The Role of the Colon Epithelium in Visceral Nociception and Gut Motility

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Visceral pain and dysmotility are major symptoms of common gastrointestinal disorders like inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Both of these symptoms arise from changes in sensory signaling in the colon; visceral pain results from increased excitability of colon extrinsic primary afferent neurons (ExPANs) and disordered motility involves changes in neurons of the enteric nervous system (ENS), including the intrinsic primary afferent neurons (IPANs) that initiate peristalsis. The colon epithelium is at the forefront of these sensory cascades, as it is the first to interact with all luminal contents and contains the machinery to detect and send sensory information. However, it is unclear exactly how epithelial cell activity influences surrounding afferent neurons in normal and pathological conditions, and this intercellular signaling has been difficult to study because of the close association between epithelial and afferent nerve endings. Therefore, the goal of my studies was to investigate how selective activation and inhibition of colon epithelial cells influences these processes. Using optogenetic tools, I demonstrated that specific activation of the colon epithelium initiates action potential firing in ExPANs in an ATP-dependent manner. Epithelial-induced ExPAN activation resulted in nociceptive behavioral responses. I then showed that inhibition of the epithelium could attenuate nociceptive signaling and reduce visceral hypersensitivity in a mouse model of IBD. Lastly, I showed that selective activation of the epithelium initiated Ca^{2+} activity in ENS neurons and initiated local colon contractions, which was also dependent on ATP signaling. Additionally, these

experiments showed that epithelial activation influenced the frequency of neuronally-mediated rhythmic contractions in the colon. Together these results demonstrate for the first time how epithelial stimulation, in the absence of any other mechanical or chemical stimuli, initiates nociceptive signaling and gut motility. The ability of epithelial inhibition to reduce visceral hypersensitivity reveals the potential of targeting the colon epithelium for treatments of pain. Understanding the biology of this epithelial-neuronal interface and how it changes in pathological conditions will provide valuable insight into why visceral pain is often co-morbid with dysmotility.

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Preface

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1.0 Introduction

1.1 Epithelial-Neuronal Signaling and Visceral Pain

Visceral hypersensitivity and pain are common debilitating symptoms of inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS). These disorders represent widespread health problems, estimated to affect up to 20% of the population (Abdul Rani, Raja Ali, & Lee, 2016). Effective treatments to control pain and resolve hypersensitivity are lacking. Pathophysiological changes associated with IBS and IBD are thought to cause sensitization of spinal afferent neurons that innervate the colon, which transmit noxious stimuli to the spinal cord and central nervous system (CNS) (Azpiroz et al., 2007; Gold & Gebhart, 2010). Pain research has largely focused on injury-evoked changes in spinal afferent and CNS neuron activity, but recent studies of skin, bladder, and colon have revealed new regulatory roles for epithelial cells in sensory transduction. In skin, epithelial cells (keratinocytes) have been shown to have direct communication with epidermal nerve fibers that is sufficient to drive action potential firing and nociceptive responses (Baumbauer et al., 2015; Moore et al., 2013; Pang et al., 2015). Augmentation of keratinocyte-neural signaling may also occur under inflammatory conditions (Kohda, Koga, Uchi, Urabe, & Furue, 2002; Shi et al., 2011; I. S. Song et al., 2000). The bladder

epithelium (urothelium) not only serves a barrier function but also has an active role in mechanotransduction via release of neuroactive substances such as ATP, acetylcholine, and nitric oxide (Winder, Tobin, Zupancic, & Romih, 2014). This release is increased in models of bladder inflammation and cystitis (Sun, Keay, De Deyne, & Chai, 2001).

In the context of the colon, changes in epithelial structure and permeability are common in IBD (i.e., ulcerative colitis, Crohn's disease). In active disease a compromised epithelium allows infiltration of bacteria, which provokes inflammatory immune cell responses (Peterson & Artis, 2014; Roda et al., 2010). Activated immune cells (e.g., macrophages and mast cells) release cytokines and neuroactivators that can affect primary afferent function and result in pain (Akbar, Walters, & Ghosh, 2009; Sharkey & Kroese, 2001). Biopsies of colon from IBD patients with pain often exhibit inflammation (Akbar et al., 2009). However, pain is reported by some patients with no evidence of inflammation or epithelial damage (Bielefeldt, Davis, & Binion, 2009). This finding and other preclinical studies suggest that epithelial regulation of sensory afferent activity may have a more significant role in pain signaling than previously thought and that subtle changes in epithelial function could lead to persistent pain. In support of this concept, several studies have shown that the intestinal epithelium produces and releases neurotransmitters such as ATP (Burnstock, 2001), glutamate (Uehara et al., 2006), serotonin (5-HT) (Gershon, 2013) and acetylcholine (ACh) (Klapproth et al., 1997), which act on colon nociceptive neurons. Data in the following chapters show that channelrhodopsin (ChR2)-mediated selective activation of the colon epithelium is sufficient to evoke action potential firing in ExPANs and a pain-related visceromotor response (Makadia et al., 2018). Thus, the epithelium alone, in the absence of applied mechanical, chemical or thermal stimuli, can initiate sensory neuron activity and pain-like behavior. At the anatomical level, studies have indicated direct synaptic communication between specialized colon

epithelial cells and surrounding neurons, providing a direct means of communication (Bellono et al., 2017; Bohorquez et al., 2015). Taken together, these observations indicate that with respect to structure and function, epithelial cells share many of the properties associated with neurons.

The first part of this introduction will summarize what is known about extrinsic primary afferent neurons (ExPANs) that innervate the colon and how they convey sensory information. The different cell types that make up the colon epithelium will be described, with a focus on enteroendocrine cells (EECs) and their role in sensory signaling. The chemical mechanisms of epithelial-neuronal communication in the colon are also discussed, with emphases on how these neurotransmitter systems are altered in colon inflammation and visceral pain disorders such as IBS and IBD.

1.2 Anatomical and Functional Organization of Colon Epithelial-Neuronal Communication

1.2.1 Extrinsic Sensory Afferents that Innervate the Colon

Colon function is coordinated by intrinsic and extrinsic subpopulations of neurons. The colon receives extrinsic innervation from autonomic and sensory pathways (Brierley, Hibberd, & Spencer, 2018). Extrinsic primary afferent neurons (ExPANs) convey sensory information from the colon to the central nervous system. They are the first in a chain of neurons that give rise to conscious sensations of pain, bloating, fullness and urgency (Brierley et al., 2018). ExPAN somata are in dorsal root ganglia (DRG) at thoracolumbar and lumbosacral spinal levels. Thoracolumbar afferents project to the colon via the splanchnic nerve and lumbosacral afferents project via the pelvic nerve (Brookes, Spencer, Costa, & Zagorodnyuk, 2013). The vagus nerve is a third source

of sensory innervation; vagal afferents that project to the colon have cell bodies in the nodose ganglion (Brookes et al., 2013). Single-unit electrophysiological recordings made in rodent *ex vivo* preparations have revealed five main classes of ExPANs, defined by their functional properties: muscular afferents, which respond to stretching of the colon, mucosal afferents, which respond to light distortion of the mucosa, muscular/mucosal afferents, which respond to stretch as well as light distortion of the mucosa, and vascular afferents, which respond to focal probing of the colon wall (serosal afferents) or mesentery (mesenteric afferents) (Brierley, Jones, Gebhart, & Blackshaw, 2004; Brookes et al., 2013). Muscular afferents include both low-threshold mechanosensors with a slowly adapting response to low-intensity stretch stimuli and high threshold fibers with response properties associated with dedicated nociceptors (Brierley et al., 2004; Hughes et al., 2009; S. A. Malin, Christianson, Bielefeldt, & Davis, 2009). The low-threshold fibers are thought to respond to physiological distension induced by the passing of fecal matter. Mucosal afferents are also low-threshold responders and thought to participate in detecting luminal contents as part of defecatory reflexes (Brierley et al., 2018). In contrast, vascular afferents are high-threshold mechanosensors and display an adapting response to noxious stimuli, making them likely to transmit mechanically-induced pain stimuli (Brierley et al., 2004; Brierley et al., 2008). Mechanically insensitive afferents or "silent afferents" represent a fifth class of ExPANs that respond to chemical stimuli and, after activation by inflammatory mediators, to mechanical stimuli (Feng & Gebhart, 2011). These five major classes of ExPANs have been demonstrated in both rodent and human (J. R. F. Hockley, Smith, & Bulmer, 2018).

The classification of ExPANs continues to be updated as more advanced techniques become available. A recent study using single-cell RNAseq analyses proposed distinct classes of ExPANs based on molecular profiles (J. R. F. Hockley, Taylor, et al., 2018). These included

peptidergic, non-peptidergic, and neurofilament-positive populations at both thoracolumbar and lumbosacral levels. This study also identified neurotransmitter receptors expressed across these populations. Ongoing molecular and functional studies aim to reveal the signaling mechanisms of these ExPAN classes and their role in visceral pain.

1.2.2 Colon Epithelial Cell Types

Epithelial cells that line the small and large intestine form a simple columnar epithelium with two major functions: absorbing nutrients and water and forming a protective barrier (**Figure 1**). Epithelial morphology (i.e., number of villi vs. crypts) and secretory cell populations vary along the GI tract (Wong, Vanhove, & Watnick, 2016). This review focuses on the colon epithelium, partly because most studies of IBD and IBS in humans examine colon biopsies. Additionally, measures and outcomes of most animal models of visceral pain target the colon epithelium and ExPANs. Most colon epithelial cells are absorptive enterocytes, with remaining types having secretory functions. Secretory cell types include goblet cells that secrete mucus for lubrication, tuft cells (also known as brush cells) that secrete opioids and immune mediators (Gerbe & Jay, 2016), and enteroendocrine cells (EECs) that secrete hormones and peptides (Leushacke & Barker, 2014). There are over 10 types of EECs. Most prevalent in the human and mouse colon are L-cells, which express peptide YY (PYY) and glucagon-like peptides 1 and 2 (GLP-1 and GLP-2), and enterochromaffin cells, which release serotonin (5-HT) (Gunawardene, Corfe, & Staton, 2011). The mouse colon also contains a small number of I-cells that express cholecystokinin (CCK) (Egerod et al., 2012) and the human colon contains a small proportion of somatostatin-expressing D-cells (Sjolund, Sanden, Hakanson, & Sundler, 1983).

Although EECs comprise about 1% of the epithelium, they are the most likely cell type to participate in epithelial-neuronal communication in the colon. EECs function as endocrine regulators; they sense luminal content and release hormones that regulate secretion, motility and satiety signals (Janssen & Depoortere, 2013). EECs express many receptors typically found on afferent neurons, e.g., taste receptors that sense glucose, amino acids and fatty acids (Latorre, Sternini, De Giorgio, & Greenwood-Van Meerveld, 2016). EECs also express receptors implicated in mechanotransduction and nociceptive signaling, such as Piezo2 and transient receptor potential ankyrin 1 (TRPA1) (Doihara et al., 2009; Murthy et al., 2018; Nozawa et al., 2009; Szczot et al., 2018; F. Wang et al., 2017). These epithelial cells are electrically excitable and can form synaptic structures with neurons (Bellono et al., 2017; Bohorquez et al., 2015). Using EEC-specific fluorescent reporter lines and transcriptional profiling, axon-like basal processes (a.k.a. neuropods) and presynaptic vesicle proteins were identified (Bohorquez & Liddle, 2011). Other studies targeted rabies virus to EECs in the colon and demonstrated synaptic connectivity between EECs (specifically, PYY-expressing cells) and surrounding neurons (Bohorquez et al., 2015), which include extrinsic primary afferents with cell bodies in lumbar DRG (Kaelberer et al., 2018). This EEC-colon ExPAN connection provides at least one potential cellular substrate for visceral pain transmission.

1.2.3 Transmitters Involved in Epithelial-Neuronal Communication

Diverse cell types within the colon express numerous signaling molecules and receptors that are likely to facilitate epithelial-neuronal communication. Some of these are highlighted in **Figure 1**.

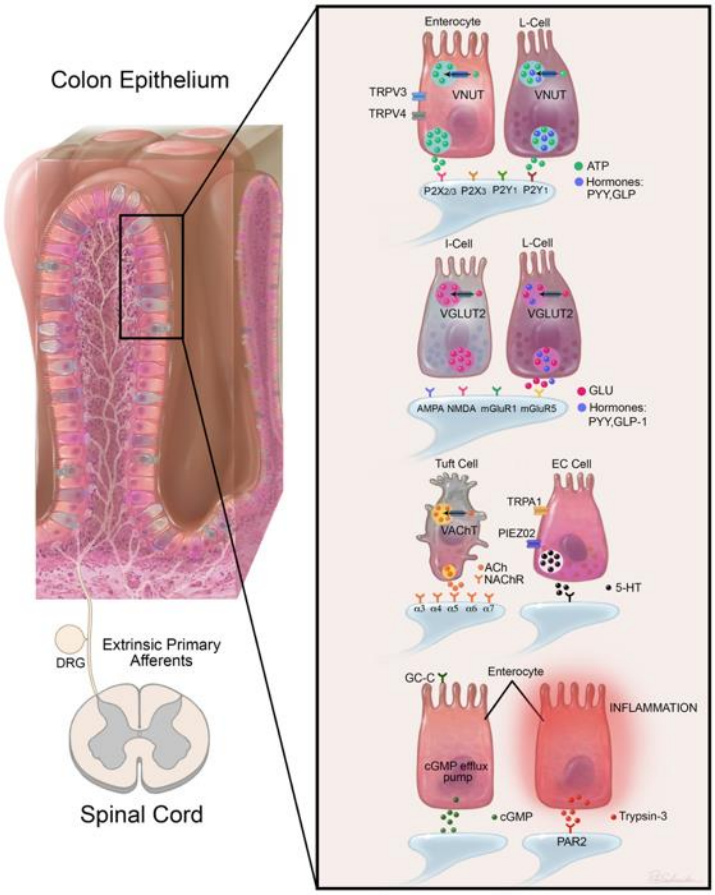


Figure 1. The mechanisms of communication between colon epithelial cells and colon extrinsic primary afferent neurons (ExPANs).

This graphic summarizes some of the key mechanisms of epithelial-ExPAN communication. Neuroactive substances released by specific epithelial cell types are illustrated, along with the corresponding receptors that are found on ExPAN terminals. In some cells, mechanisms of release are also illustrated, including the ion channels and vesicular transporters involved. 1) ATP: Both enterocytes and EECs release ATP (along with other cell types) and VNUT expression is ubiquitous in the epithelium. ATP is also co-released with hormones from L-cells. 2) Glu: VGLUT2 is expressed in I-cells and L-cells, and glutamate is co-released with hormones from L-cells. 3) ACh: VAChT is specifically expressed in tuft cells. 4) 5-HT: EC cells are a primary source of 5-HT and express the sensory receptors TRPA1 and Piezo2. Evidence shows that 5-HT may be released upon activation of these receptors. 5) cGMP: Activation of the GC-C receptor on enterocytes causes release of cGMP. 6) Trypsin-3: Epithelial cells express trypsin-3 and release it in the presence of inflammation. *Reprinted from Najjar et al. (2020), with permission.*

1.2.3.1 Adenosine Triphosphate (ATP)

One mechanism of colon mechanosensory transduction was proposed by Geoffrey Burnstock, a pioneer of the purinergic signaling field, who posited that colon distension leads to ATP release from epithelial cells, which then acts on purinergic receptors expressed on primary afferents (Burnstock, 2001). This hypothesis was supported by studies showing that mechanical stimulation of the mucosa resulted in ATP release and that ExPAN responses to mechanical stimulation were attenuated in the presence of purinergic receptor antagonists (Wynn, Rong, Xiang, & Burnstock, 2003). Distension-evoked release of ATP may be mediated, at least in part, by activation of transient receptor potential channel V4 (TRPV4), which is present in human and mouse epithelial cells (Cenac et al., 2008; D'Aldebert et al., 2011; Mihara, Uchida, Koizumi, & Moriyama, 2018). TRPV3 channel activation is also linked to ATP release; one study showed that addition of the TRPV3 agonist carvacrol to cultured colon epithelial cells increased ATP in culture supernatants (Ueda, Yamada, Ugawa, Ishida, & Shimada, 2009).

The mechanisms that underlie ATP release from epithelial cells are only partially known. As shown for neurons and adrenal chromaffin cells (Burnstock, 1999; Zhang et al., 2019), ATP may be released in combination with other neurotransmitters from enterochromaffin (EC) cells or other EECs (Winkler & Westhead, 1980). ATP can also be co-released with hormones from EECs, e.g., L-cells release ATP along with GLP-1 and PYY (Lu et al., 2019). ATP is also likely released on its own from diverse epithelial cell types. Ubiquitous expression of vesicular nucleotide transporter (VNUT) in the mouse colon epithelium and in human intestinal epithelium cell lines suggest all epithelial types are capable of ATP release. In addition, VNUT-mediated exocytosis of ATP-containing vesicles may be dependent on TRPV4 activation (Mihara et al., 2018). Other

mechanisms of ATP release from colon epithelial cells, including ATP-permeable channels (e.g., pannexins), have not been ruled out (Schwiebert & Zsembery, 2003).

Targets of ATP release on colon ExPAN endings include ionotropic $P2X_3$ and $P2X_{2/3}$ receptors (Shinoda, Feng, & Gebhart, 2009; Wynn et al., 2003) as well as metabotropic $P2Y_1$ and $P2Y_2$ receptors (J. R. Hockley et al., 2016) expressed by muscular and vascular ExPANs (J. R. Hockley et al., 2016; Shinoda et al., 2009). Findings in the following chapters support these targets, as epithelial-neuronal signaling was blocked using a cocktail of $P2X_3$, $P2X_{2/3}$, and $P2Y_2$ antagonists (Makadia et al., 2018). Additionally, ATP released during distension has been shown to activate and sensitize TRPV1 channels on ExPANs (Lakshmi & Joshi, 2005).

Models of visceral hypersensitivity suggest inflammation-induced changes occur in purinergic signaling. In the trinitro benzene sulfonic acid (TNBS) model of IBD, increased distension-evoked ATP release augmented ExPANs firing in response to ATP application and this was correlated with upregulation of $P2X_3$ receptors in lumbosacral dorsal root ganglia (Wynn, Ma, Ruan, & Burnstock, 2004). ATP signaling is also implicated in colon hypersensitivity resulting from intracolonic infusion of zymosan (a model of post-infectious IBS), based in part on the absence of hypersensitivity in $P2X_{3^{-/-}}$ mice (Shinoda et al., 2009). In addition, $P2X_3$ protein level is higher in colon biopsies from IBD subjects (which is often accompanied by persistent pain) compared with control tissue (Yiangou et al., 2001). The authors hypothesized that both submucosal enteric neurons and primary afferent nerve endings contributed to the increase in ATP. Together, these studies suggest that inflammation-induced changes in ATP release (possibly from epithelial cells) and ATP receptor activity (on colon ExPANs) contribute to hypersensitivity.

1.2.3.2 Serotonin (5-hydroxytryptophan)

Serotonin (5-HT) in the gut regulates peristalsis, secretion, and nociceptive signaling. Enterochromaffin (EC) cells make up less than 1% of the epithelium, yet they synthesize and release over 95% of the body's 5-HT (Gershon, 2013). The 5-HT release machinery, located at the basal surface of EC cells, is in close apposition to nerve fibers (Bellono et al., 2017; Mawe & Hoffman, 2013). Secretory granules at the apical surface may facilitate release of 5-HT into the gut lumen (Fujimiya, Okumiya, & Kuwahara, 1997). EC cells are the major source of 5-HT whereas virtually all epithelial cells in the gut express the serotonin-selective reuptake transporter (SERT) and thus take part in controlling 5-HT levels (Wade et al., 1996).

The excitatory ionotropic 5-HT_3 receptor (5-HT_3R) is widely expressed on colon ExPANs (J. R. F. Hockley, Taylor, et al., 2018) and has been implicated in visceral pain signaling. In the mouse model of dextran sulfate sodium (DSS)-induced colitis, an increase in 5-HT_3R-positive nerve fibers in the colon mucosa was measured (Matsumoto et al., 2012). *In vivo* studies of colon sensitivity, measured by responses to colorectal distension (CRD), have shown that i.v. administration of alosetron, a 5-HT_3R antagonist, diminishes these responses. Alosetron administration also decreased c-Fos positive neurons in the spinal cord dorsal horn in response to CRD (Kozlowski, Green, Grundy, Boissonade, & Bountra, 2000). Importantly, alosetron has been used to treat IBS-D (diarrhea-predominant) and randomized controlled trials showed pain relief. However, due to side effects of constipation and colitis, alosetron was withdrawn from the market (Fayyaz & Lackner, 2008).

A study examining both intestinal cryosections and organoids derived from small intestine tissue showed that EC cells form synapse-like contacts with 5-HT_3R-expressing nerve endings

(Bellono et al., 2017). In the same study, functional connectivity between EC cells and nerve fibers was revealed using a colon-nerve *ex vivo* preparation in which selective pharmacological activation of EC cells caused action potential firing in 5-HT$_3$ positive colon mucosal ExPANs, presumably via the release of 5-HT (Bellono et al., 2017). Communication between nerves and EC cells could be initiated via EC expressed molecules that transduce sensory stimuli. For example, studies show that some EC cells express the mechanosensitive Piezo2 receptor, localized adjacent to 5-HT vesicles (Alcaino et al., 2018; F. Wang et al., 2017). Stretching of the colon tissue can activate Piezo2, providing a link between sensations associated with colon distension and 5-HT signaling. However, it should be noted that colon mechanotransduction is likely to be a shared responsibility of EC cells and ExPANs; ExPANs also express Piezo2, most frequently in neurofilament-positive populations (J. R. F. Hockley, Taylor, et al., 2018). TRPA1 is another potential stimulus-transducing molecule expressed by EC cells. In the context of gustatory perception, this ligand-gated receptor is responsible for the oral cavity's ability to detect molecules like allyl isothiocyanate (found in mustard oil, wasabi, and horseradish) and cinnamaldehyde (found in cinnamon). The presence of TRPA1 in colon EC cells supports the concept that these cells can act as "taste buds" of the colon (Bertrand, 2009) and as sensors of chemical irritants. Like Piezo2, TRPA1 is expressed in colon ExPANs and its deletion degrades, but does not eliminates mechanosensory function (Brierley et al., 2009), indicating mechanistic redundancy for this sensory modality that could include a role for the epithelium.

Several studies have examined the role of 5-HT in visceral pain disorders. One study reported that inflamed colon tissue samples from IBD patients have decreased 5-HT compared to controls, decreased SERT levels and fewer 5-HT-immunoreactive EC cells per crypt in the mucosa (Coates et al., 2004). In contrast, animal models of colitis (TNBS and DSS) display increases in

EC cell count and mucosal 5-HT content (Bertrand, Barajas-Espinosa, Neshat, Bertrand, & Lomax, 2010; Linden et al., 2005). The discrepancy in EC cell count and 5-HT levels could be due to limitations of the models, where acute colitis does not directly compare to the chronic inflammation in human IBD (Mawe & Hoffman, 2013).

