon inflammation. Paradoxically, sympathetic postganglionic neurons have been shown to have an increase in activity but a decrease in neurotransmitter release that may be caused by reduced evoked release via increased levels of autoinhibitory presynaptic α2a-adrenoreceptors (Blandizzi et al., 2003). The vagus nerve also sends efferent innervation to the colon and the function of these fibers is not currently known in healthy or inflamed colon.

The DSS model of colon inflammation did produce some alterations in colon function, but these changes were subtle. DSS treatment caused a 61.67% increase in the frequency of contractions only in the distal colon and a 36.59% decrease in the amplitude of contractions specifically in the middle colon. DSS mice exhibited shortened colons, increased spleen weight, and bloody stool indicating that this model does produce colon inflammation. However, there are

only subtle effects on motility suggesting that the ENS is robust and can maintain motility patterns even after colon inflammation. The expulsion of waste is required for life, therefore, the need for robust circuits and potential compensatory mechanisms (i.e. extrinsic innervation) to control colon motility after visceral injury are apparent. The model of DSS used in these experiments was an acute model and more robust changes in motility may be seen after chronic DSS treatment.

5.0 Discussion

5.1 What is the Role of Sensory Neurons in Colon/Visceral Biology?

5.1.1 Anatomical Considerations

A major difference between the three levels of afferents is in their central terminals. Vagal afferents terminate in the NTS whereas spinal afferents terminate in the TL and LS spinal cord. An important reason for the different levels of innervation may be that these different central terminals all require information regarding the visceral environment to coordinate visceral function. TL DRGs are the main source of visceral information to the sympathetic branch of the autonomic nervous system. In contrast, NG and LS afferents send information to the parasympathetic branch of the autonomic nervous system, with LS afferents mainly providing information from the distal colon and NG afferents sending information throughout the colon. The vagus nerve also innervates throughout the GI tract allowing NG afferents to be involved in global regulation of colon function with the rest of the GI tract compared to the LS afferents that may be involved in more localized functions. Visceral afferents from these different levels are sending information about the state of the colon to their respective autonomic nuclei and the need for input from visceral organs to be communicated to each level of the autonomic nervous system for specific reflexes is likely one of the reasons for the different levels of sensory innervation.

In addition to autonomic reflexes, the NTS and spinal cord project to a number of brain areas to provide visceral information for higher order functions (e.g. pain, satiety). The NTS projects to several brain areas involved in emotional regulation, higher autonomic areas, and pain

processing (Rinaman, 2010; Rinaman & Schwartz, 2004). Spinal afferents (both TL and LS) not only project to autonomic regions in the spinal cord but also onto dorsal horn cells that project centrally through spinoparabrachial, spinothalamic, and the dorsal column pathway (Sikandar & Dickenson, 2012; Vizzard et al., 2000). Although it has largely been believed that spinal afferents convey nociceptive aspects of pain and vagal afferents affective aspects, both vagal and spinal afferents have projections to brain areas involved in both the nociceptive and affective components of pain (Rinaman, 2010; Vizzard et al., 2000).

There are also projections between the NTS and the spinal cord. Tracing experiments have revealed a projection from NTS neurons onto the spinal cord, specifically at cervical, thoracic, and lumbar levels in the superficial dorsal horn, lamina VII and X, ventral horn, phrenic and intercostal motor neurons, and the IML (Loewy & Burton, 1978; Mtui, Anwar, Gomez, Reis, & Ruggiero, 1993). The caudal regions of the NTS (containing the majority of GI afferents) show a stronger projection to the lower thoracic and lumbar spinal cord (Leong, Shieh, & Wong, 1984). The thoracic, lumbar, and sacral levels of the spinal cord project to NTS, largely through superficial dorsal horn neurons, the DCM, the IML, and the SPN (Esteves, Lima, & Coimbra, 1993; Menetrey & De Pommery, 1991). There is also evidence for communication between levels of the spinal cord. LS colon afferents modulate the response of thoracolumbar spinal neurons to colorectal distention by a supraspinal loop, which may be an important mechanisms involved in the TL level not processing acute pain (G. Wang, Tang, & Traub, 2007). These data suggest that spinal cord and brainstem input is reinforced via reciprocal connections between each population of the second-order neurons that are the major targets of visceral afferents.