Studies of 5-HT signaling in IBS have also shown conflicting results. One study compared patients with IBS-D (diarrhea-predominant) and IBS-C (constipation-predominant). Both groups had decreased mucosal SERT expression and no change in EC density or in tissue agitation-evoked 5-HT release (Coates et al., 2004). Another study showed that colon samples from post-infectious (PI) IBS patients had significantly more 5-HT-immunoreactive EC cells per imaging field compared to healthy controls, whereas non-PI-IBS samples showed no difference in EC cell count (Lee et al., 2008). In an analysis of samples from IBS patients of all phenotypes, no difference in EC cell count or 5-HT content was found between patients with and without hypersensitivity (Kerckhoffs, ter Linde, Akkermans, & Samsom, 2012). Thus, 5-HT signaling may be altered in IBS, but its role in colon hypersensitivity and pain remain unclear.

1.2.3.3 Glutamate

Glutamate is another epithelial-derived transmitter involved in sensory signaling. PYY- and CCK-expressing EECs have been shown to release glutamate (Kaelberer et al., 2018) and the vesicular glutamate transporter 2 (VGLUT2) colocalizes with PYY and GLP-1 throughout the intestines (Hayashi, Morimoto, Yamamoto, & Moriyama, 2003). Additional culture studies using a neuroendocrine cell line (GLUTag) showed that glutamate is co-released with GLP-1 in response to KCl or glucose (Uehara et al., 2006).

Glutamate receptors are expressed by colon ExPANs and are implicated in visceral hypersensitivity (Blackshaw, Page, & Young, 2011). Single cell RNA sequencing indicates several types of glutamate receptors are expressed across subtypes of ExPANs, including the ionotropic AMPA and NMDA receptors, and the metabotropic receptors mGluR1-5 (J. R. F. Hockley, Taylor, et al., 2018). Studies in rat showed mGluR5 antagonists diminish visceromotor responses to CRD and, in an *ex vivo* colon-nerve preparation, decrease muscular and serosal colon ExPAN responses to mechanical stimulation (Lindstrom et al., 2008). Changes in NMDA receptors (NMDAR) are also associated with IBS. Specifically, IBS patients were shown to have upregulation of NMDAR, which correlated with abdominal pain scores, whereas mice showed increased sensitivity to colorectal distension in response to intracolonic administration of NMDA (Q. Qi et al., 2017). These findings complemented previous reports showing that NMDAR antagonists prevented hypersensitivity in a mouse model of TNBS-induced colitis (Q. Q. Qi et al., 2016).

A recent study employed rabies virus retrograde tracing in mice to show that PYY- and CCK-positive EECs synapse with DRG afferent fibers (Kaelberer et al., 2018). Many EECs were shown to co-express VGLUT2 and CCK. Optogenetic activation *in vitro* confirmed that CCK-positive EECs release glutamate, causing activation of vagal neurons. These studies support the idea that EECs use glutamate to signal to ExPANs.

1.2.3.4 Acetylcholine (ACh)

The ACh synthesizing enzyme choline acetyltransferase (ChAT) is one of the commonly used indicators of ACh production. Expression of ChAT in the intestinal epithelium has been recognized for decades (Porter, Wattchow, Brookes, Schemann, & Costa, 1996). ACh may be an important transmitter between tuft cells, which express ChAT, and surrounding neurons (Gautron

et al., 2013). The vesicular acetylcholine transporter (VAChT), another indicator of ACh production, has been detected via immunohistochemistry in both human (Jonsson, Norrgard, & Forsgren, 2007) and mouse (Schutz et al., 2015) intestinal epithelium. In mice, VAChT is expressed in only about 20% of ChAT-expressing tuft cells in the proximal colon and not detected in distal colon or small intestine (Schutz et al., 2015). In VAChT+ tuft cells, ACh may be released through synaptic vesicles, but in VAChT- cells, ACh may be released via organic cation transporters (Wessler et al., 2001), or gap junctions (Huang & Roper, 2010).

Nicotinic ACh receptors (nAChR) are expressed on colon ExPANs, but their role in visceral pain is unclear. Single cell RNAseq analysis has shown high levels of nAChRα subunits 3-7 in colon ExPANs (J. R. F. Hockley, Taylor, et al., 2018). Another study showed nAChRα3 expression in about 50% of peptidergic afferents that innervate visceral organs. This study also showed that in skin, mechanically insensitive afferents (MIAs), also known as "silent" nociceptors, can be defined by expression of nAChRα3 (Prato et al., 2017), raising the possibility that MIAs that innervate the colon also express nAChRα3.

Although tuft cells represent only 0.4% of intestinal epithelial cells (in the mouse), they have an important role in mediating immune responses (Gerbe & Jay, 2016). They are a primary source of interleukin-25, which recruits helper T cells and innate lymphoid cells during infection (Fallon et al., 2006). They also express cyclooxygenase enzymes (COX1 and COX2), which produce inflammatory prostaglandins (Bezencon et al., 2008). Recent evidence suggests tuft cells also initiate a type 2 immune circuit in response to succinate, a metabolite secreted by parasitic helminths (Nadjsombati et al., 2018). Visceral hypersensitivity, which often occurs with infection, is thought to result from an inflammatory response to the parasite. As suggested by this study, this response may be initiated by succinate activation of tuft cells and subsequent activation of sensory

signaling pathways. The role of tuft cells in prolonged inflammatory conditions such as IBD is unclear, but alteration in the gut's non-neuronal cholinergic system is a possible link. Evidence consistent with this idea comes from immunohistochemical and mRNA analyses of human colon tissue, which show that patients with ulcerative colitis expressed significantly lower levels of ChAT and VAChT in the colon epithelium (Jonsson et al., 2007).

1.2.3.5 Proteases

Proteases released by colon epithelial cells are likely regulators of colon ExPANs hypersensitivity. Supernatants obtained from IBS colon biopsies increase the excitability of sensory neurons in culture, an effect that is blocked by protease inhibitors (Valdez-Morales et al., 2013). These supernatants also induce visceral hypersensitivity in mice, but not in the presence of a protease-activated receptor-2 (PAR_2) antagonist or in PAR_2-deficient mice (Cenac et al., 2007). In addition, application of a PAR_2 agonist (SLIGRL-NH_2) into the colon lumen results in hypersensitivity to colorectal distension and increased Fos expression in the spinal cord dorsal horn (Coelho, Vergnolle, Guiard, Fioramonti, & Bueno, 2002). Electrophysiology studies have shown PAR_2 sensitizes serosal ExPANs via a TRPV4-dependent mechanism (Sipe et al., 2008). Colon epithelial cells, enterocytes in particular, are a source of proteases; addition of lipopolysaccharide (LPS) to Caco-2 cells in culture caused release of the protease trypsin-3 (Rolland-Fourcade et al., 2017). Trypsin-3 has been shown to induce colorectal hypersensitivity in a PAR_2-dependent manner and is increased in epithelium of colon biopsies of human IBS patients and rat IBS models (Rolland-Fourcade et al., 2017).

Efforts to identify proteases active in IBS and IBD patients are ongoing. Functional proteomic assays of colon tissue and supernatants from IBD patients (both ulcerative colitis and

Crohn's disease), showed increased trypsin-like activity as well as overactive cathepsin G and thrombin activity in IBD patients (Denadai-Souza et al., 2018). How human colon ExPANs respond to proteases is also unclear; although PAR_2 is expressed in 40% of human sensory afferent neurons, neuronal responses to supernatants from IBS patients are mediated by PAR_1 (Desormeaux et al., 2018).

1.2.3.6 Cyclic guanosine-3',5'-monophosphate (cGMP)

Signaling molecules released from colon epithelial cells may also inhibit neural activity. One such molecule is cGMP, which is released upon activation of the epithelial guanylate cyclase C (GC-C) receptor (Huott, Liu, McRoberts, Giannella, & Dharmsathaphorn, 1988). Linaclotide, a drug prescribed for IBS-C for relief of pain and constipation, is a peptide agonist of GC-C, a transmembrane receptor expressed on the luminal aspect of the intestinal epithelium that also binds bacterial enterotoxins (responsible for traveler's diarrhea) and peptide hormones (e.g., guanylin) (Hannig et al., 2014). In randomized controlled trials, linaclotide reduced abdominal pain in over 60% of patients (Castro et al., 2013). Linaclotide's binding to GC-C stimulates the synthesis and release of cGMP from epithelial cells, which has effects that are twofold: stimulation of fluid production in the intestinal lumen and inhibition of colon ExPAN activity (Busby et al., 2010). The increase in epithelial cGMP initiates a protein kinase-dependent pathway which activates the cystic fibrosis transmembrane regulator (CFTR), increasing secretion of bicarbonate and chloride into the lumen. These secretions inhibit the sodium/hydrogen exchanger, leading to fluid secretion into the lumen (Lacy, Levenick, & Crowell, 2012). The increased production of cGMP in the epithelial cells leads to more extracellular cGMP, which has been shown to inhibit colon ExPAN firing via a membrane receptor target, though little is known about the identity of this target

(Grundy et al., 2018). Linaclotide itself does not reduce ExPAN excitability; only cGMP released from the epithelium has this effect.

In rodent *ex vivo* preparations, cGMP applied to the colon lumen decreased colon ExPAN firing rates (Castro et al., 2013; Feng et al., 2013). This effect was more robust in a mouse model of TNBS-induced colon inflammation (Grundy et al., 2018). Patch clamp analysis of cultured human DRG neurons also showed greater effectiveness of cGMP in the presence of inflammatory mediators (Grundy et al., 2018). Although the mechanisms involved in cGMP-induced inhibition remain unclear, further investigation of cGMP targets is warranted as this approach has significant therapeutic potential.

1.2.4 Summary of Epithelial-Sensory Afferent Communication

Colon epithelial cells, EECs in particular, are likely to have a significant influence on the activity of colon ExPANs and visceral pain signaling. This idea is explored in Chapter 2, which describes studies in which colon ExPAN activity and nociceptive responses are measured in response to colon epithelium activation. Chapter 3 also proposes a role for the colon epithelium in pain signaling, demonstrating the effects of colon epithelium inhibition on nociceptive responses.

The neuroactivators released by epithelial cells that are most likely to be involved in this epithelial-neuronal communication are summarized in Table 1. This table shows each neurotransmitter and its epithelial cell sources, as well as the model systems and techniques that have been used to identify the sources of each transmitter.

As summarized in **Figure 1**, colon epithelial cells express some of the same sensory receptors that are found in colon ExPANs, indicating that they have a role in monitoring the environment in the gut lumen. This is supported by studies showing that colon ExPAN responses

to mechanical stimuli are diminished when epithelium-released neurotransmitters are blocked (Makadia et al., 2018; Wynn et al., 2003). In many cases, transmission from epithelial cells may be diffuse and have slow or indirect actions on colon ExPANs terminals. However, communication from some EECs to colon ExPANs may be achieved through direct synaptic transmission. Enterochromaffin cells (ECs) are one type of EEC that show evidence of these synapses and they contain the receptors TRPA1 and Piezo2, which are critical in mechanosensation and pain signaling (Alcaino et al., 2018; Bellono et al., 2017). This receptor expression profile and connectivity with surrounding colon ExPANs may indicate that ECs have a more salient role in sensory signaling than other cell types.

Table 1. Major regulators of epithelial-neuronal communication in the small intestine and colon.

Transmitter	*Epithelial Cell Source*	*Model System*	*Techniques Used to Identify Source of Transmitter*	*Ref*
Acetylcholine (ACh)	Tuft cells	Mouse: small intestine and colon tissue	Immunohisto-chemistry (IHC), *in situ* hybridization, transgenic fluorescent reporter	(Porter et al., 1996)
Adenosine triphosphate (ATP)	All	Rat: colon tissue	Luciferin-luciferase, ATP release assay	(Wynn et al., 2003)
	All	Human: CCD 841 cell line	Luciferin-luciferase, ATP release assay	(Mihara et al., 2018)
	All	Mouse: colon epithelium primary culture	Luciferin-luciferase, ATP release assay	(Ueda et al., 2009)
	L-cells	GLUTag cell line Mouse: cultures of small intestine epithelium Human: culture of colon epithelium	Epithelial-neuronal co-cultures, luciferin-luciferase ATP release assay, sniffer patch IHC IHC	(Lu et al., 2019)
	All	CCD 841 cell line Mouse: colon tissue	qRT-PCR, IHC, luciferin-luciferase assay qRT-PCR, IHC	(Mihara et al., 2018)
Glutamate (Glu)	L-cells	Rat: small intestine tissue	IHC	(Hayashi et al., 2003)
	L-cells	GLUTag cell line	*In vitro* chemically evoked release	(Uehara et al., 2006)
	I-cells	Mouse: small intestine tissue	IHC, *in vitro* optogenetics	(Kaelberer et al., 2018)
Cyclic guanosine-3',5'-monophosphate (cGMP)	All	Human: T84 cell line	Enzyme immunoassay	(Busby et al., 2010)

Table 1 Continued

Serotonin (5-HT)	EC cells	Rat: small intestine tissue	Immunoelectron microscopy	(Fujimiya et al., 1997)
	EC cells	Mouse: small intestine, cultured organoids, *ex vivo* colon-nerve preparation	IHC, *in vitro* electrophysiology, 5-HT biosensors (sniffer patch)	(Bellono et al., 2017)
	EC cells	Mouse: small intestine and colon, cultured organoids	IHC, *in vitro* calcium imaging, 5-HT biosensors (sniffer patch)	(Alcaino et al., 2018)
Trypsin-3	All	Rat: colon tissue Human: colon tissue, Caco-2 cell line	IHC, Western blot IHC, Western blot	(Rolland-Fourcade et al., 2017)

1.3 Communication Between the Colon Epithelium and Enteric Nervous System

1.3.1 Intrinsic Innervation of the Colon

The colon contains intrinsic neurons as part of the enteric nervous system (ENS), which are essential for peristalsis, mucous and acid secretion, and nutrient absorption (Furness, 2012). The ENS has been referred to as the "brain-in-the-gut" or the "second brain" because of its expansive network of neurons (numbering over 500 million) which carry out complex sensory and motor functions (Gershon, 1999; Wood, 2016). Enteric neurons control motility in the small intestine and colon through coordination of smooth muscle contractions that propel food and fecal contents along the gut. Interconnected enteric neurons and glia form two plexuses within the colon

wall: the myenteric plexus resides between the longitudinal and circular muscle layers and the submucosal plexus between the circular muscle layer and mucosal layer (Costa, Brookes, & Hennig, 2000).

Proper gut motility relies on ENS reflexes, which are initiated by sensory neurons that are unique to the GI tract, called intrinsic primary afferent neurons (IPANs). IPANs sense chemical and mechanical (e.g., stretch) stimuli in the lumen and send this sensory information to other enteric neurons to facilitate digestion (Furness, 2012). These neurons include excitatory and inhibitory motor neurons, and ascending and descending interneurons, which coordinate functions such as secretion, vasodilation, and smooth muscle contraction (Furness, 2000). Propulsion of food and fecal contents requires coordinated motor patterns in different segments along the gut involving both contraction and relaxation of smooth muscle, hence the diversity of excitatory and inhibitory cell types in the ENS (**Figure 2**). Most of the ENS neurons associated with motor function are located in the myenteric plexus and IPANs originating in these ganglia have processes that extend into the mucosa, enabling detection of luminal contents (Furness, Robbins, Xiao, Stebbing, & Nurgali, 2004). Considering how important gut motility is to overall health, it is critical to understand this sensory component of ENS reflexes.

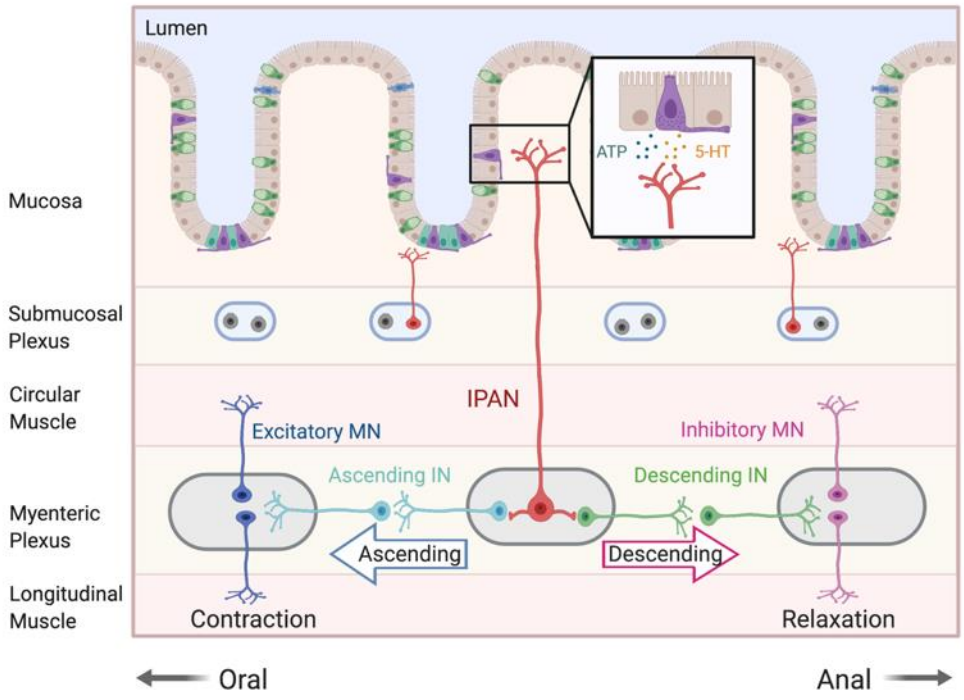


Figure 2. Colon enteric nervous system reflexes and the role of the epithelium.

Intrinsic primary afferent neurons (IPANs) in the myenteric plexus project to the mucosa to detect luminal contents. In response to chemical and mechanical stimuli, IPANs signal to interneurons (INs) and motor neurons (MNs) to produce propulsive smooth muscle contractions. In this example, a myenteric IPAN signals to ascending INs which synapse with excitatory MNs to produce contraction in the oral direction. The IPAN also signals to descending INs which synapse with inhibitory MNs to produce relaxation in the anal direction. Epithelial cells in the mucosal layer also sense luminal contents and release neurotransmitters in response (inset). In this example, enterocytes and an enterochromaffin cell release ATP and 5-HT, respectively, which can act on local IPAN terminals to initiate reflexes. Image created in BioRender.

1.3.2 Role of the Colon Epithelium in ENS Functions

Reciprocal interactions between the epithelium and ENS neurons are thought to have an important role in colon homeostasis. This epithelial-neuronal communication is thought to be

paracrine, where molecules diffuse from one cell and act on nearby receptors (Walsh & Zemper, 2019). Although studies show synaptic connections between EEC neuropods and extrinsic neurons in the gut (Kaelberer et al., 2018), it has not yet been determined whether neuropods form synapses with intrinsic ENS neurons. Studies performed in intestinal organoids show that the ENS influences proliferation and differentiation of cell types in the epithelium (Workman et al., 2017). ENS neurons also influence the function of epithelial cells; namely, cholinergic ENS neurons innervating the epithelium trigger mucous secretion (Specian & Neutra, 1980).

Conversely, colon epithelial cells are influential in ENS function, particularly in sensory signaling. In the mucosa, IPAN nerve endings lie in close apposition to epithelial cells. Evidence shows that IPANs can sufficiently detect mechanical stimuli in the lumen to initiate enteric reflexes (Bertrand, Kunze, Bornstein, & Furness, 1998), but it has been proposed that the epithelium acts as an intermediary between luminal stimuli and ENS neurons. ATP and 5-HT are the two major epithelial-released neurotransmitters thought to have excitatory actions on ENS neurons (Bertrand, 2003) (**Figure 2**). Enterochromaffin (EC) cells are thought to be the main epithelial cell type involved, as they are the body's main source of 5-HT, an important signaling molecule in the ENS. As previously discussed, EC cells express receptors for chemical irritants (TRPA1) (Nozawa et al., 2009), and mechanical stimulation (Piezo 2) (Alcaino et al., 2018). EC cells release 5-HT in response to activation of these receptors, which is thought to act on local ENS neurons and initiate colon motility (Grider, Kuemmerle, & Jin, 1996; Kadowaki, Wade, & Gershon, 1996; Nozawa et al., 2009).

Despite the evidence of the epithelium's role in sensory signaling, the extent to which endogenous neurotransmitter release from colon epithelial cells activates IPAN nerve terminals is a major unanswered question. Additionally, it has been debated whether the epithelium has an

essential role in colon motility, particularly in colonic migrating motor complexes (CMMCs), which are rhythmic motor patterns that facilitate the movement of fecal matter through the colon (Spencer, 2001). Some researchers report that mucosal 5-HT is essential for normal propagation of motor patterns in the colon (Heredia, Dickson, Bayguinov, Hennig, & Smith, 2009; Heredia et al., 2013; Smith & Gershon, 2015), while others report that motor patterns are generated in the myenteric plexus and/or smooth muscle layers and do not require signaling from the mucosa, though mucosal serotonin may have a modulatory role (Keating & Spencer, 2010; Spencer, Sia, Brookes, Costa, & Keating, 2015). Given these discrepancies, more specific tools are needed to discern how epithelial activation affects ENS activity and whether the epithelium has a direct or modulatory role. The experimental techniques described in Chapter 4 provide a means to address these questions.

1.4 Optogenetic Tools for Activating Neuronal and Non-Neuronal Cells

Optogenetics refers to the expression of light-activated proteins, called opsins, in specific cell populations to enable manipulation of activity in those cells. The first successful expression of the algae-derived protein Channelrhodopsin-2 (ChR2) in mammalian neurons was demonstrated in 2005 (Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005) and, since then, many types of excitatory and inhibitory opsins have been engineered. ChR2 is a blue light-activated non-selective cation channel that, when genetically expressed in the cell membrane, enables depolarization of the cell (Nagel et al., 2003) (**Figure 3**). This opsin is still commonly used to excite specific cell types with temporal precision. Conversely, inhibitory opsins are used to silence the activity of specific cell types. For example, the yellow light-activated archaerhodopsin (Arch) is an outward

proton pump that can mediate hyperpolarization of neurons upon activation (Chow et al., 2010) (**Figure 3**). Among many other purposes, optogenetic tools have been useful in interrogating peripheral pain circuits and ENS function.

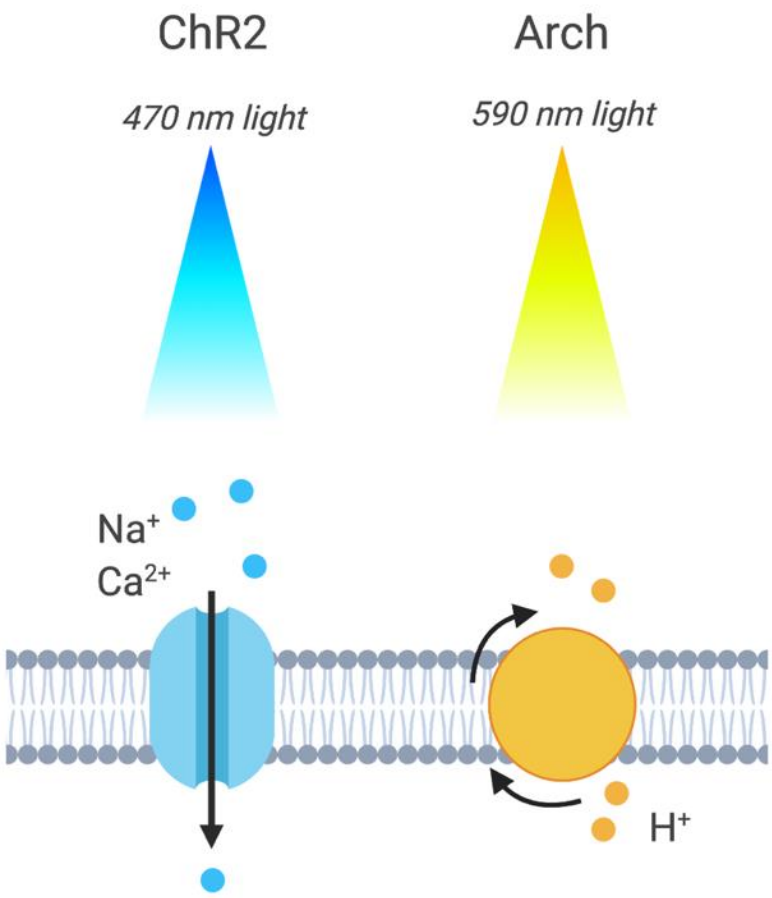


Figure 3. Excitatory and inhibitory opsins.

Opsins used in the following studies include the excitatory channelrhodopsin-2 (ChR2), a non-selective cation channel activated by ~470 nm light and the inhibitory archaerhodopsin (Arch), an outward proton pump activated by ~590 nm light. These opsins can be transgenically expressed in specific cell types using Cre/Lox recombination technology.

Opsins can be genetically targeted using viral techniques or transgenic methods using Cre/Lox recombination technology. Both have been used to investigate specific populations of sensory neurons (Mickle & Gereau, 2018). Studies in mice have demonstrated that stimulating

ChR2 targeted to neurons expressing TRPV1 (Baumbauer et al., 2015), Nav1.8 (Daou et al., 2013), or Advillin (Park et al., 2015) can evoke nociceptive-live behaviors. On the other hand, expression of Arch in these neuron populations enables an analgesic effect. For example, silencing of Nav1.8-expressing peripheral neurons attenuated inflammatory pain (Daou et al., 2016). Many of these studies were performed by shining blue light on the skin or along the nerve.

Peripheral optogenetic manipulation of visceral organs is also possible, for example Advillin-ChR2 has been used to interrogate extrinsic primary afferents innervating the colon (Feng, Joyce, & Gebhart, 2016). In a study of bladder afferents, ChR2 was targeted to both TRPV1- and Nav1.8-expressing neurons to reveal the role of each subpopulation on bladder nociception and voiding function (DeBerry et al., 2018). Activation of Arch expressed in bladder afferents attenuated nociceptive responses in a visceral inflammatory pain model (Samineni et al., 2017). More recently, these tools have been employed in gut motility studies, where subpopulations of ENS neurons are targeted with ChR2 to reveal their contributions to neurogenic motor patterns (Hibberd, Feng, et al., 2018).

Excitatory and inhibitory opsins can also be expressed in non-neuronal cells. While there is evidence of intercellular communication between neurons and epithelial cells, endothelial cells, and glia, this intercellular signaling has been difficult to study because of their close association with neurons. Optogenetic tools enable activation of these cell types without influencing the surrounding neurons and enable measurement of their impact on neural activity and behavioral outputs (Mickle & Gereau, 2018). Studies have shown that ChR2-mediated activation of Merkel cells and keratinocytes result in sensory afferent action potential firing and nociceptive responses (Baumbauer et al., 2015; Maksimovic et al., 2014). Optogenetic inhibition of keratinocytes inhibited sensory responses to mechanical stimulation (Baumbauer et al., 2015; Moehring et al.,

2018). More recently, a study showed that the Schwann cells that ensheathe peripheral nerve endings also contribute to sensations from the skin (Abdo et al., 2019).

In studies of this dissertation, ChR2 and Arch were employed to modulate both colon ExPANs and colon epithelial cells. In Chapter 2, ChR2 was used to investigate the role of the colon epithelium in sensory signaling and responses were compared to direct ChR2-mediated activation of colon ExPANs. In Chapter 3, Arch was targeted to the colon epithelium and ExPANs to investigate the role of the colon epithelium in visceral hypersensitivity associated with inflammation. Finally, in Chapter 4, ChR2 was targeted to the colon epithelium to examine its effects on ENS neuron activity and gut motility.

2.0 Optogenetic Activation of Colon Epithelium Produces High-Frequency Bursting in Extrinsic Colon Afferents and Engages Visceromotor Responses

2.1 Introduction

The epithelial-lined mucosa of the colon is innervated by autonomic and sensory nerve fibers of intrinsic and extrinsic origin (Spencer, Kyloh, & Duffield, 2014). Understanding how activation of these nerve fibers is regulated at this important interface is of significant clinical and biological interest, particularly for understanding their role in pain signaling in chronic inflammatory conditions of the gut. In recent studies of the epithelium covering the skin it has been possible to demonstrate that excitation of epithelial cells alone can directly produce action potentials in primary sensory neurons in the absence of naturalistic mechanical, thermal, or chemical stimulation (Baumbauer et al., 2015; Maksimovic et al., 2014; Pang et al., 2015). Epithelial cells, genetically modified to express channelrhodopsin (ChR2) and stimulated with blue light, were shown to elicit firing in diverse types of cutaneous sensory neurons, including those that sense pain and mechanical stimuli (Baumbauer et al., 2015). The colon epithelium, comprised of absorptive enterocytes, endocrine cells, and goblet cells has features in common to the skin epithelium; it interfaces with the internal environment of the gut and is the first line of defense for the colon, protecting it from a wide range of pathogenic and chemical insults within the colon lumen. Similar to keratinocytes of the skin, colon epithelial cells can release several types of neuro-activator compounds, including classic neurotransmitters such as acetylcholine (ACh), ATP, and 5-hydroxytrypamine (5-HT/serotonin), and peptides and hormone transmitters, such as somatostatin and peptide YY (Bertrand, 2009; Gunawardene et al., 2011; Rindi, Leiter,

Kopin, Bordi, & Solcia, 2004). Released neuromodulators are thought to both stimulate and inhibit neurons of the myenteric plexus, as well as act on primary sensory afferents from the dorsal root ganglia (DRG), through activation of metabotropic and ionotropic receptor proteins, e.g., muscarinic or nicotinic acetylcholine receptors, purinergic receptors, and/or serotonergic receptors (Bertrand, 2003; Bornstein, 2006; Burnstock, 2014; King, 2015; Linan-Rico et al., 2015; Linden, Chen, Gershon, Sharkey, & Mawe, 2003; Mawe, 2015; Wynn et al., 2004). Few studies have examined how compounds, potentially derived from the colon epithelium, can initiate neuronal action potentials in the absence of other naturalistic stimuli (Bellono et al., 2017; J. R. F. Hockley, Taylor, et al., 2018; Wynn et al., 2004). Given the inconclusive evidence for direct activation of nerve fibers by colon epithelial cells and the recent finding that skin epithelial cells can independently initiate action potentials in DRG sensory fibers, we examined the contribution of the colonic epithelium to generation of action potentials in colon ExPANs. To allow epithelial-specific activation we targeted ChR2 to colonic epithelial cells to determine whether light-induced activation of these cells could generate action potentials in ExPANs. Our results show that almost one-half of all colon ExPANs fired high-frequency trains of action potentials in response to light activation of the epithelium, often in patterns similar to those evoked by mechanical stimulation. In addition, the response of 70% of the epithelial-activated ExPANs exhibited a significant decrease in firing frequency when stimulated in the presence of blockers of P2X- and P2Y-receptor-mediated neurotransmission, suggesting that ATP and/or UTP are major components of epithelial cell – colon ExPAN excitation coupling. Using the visceromotor response (VMR) as an *in vivo* assay, it was also determined that light stimulation of epithelial lining cells alone is sufficient to elicit behavioral changes similar to those obtained in response to colon distension. That colon epithelial cells alone have the ability to directly initiate ExPAN firing and cause

behavioral response supports a central role for these cells in colon function and physiology and indicate that the epithelium not only serves as a barrier and source of chemical modulators, but also provides a means for direct transmission of stimuli from the colon lumen to the nervous system.

2.2 Methods

2.2.1 Animals

Male and female mice 6 –10 weeks of age were analyzed. Mice with a fusion protein of ChR2 and EYFP [ChR2(H134R)-EYFP] in the *Rosa26* locus downstream of a floxed-STOP cassette (Ai32 mice) were crossed with either TRPV1-Cre or villin-Cre mice. Littermates with ChR2-EYFP but lacking Cre were used as controls. Animals were maintained in an association for assessment and accreditation of laboratory animal care (AAALAC) approved facility and handled following protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2.2 Tissue immunolabeling

L5, L6, and S1 DRG and distal colon segments were fixed in 4% paraformaldehyde, cryoprotected in 25% sucrose, embedded in optimal cutting temperature (OCT) compound and sectioned on a cryostat at either 14 µm (for DRG) or 20 µm (for colon). Air dried sections were washed in phosphate buffered saline (PBS), incubated overnight at room temperature in

anti-PGP 9.5 (1:500; rabbit polyclonal, ThermoFisher, PA1-21097) made in PBS/0.25% Triton X-100, washed, incubated in donkey anti-rabbit-Cy3 (1:500; Jackson ImmunoResearch), washed, coverslipped, and images captured using a digital camera attached to a Leica DM4000B microscope.

2.2.3 Reverse-transcriptase polymerase chain reaction analysis

The relative expression level of ChR2-YFP mRNA was determined using reverse transcriptase PCR analysis. Total RNA isolated from the distal colon was purified using the Quick-RNA miniprep kit (Zymo Research), DNase (Invitrogen), and reverse-transcribed using Superscript II (Invitrogen). Primers for ChR2 (5'TGG CTC TGT ACT TGT GCC TG3' and 5'TGA CCA TCT CGA TAG CGC AC3') and GAPDH (5'ATG TGT CCG TCG TCG TGG ATC TGA and 5'ATG CCT GCT TCA CCA CCT TCT T3') were used in SYBR Green PCR amplification reactions performed using a Bio-Rad CFX system. Relative fold-change was calculated using the $\Delta\,\Delta C_t$ method with GAPDH as standard.

2.2.4 *In vitro* single-fiber recording from colon-pelvic nerve

Mice killed with isoflurane were transcardially perfused with carbogenated (95% O_2, 5% CO_2) ice-cold artificial CSF (ACSF) containing the following (in mM: 117.9 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4$-7 H_2O, 2.5 $CaCl_2$, 11.1 D-glucose) and the distal colorectum (~3 cm) removed with the attached major pelvic ganglion and pelvic nerve (PN). Tissues were transferred to ice-cold modified Krebs' solution (Krebs' solution containing 2 mM butyrate, 20 mM sodium acetate, 4 µM nifedipine, and 3 µM indomethacin bubbled with

carbogen (Brierley et al., 2004; Feng, Brumovsky, & Gebhart, 2010) and then to an acrylic organ bath consisting of two adjacent chambers separated by a plastic gate with a small opening. The colon was opened longitudinally along the mesenteric border, placed in a Sylgard 182 (Dow Corning) lined chamber with circulating Krebs' maintained at 34°C and pinned flat with mucosal side up. The spinal nerve containing afferents from the L6 DRG, which contributes to both the pelvic nerve and lumbar splanchnic nerve, was dissected and followed out to the fat pad located near the junction of the bladder and the colon (see **Figure 5A,B**). For the purpose of recording, the entire L6 ventral ramus that gives off branches that run into the fat pad was placed in the recording chamber and the fat pad and nerves contained within positioned under the Plexiglas divider that separates the recording chamber from the colon. To obtain nerve fascicles that contain afferent fibers that innervate the colon, the L6 ventral primary ramus (i.e., the PN) is placed onto a mirror in the recording chamber containing mineral oil. Under a dissection microscope, the nerve sheath was removed and the nerve trunk teased into fine bundles (~10 µM thick) that were placed onto a platinum-iridium recording electrode for single-fiber electrophysiological recordings.

2.2.5 Characterization of pelvic afferents

Teased fibers of the PN were characterized using the classification system previously described (Brierley et al., 2004; Lynn & Blackshaw, 1999). Fibers that were activated by circumferential stretch were designated as "muscular", or "muscular/mucosal" if they also responded to gentle stroking of the mucosa. Fibers that only responded to blunt probing were classified as "serosal", whereas those that only responded to gentle mucosal stroking were designated as "mucosal". Receptive fields were identified by first stroking the mucosal surface with a brush to locate mechanosensitive ExPANs. Responsiveness to blue-light stimulation was then

determined using a 473 nm wavelength laser (Laserglow Technologies) and power meter readings taken to estimate the average wattage reaching the tissue (40 mW at maximum power) over a 200 µm spot size (delivered via a 100 µm fiber-optic light guide). Mechanical stimuli were then applied to further classify ExPANs as serosal, muscular, mucosal, or muscular/mucosal: probing with nylon monofilaments (von Frey filaments, 1–1.4 g force), mucosal stroking with calibrated nylon filaments (10 mg force) and circumferential stretch generated using a servo-controlled force actuator (Aurora Scientific). Custom-made claws (1 mm interval) were attached along a mesenteric edge of the colorectum to allow for uniform circumferential stretch by slow ramped force (0–170 mN at 5 mN/s). The circumferential stretch generated corresponds to intraluminal pressures of 0–45 mmHg (Feng et al., 2010).

2.2.6 ATP antagonist pharmacology

2.2.6.1 Villin-ChR2 responsive ExPANs

Once receptive fields of fibers were identified with blunt probing, blue-light laser at a minimal intensity (1–2 mW) was placed in the field. Light stimulation was removed for 3–5 min to allow the fiber to equilibrate and then turned on at a maximal intensity of 40 mW for at least 10 s (but no longer than 60 s) to determine whether each ExPAN responded to light activation of the epithelium. For light responsive fibers, a combination of three antagonists was then added to the bath: pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS; Sigma-Aldrich); P2X antagonist (and $P2Y_1$ antagonist at higher concentrations than used here); 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5' triphosphate tetrasodium salt (TNP-ATP; Sigma-Aldrich); $P2X_1$, $P2X_3$, $P2X_{2/3}$ antagonist; and AR-C118925XX (selective $P2Y_2$ antagonist; Tocris Bioscience) at final concentrations of 100, 3, and 10 mM, respectively. After 10 min of drug

incubation, the fiber was stimulated with blue light for 10 s. The drug cocktail was then washed out for 10 min or until 600 mL of fresh ACSF had flowed through the recording chamber (~20 times the chamber volume). The field was again stimulated with blue light and response recorded. Response to drug mixture was defined as ≥25% reduction in average firing rate with drug application and recovery of firing rate by ≥25% (Brierley et al., 2005; Hicks et al., 2002).

2.2.6.2 Stretch-responsive ExPANs

Effects of the ATP antagonist mixture on stretch activation of TRPV1-ChR2 colon ExPANs used the same pharmacological protocol but without laser stimulation. Stretch-sensitive fibers were identified and baseline circumferential stretch obtained followed by 10 min of exposure to the mixture of ATP receptor antagonists. Drugs were washed out and circumferential stretch repeated and the response recorded. For the ATP experiments, five Vil-ChR2 mice (3 males, 2 females) and three TRPV1-ChR2 mice (2 males, 1 females) were used.

2.2.7 Data recording and analysis

Action potentials generated by PN afferents were recorded using a low-noise AC differential amplifier (DAM80; World Precision Instruments) as described previously (Feng et al., 2010; Feng & Gebhart, 2011). Electrical signals were filtered (0.01–10 kHz), amplified (×10,000), digitized at 20 kHz using a 1401 interface [Cambridge Electronic Design (CED)], monitored online by an audio monitor (Grass AM10; Astro-med) and stored on a personal computer. Action potentials were analyzed using Spike2 software (CED). Action potentials were discriminated as single units based on principle component analysis of individual spike waveform. To avoid errors in discrimination, no more than two discernable active units in any record were studied. The fiber

stretch threshold (T_{force}) was defined as the force that evoked the first action potential during a stretch stimulus (Feng et al., 2010; Feng and Gebhart, 2011). Latency (T_{time}) was the time when the first action potential was fired. Analysis of firing rate (hertz) and instantaneous frequency (IF; average and peak) were done over the first 10 s from the first action potential. To ensure that light responses in sensory fibers were not due to laser generated heating of the tissue, similarly timed light exposures were done using preparations from littermate control mice. Light stimulation in controls did not produce action potential firing.

2.2.8 Culture of primary colon epithelial cells

Colon epithelial cells were acutely dissociated and cultured from the isolated distal colon. The longitudinal muscle layer was teased apart, stripped off, and the remaining tissue was digested with collagenase P (Sigma-Aldrich; 0.09 mg/ml dissolved in Eagle's Essential Medium containing 1% glutamine and 1% bovine serum albumin) for 20 min at 37°C, followed by 0.25% trypsin-ethylenediamine acid solution for 10 min at 37°C. Tissue was then resuspended in Basal Medium Eagle containing 1% glutamine and 2% penicillin/streptomycin and mechanically dissociated with fire-polished Pasteur pipettes. Dissociated cells were plated on 15 mm glass coverslips and maintained at 37°C in 5% CO_2. Patch-clamp recordings were performed after 2 h of incubation.

2.2.9 Whole-cell patch-clamp electrophysiology

Patch-clamp recordings were obtained from ChR2-expressing colon epithelial cells exposed to blue light. Coverslips with plated epithelial cells were transferred to a recording chamber bath maintained at 22°C in a solution containing the following (in mM): 117.9 NaCl, 4.7

KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 11.1 D-glucose, 2 sodium butyrate, and 20 sodium acetate. Patch pipettes (2–3 MΩ) were fabricated from Corning 8161 glass tubing and filled with (in mM) 145.6 CsCl, 0.6 $CaCl_2$, 2.0 EGTA, 15.4 glucose, and 5.0 Na-HEPES, pH 7.3. Currents were acquired from epithelial cells in whole-cell configuration using an Axopatch 200B patch-clamp amplifier (Molecular Devices) and digitized using an ITC-16 interface and Pulse 8.6.3 software (InstruTECH/HEKA). Cells were visualized using an Olympus BX50 microscope with differential interference contrast optics. The currents were clamped at 70 mVand blue light was delivered from a mercury arc lamp and sustained for 5–7 s using a 40× water-immersion objective.

2.2.10 Electromyographic recording of VMR to colorectal distension and light stimulation

Visceral sensitivity to colorectal distension (CRD) and light stimuli was measured using protocols detailed previously (Christianson, Bielefeldt, Malin, & Davis, 2010; DeBerry, Saloman, Dragoo, Albers, & Davis, 2015; Sadler et al., 2017). Mice were anesthetized in an induction chamber and then moved to a nose cone administering 2% isoflurane (vaporized with 95% O_2/5% CO_2). An incision was made in the skin of the lower abdomen and two silver wires were implanted in the external oblique abdominal muscle and attached to a differential amplifier (A-M Systems). A grounding electrode was adhered to the tail using Signagel (Parker Laboratories). A custom-made laser-balloon device was inserted through the anus. This device contains polyethylene tubing (diameter, 0.8 cm), enclosing an optical fiber (400 µm core; Thor Labs) connected to a 473 nm laser power source (Laserglow Technologies) with an inflatable 1 cm plastic balloon on one end. After electrode implantation and balloon insertion, the level of isoflurane was lowered to 1.5% and then lowered by 0.125% every 10 min, down to 0.8%. Toe-pinch reflex was tested as isoflurane was slowly lowered until mice were responsive to toe pinch but were not ambulating. Once a

steady level of anesthesia was reached, CRD testing began. CRD was produced by inflating the balloon with air from a compressed nitrogen tank equipped with a pressure regulator and a separate pressure monitor used to regulate the pressure inside of the balloon. EMG signals (indicative of VMR) were amplified, filtered, recorded using Spike2 software (CED), and saved to a PC. Initial 60 mmHg distensions were delivered every 4 min until mice displayed consistent responses to CRD. An additional three distensions were then applied, at 10 s each, with a 4 min rest period in between. The laser stimulation was then applied three times, at 20 s each, with a 4 min break between stimuli. Colorectal responses were quantified by measuring the area under the curve (AUC) for the entire distension or laser period and then subtracting the baseline AUC before application of the stimulus. These measures were then normalized to baseline.

2.2.11 Experimental design and statistical analysis

For single-fiber experiments, the mean values measured in firing properties between two groups (e.g., TRPV1-ChR2 vs. Vil-ChR2, homozygous vs heterozygous mice, or non-stretch-sensitive fibers and stretch-sensitive fibers) were compared using unpaired two-tailed t tests with $p < 0.05$. Analysis of distension and laser VMR data across control, TRPV1-ChR2, and Vil-ChR2 groups was performed using nonparametric Kruskal–Wallis and Mann–Whitney tests. The number of animals used for each analysis and statistical values are reported in Results. All data were analyzed using Prism software (GraphPad).

2.3 Results

2.3.1 Expression of ChR2-EYFP in colon ExPANs and colon epithelial cells

We examined expression of the ChR2-EYFP fusion protein in DRG sensory neurons and distal colon epithelial cells using TRPV1-ChR2 and villin-ChR2 mice, respectively. Many ChR2-YFP positive neurons were present in DRG of TRPV1-ChR2 mice (**Figure 4A**), including those with ExPANs that project to the colon (**Figure 4B**). Previous analysis of the TRPV1-Cre mouse line showed that transgene expression occurs in the majority of C-fibers and a subset of A**δ** ExPANs (Cavanaugh, Chesler, Jackson, et al., 2011). This distribution is broader than that seen for TRPV1 in wildtype adult DRG because many neurons express TRPV1 during embryonic development (which would activate the Cre recombinase) but downregulate expression postnatally (Cavanaugh, Chesler, Jackson, et al., 2011).

In colon tissue of TRPV1-ChR2 mice, ChR2-EYFP-positive fibers were observed in muscular and mucosal layers of the organ (**Figure 4B**). TRPV1 expression has been reported in a subset of vagal afferents (Bielefeldt, Zhong, Koerber, & Davis, 2006; Patterson, Zheng, Ward, & Berthoud, 2003; Taylor-Clark et al., 2008) and thus, some of these endings could be vagal (Berthoud, Carlson, & Powley, 1991; Berthoud, Jedrzejewska, & Powley, 1990; F. B. Wang & Powley, 2000). In addition, there are reports of occasional TRPV1-expressing neurons in the myenteric plexus (Buckinx et al., 2013; Matsumoto et al., 2012) but in general, TRPV1-expressing neurons within the enteric nervous system are rare (Sharrad, Hibberd, Kyloh, Brookes, & Spencer, 2015).

Cre-recombinase expression, driven by the promoter of the actin binding protein villin, targeted ChR2 expression to epithelial lining cells of the colon (**Figure 4C,D**). YFP labeling

indicated expression occurred in all lining epithelial cells but not in other cell types within the colon (e.g., immune, vascular, mesenchymal), cells within the enteric nervous system, or DRG afferents (data not shown). To ensure epithelial cells were responsive to blue light, patch-clamp analysis of YFP-positive cells isolated from enzymatically dissociated colon was carried out. YFP-labeled cells, but not unlabeled cells, showed membrane depolarization in response to blue light, indicating light-activation of the membrane localized ChR2 ion channel (**Figure 4E–G**).

As colon tissue from homozygous mice showed higher levels of ChR2 mRNA relative to heterozygotes (determined using reverse transcriptase real time PCR) and this increase correlated with stronger physiologic responses (data below), the majority of experiments were conducted on mice homozygous for the ChR2 transgene. The number of Cre alleles did not affect the response to light-activation of ExPANs or epithelium.