5.1.2 Molecular Phenotypes – Are They Useful?

The development of single cell RNA sequencing in recent years has allowed researchers to begin to define molecular phenotypes of primary afferents on a larger scale than ever before. It has long been a goal to be able to find marker genes that define subtypes of primary afferents with specific functions. RNA sequencing has now been performed on unidentified DRGs (Chiu et al., 2014; C. Li, Wang, Chen, & Zhang, 2018; Usoskin et al., 2015; Zeisel et al., 2018), unidentified vagal afferents (Kupari et al., 2019; J. Wang et al., 2017), and colon specific afferents (Hockley et al., 2018). These studies show that there are distinct molecular clusters of afferents and the authors tried to match putative functional subtypes of afferents with distinct clusters. However, it is unclear if the genes that are used to define clusters of afferents are functionally relevant to their subtypes as most RNA sequencing studies do not perform functional experiments. Studies that have performed functional experiments with RNA sequencing or single cell RT-qPCR suggest this is not the case for many genes (Adelman et al., 2019; Hockley et al., 2018; Meerschaert et al., 2020). Select genes do seem to correlate gene expression and function; for example, the percentage of TRPV1 mRNA expressing colon afferents was correlated with the percentage of colon afferents that responded to capsaicin (Chapter 2). However, the absence of a gene used to functionally define a cluster does not mean a cluster without that gene does not have that function. For example, piezo2 is a mechanically sensitive channel and LS colon afferents had the highest expression of piezo2 implying LS afferents are mechanosensitive. However, electrophysiological recordings from TL colon afferents show these afferents are also mechanosensitive (Brierley et al., 2005; Brierley et al., 2004) therefore, piezo2 expression cannot be used to define mechanosensitive colon afferents. mRNA expression does not always correlate to functional protein expression and using RNA sequencing to predict putative functional subtypes may lead to functional misclassification.

The mRNA analysis from the current study revealed some key differences between colon and bladder afferents compared to studies examining unidentified (i.e., not back-labeled from a specific target) DRG afferents (Usoskin et al., 2015; Zeisel et al., 2018). Almost all spinal afferents projecting to bladder and colon expressed some levels of *Calcα* including neurofilament clusters more associated with non-peptidergic markers (*Asic1, Trkb, Gfra2, Piezo2*) whereas previous studies of unidentified afferents reported 5/16 clusters with low expression and 6/16 with no *Calcα* expression (Zeisel et al., 2018). Like the *Calcα*-expressing clusters in the studies of unidentified afferents, peptidergic colon and bladder afferents also express *Trpv1* and *Gfra3*. However, greater than 80% of colon and bladder afferents also express *Th*, a gene that the authors of these previous studies proposed to be expressed in a single cluster of nonpeptidergic cutaneous afferents (putative C-LTMRs) (Usoskin et al., 2015; Zeisel et al., 2018). There were also differences between colon vagal afferents and unidentified vagal afferents, the most obvious being only four clusters of colon NG afferents were found compared to 18 clusters in unidentified afferents (Kupari et al., 2019). The colon clusters also didn't match with the clusters identified in Kupari et al., several genes that defined colon clusters were widely expressed by unidentified NG afferents (*Ptgir, Ifngr2*) or had low expression in all clusters (*Cxcr2, Oprk1*). Observations like these highlight the importance of information regarding innervation target for the function of neurons used in these big data, transcriptomic studies.