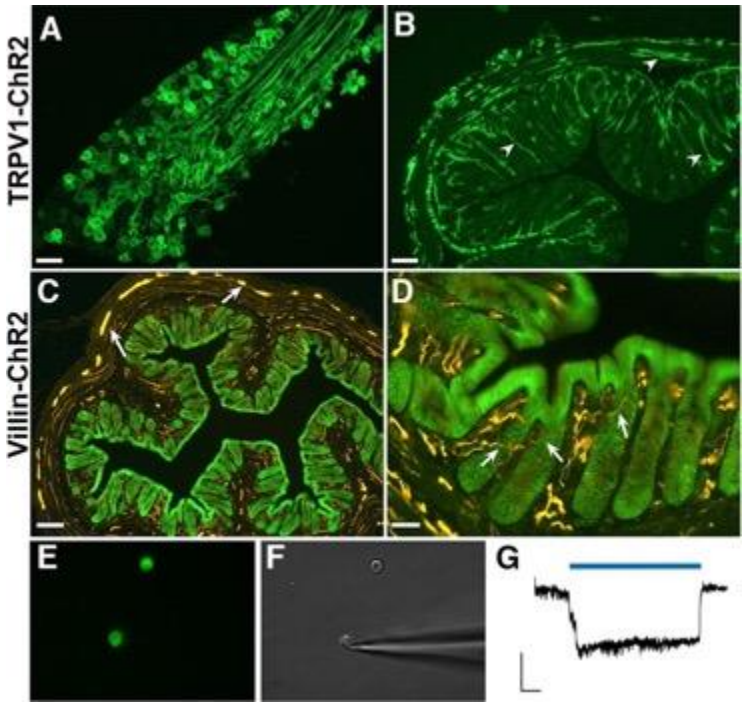


Figure 4. Cell-specific expression of Cre recombinase targets ChR2 to sensory neurons and colon epithelium

(A) ChR2-YFP (green)-positive neurons and fibers are present in the L6 DRG of TRPV1-ChR2 mice. **(B)** Low -power image shows distribution of ChR2-YFP in the distal colon of TRPV1-ChR2 mice. Arrows indicate nerve bundle in muscular layers and nerve fibers penetrating submucosal layers of the colon. **(C)** ChR2-YFP (green) expression in villin-ChR2 mice is confined to epithelial cells of the colon mucosal layer. Anti-PGP9.5 (yellow) labels nerve fibers that extend into mucosal regions and regions of nerve ganglia in the muscular layer (arrows). **(D)** High-power image illustrates close association between PGP9.5-positive nerve fibers (arrows) and ChR2-YFP labeled epithelial cells. **(E, F)** Patch-clamp analysis of dissociated YFP colon epithelial cells show production of inward currents **(G)** in response to 488 nm blue-light stimulation. YFP+ cell diameter diameters were ~5 µm. Calibration bars: **A, D, F**, 50 µm; B, E, 200 µm; **C**, 20 µm; **G**, 50 pA, 1 s.

2.3.2 ChR2-YFP expression does not change intrinsic firing properties of colon ExPANs

A total of 207 colon ExPANs (light-responsive and non-light-responsive) were recorded from TRPV1-ChR2 (69 fibers) and villin-ChR2 (138 fibers) mouse lines. There were no obvious

effects of transgene expression on normal sensory responses (i.e., to brushing, probing, or stretching). For stretch, single colon ExPAN fibers in both TRPV1-ChR2 mice and villin-ChR2 mice exhibited no significant difference in the IF_{avg} in response to circumferential distension [firing (spike) frequency of TRPV1-ChR2 colon ExPANs, 4.74 ± 0.83 Hz (n = 28) vs. villin-ChR2 ExPANs, 5.91 ± 0.66 Hz (n = 50); p = 0.28, t = 1.09].

2.3.3 Activation of ChR2 in colon ExPANs phenocopies responses to natural stimuli

Electrophysiological recordings of teased fibers from the PN of TRPV1-ChR2 mice were used to compare colon ExPAN responses to blue light with their response to applied natural stimuli (blunt probing, stroking, stretch). The dissection and *ex vivo* preparation used for this analysis are described in Materials and Methods and **Figure 5**, A and B. Examples of single-fiber recordings for each ExPAN type are shown in **Figure 5C**. From 21 TRPV1-ChR2 mice (9 males, 12 females), recordings from 69 sensory fibers were obtained. Of these, 60 were blue light-sensitive, or 86%, which is virtually identical to the number of capsaicin responsive colon ExPANs reported from intracellular recording from L6 colon ExPANs (S. A. Malin et al., 2009). Among the light-responsive fibers, 33 were stretch-sensitive whereas 27 were not (**Figure 6A**).

We also noted that the IF_{avg} in response to continuous blue light illumination (at least 10 s) was significantly higher in fibers from homozygous (n = 12) animals compared with their heterozygous (n = 38) littermates (homozygous 5.49 ± 1.31 Hz vs. heterozygous 3.3 ± 0.45 Hz; p = 0.05, t = 2.02), which suggests that increased expression and membrane insertion of the ChR2 protein leads to enhanced ExPAN firing.

ExPAN responses to blue light in TRPV1-ChR2 preparations produced variable firing patterns that were not unique to fiber type. Patterns ranged from firing in single or multiple bursts

to continuous tonic firing (**Figure 5C**). The average firing rate and latency to fire in response to light onset was similar for stretch and non-stretch-sensitive fibers [(IF_{avg} = 3.8 ± 0.57 Hz (stretch-sensitive, n = 28) vs. 3.86 ± 0.82 Hz (non-stretch-sensitive, n = 22); p = 0.95, t = 0.06; latency to first spike = 0.4 ± 0.17 s (stretch-sensitive, n = 27) vs. 0.28 ± 0.15 s (non-stretch-sensitive, n = 20) p = 0.62, t = 0.5)].

The ability of TRPV1-ChR2 fibers to follow pulsed blue light was also assessed at 1, 5, 10, and 20 Hz in 24 fibers. All TRPV1-ChR2 fibers that did respond to light were able to fire action potentials at 1 Hz, but the number of action potentials decreased with increasing frequency. At 1 Hz, 86.4% of the 50 ms light pulses induced action potentials in all ExPANs. With increasing frequency this percentage decreased to 20.7% at 20 Hz (Table 1). This pattern was observed across all fiber types.

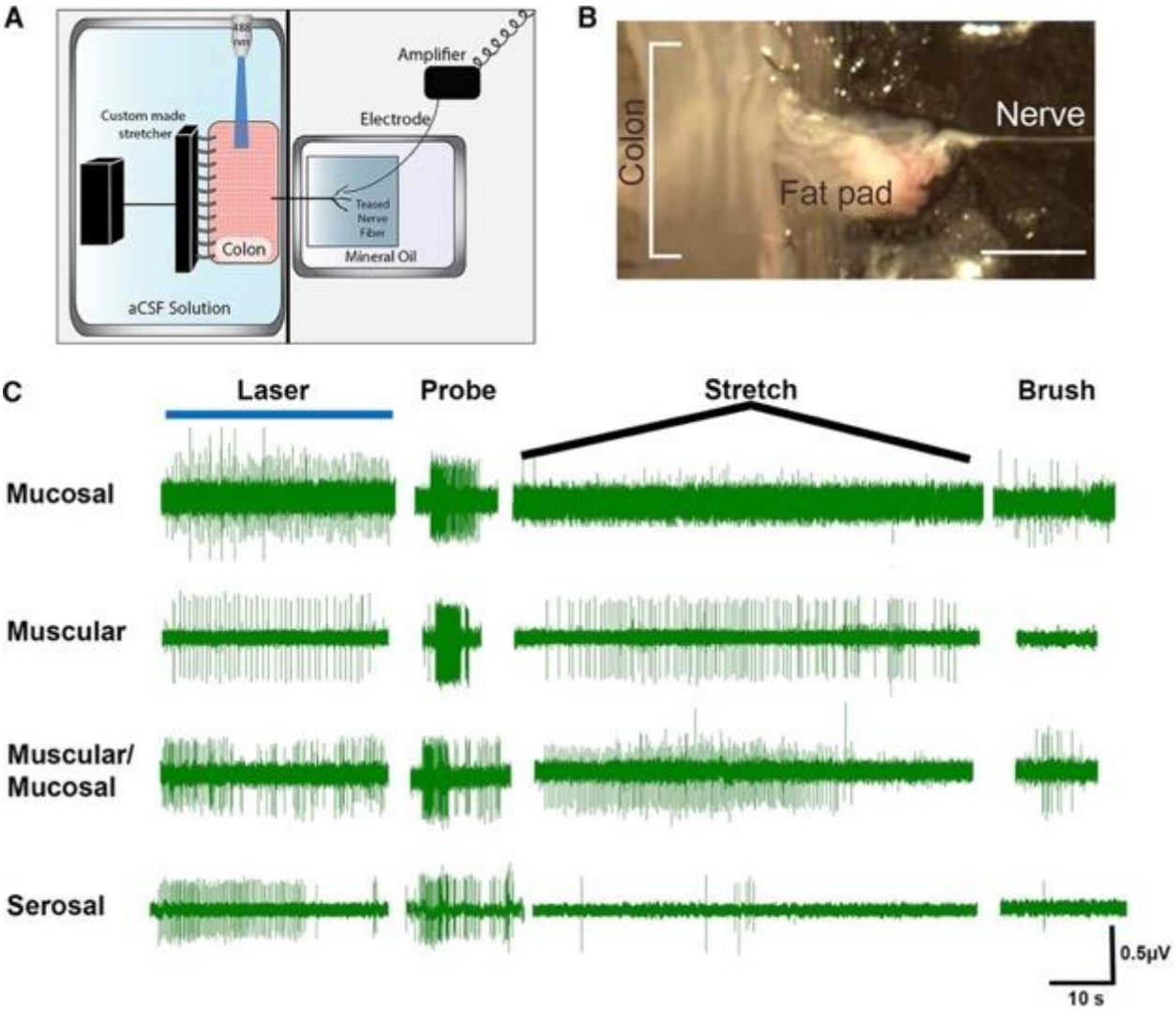
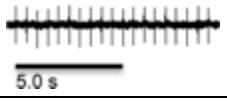
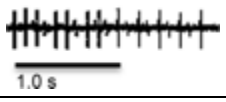
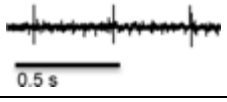
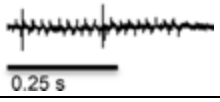


Figure 5. Examples of teased fiber recordings from TRPV1-ChR2 mice show activation of all fiber types in response to blue light.

(A) Diagram illustrates the components of the *ex vivo* preparation used to obtain single-fiber recordings of colon ExPANs. **(B)** Image shows colon tissue with attached fat pad and pelvic nerve from which recordings were made. **(C)** All classes of mechanically sensitive colon ExPANs (mucosal, muscular, muscular/mucosal, and serosal) exhibited robust action potential firing in response to blue laser light. Scale bar, 4 mm.

Table 2. TRPV1-ChR2 fibers responses to 1, 5, 10, and 20 Hz light stimulation

	1 Hz (n = 24)	5 Hz (n = 23)	10 Hz (n = 9)	20 Hz (n = 12)
Light pulses that generate an action potential, % (avg)	86.4 % 	50.3 % 	24.4 % 	20.7 % 

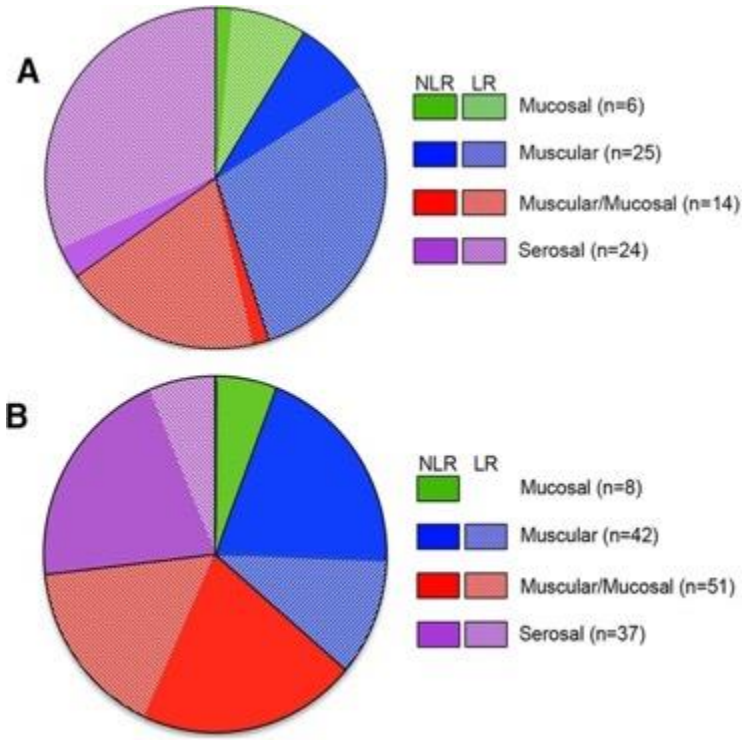


Figure 6. Distribution of blue light-responsive (LR) and non-light-responsive (NLR) fiber types in TRPV1-ChR2 and villin-ChR2 mice

(A) Of 69 sensory fibers recorded from TRPV1-ChR2 mice (N = 20 mice), 86% were responsive to blue light. Of the 69 fibers, 39 were stretch-sensitive (muscular or muscular/mucosal) and 30 were not stretch-sensitive (mucosal and serosal). **(B)** For villin-ChR2 mice (N = 27 mice) a total of 138 fibers were characterized and of these, 50 (36%) were responsive to blue-light activation of colon epithelium.

2.3.4 Optogenetic activation of ChR2 in colon epithelial cells generates action potentials in colon ExPANs

Using the *ex vivo* colon preparation we then determined the effect of light activation of ChR2 in epithelial cells on colon ExPAN firing. Teased fiber analysis of the pelvic nerve showed that blue light generated robust action potential firing in DRG sensory fibers, similar to responses elicited by stretch stimulation (**Figure 7**). Responses of teased fibers to mechanical or blue-light stimulation were recorded in preparations from 27 villin-ChR2 mice (14 males, 13 females; n = 138 fibers). Comparison of response properties of light-sensitive and light-insensitive fibers showed no difference in the IF_{avg} for circumferential stretch in stretch-sensitive ExPANs [light-sensitive (n = 8; mean stretch $IF_{avg} = 4.52 \pm 1.04$) vs. light-insensitive ExPANs (n = 42; mean stretch $IF_{avg} = 6.18 \pm 0.75$, $p = 0.36$, $t = 0.92$)]. IF_{avg} values for blunt probing were also similar between light-sensitive and light-insensitive serosal ExPANs (those ExPANs that did not respond to stretch but did respond to probing: light-sensitive, n = 9, mean probe $IF_{avg} = 21.76 \pm 4.11$ vs. light-insensitive, n = 13, mean probe IF_{avg} 21.6 ± 3.52; $p = 0.98$, $t = 0.03$).

The increased level of ChR2 mRNA in homozygous villin-ChR2 mice, as indicated by RT-PCR measures, translated to increased fluorescent intensity of ChR2-EYFP expression in the colon. Colon preparations from homozygous villin-ChR2 mice also had a greater percentage of action potentials generated in response to epithelial activation; 50.5% of fibers recorded from villin-ChR2 homozygous mice (46/91) responded to light compared with 8.5% (4/47) in heterozygous mice.

Of the 50 fibers that responded to light stimulation of colon epithelium, 39 were stretch-sensitive (i.e., muscular and muscular/mucosal subtypes; **Figure 6B**). Evoked responses had a wide range of latencies (0.03 – 60.3 s, mean 15.5 ± 2.09 s; **Figure 8B**), and the majority of

responses exhibited robust firing that adapted over time. Comparison of stretch-sensitive and stretch-insensitive (serosal) ExPANs in which firing latency to light could be accurately measured, showed that action potentials elicited in stretch-sensitive fibers (n = 27) had shorter latencies (latency = 12.2 ± 1.98 vs. 24.03 ± 6.14, p = 0.02, t = 2.43) and higher IF_{avg} values (5.37 ± 0.56 vs. 3.13 ± 0.97, p = 0.058, t = 1.96) compared with non-stretch-sensitive fibers (n = 8).

Interestingly, of the four types of extrinsic colon ExPANs, light activation of the epithelium produced robust action potential firing in all but mucosal ExPANs (**Figure 6B**). This was surprising given that this type of fiber, identified by its ability to respond exclusively to light brushing of the epithelium, is thought to terminate in or nearby the colonic epithelium and therefore should be in an ideal location to respond to activation of ChR2 in the epithelium. It should be noted that this anatomical relationship is only inferred based on stimulus response properties; there are no reports in which anatomical features have been determined for physiologically characterized ExPANs. Thus, mucosal fibers may communicate with the epithelium in a manner that is unique from other types of ExPANs (and one not engaged by ChR2 activation) and/or their ability to respond to brushing of the epithelium is via mechanical sensory transducers intrinsic to this population of ExPANs in a manner that does not rely on communication with the epithelium.

ExPAN firing rates in response to light stimulation of epithelial cells was similar to rates recorded from TRPV1-ChR2 ExPANs [mean villin: IF_{avg} = 4.86 ± 0.5 Hz (n = 35) vs. TRPV1: 3.83 ± 0.48 Hz (n = 50), respectively; p = 0.15, t = 1.46]. However, there was a significantly longer latency for the first action potential with light-activation of villin-ChR2 epithelial cells than that measured for light activation of TRPV1-ChR2 ExPANs [latency: villin 14.9 ± 2.2 s (n = 35); TRPV1 0.35 ± 0.12 s (n = 47); p < 0.0001, t = 7.67; compare **Figures 5C and 7**]. In addition, the latency of ExPAN firing in villin-ChR2 mice increased with repeated light stimulation.

Interestingly, when comparing afferent firing induced by light activation of epithelial cells to that induced by natural stimuli (stretch) in the same fiber, the latencies to first action potential were not significantly different [stretch-sensitive fibers (n = 8), latency in response to light = 4.73 ± 1.07 s; latency in response to stretch = 4.52 ± 1.04 s, p = 0.89, t = 0.14]. This suggests that the mechanism(s) underlying epithelial activation of colon ExPANs shares temporal characteristics with those for stretch induced activation. Thus, epithelial activation could be a contributor to stretch-induced ExPAN activation.

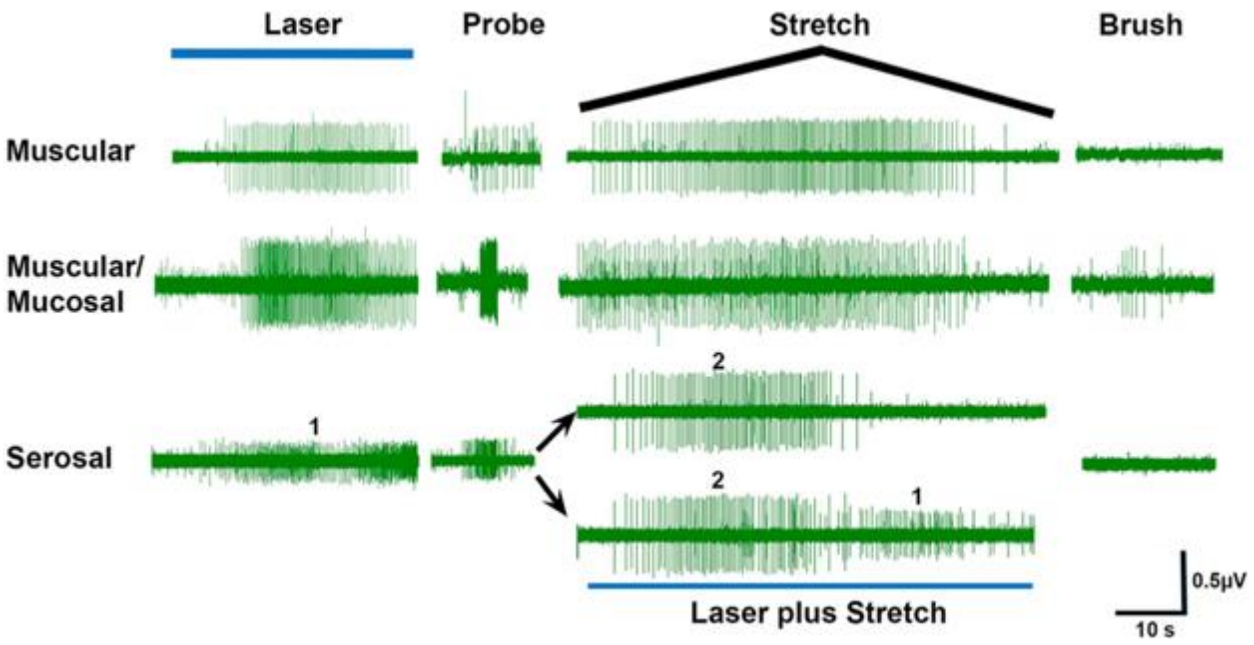


Figure 7. Examples of teased fiber recordings from villin-ChR2 mice show activation of all fiber types in response to blue light

With the exception of mucosal ExPANs, all classes of mechanically sensitive colon ExPANs are blue light-sensitive. In the example provided for serosal ExPANs, two fibers were recorded together. One fiber was classified as serosal and light responsive, due to its responses to probe and laser stimulation (1). A second fiber was sensitive to stretch stimulation but not light (2). The two different fibers are apparent in the bottom trace, where stretch and laser are applied at the same time.

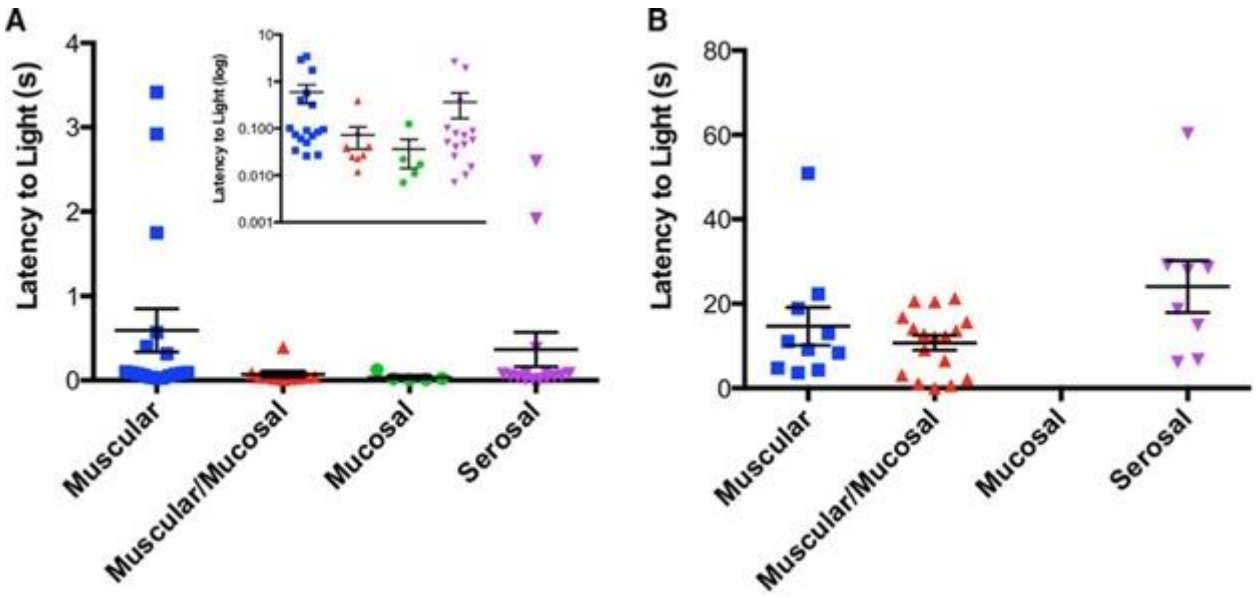


Figure 8. ExPAN of TRPV1-ChR2 and villin-ChR2 mice have different latencies to blue-light stimulation.

(A) Nearly all mechanically sensitive ExPANs from TRPV1-ChR2 mice had firing latencies (time between start of light stimulus and ExPAN firing) <1 s (avg = 0.35 s). Inset, Log plot of values on an expanded scale. **(B)** Mechanically sensitive fibers from villin-ChR2 mice exhibit much longer latencies (avg = 14.9 s) in response to light activation of the epithelium. Mucosal fibers that respond to light activation were rare compared with all other fiber types.

2.3.5 Antagonism of ATP signaling inhibits stretch- and light-induced ExPAN firing

The increased latency of firing in generation of action potentials by light-mediated activation of epithelial cells suggests that communication to nerve fibers occurs through release of a chemical mediator(s). To explore this possibility, we bath applied an ATP antagonist drug mixture (PPADS, TNP-ATP, and AR-C118925XX) to the preparation and assessed firing properties. In response to a ramp stretch stimulus, 4 of 6 (66.7%) TRPV1-ChR2 stretch-sensitive fibers had reduced responses in the presence of drug mixture (**Figure 9A**). No fibers showed complete inhibition of firing with drug application. The ability of the ATP antagonist mixture to

block the response to light stimulation of the epithelium was also tested in 11 fibers of villin-ChR2 mice. Of these, 8 (72.7%) responded to drug application, which is comparable to the inhibitory effects on the TRPV1-ChR2 stretch-sensitive fiber population (**Figure 9B**). All fibers tested were stretch-sensitive. Of the eight drug-sensitive fibers, 37.5% (3/8) had a complete block in firing, whereas 62.5% (5/8) had a partial block to drug application.

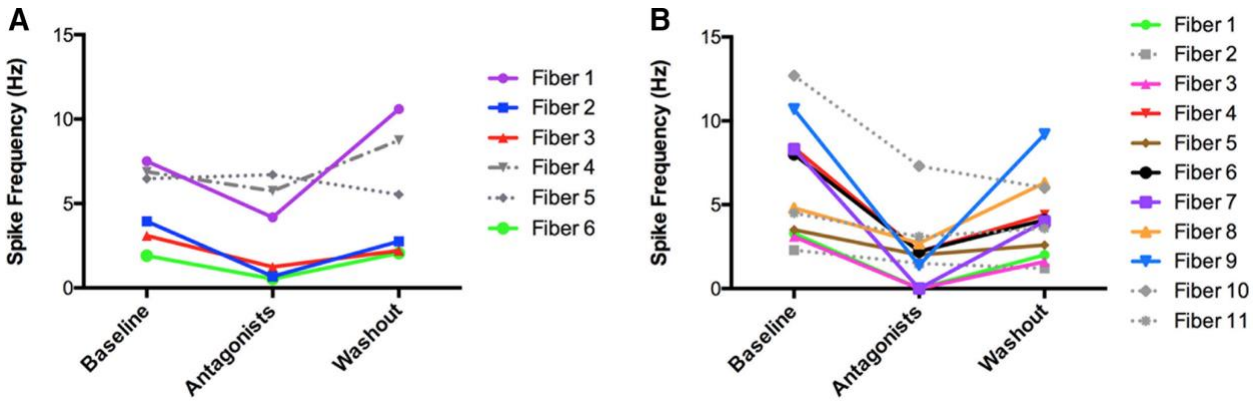


Figure 9. Antagonism of ATP signaling causes reduction in firing to stretch and light stimuli

A drug mixture containing PPADS, TNP-ATP, and AR-C118925XX was used to block responses to stretch **(A)** or laser **(B)** stimuli. Response to the drug mixture was defined as a ≥25% reduction in average firing rate upon drug application and a recovery of firing rate by ≥25% upon washout. Gray dotted lines in each graph represent nonresponders. **(A)** Fiber responses to stretch were recorded at baseline, in the presence of the antagonist mixture, and after a 10 min washout. Four of six fibers tested (66.7%) responded to the antagonist mixture and showed reduction in spike frequency in response to stretch. **(B)** In villin-ChR2 mice, fibers that were identified as stretch-sensitive were also evaluated. Fiber responses to laser stimulation of colon epithelium were recorded at baseline, in the presence of the antagonist mixture, and after a 10 min washout. Application of the ATP antagonist mixture caused a reduction in firing frequency to light in 8 of the 11 fibers tested (72.7%).

2.3.6 Light activation of colon epithelium elicits behavioral responses

To determine whether activation of ChR2 in colon epithelial cells engages neural signaling pathways *in vivo*, we assessed behavioral responses to colon distention and light activation in anesthetized animals. Measures of the VMR were obtained as a surrogate for sensory-induced activation of the colon neural circuitry. Colon distension, via computer-controlled inflation of a balloon in the distal colon, was combined with laser illumination of the colon via a fiber optic contained within the balloon. In littermate control mice (4 males, 2 females), balloon distension to 60 mmHg induced a strong VMR response (**Figure 10A**) but no change in VMR in response to light illumination of the colon (**Figure 10B**). As a positive control for ChR2 activation of sensory neurons, TRPV1-ChR2 mice were tested (1 male, 3 females). As expected, TRPV1-ChR2 mice exhibited a strong VMR in response to light activation similar to that evoked with balloon distention of the colon (**Figure 10C**). For Vil-ChR2 mice (4 males, 1 female), light activation of ChR2 in the epithelium also produced robust and reproducible VMRs (Figure 10D). Comparison of the response rate to light stimuli shows that relative to control littermates (n = 6 mice), a significant increase occurs in the percentage of responses for both TRPV1-ChR2 ($p = 0.005$, n = 4) and Vil-ChR2 ($p = 0.038$, n = 5) groups. There was no difference in the percentage of response between TRPV1-ChR2 and Vil-ChR2 groups (**Figure 10E**). The average VMR values for the TRPV1-ChR2 and Vil-ChR2 groups also were significantly greater relative to the control group (TRPV1-ChR2, $p = 0.006$; Vil-ChR2, $p = 0.043$; Kruskal–Wallis test), but there was no difference between the two ChR2-expressing mouse lines (**Figure 10F**).

Finally, though the response rates were not significantly different between the TRPV1 and Vil-ChR2 groups, the latency to respond was significantly faster in TRPV1-ChR2 mice [latency

Vil-ChR2, 7.9 ± 1.9 s (n = 6 mice) vs. TRPV1-ChR2, 0.31 ± 0.2 s (n = 4 mice); p = 0.010, t = 3.957; unpaired t test with Welch's correction]. This difference well parallels the increased latency of firing measured using the teased fiber preparation and likely reflects the more direct activation of ChR2 in ExPAN terminals versus the epithelium.

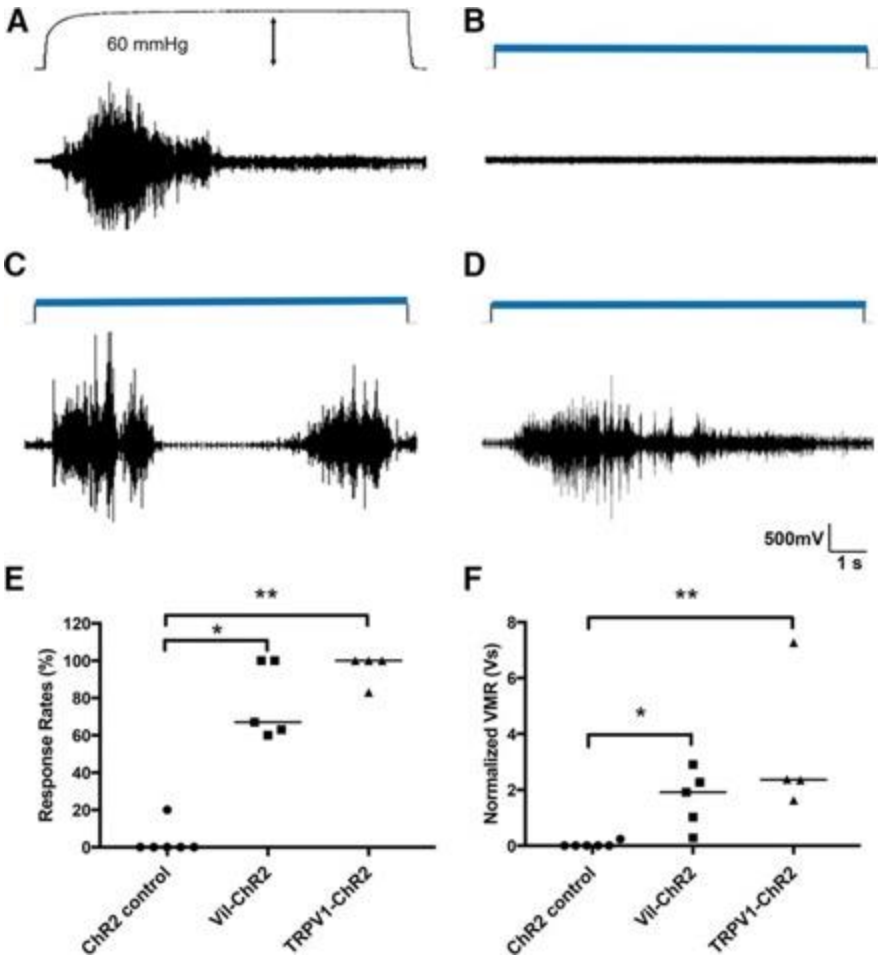


Figure 10. ***In vivo*** **light-mediated activation of colon epithelium generates visceromotor responses**

(A) Visceromotor responses (VMRs) to noxious colon distention (60 mmHg) were elicited in all mouse strains analyzed: Vil-ChR2, TRPV1-ChR2 and littermate controls. Example shown is a recording from a control mouse. **(B)** In control mice, blue-light stimulation produces no VMR in response. **(C)** In TRPV1-ChR2 mice, blue-light activation of ChR2 expressed in colon ExPANs elicits VMRs comparable to those measured using 60 mmHg of balloon pressure. **(D)** Vil-ChR2 mice exhibit robust VMRs in response to blue-light stimulation of ChR2 expressed in epithelial cells. **(E)** Plot shows percentage of VMR responses per animal obtained in response to light stimulation of the colon (n = 4 TRPV1 mice, n = 5 Vil-ChR2 mice, and 6 littermate controls). There was no difference in response rate between TRPV1- and Vil-ChR2 groups. **(F)** Plot shows average VMR values obtained from TRPV1-ChR2 (n = 4), Vil-ChR2 (n = 7), and control (n = 6) groups. Values were obtained by normalizing to baseline measures from individual animals. Asterisks indicate significant increases relative to control group for TRPV1- and Vil-ChR2 groups. There was no difference in VMRs between TRPV1- and Vil-ChR2 groups.

2.4 Discussion

Results from this study show that ChR2-mediated activation of colon epithelium can directly engage neural circuitry involved in regulating neural pathways critical for colon function. Results of teased fiber analysis of an intact colon preparation and *in vivo* measures of VMRs in response to light stimuli indicate that selective activation of epithelial cells generates action potentials in ExPANs that have functional consequences. Epithelial–nerve communication was evident across all functional subtypes of colon ExPANs with the exception of mucosal ExPANs. The absence of this fiber type may be due to the small number of mucosal sensory neurons sampled by our study (6 of 138 total fibers). The response parameters of sensory fibers activated by light/epithelial stimulation were often similar to those elicited by mechanical stimulation (designed to mimic natural stimuli). Importantly, the neuronal response to light stimulation of epithelial cells could be blocked or diminished in the majority of responsive sensory fibers with the application of a mixture of ATP receptor antagonists. These results indicate that the intestinal epithelium alone, e.g., without additional mechanical stimuli, can directly activate colon ExPANs and that ATP is likely to have a critical role in this epithelial cell–nerve communication.

Light activation of epithelial cells was sufficient to cause firing in ExPANs functionally classified as muscular, muscular/mucosal, and serosal. Because fiber classifications were made based on responses to stroking, probing, and stretch, as is standard in the field, the exact anatomical location of these endings is not known. For example, anatomical tracing studies (X. Song et al., 2009; Spencer et al., 2014) find no evidence of fibers that actually end in the serosa, making the terminology misleading. This makes it difficult to accurately define a mechanistic link between epithelial activation and firing in ExPAN types, e.g., muscular and serosal. With this limitation in mind, one mechanism of activation could involve an intermediary cell; for example, a neuron in

the submucosal and/or myenteric plexus that has projections near the epithelium. In this scenario, the location of a responsive ExPAN relative to the epithelium would not matter, provided that it connects with another neuron(s) that is near the mucosa. A synaptic delay might, in part, explain the longer latency to firing that follows laser exposure when ChR2 is expressed in the epithelium compared with when it is expressed within the sensory fiber itself. There is also the question as to whether a response to brushing really means that the fiber is terminating at the mucosa. It is entirely possible that this response simply represents a colon ExPANs with a very low mechanical threshold whose termination could be in more distant layers.

The epithelium has long been implicated as a modulator of neural activity. A recent example comes from Bellono et al. in which application of norepinephrine to the mucosa was shown to induce action potential firing in mucosal ExPANs that could be blocked by 5-HT_3 receptor antagonism. Bellono et al. also showed that a subset of colon epithelial cells, enterochromaffin cells, which are electrically excitable, express voltage-gated ion channels and release 5-HT in response to depolarization (Bellono et al., 2017).

Of particular relevance for the present work is the purinergic mechanism hypothesis, in which Burnstock proposed that intestinal epithelial cells release endogenous ATP in response to stretch. This hypothesis proposed that released ATP activates $P2X_3$ or $P2X_{2/3}$ receptors localized on ExPAN terminals, resulting in increased firing (Burnstock, 1999, 2001, 2013). Using an *ex vivo* preparation of rat colorectum, Wynn et al. provided support for this hypothesis by showing that serosal application of ATP could activate mechanosensitive ExPANs, increasing their peak responses (Wynn et al., 2003). However, missing from these previous studies is direct evidence that activation of the colonic epithelium can produce action potentials *de novo* in extrinsic primary sensory neurons, e.g., without simultaneous mechanical stimuli. Until the advent of optogenetic

tools these data have been difficult to obtain since it was not possible to stimulate colonic epithelium without also engaging putative transduction mechanisms resident in sensory fibers themselves, i.e., because of the intimate anatomical relationship of the colonic epithelium and the nerves innervating these cells, mechanical, chemical, or thermal stimulation could not be applied without simultaneously affecting both compartments. The selective expression of opsin genes by tissue-specific gene promoters as used here allows specific activation of epithelial cells. As seen in cutaneous sensory neurons (Baumbauer et al., 2015), light activation of ChR2 expressed in colon ExPANs (i.e., in TRPV1-ChR2 mice) did not completely mimic the response to naturalistic colon stimuli (i.e., stretch or probing).

As in cutaneous afferents, light activation of ChR2 in colon ExPANs produced almost instantaneous action potentials and continuous exposure to 473 nm light produced repetitive firing with relatively stable instantaneous frequency (although desensitization was seen at longer exposure times). This pattern of action potential firing differs from the patterns observed when action potentials were induced by light activation of the colon epithelium; for epithelium, neuronal firing patterns were much more complex and often similar to those produced by mechanical stimulation of the colon. These more complex patterns of neuronal activation included variable delays in the onset of firing, which were comparable to delays that occur during the induction of circumferential stretch and included high-frequency bursting and desensitization. The relatively long delay in ExPANs activation following light stimulation of the epithelium may reflect multiple factors. First, the intestinal epithelium is constantly moving upward from the stem-cell zone in the crypts toward the lumen as part of the dynamic process of epithelial cell turnover (J. J. Chen et al., 2001; Heath, 1996). As a result, the anatomic relationship between the epithelial cells and the more static neuronal terminals varies at any given point in time (J. J. Chen et al., 2001) making it likely

that any neuromodulatory substance released by epithelial cells may have significant distances to diffuse before encountering neurotransmitter receptors on sensory endings. Another possibility is that epithelial activation releases activators that stimulate other cells in colon tissue, including immune cells or neurons in the enteric nervous system, that subsequently release neurotransmitters that activate colon ExPANs.

As previous data show that epithelial-released ATP may modulate colon ExPAN activity (Ueda et al., 2009; Wynn et al., 2003), we further investigated epithelial–nerve signaling by combining ATP antagonists with light stimulation of the epithelium. As a positive control, we were able to confirm that ATP is involved in modulation of action potentials in colon ExPANs in response to physiologic stretch by demonstrating that ATP receptor antagonists reduce firing during a ramp stretch. A similar decrease in firing was seen when the same ATP antagonists were combined with light activation of ChR2-expressing epithelial cells, supporting the hypothesis that colonic epithelium can directly initiate action potentials in colon ExPANs via the release of ATP. It should be noted that although some epithelial-induced firing of colon ExPANs was completely blocked by our ATP antagonist mixture, most fibers exhibited only partial reduction in firing. This suggests that additional mechanisms are likely involved in neuronal activation. In addition, our drug mixture of ATP antagonists targeted ATP receptors known to be expressed in colon ExPANs, including $P2X_{2/3}$ and $P2Y_2$ (Brierley et al., 2005; Giaroni et al., 2002; Robinson, McNaughton, Evans, & Hicks, 2004; Shinoda, La, Bielefeldt, & Gebhart, 2010). However, Hockley et al. recently described the expression of additional P2Y receptors including $P2Y_1$, $P2Y_4$, $P2Y_{12}$, and $P2Y_{13}$ on colon ExPANs (although $P2Y_{12}$, and $P2Y_{13}$ are $G_{i/o}$ GPCRs and therefore likely to be inhibitory). Hockley et al. found that $P2Y_1$, an excitatory receptor that binds ADP (a rapid metabolite of ATP) and UTP, was expressed in a majority of dissociated colon sensory neurons (J.

R. Hockley et al., 2016). Our studies used PPADS, but at a concentration that may not completely block $P2Y_1$, and therefore, some of the ExPAN activity not blocked following ChR2 activation of the epithelium could be due to binding of either ADP or UTP to $P2Y_1$ expressed on colon ExPANs. Also to be considered is that colon ExPANs express several additional ligand-mediated excitatory channels including TRPV1 (Brierley et al., 2005; S. Malin et al., 2011; S. A. Malin et al., 2009; Matsumoto et al., 2009; Sugiuar, Bielefeldt, & Gebhart, 2004), TRPA1 (Brierley et al., 2009; Christianson et al., 2010; Engel et al., 2011; S. Malin et al., 2011), TRPM8 (Mueller-Tribbensee et al., 2015), and 5-HT_3 and 5-HT_4 receptors (Coldwell, Phillis, Sutherland, Howarth, & Blackshaw, 2007; Feng, La, Schwartz, & Gebhart, 2012; Gershon, 2013; Hicks et al., 2002; Mawe & Hoffman, 2013).

The present study fills a major gap in the purinergic hypothesis in that it shows using an intact preparation that it is possible to stimulate colonic epithelium without stimulating any surrounding cells, including neurons. This stimulation produces robust firing of colon extrinsic sensory neurons in a manner that is at least partially ATP dependent. However, numerous questions remain. Studies by Bohorquez et al. (Bohorquez, Chandra, Samsa, Vigna, & Liddle, 2011; Bohorquez et al., 2014) have revealed molecular and anatomical specializations typically present at synapses (including dense and clear core vesicles) in unique populations of neuroendocrine cells in the colon and that these specializations are found adjacent to colon ExPANs. Although these cells represent a minority of colonic epithelial cells, they provide a "proof of concept" for the type of structure that could be responsible for the epithelial based activation of colon ExPANs described here. However, it is important to note that other authors provide evidence that stretch-evoked activation of colon ExPANs is Ca^{2+}-independent (Zagorodnyuk, Lynn, Costa, & Brookes, 2005), indicating that vesicular release (which is typically

Ca^{2+}-dependent) is not an essential requirement for mechanotransduction. Thus, further work is necessary to identify the mechanism underlying epithelial–nerve communication and to better understand the role of the colon epithelium in modulation of colon ExPANs activity under normal and pathological conditions.

3.0 Optogenetic Inhibition of Colon Epithelium Reduces Hypersensitivity in a Mouse Model of Inflammatory Bowel Disease

3.1 Introduction

In the gut, extrinsic primary afferent neurons (ExPANs) convey mechanical and chemical stimuli to neurons in the central nervous system (Brierley et al., 2018). In response to inflammatory and other pathophysiological changes in the gut, sensory afferents can become sensitized. Evidence suggests this sensitization significantly contributes to the chronic pain and decreased quality of life that accompany inflammatory bowel disease conditions (IBD; e.g., Crohn's disease (CD) and ulcerative colitis (UC)) (Bielefeldt, Lamb, & Gebhart, 2006; Farrell, Callister, & Keely, 2014; S. A. Malin et al., 2009; Mawe & Hoffman, 2013).

While there is evidence to suggest that the intrinsic changes in ExPANs underlies the increase in excitability observed following inflammation (Azpiroz et al., 2007; Gold & Gebhart, 2010; Gold & Traub, 2004; Gold, Zhang, Wrigley, & Traub, 2002), a growing body of evidence suggestions changes in other cell types may also contribute to this process. Colon epithelial cells are one of these cell types. The epithelium not only maintains the mucosal barrier, but also synthesizes and releases neurotransmitters such as adenosine triphosphate (ATP) and 5-hydroxytrypamine (5-HT/serotonin) (Najjar, Davis, & Albers, 2020). The spatially- and temporally-controlled release of these neurotransmitters is thought to facilitate communication between the epithelial lining and ExPAN terminals (Bertrand, 2009; Latorre et al., 2016), supporting an important role for the epithelium in regulating primary afferent activity and

nociceptive responses (Baumbauer et al., 2015; Bellono et al., 2017; Makadia et al., 2018; Moore et al., 2013; Pang et al., 2015; Sun et al., 2001).

In the context of IBD, most studies of epithelial cells have focused on a failure of the epithelium to maintain a barrier, enabling bacterial infiltration and ExPAN sensitization secondary to the resulting immune responses (Peterson & Artis, 2014; Roda et al., 2010). However, with evidence of a direct role in pain signaling, it is possible that the epithelium contributes to persistent ExPAN sensitization. Studies in animal models as well as in colon tissue from IBD patients suggest that inflammation affects enteroendocrine cell signaling (Lomax, Linden, Mawe, & Sharkey, 2006), thus it is possible that inflammation changes normal communication between the epithelium and ExPANs. This may contribute to the sensitization of ExPANs and consequently the pain and hypersensitivity associated with IBD. To begin to address this possibility we tested whether optogenetic-mediated inhibition of the epithelium alters nociceptive signaling. We examined epithelial-sensory afferent communication under normal and inflamed conditions using a mouse model in which the inhibitory, yellow light activated archaerhodopsin (Arch) protein was targeted to epithelial cells. Colon sensitivity was assessed using the visceromotor response (VMR) to colorectal distension (CRD) measured with and without the light-induced inhibition of epithelial cells. We measured the efficacy of Arch-mediated inhibition of epithelial cells in reducing the VMR to CRD and then compared these results to those obtained in parallel using mice that express Arch in transient receptor potential vanilloid 1 (TRPV1)-lineage ExPANs. Results show that inhibition of the colon epithelium is effective in reducing inflammation-induced hypersensitivity.