Unique subtypes of visceral afferents may not be as well defined as somatic afferents. Electrophysiological studies of visceral afferents show most can be classified as polymodal nociceptors (Bielefeldt et al., 2006; Brierley et al., 2004; Christianson et al., 2006), whereas cutaneous afferents have both polymodal and unimodal subtypes (McGlone & Reilly, 2010). This may be unsurprising, given the difference in the number of afferents innervating skin compared to

viscera and the more specific sensations arising from the skin (e.g. light touch, vibration, temperature, pressure). Normal homeostasis sensations from the gut are largely not perceived and only after visceral disease or dysfunction does afferent activity arising from the gut reach conscious perception. The need for unique subtypes of somatic afferents may be due to the large variety of sensations arising from the skin, however, visceral afferents may be on more of a continuum with clusters of afferents being better tuned for specific modalities or certain aspects of modalities but all being polymodal in nature. This idea is supported by electrophysiological recordings from TL and LS colon afferents; both TL and LS respond to mechanical and chemical stimuli but to different qualities of the stimuli (Brierley et al., 2005; Brierley et al., 2004).

There is evidence that the visceral afferent system evolved earlier than the somatic system. Hemichordates and early chordates are simple animals, and their structure is little more than a food gathering and digestive system (Romer, 1972). As chordates became more evolved through time, a need for a locomotor system to interact with the environment was required. Somatic function and behavior have continued to evolve over time whereas visceral function has largely remained the same (Romer, 1972). This suggests that visceral and somatic neurons represent the descendant afferents of old and new nervous systems, respectively, the old one being originally charged with internal homeostatic functions and the new one charged with the surveillance and behavioral processes necessary for active animal-environment external interactions (Prechtl & Powley, 1990).

5.1.3 Efferent Function of Afferents

The ability of primary afferent neurons to release neurotransmitters and peptides at their peripheral terminals provides these neurons with unique afferent and efferent functions. It had been hypothesized that ExPANs communicate with myenteric neurons via this local effector

function, however, previous data from our lab (Smith-Edwards et al., 2019) and Chapter 3 of this dissertation show this direct communication does not occur in the naïve state or after colonic inflammation at the LS level. This still leaves the possibility that the TL and/or NG level may have this direct communication. TL afferents are likely candidates as the colon vagal afferents were non-peptidergic and TL had the highest level of peptide expression (e.g. CGRP, SP) (However, it should be noted that vagal afferents release glutamate which is a major transmitter system for myenteric neurons and resident immune cells (Swaminathan, Hill-Yardin, Bornstein, & Foong, 2019; Zhang et al., 2021)). ExPANs may also be releasing peptides and affecting different cell types in the colon. For example, IMA (intramuscular arrays) arising from vagal afferents are closely associated with ICC and it has been speculated that ExPANs and ICC may form functional complexes, although no electrophysiological recordings have been confidently attributed to IMAs (S. J. Brookes et al., 2013). Vascular afferents give rise to fine branching peri-arterial axons that are preferentially associated with arterial branch points and are thought to play an important vasodilatory role based on vasodilation that can produced by stimulation of spinal ganglia (Bayliss, 1901). Moreover, electrical stimulation or capsaicin causes hyperpolarization and dilatation of mesenteric vessels (Meehan, Hottenstein, & Kreulen, 1991) that is mimicked by CGRP and blocked by a CGRP antagonist (Kawasaki, Takasaki, Saito, & Goto, 1988; Uddman, Edvinsson, Ekblad, Håkanson, & Sundler, 1986). Substance P (and neurokinin A) is also released onto blood vessels, where it increases vascular permeability and thereby causes plasma extravasation associated with neurogenic inflammation in many tissues throughout the body (Lembeck & Holzer, 1979). Vascular afferents have been described in TL and LS afferents, with a higher proportion at the TL level, but not in NG afferents.