3.2 Methods

3.2.1 Animals

Male and female mice aged 6-8 months were analyzed. Two mouse lines were used: Vil-Arch, in which the inhibitory opsin ArchT was targeted to intestinal epithelium, and TRPV1-Arch, in which ArchT was targeted to primary afferent neurons innervating the colon. ArchT, an archaerhodopsin from *Halorubrum* strain TP009, is a yellow light driven outward proton pump that causes cellular hyperpolarization (Han et al., 2011).

Mice with an ArchT-EGFP fusion protein in the *Rosa26* locus (Ai40D mice; RRID: IMSR_JAX:021188) were crossed with villin-Cre mice (RRID: IMSR_JAX:004586) and TRPV1-Cre mice (RRID:IMSR_JAX:017769). Littermates with Cre only or ArchT-EGFP only were used as controls. Animals were housed in an AAALAC-approved facility and handled in accordance with protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2.2 Laser-balloon device construction

To enable balloon distension simultaneous with optogenetic stimulation a 100-cm optical fiber (400 µm core with 1.25 mm ceramic ferrule and 440 µm FC/PC connector; Thor Labs, Newton, NJ) was threaded through a 15-cm piece of PE-200 polyethylene tubing connected to a 3-way stopcock (Makadia et al., 2018). To make the balloon, a 3 cm x 3 cm piece of Saran wrap was secured around the tubing using silk sutures (Ethicon, Somerville, NJ). The balloon length was 1.5 cm and when inflated, its diameter was 0.9 cm. The fiber optic was attached to a 589 nm

laser and power source (Laserglow Technologies, Toronto, ON, Canada). The laser power measured when illuminated through the plastic balloon was 12 mW/mm2.

3.2.3 Electromyographic recording of visceromotor responses

Mice were fasted overnight and anesthetized using an intraperitoneal injection of urethane (Sigma; 1.2 g/kg). Mice were then placed on a heating pad and into a nosecone attached to an isoflurane vaporizer delivering 1% isoflurane (vaporized with 95% O2/5% CO2) (Parkland Scientific, Coral Springs, FL). The lower left quadrant of the abdomen was shaved, and a 1.5-cm incision was made in the skin, revealing abdominal musculature. Two clip electrodes (Pomona Electronics, Pomona, CA) were attached to the abdominal muscle, approximately 3 mm apart, to enable electromyographic (EMG) recording. The laser-balloon device was inserted into the colorectum and secured with tape to the tail. A ground electrode was secured with SignaGel (Parker Laboratories, Fairfield, NJ) to the mouse's tail. Electrodes were attached to a differential amplifier (A-M Systems, Sequim, WA) connected to an A-D converter (Micro 1401, Cambridge Electronic Design (CED), Cambridge, UK). EMG signals were amplified (10,000x), filtered (0.3-10 kHz band pass), and sampled at 20 kHz with the 1401 interface. Signals were recorded using Spike2 software (CED) and saved to a PC. Colorectal distension (CRD) was performed by balloon inflation using a compressed N2 tank equipped with a pressure regulator and connected to an air valve box enabling computer control. Prior to testing isoflurane level was slowly lowered to 0.25%. Mice were held at this level of combined anesthesia (isoflurane with previous urethane injection) until they showed no signs of ambulation but were responsive to toe pinch. After the mice displayed consistent responses to 60 mmHg CRD, the stimulus protocol was administered. This consisted of 3-5 trials of 10 s 60 mmHg CRD, followed by 3-5 trials of 10 s 60 mmHg CRD

plus yellow laser; laser was turned on 1 s before the distension stimulus and remained on throughout the 10 s stimulus. All stimuli were delivered at 4-min intervals. EMG waveforms were rectified in Spike2 and the integral of the waveform was used to quantify VMRs. The resting activity 10 s before distension was subtracted from the distension-evoked activity. EMG waveform extraction and data analysis were performed in a blinded manner.

3.2.4 Dextran sulfate sodium inflammation protocol

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 autoclaved water that was provided *ad libitum* via water bottle for 5 days. Vehicle control mice were provided water without DSS. VMR analysis was performed immediately after 5 days of DSS treatment.

3.2.5 Fluorescent microscopy and histopathological scoring

Distal colon segments and L6 DRGs were isolated and fixed in 4% paraformaldehyde for at least 30 min. Tissues were cryopreserved in 25% sucrose overnight, embedded in optimal cutting temperature (OCT) compound, and sectioned at 14 μm thickness. Sections were coverslipped and EGFP expression imaged using a Leica DM4000B microscope equipped with a Leica DFC7000 T digital camera.

DSS- and vehicle-treated mice were euthanized with isoflurane after VMR experiments. A 1-cm piece of colon was removed and fixed for at least 30 min in 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) and cryoprotected in 25% sucrose in PBS at 4°C for 24 hours. The tissue was embedded in optimal cutting solution (OCT), sectioned on a cryostat at 14 μm, and

mounted on microscope slides. Sections were stained with hematoxylin and eosin (H&E) and brightfield images obtained using a Leica DM4000B microscope with a digital camera attachment.

For disease scoring, a modified method of histopathological analysis was used (Kim, Shajib, Manocha, & Khan, 2012) The extent of damage to the tissue was determined by goblet cell depletion (0 = absent, 2 = severe), inflammatory cell infiltration (0 = normal, 2 = dense), and thickening of the submucosal layer (0 = base of crypt sits on muscularis mucosa, 2 = marked muscle thickening present). The disease score is the sum of the scores from each of these three categories, with a max score of 6 signifying greater pathological damage to the colon tissue. The experimenter determining disease scores was blinded to the treatment the mice received (DSS vs. vehicle).

3.2.6 Data analysis

Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA). For VMR experiments, mice received 3-5 trials of 60 mmHg CRD to establish baseline responses and then 3-5 trials of 60 mmHg CRD + Laser. Trials with laser were considered inhibited when there was a significant (>2 standard deviations) decrease in VMR from mean baseline responses. Comparisons between Vil-Arch and TRPV1-Arch mice that displayed yellow-light induced inhibition in VMR (i.e., “responders”) were performed using 2-way ANOVA and Mann-Whitney tests. Histopathological scores and spleen weights of the DSS-treated colons were compared to vehicle-treated colons using unpaired t-tests. VMR to CRD at various pressures was tested in DSS-treated and untreated mice using a two-way ANOVA. Statistical tests are specified in the results section and significance was defined as $p < 0.05$. Data are plotted as mean ± standard error of the mean.

3.3 Results

3.3.1 Optogenetic inhibition of colon epithelial cells reduces VMR to CRD

To enable specific inhibition of the colon epithelium, the yellow light-activated proton pump Arch was expressed under control of the villin-Cre driver. In Vil-Arch animals, Arch is fused to enhanced green fluorescent protein (EGFP), enabling visualization of the fusion protein in the villin-positive lining epithelial cells of the colon (**Figure 11A**). Arch-EGFP expression was restricted to the colon epithelium and not present in the L6 DRG (**Figure 11B**). A laser-balloon device was constructed to enable colorectal distension (CRD) simultaneous with light illumination of the colon lumen (**Figure 11C**). Visceromotor responses (VMR) to CRD were recorded, as they provide a reliable indication of visceral nociception (Christianson & Gebhart, 2007). VMRs to noxious distension (3-5 trials of 60 mmHg CRD) were recorded in Vil-Arch and control littermate mice before (baseline) and during yellow light application (**Figure 11D**). Out of 7 Vil-Arch mice, 4 displayed inhibition with yellow light. Inhibition was defined as VMR <2 standard deviations from average baseline responses. In the CRD trials that were inhibited, yellow light reduced the VMR by 67 ± 19% (VMR to CRD baseline: 2.67 ± 0.72 V*s vs. VMR to CRD + laser: 1.14 ± 0.88 V*s; **Figure 11E**). This inhibition occurred in an average of 66% of CRD + laser trials; **Figure 11E inset**). The remaining 3 Vil-Arch mice that were tested did not exhibit a decrease in VMR in response to light stimulation that met our threshold of <2 standard deviations from average baseline responses (VMR to CRD baseline: 1.4 ± 0.52 V*s vs. average VMR to CRD + laser: 1.39 ± 0.60 V*s; **Figure 11F**). The littermate control mice (n = 5) did not display inhibition with yellow light in any trial (VMR to CRD baseline: 1.65 ± 0.27 V*s vs. average VMR to CRD + laser: 1.71 ± 0.32 V*s; **Figure 11G**).

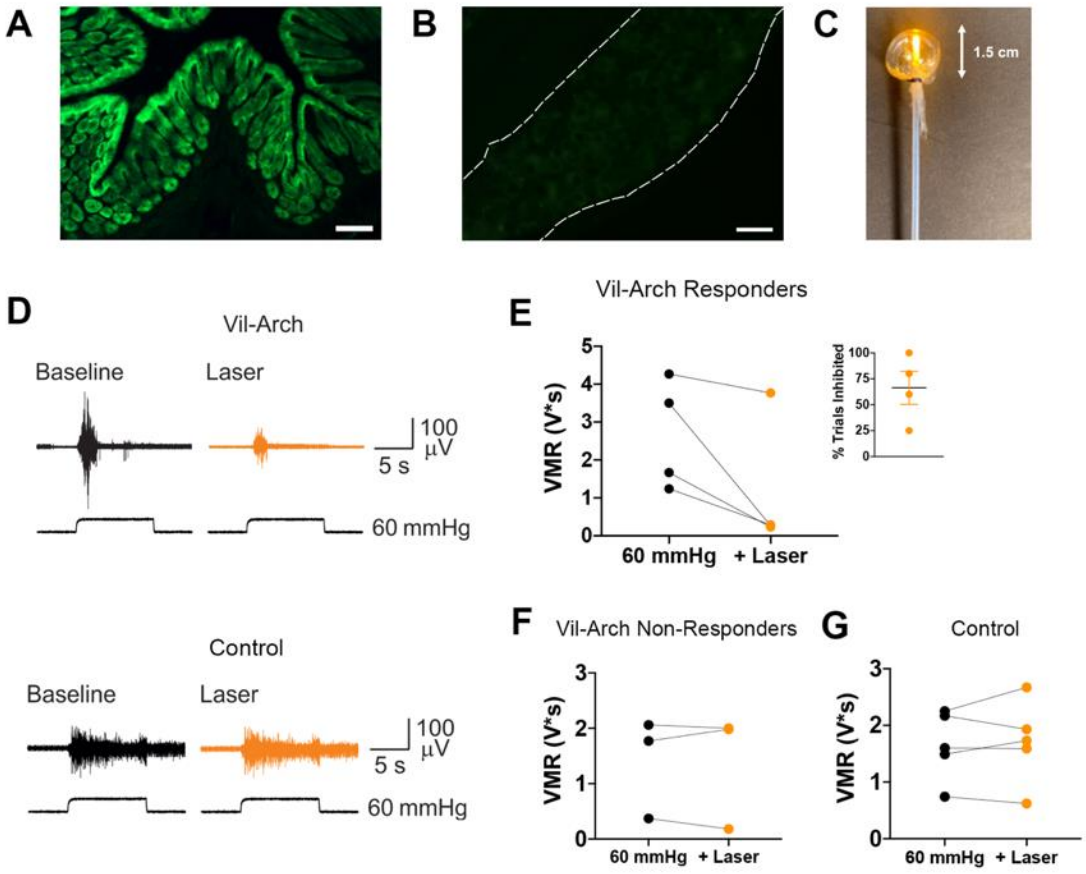


Figure 11. Inhibition of the colon epithelium reduces visceromotor responses VMR to CRD

Inhibitory opsin archaerhodopsin (Arch) is conjugated to enhanced green fluorescent protein (EGFP) to enable visualization. Arch-EGFP (green) is specifically expressed in colon epithelial cells under the villin-cre driver **(A)** and not in the L6 dorsal root ganglion (DRG) **(B)**. **(C)** A laser-balloon device was constructed to enable colorectal distesion (CRD) simultaneous with light illumination of the colon lumen. **(D)** Visceromotor responses (VMRs) to 60 mmHg CRD were recorded in villin-Archaerhodopsin (Vil-Arch) and control littermate mice before (Baseline) and during yellow light application (+ Laser). **(E)** Out of 7 Vil-Arch mice, 4 displayed a significant decrease in VMR to CRD with the addition of yellow light, which occurred in an average of 66% of trials (inset). **(F)** In the remaining 3 Vil-Arch mice that were tested, none of the CRD + Laser trials met the threshold for a decrease. **(G)** Littermate control mice (n = 5) did not display inhibition with yellow light in any trial. Scale bars = 100 μM (A), 50 μM (B).

3.3.2 Optogenetic inhibition of colon ExPANs reduces VMR to CRD

For comparison, I next measured changes in distension-induced VMRs due to yellow light stimulation in TRPV1-Arch mice. In these experiments, Arch was targeted to colon extrinsic primary afferent neurons (ExPANs) using the TRPV1-Cre driver, as TRPV1 is expressed in nearly 90% of colon afferents (S. A. Malin et al., 2009). Arch-EGFP expression was observed in nerve terminals in the colon (**Figure 12A**) and in cell bodies in the DRG (**Figure 12B**). VMRs to 60 mmHg CRD were recorded in TRPV1-Arch and control littermate mice before and during yellow light stimulation (**Figure 12C**). The same criteria were employed to identify mice responsive to light stimulation. Out of 6 TRPV1-Arch mice, 4 responded to yellow light stimulation. In the trials in which CRD was inhibited, yellow light reduced the VMR by 84 ± 8% (VMR to CRD baseline: 1.22 ± 0.12 V*s vs. VMR to CRD + laser: 0.18 ± 0.08 V*s; **Figure 12D**). This inhibition occurred on average in 82% of trials (**Figure 12D inset**). The remaining 2 TRPV1-Arch mice did not respond to yellow light (**Figure 12E**). In the control littermate mice (n = 4), no reduction in VMR in response to light stimulation was observed (baseline VMR to CRD: 1.84 ± 0.23 V*s vs. average VMR to CRD + laser: 1.82 ± 0.33 V*s; **Figure 12F**). When comparing the Vil-Arch and TRPV1-Arch mice that responded to yellow light, there was no statistical difference between groups in either were detected in the extent of inhibition in which a response was detected [main effect of yellow light ($p = 0.01$), no effect of genotype ($p = 0.16$); 2-way ANOVA; **Figure 12G**] or in the percentage of trials in which inhibition was detected ($p = 0.57$; Mann-Whitney test; **Figure 12H**).

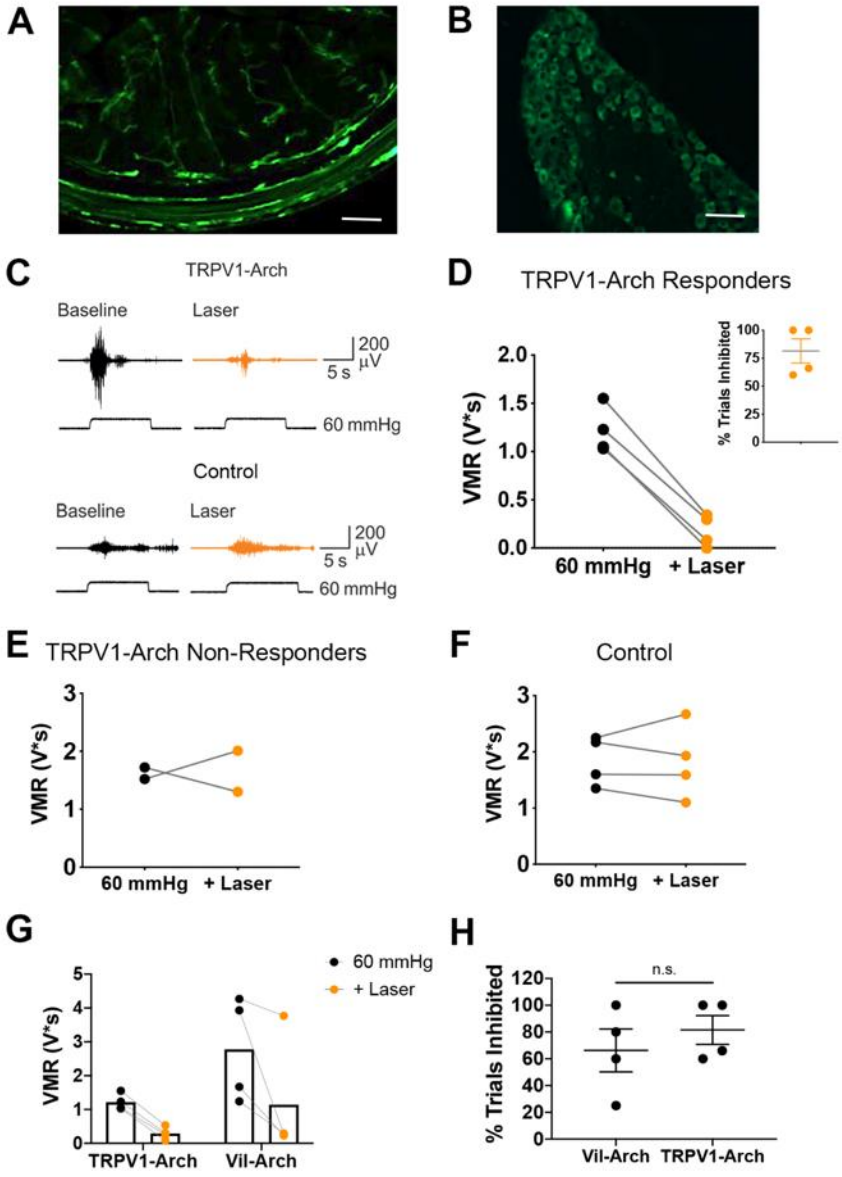


Figure 12. Inhibition of colon extrinsic primary afferent neurons reduces VMR to CRD

Inhibition of colon extrinsic primary afferent neurons reduces visceromotor responses (VMR) to colorectal distention (CRD). **(A)** Archaerhodopsin-enhanced green fluorescent protein (Arch-EGFP) was targeted to colon afferents using the transient receptor potential vanilloid 1 (TRPV1)-cre driver. Arch-EGFP-positive nerve terminals are found in the distal colon. **(B)** Arch-EGFP-positive (green) nerve cell bodies are present in the L6 dorsal root ganglion (DRG). **(C)** VMRs to 60 mmHg CRD were recorded in TRPV1-Arch and control littermate mice before (Baseline) and during yellow light illumination (+ Laser). **(D)** Out of 6 TRPV1-Arch mice, 4 displayed a significant decrease in VMR to CRD with the addition of yellow light, which occurred in an average of 82% of trials (inset). **(E)** The remaining 2

TRPV1-Arch mice did not display yellow light-induced reduction in VMR. **(F)** In the control littermate mice (n = 4), no reduction in VMR in response to yellow light was observed. **(G)** In comparing the Vil-Arch and TRPV1-Arch mice that responded to yellow light (n = 4 per group), no statistical differences were detected in the extent of inhibition in responding trials [main effect of yellow light (p = 0.01), no effect of genotype (p = 0.16); 2-way ANOVA]. **(H)** There was also no significant difference in the percentage of inhibited trials in Vil-Arch vs. TRPV1-Arch responders (p = 0.57; Mann-Whitney test).

3.3.3 Dextran sulfate sodium-mediated inflammation causes visceral hypersensitivity

To examine the role of epithelial cells in inflammation-induced hypersensitivity, we used the dextran sulfate sodium (DSS) model of inflammatory bowel disease. DSS damages the mucosal barrier, causing inflammation and other symptoms that mimic human ulcerative colitis (Chassaing, Aitken, Malleshappa, & Vijay-Kumar, 2014). After testing dose and time variables, we used a protocol in which 3% DSS is administered in the drinking water for 5 days prior to VMR analysis, as it consistently produced hypersensitivity. Histopathological comparison of vehicle-treated (**Figure 13A**) versus DSS-treated (**Figure 13B**) colons showed that this DSS protocol did not cause notable loss of lining epithelial cells, which allowed further examination of epithelial-neuronal communication. In addition, fluorescent images of vehicle-treated (**Figure 13C**) versus DSS-treated (**Figure 13D**) Vil-Arch colons showed that Arch-EGFP expression was not reduced by DSS treatment.

Though the epithelial lining is intact, histopathological analysis (Kim et al., 2012) showed that DSS-treated mice had a higher inflammation score, as indicated by fewer mucous-secreting goblet cells, more infiltrating immune cells, and thickening of the submucosal layer (n = 10 in vehicle-treated group and n = 12 in DSS-treated group; p < 0001, unpaired t-test; **Figure 13E**). Increased spleen weights in DSS-treated mice, an indicator of inflammation, were also present (n

= 11 in vehicle-treated group and n = 11 in DSS-treated group; p = 0.007; unpaired t-test with Welch's correction; **Figure 13F**). These data were pooled from vehicle- and DSS-treated Vil-Arch, TRPV1-Arch, and control mice, as previous analysis showed that genotype did not have an effect on inflammation. When comparing disease scores in DSS-treated Vil-Arch, TRPV1-Arch, and control mice, there was a main effect of DSS ($p < 0.0001$) but not genotype ($p = 0.09$), and there was no interaction between DSS and genotype ($p = 0.16$; two-way ANOVA). When comparing spleen weights, there was a main effect of treatment ($p = 0.01$) but not genotype ($p = 0.91$), and there was no interaction ($p = 0.99$; two-way ANOVA).

Previous studies have shown that DSS treatment at various doses can result in visceral hypersensitivity (Kalra et al., 2018; Scanzi et al., 2016; Verma-Gandhu et al., 2007). To confirm that our DSS protocol produced hypersensitivity, VMR analysis was conducted in Vil-Arch mice. Mice were stimulated with CRD pressures of 40, 60, and 80 mmHg. There were main effects of DSS treatment ($p = 0.02$) and distension pressure ($p < 0.0001$) with a significant interaction ($p = 0.002$; two-way ANOVA). In addition, the VMR in response to 60 and 80 mmHg was significantly greater in DSS treated than vehicle-treated mice ($p = 0.01$ and $p < 0.001$, respectively; Sidak's test; n = 5 mice per group) (**Figure 13G**).