Primary afferent release of peptides to modulate the immune system has been a growing topic in recent years. The release of peptides from both vagal and spinal afferents has been shown to modulate the immune system in small intestine and lung (Baral et al., 2018; N. Lai et al., 2020). Interestingly, CGRP release was implicated in both of these studies, however, ablation of vagal afferents was protective in the lung whereas ablation of spinal afferents increased bacterial load and disease activity in the small intestine. Possibilities for this difference in immune response may be due to vagal vs. spinal afferents, different organ systems, or differing pathogens.

Although specific colon afferent neuroimmune interactions are not as well characterized, there is evidence implicating a role for colon afferent modulation of the immune system. Substance P induces immune cells to secrete proinflammatory cytokines (e.g. IL-1b, IL-6, IL-8, TNFa) (Shimizu, Matsuyama, Shiina, Takewaki, & Furness, 2008). Studies suggest a pro-inflammatory role for SP and NK1R activation in DSS- and TNBS-induced colitis. Tac1-/- and NK1R antagonist-treated mice showed a decreased in body weight, increased myeloid peroxidase activity, higher histopathological scores, and pro-inflammatory cytokine production compared to wild-type mice (Engel, Khalil, Mueller-Tribbensee, et al., 2012; Engel et al., 2011; Stucchi et al., 2000; Ursino, Vasina, & De Ponti, 2009). A small number of studies showed no difference or more severe colitis after blockade of NK1R signaling (Castagliuolo et al., 2002; Reinshagen et al., 1998). CGRP immunoreactivity and release is increased in distal colon after colonic inflammation (Engel, Khalil, Mueller-Tribbensee, et al., 2012). Exogenous administration of CGRP showed anti-inflammatory effects, leading to amelioration of ulcerative lesions, and attenuation of colonic weight increase (Evangelista & Tramontana, 1993; Mazelin, Theodorou, Fioramonti, & Bueno, 1999). Blockade of CGRP signaling by antagonists in mice increased macroscopic damage, ulcers, and myeloperoxidase activity (Engel, Khalil, Siklosi, et al., 2012; Mazelin et al., 1999; Reinshagen

et al., 1998). CGRP and SP can be released by both intrinsic ENS neurons and ExPANs, and the origin of the peptide release was not investigated in these studies. However, mechanical and chemical denervation removing ExPAN input have been shown to modulate the severity of colitis (Di Giovangiulio et al., 2016; Engel, Khalil, Mueller-Tribbensee, et al., 2012; Engel et al., 2011; Ghia, Blennerhassett, Kumar–Ondiveeran, Verdu, & Collins, 2006; Jancso, JANCSÓ-GÁBOR, & Szolcsanyi, 1967) implying ExPANs may be involved in the development of colitis through peptide release.

5.1.4 Changes with Inflammation (DSS)

Colitis is associated with visceral hypersensitivity and dysmotility and has been shown to effect intrinsic and extrinsic innervation of the colon. The results from our studies investigating the LS level showed no functional change in LS afferent to myenteric neurons communication after DSS. Furthermore, the LS spinal cord only showed increased c-Fos activation in the dorsal horn but not areas associated with autonomic nuclei. This supports previous research that indicates that LS afferents are involved in acute and inflammatory pain (Traub, 2000; Traub et al., 1993; G. Wang et al., 2005). We also found the pelvic nerve stimulation effects on colon motility were not changed after DSS, which supports data showing that c-Fos activation of LS autonomic nuclei was not changed after DSS. In contrast, the TL spinal cord had little to no c-Fos staining with colon optogenetic afferent activation in vehicle treated mice (which was seen in LS autonomic nuclei), but after DSS-induced inflammation, c-Fos staining was found in both dorsal horn and TL autonomic nuclei. TL afferents have previously been hypothesized to only be involved in inflammatory pain and not acute pain, matching our results (Traub, 2000; G. Wang et al., 2005).

The activation in TL autonomic nuclei implies that sympathetic output is altered after inflammation. In naïve mice, sympathetic nerve stimulation decreases CMMC frequency (Smith-Edwards et al., 2021) and the amplitude of contractions but has no effect on the overall frequency of contractions (presumably due to a 'compensatory' increase in ripples and non-propagating contractions). However, in DSS-treated mice, sympathetic nerve stimulation decreases amplitude and frequency of contractions, which suggests a loss of these compensatory non-propagating contractions. There is a large body of evidence suggesting that GI inflammation affects the post-ganglionic sympathetics as there are marked changes in sympathetic neuron excitability (Dong, Thacker, Pontell, Furness, & Nurgali, 2008), neurotransmitter release (Blandizzi et al., 2003; Swain, Blennerhassett, & Collins, 1991), and structure (Dvorak & Silen, 1985; Magro et al., 2002; Straub et al., 2008). In addition, patients with active UC display increased sympathetic activity at rest (Furlan et al., 2006; Maule et al., 2007), while Crohn's disease patients and animal models typically have decreased sympathetic activity (Swain et al., 1991). The NTS also showed an increase in c-Fos staining after colon afferent stimulation is DSS treated mice. The increase at the TL and NG level show that one level of parasympathetic and the sympathetic level are modulated after DSS. A common feature of these two systems is that both TL and NG afferents project to both the proximal and distal colon, whereas LS afferent project overwhelmingly (98%) to the distal colon. These data suggest that collaboration between NG and TL neurons and their second order neurons are central players in autonomic responses to colon inflammation.

The intrinsic ENS was also modulated after DSS-induced inflammation. DSS specifically effected myenteric neurons only responding to anal stimulation, suggesting ascending circuits are attenuated during inflammation. Previous work has shown colitis induces an indiscriminate loss of enteric neurons, a specific loss of intestinofugal afferents, hyperexcitability of IPANs and

ascending neurons, and facilitated synaptic transmission (Brierley & Linden, 2014). These changes lead to a mixture of overlapping excitatory and inhibitory motor outputs in the inflamed regions and halts propagating contractions (Mawe, 2015). DSS also lowers the frequency of ICC-SM slow waves specifically in the middle colon. This alteration of ICC frequency in the middle colon will alter the frequency gradient of ICC along the proximal-distal extent of the colon. ICC have been implicated to play a major role in dysmotility following inflammation in the small intestine (Der et al., 2000). With this protocol of DSS, dysmotility is likely mediated through modulation of enteric circuits, ICC, and sympathetic post-ganglionic neurons. The pelvic nerve parasympathetics do not seem to be affected with this model.

Although statistically significant changes were seen in different components of the ENS following DSS, it was noted that for the most part, these changes were mild, especially given the extent of colon inflammation; DSS treatment resulted in increased spleen weight, edema, shortening of the colon, and bloody stool. Yet despite this significant pathology changes in CMMC frequency and amplitude were restricted to specific areas of the colon and constituted a 61.67% increase in the frequency of contractions only in the distal colon and a 36.59% decrease in the amplitude of contractions in the middle colon. Furthermore, alterations in the ENS following DSS were restricted to a 26.67% decrease in ICC frequency in the middle colon and a decrease in the percentage of myenteric neurons responding to anal stimulation that required further breakdown of myenteric populations that specifically responded to anal stimulation. The processing and expulsion of waste is required for life and the need to continue expulsion during colon disease is a necessity. Therefore, it is likely that the ENS circuits underlying motility are robust and compensatory mechanisms are in place to continue the expulsion of waste even after

visceral inflammation. The DSS model used in these experiments was an acute model and a chronic model of inflammation may produce more robust changes in motility.