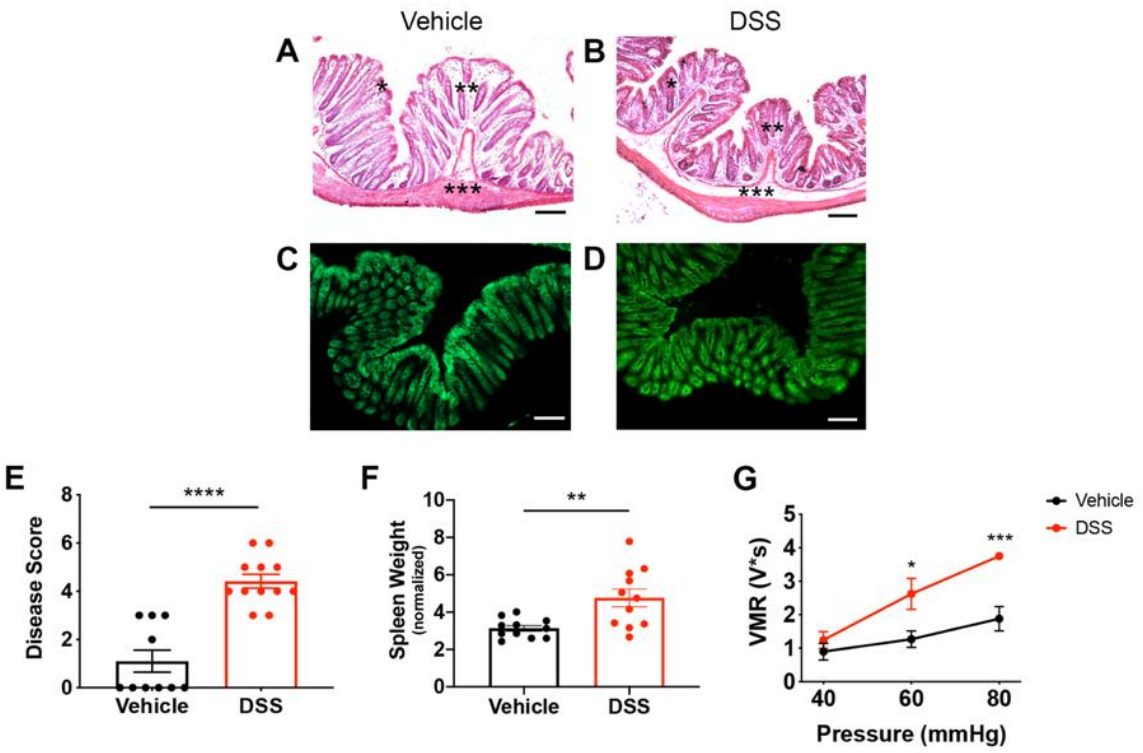


Figure 13. Dextran sulfate sodium (DSS) treatment induces colon inflammation and visceral hypersensitivity

Dextran sulfate sodium (DSS) treatment induces colon inflammation and visceral hypersensitivity. Mice were administered 3% DSS for 5 days or vehicle treatment. Histopathological analysis of vehicle-treated **(A)** vs. DSS-treated **(B)** colons showed that the DSS protocol did not cause a notable loss in lining epithelial cells. There was a depletion of goblet cells (indicated by *), increase in infiltrating cells (indicated by **), and thickening of the submucosal layer (indicated by ***). Fluorescent images of vehicle-treated **(C)** and DSS-treated **(D)** villin-Archaerhodopsin (Vil-Arch) colons showed comparable enhanced green fluorescent protein (EGFP) expression, indicating that Arch-EGFP expression is not ablated after DSS treatment. **(E)** DSS-treated mice had a higher inflammation disease score, indicating fewer mucous-secreting goblet cells, more infiltrating cells, and expansion of the submucosa (n = 10 in vehicle-treated group and n = 12 in DSS-treated group; $p < 0.0001$, unpaired t-test). **(F)** DSS-treated mice also had significantly higher spleen weights indicating inflammation n = 11 in vehicle-treated group and n = 11 in DSS-treated group; $p = 0.007$; unpaired t-test with Welch's correction. **(G)** Visceromotor responses (VMRs) to colorectal distension (CRD) were measured in vehicle- and DSS-treated mice. Mice received multiple CRD pressures (40, 60, and 80 mmHg) and showed significantly greater VMRs at 60 ($p = 0.01$) and 80 mmHg ($p < 0.001$; two-way ANOVA with Sidak's test; n = 5 mice per group). Scale bars = 100 μM.

3.3.4 Inhibition of the colon epithelium reduces DSS-induced hypersensitivity

As our DSS protocol produced hypersensitivity to CRD in response to 60 mmHg pressure, we used this stimulus intensity to assess the impact of light stimulation on the VMR in DSS-treated control and Vil-Arch mice. VMRs to CRD were recorded in Vil-Arch and control littermate mice, both treated with DSS. Measures were made before (baseline) and during application of light stimulation (**Figure 14A**). Yellow light produced effective inhibition in 4 out of 5 Vil-Arch mice, reducing the VMR by 51 ± 9% (baseline VMR to CRD: 2.66 ± 0.57 V*s vs. VMR to CRD + laser: 1.22 ± 0.41 V*s; **Figure 14B**). Yellow light was effective in inhibiting the VMR in 60 ± 9% of trials (**Figure 14B inset**). In the 1 Vil-Arch mouse that did not respond to yellow light, the baseline VMR to CRD was 2.58 V*s and the average VMR to CRD + laser was 2.49 V*s. In the control littermate mice (n = 4), no reduction in VMR in response to light stimulation was observed (baseline VMR to CRD: 2.1 ± 0.2 V*s vs. average VMR to CRD + laser: 2.68 ± 0.42 V*s; **Figure 14C**). Yellow light induced inhibition in the DSS-treated Vil-Arch mice to a similar extent compared to untreated Vil-Arch mice (vehicle: 67 ± 19% inhibition vs. DSS: 51 ± 9%; p = 0.57; unpaired t-test; **Figure 14D**).

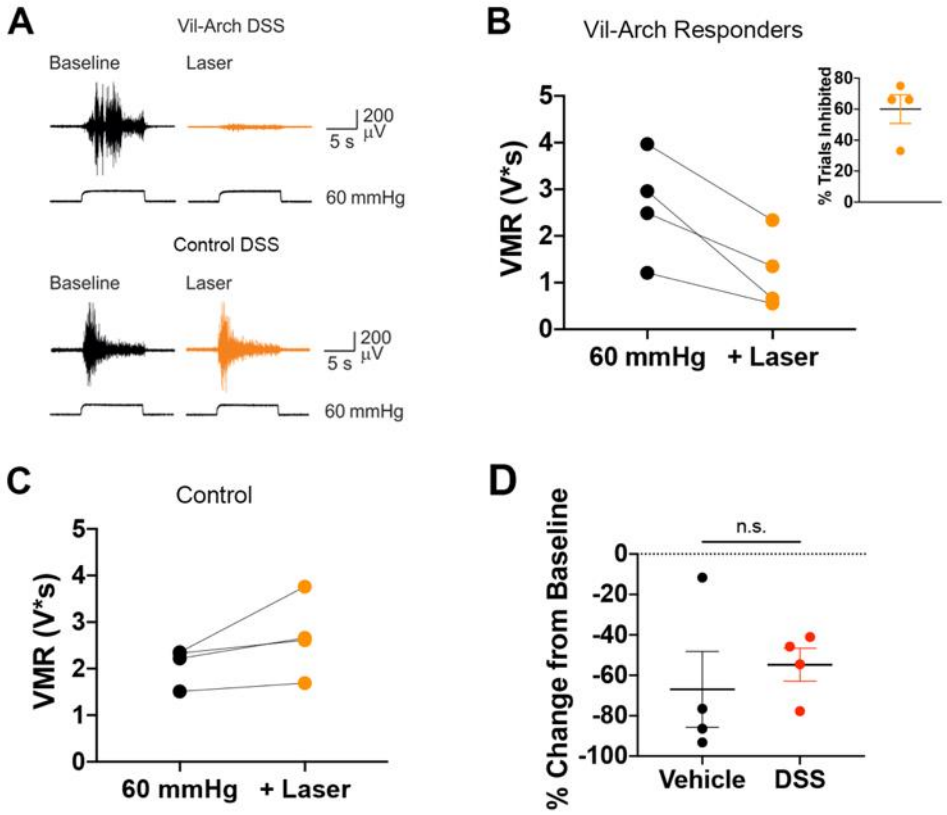


Figure 14. Inhibition of colon epithelium reduces DSS-induced visceral hypersensitivity

Inhibition of colon epithelium reduces dextran sulfate sodium (DSS)-induced hypersensitivity. **(A)** Visceromotor responses (VMRs) to 60 mmHg colorectal distension (CRD) were recorded in DSS-treated villin-Archaerhodopsin (Vil-Arch) and control littermate mice before (Baseline) and during yellow light stimulation (+ Laser). **(B)** Yellow light produced effective inhibition in 4 out of 5 Vil-Arch mice, in an average of 60% of CRD + laser trials (inset). **(C)** In control littermate mice (n = 4), no reduction in VMR in response to light stimulation was observed. **(D)** Yellow light-induced inhibition in DSS-treated Vil-Arch mice was similar to inhibition in untreated Vil-Arch mice (n = 4 per group; p = 0.57; unpaired t-test).

3.3.5 Inhibition of colon ExPANs reduces DSS-induced hypersensitivity

I next examined the effects of inhibiting colon afferents on DSS-induced hypersensitivity. VMRs to CRD were recorded in TRPV1-Arch and control littermate mice before (baseline) and

during yellow light stimulation (**Figure 15A**). Yellow light was effective in inhibiting the VMR to CRD in 5 out of 6 TRPV1-Arch mice. In the CRD trials that were inhibited, yellow light reduced the VMR by 62 ± 8% (baseline VMR to CRD: 2.17 ± 0.39 V*s vs. VMR to CRD + laser: 1.82 ± 0.31 V*s; **Figure 15B**). Inhibition occurred in an average of 54% of the CRD + laser trials in these mice (**Figure 15B inset**). In the 1 TRPV1-Arch mouse that did not respond to yellow light, the baseline VMR to CRD was 2.07 V*s and the average VMR to CRD + laser was 2.09 V*s. In control littermate mice (n = 4), yellow light-induced reduction in VMR only occurred in 1 CRD + laser trial in 1 mouse. In all other trials, this reduction was not observed (baseline VMR to CRD: 1.93 ± 0.45 V*s vs. average VMR to CRD + laser: 2.07 ± 0.39 V*s; **Figure 15C**). Yellow light induced inhibition in the DSS-treated TRPV1-Arch was compared to inhibition in untreated TRPV1-Arch mice (vehicle: 83 ± 8% inhibition vs. DSS: 53 ± 11%; p = 0.11; Mann-Whitney test; **Figure 15D**). There was a trend towards a decrease in the extent of inhibition in DSS-treated mice, but this was not significant. Finally, comparison between inhibition via Arch-expression in the epithelium versus inhibition in colon afferents showed that both were similarly effective at reducing the VMR response in inflamed mice. No statistical differences were detected in the extent of inhibition in responding trials [main effect of yellow light (p = 0.001), no effect of genotype (p = 0.57), 2-way ANOVA; **Figure 15E**] or in the rate of inhibited trials (p = 0.62, Mann-Whitney; **Figure 15F**).

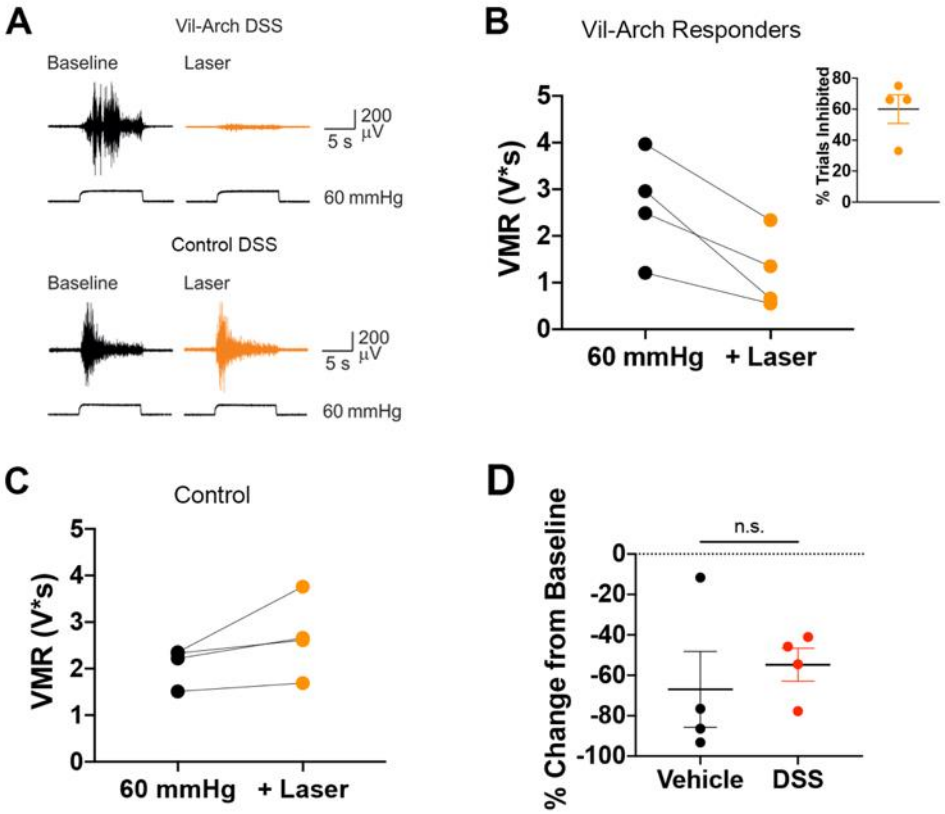


Figure 15. Inhibition of colon ExPANs reduces DSS-induced visceral hypersensitivity

(A) Visceromotor responses (VMRs) to colorectal distension (CRD) were recorded in DSS-treated transient receptor potential vanilloid 1 (TRPV1)-Archaerhodopsin (Arch) and control littermate mice before (Baseline) and during yellow light stimulation (+ Laser). **(B)** Yellow light produced effective inhibition in 5 out of 6 TRPV1-Arch mice, in an average of 45% of CRD + laser trials (inset). **(C)** In the control littermate mice (n = 4), no reduction in VMR in response to light stimulation was observed. **(D)** Yellow light-induced inhibition in the DSS-treated TRPV1-Arch mice (n = 5) was compared to inhibition in untreated TRPV1-Arch mice (n = 4). There was a trend for a decrease in inhibition in DSS-treated mice, but this was not significant (p = 0.11; Mann-Whitney test). **(E)** Comparing DSS-treated Vil-Arch (n = 4) and TRPV1-Arch (n = 5) mice that responded to yellow light shows no statistical differences in the extent of inhibition in responding trials [main effect of yellow light (p = 0.001), no effect of genotype (p = 0.57); 2-way ANOVA]. **(F)** There was also no significant difference in the percentage of inhibited trials in Vil-Arch vs. TRPV1-Arch responders (p = 0.62; Mann-Whitney test).

3.4 Discussion

Results from this study show that Arch-mediated inhibition of colon epithelial cells diminishes visceral nociceptive responses in both healthy mice and mice with DSS-induced inflammation. In Vil-Arch animals that displayed yellow light-induced decreases in VMR, inhibiting the epithelium reduced VMRs by more than 80%. Notably, epithelial inhibition was similarly effective as colon afferent inhibition in reducing the VMR. When combined with our previous findings that ChR2-mediated excitation of the colon epithelium evokes VMRs (Makadia et al., 2018), these results support the role of the epithelium as a critical component of nociceptive signaling.

How changes in the epithelium specifically impact the response to noxious stimuli has proven difficult to study because of the complexity of neural and epithelial cell types and the intimate association of nerve terminals and the epithelium, e.g., chemical or mechanical stimulation of the colon simultaneously activates both nerves and epithelial cells, making the relative contribution of each cell type undecipherable. To overcome this limitation, we used villin-Cre driver mice to genetically target the inhibitory Arch opsin to all epithelial cells lining the colon. This allowed a specific assessment of the contribution of the epithelium to distension evoked VMRs and revealed that inhibition of the epithelium reduces visceral hypersensitivity. However, because the villin-Cre driver targets enterocyte, goblet, enterochromaffin and enteroendocrine cell subpopulations it is not possible to determine which of these subpopulations of epithelial cell types mediates the effects of light stimulation. Thus, future studies will employ additional Cre-lines will reveal the contribution of different epithelial cell types to visceral nociception.

Although VMR is a reliable way to assess visceral sensitivity (Christianson & Gebhart, 2007), we observed variability in responses to CRD within the Vil-Arch and TRPV1-Arch groups.

Some Arch-expressing mice did not display yellow light mediated inhibition of the VMR in any CRD + laser trials. This variability in VMR is likely due to inappropriate positioning of the laser or excessive luminal contents that occluded the laser. The lack of a reliable response could also be due to the interstimulus interval used in these experiments. We consistently waited 4 min in between distension and distention + laser trials, and this may not have been enough time for the Arch proton pump to reset properly. Variability could also be attributed to the possibilities that different epithelial cell types play different roles in the regulation of ExPAN activity, or Arch activation has different effects in subpopulations of epithelial cells. That is, while previous studies showed light activation of Arch leads to proton release and membrane hyperpolarization, preventing action potential firing in neurons (Chow et al., 2010), it remains to be determined how Arch-mediated hyperpolarization of colon epithelial cell membranes results in the 'silencing' of epithelial cells and inhibition of distension-induced VMRs. One possibility is that hyperpolarization blocks the distension (stretch)-mediated release of ATP from the epithelium, which is known to activate purinergic receptors expressed on extrinsic afferent terminals (Burnstock, 2001; Makadia et al., 2018). Release of 5-HT from the subset of electrically excitable enterochromaffin cells, as shown in cultured organoids (Alcaino et al., 2018; Bellono et al., 2017) may also be changed by Arch-mediated hyperpolarization.

Our studies indicate limitations in the extent to which inhibition of colon epithelial cells or ExPANs can reduce VMRs to CRD. Post DSS treatment, both epithelial and ExPAN inhibition were effective in reducing hypersensitivity. However, averaging of VMR values showed that neither could by itself completely *reverse* the hypersensitivity, i.e., blocking peripheral components of the VMR could not normalize sensitivity to CRD after inflammation. The inability to completely reverse hypersensitivity suggests that Arch-mediated inhibition did not sufficiently

inhibit colon afferents. There could also be a role for central sensitization in this inflammatory state. Future studies will investigate how other components involved in the VMR circuit contribute to visceral hypersensitivity.

Visceral pain is notoriously difficult to treat, often persisting long after the precipitating injury or disease is no longer evident. Chronic disruptive changes in the epithelial lining may affect normal epithelial-neural signaling leading to persistence in hypersensitivity. Indeed, changes were identified in muscarinic signaling in urothelial cells isolated from the bladder of patients with chronic interstitial cystitis (Gupta, Lu, Gold, & Chai, 2009), suggesting injury can evoke long-term changes in epithelial signaling properties. It remains to be determined whether there are similar long-term changes in colon epithelial cells, though there is some research that shows inflammation can affect the abundance and neurotransmitter content of enteroendocrine cells (Lomax et al., 2006). These changes with inflammation should be further studied, as the work here confirms that the epithelium is a powerful component in nociceptive signaling, suggesting that it is a potential target for treatment of visceral pain.

4.0 Optogenetic Activation of the Colon Epithelium Engages Enteric Nervous System Circuits to Initiate Colon Motility

4.1 Introduction

The enteric nervous system (ENS) is a network of neural ganglia intrinsic to the gastrointestinal (GI) tract that controls motility, local blood flow, mucosal transport and secretion. Dysregulation of motility patterns are associated with constipation and diarrhea and can result in a significant reduction in quality of life. Dysregulation of gut motility is also now recognized as a symptom of diseases previously thought to only affect the brain, e.g., autism, diabetes and Parkinson's (Bessac, Cani, Meunier, Dietrich, & Knauf, 2018; Chalazonitis & Rao, 2018; Dinan & Cryan, 2017). Colon motility is dependent on controlled sensory-motor reflexes in the ENS, in which neurons responsive to mechanical or chemical changes in the intestinal lumen generate propulsive contractions. Most ENS neurons associated with motor function are located in the myenteric plexus whereas the neurons that initiate these reflexes are intrinsic primary afferent neurons (IPANs) (Costa et al., 2000).

IPANs are specialized sensory neurons unique to the GI tract that respond to mechanical and chemical stimuli in the lumen (Furness, Jones, Nurgali, & Clerc, 2004). In mouse colon, IPANs that originate in the myenteric plexus have processes that can extend into the mucosal layer and terminate in close apposition to colon epithelial cells (Furness, Robbins, et al., 2004). IPANs detect mechanical stimuli and initiate enteric reflexes (Bertrand et al., 1998), but how this signaling occurs and the role of the epithelium in this process is not clear. A role for colon epithelial cells as an intermediary between luminal stimuli and enteric neuron activation is supported by the

anatomical localization of terminals and by numerous studies of epithelial cells, enteroendocrine cells (EECs) in particular, that show expression of neuroactive compounds and their receptors (Najjar et al., 2020). Receptors expression would allow the epithelium to directly detect and respond to changes in luminal stimuli, perhaps through release of neurotransmitters such as ATP (Burnstock, 2014; Makadia et al., 2018) and serotonin (5-HT) (Gershon, 2013). Enterochromaffin cells, a type of EEC, are the main source of 5-HT in the body. These cells express receptors for chemical irritants (transient receptor potential ankyrin 1) (Nozawa et al., 2009) and mechanical stimulation (Piezo2) (Alcaino et al., 2018). In response to activation of these receptors, enterochromaffin cells release 5-HT, which binds 5HT receptors on local neuron terminals and is so doing, activate circuits controlling motility (Grider et al., 1996; Kadowaki et al., 1996; Nozawa et al., 2009).

The extent to which colon epithelial cells regulate ENS circuits is a major unanswered question. It is debated whether the epithelium has a role in colon motility, particularly for colonic migrating motor complexes (CMMCs), which are rhythmic motor patterns that facilitate the movement of fecal matter through the colon (Spencer, 2001). Evidence supports a role for mucosal 5-HT as essential for normal propagation of motor patterns in the colon (Heredia et al., 2009; Heredia et al., 2013; Smith & Gershon, 2015). Other studies contend that signaling from the mucosa is not required for generation of motor patterns, and rather that mucosal-derived serotonin has a modulatory role (Keating & Spencer, 2010; Spencer et al., 2015). Given these discrepancies, the aim of this study was to investigate the role of the epithelium in motility by assessment of ENS neuron activity and colon motility in response to direct epithelial activation.

To enable specific activation of the epithelium we used an *ex vivo* colon preparation of the mouse in which the blue light activated excitatory channelrhodopsin protein (ChR2) is expressed

in colon epithelial cells. Simultaneous with epithelial cell activation, we measured changes in calcium (Ca^{2+}) in neurons of the myenteric plexus using a red-shifted genetically encoded Ca^{2+} indicator (GECIs) and measured local colon motility and CMMCs. Our studies show that light stimulation of the epithelium caused activation of a subpopulation of myenteric neurons that led to local motility changes in the distal colon and increased the rate of CMMCs. Antagonism of ATP signaling, but not of 5-HT, caused reduction in epithelial-evoked changes in motility. Overall, our findings indicate that colon epithelial cells induce neuronally-mediated changes in motility through purinergic signaling.

4.2 Methods

4.2.1 Animals

Adult male and female mice 2-12 months old were used. As described previously (Makadia et al., 2018), channelrhodopsin (ChR2) was targeted to the intestinal epithelium under control of the villin-Cre driver. Mice with a ChR2-EYFP fusion protein [ChR2(H134R)-EYFP] in the *Rosa26* locus downstream of a floxed-STOP cassette (Ai32 mice; RRID: IMSR_JAX:012659) were crossed with villin-Cre mice (RRID: IMSR_JAX004586). Littermates with ChR2-EYFP but lacking Cre were used as controls. Animals were housed in an AAALAC-approved facility and handled in accordance with protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

4.2.2 RCaMP/R-GECO expression in enteric neurons

P2-P4 neonatal mice were injected intraperitoneally with RCaMP or R-GECO virus to enable expression throughout the peripheral nervous system. Insulin syringes (3/10 cc, 31 G; Allison Medical, Littleton, CO) were used to inject 10 µL of RCaMP virus (pAAV.Syn.NES.jRCaMP1b.WPRE.SV40, Addgene, Watertown, MA) or R-GECO virus (AAV9.Syn.NES-jRGECO1a.WPRE.SV40; Addgene). Mice were analyzed at least 6 weeks after injection.