5.2 The Function of Colon Afferents from Different Levels of the Neuraxis

5.2.1 LS level

LS spinal cord segments to the colon via the pelvic nerve and are traditionally associated with both afferent input and parasympathetic output. Teased fiber recordings in *ex vivo* colon preparations indicate an even distribution of LS afferents sensitive to different types of mechanical stimuli applied to the colon (e.g., stretch, probe, and mucosal brushing) (Brierley et al., 2004; Feng, Brumovsky, & Gebhart, 2010; Malin et al., 2009). The single cell RT-qPCR experiments revealed that a major distinction between LS and other extrinsic colon afferents is the expression of Piezo-2 (Meerschaert et al., 2020), a mechanosensitive channel, indicating an important role for LS afferents in monitoring the mechanical state of the colon. Afferents in the LS pathway respond to both noxious and innocuous distension, and activate second-order neurons in the dorsal horn of the spinal cord (Harrington et al., 2019b; Traub, 2000; Traub et al., 1993). However, during conditions of inflammation in rodents, afferent input to the LS spinal cord is increased in the dorsal horn but not autonomic nuclei, indicating that LS afferents are involved in mechanical sensation, acute visceral pain, and inflammatory pain.

Dual-color back-labeling from the colon showed that LS afferents almost exclusively project to distal regions (98% of colon-projecting LS afferents), whereas afferents of other

pathways innervate both the proximal and distal colon to a similar extent. Therefore, despite also receiving sensory innervation from TL and vagal pathways, the distal colon is innervated by a spatially restricted group of afferents completely dedicated to monitoring the mechanical state of this particular colon region. Spinal segregation of LS-specific input coming from distal colon likely helps the central nervous system manage or compartmentalize the large amounts of sensory information it continuously processes, but more importantly, probably corresponds to a distinct function specifically carried out by the LS pathway.

In the late 1970's, in vivo electrophysiology was used in cats to show that the LS parasympathetic reflexes to the large intestine are mediated via a spinal pathway and have an essential role in the initiation of propulsive activity during defecation (De Groat & Krier, 1978). More recent studies have confirmed and extended our understanding of the neural circuits involved. In an ex vivo preparation that keeps the colon intact with the LS pathway, GCaMP calcium imaging was used to monitor the activity in myenteric neurons and colon motility produced by stimulating afferent and efferent fibers of the LS pathway (i.e., L6 DRG/dorsal root and ventral root) (Smith-Edwards et al., 2019). Activating preganglionic parasympathetic neurons via ventral root stimulation increased calcium signals in myenteric neurons and evoked contractions, regardless of whether spinal cord circuitry was intact. By contrast, capsaicin and electrical stimulation applied to LS afferents caused a similar effect on myenteric neurons and motility only when the spinal cord was present in continuity. In vivo, optogenetic stimulation of TRPV1+ afferents by application of blue light to the colon lumen was associated with increased activation of autonomic nuclei in LS spinal cord segments (Smith-Edwards et al., 2019). In follow-up experiments, electrical stimulation of the pelvic nerve activated myenteric neurons in the distal colon and also produced delayed, time-locked activation of myenteric neurons in the proximal

colon that consistently initiated a colonic motor complex (Chapter four). The organization of sensory input, response patterns to mechanical stimulation in LS afferents, combined with the engagement of ENS circuits that produce contractions, are all consistent with the primary function of the LS pathway as mediating defecation or expulsion of formed fecal contents by monitoring the mechanical status of the distal colon.

5.2.2 TL level

The neural circuits of the TL pathway to the colon, associated with afferent input and sympathetic output, appear to be more complex in anatomy, cellular targets, and functional effects on colon motility patterns than the LS. As a population, TL colon afferents do not respond well to stretch or mucosal brushing; rather, the majority are activated by blunt probing of their receptive fields, 50% of which are located within or adjacent to the mesenteries that hold the colon in place within the abdominal cavity (Brierley et al., 2004; Lynn & Blackshaw, 1999). This has led to the hypothesis that TL afferents detect twisting or torsion of the colon and are involved in producing sharp, transient pain associated with spasm or distension. During conditions of inflammation induced by mustard oil (Traub, 2000), as well as a 5-day treatment of DSS, input from colon afferents to TL spinal cord neurons was significantly increased. Neurogenic inflammation is mediated via sensory neurons that release neuropeptides from their peripheral terminals, and a high proportion of TL afferents were found to have a peptidergic molecular profile, with expression of calcitonin-gene related peptide (*Calca*), preprotachykinin-1 (*Tac1*), and growth factor receptor 3 (*Gfra3*). Therefore, one would predict that TL afferents have a large role in mediating and transducing signals of inflammation and inflammatory pain.

From the colon, TL afferents course through sympathetic prevertebral ganglia on their way to TL spinal cord segments, and likely provide direct sensory input to sympathetic postganglionic neurons via axon collaterals along the way. Sensory neurons within the colon (intestinofugal afferent neurons) also have axons that project and form functional synapses onto sympathetic postganglionics (Miller & Szurszewski, 1997). Therefore, direct sensory-to-sympathetic communication occurs outside of the spinal cord (i.e., CNS) on a regular basis, and based on evidence described above, it appears that TL spinal cord neurons become more involved during inflammatory or noxious conditions.

Unlike sympathetic innervation of the vasculature, the effects of sympathetic input on colon motility occur via the enteric nervous system (ENS) rather than directly on smooth muscle cells. Early studies using distal colon muscle recordings, sympathetic nerve stimulation, and neuronal blockers reported that sympathetic input has an inhibitory effect on ascending ENS reflex responses to mucosal stimulation (Spencer, McCarron, & Smith, 1999); sympathetic stimulation also affected the resting inhibitory tone of colon smooth muscles (Spencer et al., 1999) and altered muscle activity in a context-dependent manner by acting on the ENS (Spencer, Bywater, & Klemm, 1998). More recent work has provided evidence that sympathetic postganglionic neurons also target non-neuronal cells that help to shape motility patterns (e.g., ICC, glia), regulate neuroinflammation (e.g., glia, immune cells), mediate responses to infection (e.g., immune cells) and make up the ever-important epithelial barrier (e.g., epithelial cells) (Bellono et al., 2017; Gulbransen, Bains, & Sharkey, 2010; Matheis et al., 2020; Smith-Edwards et al., 2021) . Interestingly, sympathetic input has been shown to have region-specific effects on activity in myenteric neurons, ICC and epithelial cells, causing the inhibition of propulsive contractions in distal colon, and facilitating motor patterns for mixing and processing fecal contents in proximal

colon (Smith-Edwards et al., 2021). Overall, these findings are consistent with a primary function of the TL pathway as moderating neuro- and myogenic motility, as well as coordinating the timing and propulsion of movement from proximal to more distal colon regions, which must adapt to inflammatory conditions in order to re-establish homeostasis. Therefore, whereas the LS pathway mediates motility based on mechanical input specifically from the distal colon, the TL pathway mediates motility based on mechanical and inflammatory input from the entire length of colon, and likely other regions of the GI tract.

5.2.3 NG level

Several labs have used optogenetic, electrophysiological, and tracing approaches to map functional connections between vagal neurons and visceral organs, but few reports have included vagal innervation of the colon. The extent to which the vagus nerve innervates the colon has been debated throughout history (Bayliss & Starling, 1900; Gray et al., 1955; Schmidt, 1933; Wells et al., 1942) , with more recent studies finding that contrary to the previous consensus, extrinsic neurons of the vagus nerve innervate and form functional synapses throughout the entire length of colon, including distal regions (Altschuler et al., 1993; Berthoud et al., 1991; Dapoigny et al., 1992; Larauche et al., 2020; Tong et al., 2010) . In Chapter 2, back-labeling from proximal and distal colon indicate a relatively similar extent of vagal innervation of the two regions (Meerschaert et al., 2020). Interestingly, vagal colon afferents were molecularly distinct from spinal colon afferents, mainly due to higher mRNA expression of the purinergic receptors, *P2rx2* and *P2rx3*, and low expression of *Calca* and *Tac1*, suggesting that vagal afferents innervating the colon are relatively non-peptidergic and sensitive to metabolites, such as ATP. Although data are currently

limited, these findings are consistent with the vagal pathway as being a major source of specific types of sensory input from both proximal and distal colon.

Vagovagal reflexes refer to the vagal afferent, brainstem, vagal efferent neuron circuits that coordinate the functions of visceral organs. Information from the vagus nerve converges within the nucleus tractus solitarius (NTS) of the brainstem, which projections to other brain structures that are involved in setting autonomic control, metabolism, satiety, and affect (e.g., limbic system, hypothalamus). Therefore, vagal afferents appear to be responsible for providing the brain the information required for major "top-down" decision-making regarding intestinal digestion and motility, either via vagal efferents or descending pathways to the spinal cord. The experiments performed in this dissertation suggest that TL and vagal afferents may be the more interactive compared to TL and LS.

5.3 Future Directions

Previous research has well characterized the functional properties of LS and TL afferents innervating the colon. In contrast, NG afferents innervating the colon have not been well characterized and the stimuli NG afferents respond to is currently unknown. To begin to understand what role NG afferents have in colon function, identifying what stimuli NG respond to is a key first step. *In vivo* calcium imaging of the NG has previously been utilized to identify NG afferents that respond to gastric and small intestine stimuli (Williams et al., 2016); similar experiments could be performed with colon distention and instillation of chemicals in the colon in naïve mice and after colonic inflammation. Vagal afferents have peripheral terminals that are speculated to be mechanosensitive (IGLE, IMA) and chemosensitive (mucosal). Additionally,

colon NG afferents are uniquely high in purinergic receptors implying ATP should produce activation of NG afferents. In addition to understanding which stimuli NG afferents innervating the colon respond to, examining the how activation of vagal afferent and efferent fibers modulates colon function is also important. Injection of viral ChR2 into the nodose of TRPV1-Cre mice would allow specific activation of vagal afferents. Optogenetic activation of NG afferents can be combined with motility measures as well as calcium imaging of ENS neurons to investigate how vagal afferents influence colon function. Additionally, to explore central projections of NG colon afferents, optogenetic activation and calcium imaging in brain regions thought to receive vagal GI afferents can be implemented to discover colon afferent specific vagal projections.

Another intriguing result from these experiments was the wide-spread expression of cytokine and immune related receptors on colon afferents. Visceral afferents have been revealed to be involved in the immune response in lungs and small intestine (Baral et al., 2018; N. Lai et al., 2020). Vagal and spinal afferents were shown to be differentially involved in these studies, suggesting vagal and spinal afferents may also have different roles in neuroimmune interactions in the colon. Specific optogenetic activation and/or inhibition of vagal and spinal afferents can be examined by nodose injections or intraspinal injection of virus in TRPV1-Cre mice. The ability of vagal and spinal afferents to modulate the immune system during homeostasis and after colon disease would be explored. In addition to comparisons of vagal and spinal afferents in neuroimmune responses, the results from Chapter 2 imply TL and LS afferents may play unique roles in colon immune responses. LS afferents had higher expression of immune receptors associated with a Th1-type response including interferon-gamma, whereas TL afferents had higher expression cytokine receptors associated with of Th2-type responses including IL-4.

The results from Chapter 3 and 4 implied that DSS had the greatest effects on TL afferents and sympathetic neurons. Optogenetic activation of TRPV1-ChR2 colon afferents after DSS produced c-Fos activation in TL autonomic nuclei implying TL afferent input to sympathetic preganglionic neurons is increased after inflammation. DSS also impaired stimulation of postganglionic sympathetic neuron evoked motility. To better understand how TL afferent inputs may modulate sympathetic neurons after inflammation, optogenetic activation of TL afferents can be combined with two-photon calcium imaging of the IML in the spinal cord and prevertebral ganglia. To specifically target TL afferents, intraspinal injections of viral ChR2 and lumbosacral dorsal rhizotomy would be employed. Furthermore, the studies investigated here utilized an acute inflammatory model and future studies identifying how chronic inflammation or recovery affect the different levels of innervation.

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