4.2.3 Imaging Ca^{2+} transients and local motility

After euthanasia with isoflurane, distal colons were isolated and placed in a Sylgard-lined dish containing artificial cerebrospinal fluid (ACSF), containing (in mM): 117.9 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4$-$7H_2O$, 2.5 $CaCl_2$, 11.1 D-glucose, 2 sodium butyrate, and 20 sodium acetate. Fecal contents were flushed from the lumen and the colon was opened longitudinally and pinned flat with serosal side up. During imaging, the dish was superfused with ACSF and maintained at 35º C. Nifedipine (4 µM, Sigma-Aldrich) was added to the ACSF to prevent spontaneous colon movement. The myenteric plexus was visualized at 40x using an upright Leica DM6000FS fluorescent microscope (Leica, Buffalo Grove, IL). Activity in myenteric neurons and movements of the colon were recorded using a CMOS camera (Prime 95B Photometrics; Roper Scientific, Tucson, AZ) at a 20 Hz sampling rate, 50 ms exposure time. Image stacks were collected in Metamorph software (Molecular Devices, San Jose, CA). Activity was recorded before, during, and after blue light stimulation. Stimulation was delivered using a 473 nm wavelength laser (Laserglow Technologies, Toronto, Canada) with a 1.5 mm optical fiber

(ThorLabs, Newton, NJ). The optical fiber was positioned 2-3 mm away from the imaging field, in the aboral direction, and 3 mm above the colon, enabling the optical fiber to deliver 20 mW of laser power to the colon tissue. Light stimulation was delivered for 20 s (Makadia et al., 2018).

4.2.4 Drugs used

Tetrodotoxin (TTX) 0.5 µM and hexamethonium (HEX) 300 µM (both from Sigma-Aldrich, St. Louis, MO) were dissolved in oxygenated ACSF on the day of the experiment. Drug concentrations were chosen based on previous studies (Hibberd, Travis, et al., 2018; Smith-Edwards et al., 2019). The ATP receptor antagonist cocktail consisted of the following drugs (all from Tocris Bioscience, Minneapolis, MN): TNP-ATP triethylammonium salt ($P2X_1$, $P2X_{2/3}$, and $P2X_3$ antagonist, dissolved in H_2O for a 100 µM stock solution), 5-BDBD [$P2X_4$ antagonist, dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich) for a 30 mM stock solution], MRS 2500 ($P2Y_1$ antagonist, dissolved in H_2O for a 1 mM stock solution), AR-C 118925XX ($P2Y_2$ antagonist, dissolved in DMSO for a 5 mM stock solution), and MRS 2578 ($P2Y_6$ antagonist, dissolved in DMSO for a 5 mM stock solution). Drugs were added to ACSF for final concentrations of 20 nM TNP-ATP, 20 µM 5-BDBD, 10 µM MRS 2500, 10 µM AR-C118925XX, and 1 µM MRS 2578. The 5-HT receptor antagonist cocktail consisted of the following drugs: alosetron (Sigma-Aldrich, $5\text{-}HT_3$ antagonist, dissolved in H_2O for a 20 mM stock solution), ketanserin (Tocris, $5\text{-}HT_2$ antagonist, dissolved in H_2O for a 1 mM stock solution), and GR 113808 (Tocris, $5\text{-}HT_4$ antagonist, dissolved in HCl for a 20 mM stock solution). Drugs were added to ACSF for final concentrations of 20 µM alosetron, 2 µM ketanserin, and 2 µM GR 113808. Colon tissue was imaged after 15 min of antagonist drug incubation.

4.2.5 Whole colon motility

After euthanasia with isoflurane, entire colons were isolated and placed in a Sylgard-lined dish containing ACSF. After fecal contents were flushed, the colon was gently pinned at the mesentery to the dish. The dish was superfused with ACSF and maintained at 35º C. Measures of motility began as soon as spontaneous colonic migrating motor complexes (CMMCs) were observed (about 10-30 min after dissection from the mouse). Spontaneous contractions were video recorded (Sony, HDR-CX440) for 20 min. In some experiments, colons were connected to a force transducer to measure changes in tension (mN) during CMMC propagation. To test the effects of blue light stimulation on CMMCs, a fiber optic was secured directly above the colon at 1 cm from the anus (location determined from local motility studies). Pulsed blue laser stimulation on the colon was done for 20 s every 2 min, for 20 min total, while CMMCs were video recorded. Video analysis done blinded to the mouse genotype counted the number of CMMCs observed over the course of the 20-min videos. CMMCs were defined as circular contractions initiated in the proximal colon that propagated at least 1 cm; only anterograde CMMCs (i.e., those traveling in the oral to aboral direction) were included for analysis.

4.2.6 Data analysis

Analyses of imaging files and video recordings of colon motility were conducted in a blinded manner. Images collected in Metamorph were exported to ImageJ (National Institutes of Health, Bethesda, MD). Local motility, i.e., colon tissue movement, was quantified using the Template Matching plugin in ImageJ as previously described (Smith-Edwards et al., 2019). The amplitudes of calcium indicator signals were quantified by calculating $\Delta F/F_0$ as % = [($F - F_0/F_0$)]

x 100, where F is the peak fluorescence signal and F_0 is the baseline fluorescence signal. Statistical analyses were performed in Prism (GraphPad, San Diego, CA) and included unpaired Student's t-test, one-way and two-way analysis of variance (ANOVA) with post-hoc tests as indicated in results. Data is represented as mean ± standard error of the mean and results were considered significant when $p \leq 0.05$.

4.3 Results

4.3.1 Optogenetic stimulation of colon epithelium changes activity in myenteric neurons

To determine whether stimulation of colon epithelium affects myenteric neuron activity, we virally injected red calcium indicators (RGECO, RCaMP) into Vil-ChR2 mice (n=5). This strategy allowed for an all-optical approach to investigate intercellular communication in the colon (**Figure 16A**). Myenteric neuron activity was recorded before, during, and after laser stimulation (20 s) of the epithelium (**Figure 16B**). Tracking of activity changes in individual neurons revealed a small population of myenteric neurons in the distal colon that were activated by laser stimulation. Activated myenteric neurons initially displayed no activity and only began to exhibit calcium signals after optogenetic stimulation of the epithelium. Fewer neurons per ganglion responded to laser compared to electrical stimulation applied to the same location (Electrical: 14.71 ± 1.43% vs. Laser: 6.7 ± 0.89%; $p = 0.008$; Mann-Whitney test; **Figure 16C**) indicating that a smaller subpopulation of myenteric neurons receives epithelial input. The average amplitude of response was not significantly different between laser and electrical stimulation (Electrical: 9.67 ± 1.65 ΔF vs. Laser: 8.16 ± 1.61 ΔF; $p = 0.53$; unpaired t-test; **Figure 16D**), suggesting the strength of

activation in neurons due to laser stimulation was comparable to when neural circuits were directly activated. As would be expected in a multi-cell circuit, the latency of response to laser was significantly longer than electrical stimulation (Electrical: 0.13 ± 0.02 s vs. Laser: 6.51 ± 0.4; $p<0.0001$, unpaired t-test; **Figure 16E**). Latencies ranged from 2 – 16 s with the majority of responses within 4 s from the start of laser stimulation (**Figure 16F**).

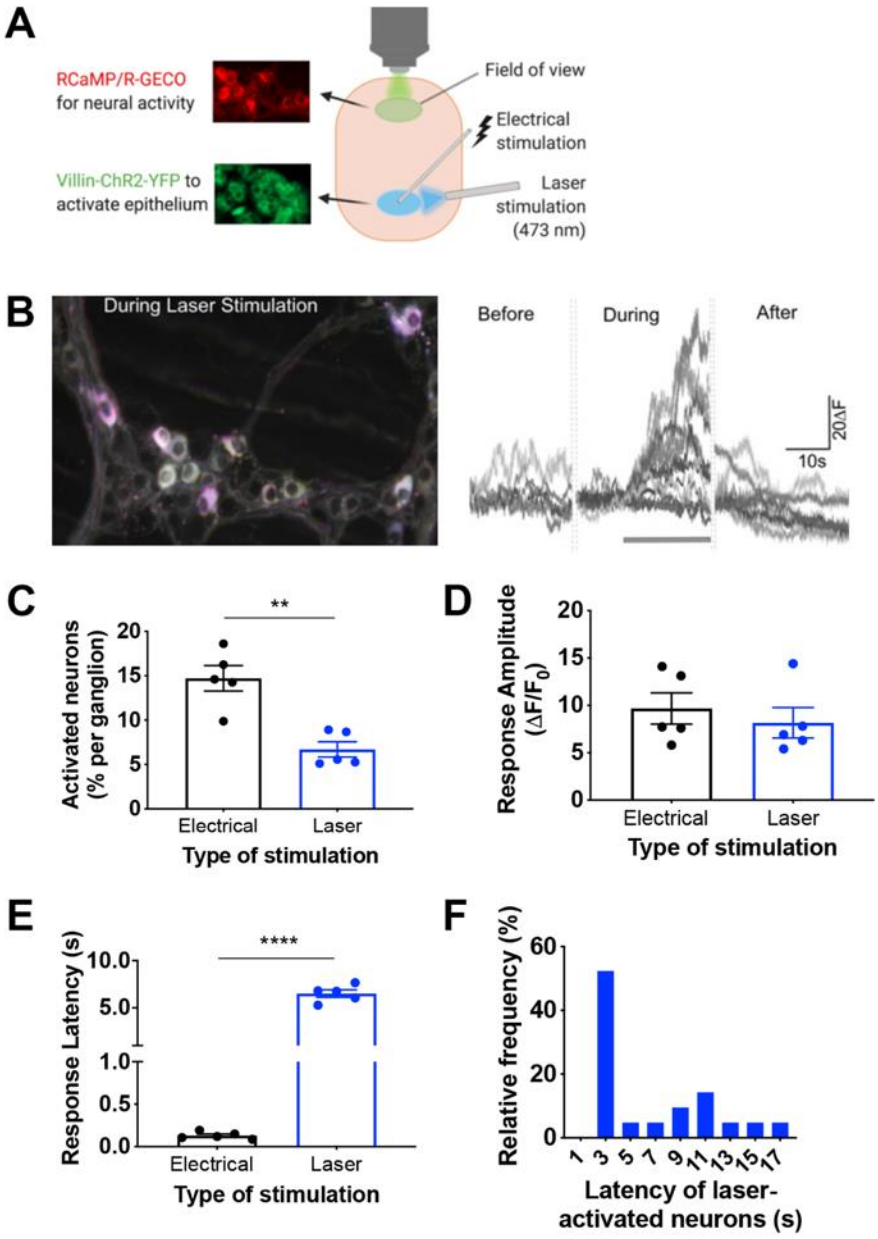


Figure 16. Optogenetic stimulation of colon epithelium initiates activity in myenteric neurons

(A) Experimental setup: RCaMP/R-GECO was virally expressed in myenteric neurons in Villin-ChR2 mice. Laser and electrical stimulation were applied 3 mm below the imaging field. (B) Myenteric neuron activity was recorded before, during, and after laser stimulation (20 s) of the epithelium. (C) Electrical stimulation in Vil-ChR2 mice (n = 5), initiated Ca2+ transients in a greater percentage of myenteric neurons than laser stimulation (p = 0.008; Mann-Whitney test). (D) The average amplitude of response observed in myenteric neurons was not significantly different between laser and electrical stimulation (p = 0.53; unpaired t-test). (E) The latency of response to laser was

significantly longer than electrical stimulation ($p < 0.0001$, unpaired t-test). (F) Latencies ranged from 2-16 s with the majority of responses within 4 s from the start of laser stimulation.

4.3.2 Optogenetic stimulation of colon epithelium initiates changes in local motility

Epithelial activation also evoked movement patterns in the imaging field, characterized by tissue movement in the longitudinal and circular directions (**Figure 17A**). Movement patterns were observed in fields throughout the distal colon, 0.8 – 2.8 cm from the anus (**Figure 17A inset**). Example traces illustrate the epithelium-evoked movement in Vil-ChR2 mice (n = 7) compared to the minimal movement in colons of control mice (n = 4; **Figure 17B**). On average, the colon was displaced 81.15 ± 15.68 µm in the longitudinal direction (compared to 2.88 ± 1.12 µm in control animals; $p = 0.006$; Mann-Whitney test; **Figure 17C**) and 38.17 ± 12.43 µm in the circular direction (compared to 0.38 ± 0.30 µm in control animals; $p = 0.006$; Mann-Whitney test; **Figure 17D**). As seen for myenteric neuron activation (**Figure 16E**), there was a latency between the start of blue light stimulation and colon movement which was significantly longer than the latency to movement with electrical stimulation (Electrical: 1.04 ± 0.18 s vs. Laser: 7.83 ± 1.04 s; $p = 0.0005$; Mann-Whitney test; **Figure 17E**). Latencies ranged from 2 – 40 s, with the majority of responses occurring within 6 s from the onset of laser (**Figure 17F**).

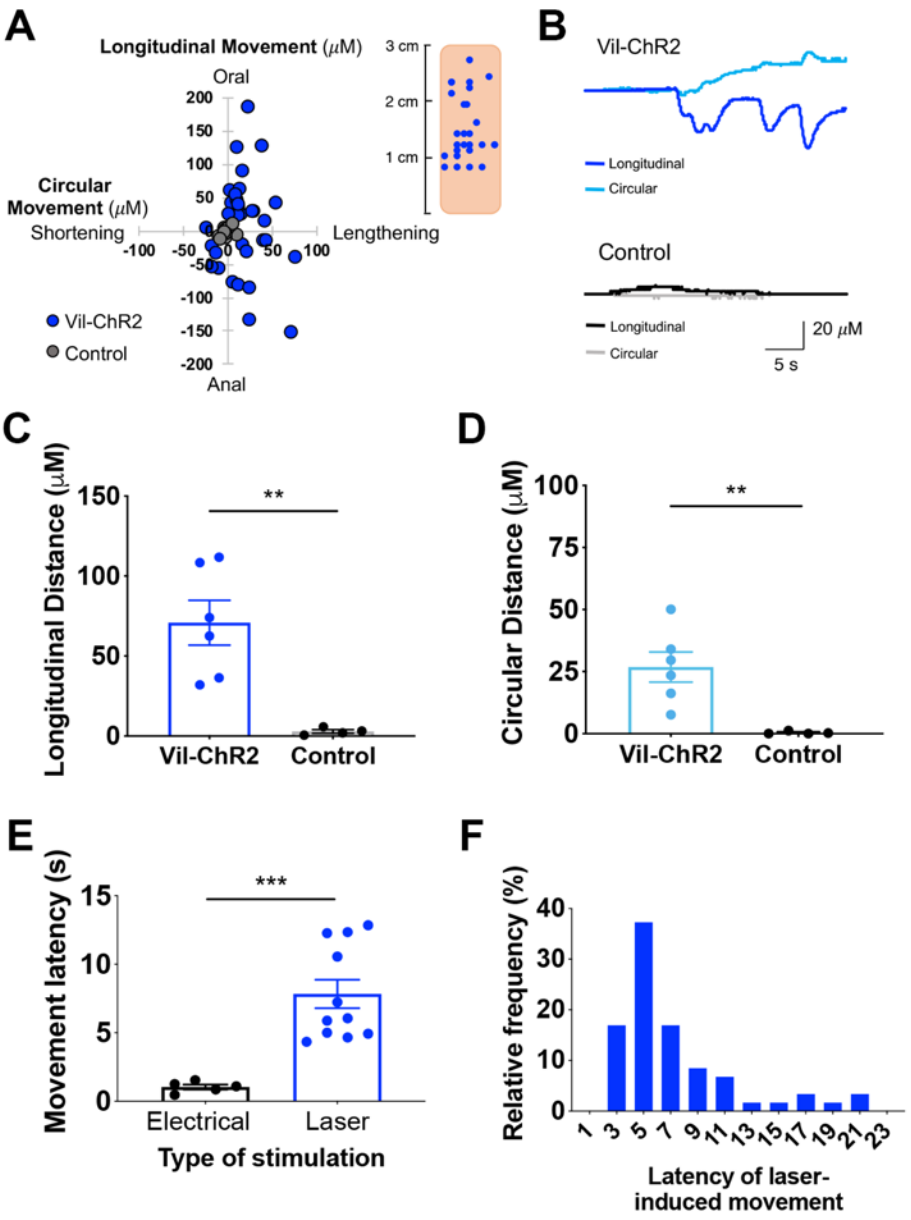


Figure 17. Optogenetic stimulation of colon epithelium initiates changes in local motility

(A) Epithelial activation evoked movement patterns in the imaging field, characterized by tissue movement in the longitudinal and circular directions, observed in fields throughout the distal colon (inset). (B) Example traces illustrate the epithelium-evoked movement in Vil-ChR2 mice (n = 7) compared to the minimal movement in colons of control mice (n = 4). (C) In the longitudinal direction, laser-evoked movement was significantly greater in Vil-ChR2 mice compared to controls (p = 0.0006; Mann-Whitney test). (D) Laser-evoked movement in the circular direction was also

greater in Vil-ChR2 mice ($p = 0.006$; Mann-Whitney test). (E) There was a latency between the start of blue light stimulation and colon movement which was significantly longer than the latency with electrical stimulation ($p = 0.0005$; Mann-Whitney test). (F) Latencies ranged from 2 – 40 s, with the majority of responses occurring within 6 s from the onset of laser.

4.3.3 Epithelium-induced motility is mediated by neuronal activity

We hypothesized that the epithelium, upon activation, signals to enteric neurons to produce the observed movement patterns. Acetylcholine (ACh) is the major neurotransmitter for synaptic transmission in the ENS and is released from enteric motor neurons to produce smooth muscle contractions (Sang & Young, 1998). Therefore, the nicotinic ACh receptor antagonist hexamethonium (HEX, 300 µM) was added to the bath to test whether laser-induced movement was reduced by blocking synaptic transmission in enteric neural circuits. We also used tetrodotoxin (TTX, 0.5 µM), a voltage gated Na^+ channel blocker, to confirm that laser-induced movement depended on action potential firing in neurons. As shown in the example traces, epithelial-evoked movement of the colon (**Figure 18A**) was effectively reduced in the presence of HEX (**Figure 18B**) and TTX (**Figure 18C**). There was a significant decrease in colon movement when either HEX or TTX was applied (vehicle: 79.76 ± 18.31 µm vs. HEX: 14.89 ± 7.14 µm; $p = 0.03$ and TTX: 12.17 ± 3.88 µm; $p = 0.02$; one-way ANOVA; **Figure 18D**), confirming that blue light-evoked colon movement observed in Vil-ChR2 mice is neuronally-mediated.

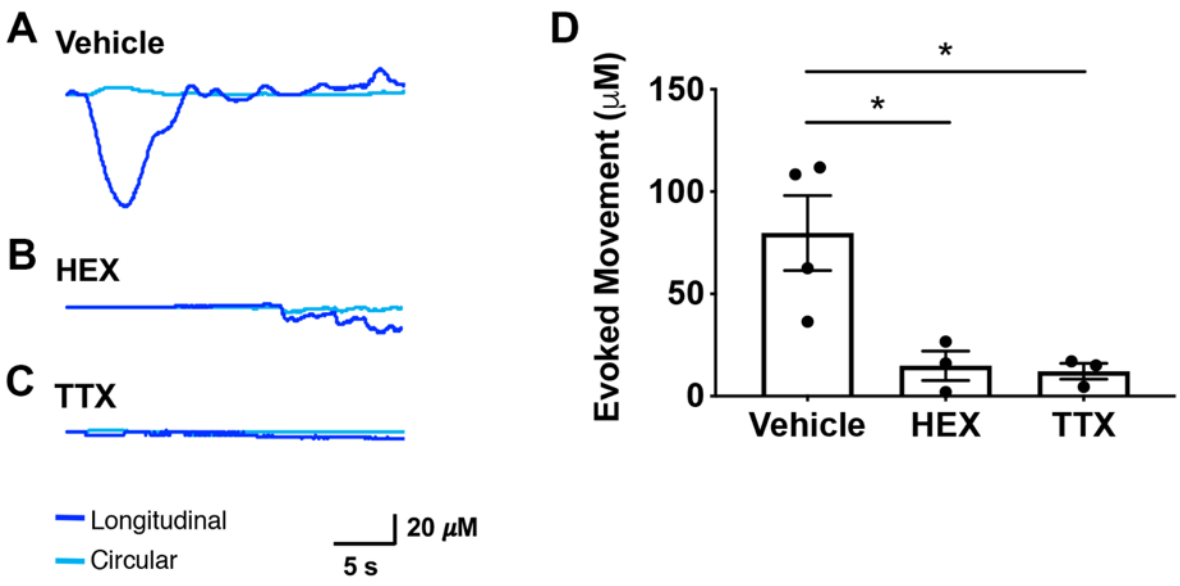


Figure 18. Epithelium-induced motility is neuronally mediated

Epithelial-evoked movement of the colon (A) was effectively reduced by the presence of HEX (B) and TTX (C). (D) There was a significant decrease in colon movement when either HEX or TTX was applied (n = 4; HEX: p = 0.03 and TTX: p = 0.02; one-way ANOVA), confirming that blue light-evoked colon movement observed in Vil-ChR2 mice is neuronally-mediated.

4.3.4 ATP mediates epithelium-ENS interactions

Pharmacological approaches were applied to begin to define the mechanism by which the epithelium initiates colon motility. Two candidate neurotransmitters were tested: 5-HT and ATP, both important signaling molecules in the ENS. A cocktail of 5-HT receptor antagonists (alosetron, ketanserin, and GR113808) was applied to the bath and local motility was assessed. Tissue movement evoked by blue light and spontaneous colon movement in the presence of the antagonists were recorded. The total spontaneous movement (combined x- and y-directions) did not change with the addition of 5-HT receptor antagonists (vehicle: 13.95 ± 3.95 μm vs. 5-HT receptor antagonists: 14.04 ± 5 μm; p = 0.99; paired t-test; **Figure 19A**). Example traces show

similar spontaneous movement in the presence of vehicle (**Figure 19B$_{i}$**) and 5-HT receptor antagonists (**Figure 19B$_{ii}$**). Surprisingly, the blue light-evoked movement also did not change after the addition of 5-HT receptor antagonists (vehicle: 23.4 ± 5.41 µm vs. 19.57 ± 2.77 µm; p = 0.48; paired t-test; **Figure 19C**). Example traces show similar evoked movement in the presence of vehicle (**Figure 19D$_{i}$**) and 5-HT receptor antagonists (**Figure 19D$_{ii}$**). However, the ATP receptor antagonist cocktail (TNP-ATP, 5-BDBD, MRS 2500, AR-C118925XX, and MRS 2578) did reduce local motility. Spontaneous movement significantly decreased after addition of ATP receptor antagonists (vehicle: 24.81 ± 3.73 µm vs. ATP receptor antagonists: 11.87 ± 0.75 µm; p = 0.05; paired t-test; **Figure 19E**). Example traces show spontaneous movement with vehicle treatment (**Figure 19F$_{i}$**) that is decreased in the presence of ATP receptor antagonists (**Figure 19F$_{ii}$**). Evoked movement was also significantly reduced (vehicle: 42.37 ± 10.48 µm vs. ATP receptor antagonists: 7.53 ± 1.86 µm; p = 0.03; paired t-test; **Figure 19G**). Example traces show evoked movement with vehicle treatment (**Figure 19H$_{i}$**) that is decreased in the presence of ATP receptor antagonists (**Figure 19H$_{ii}$**).

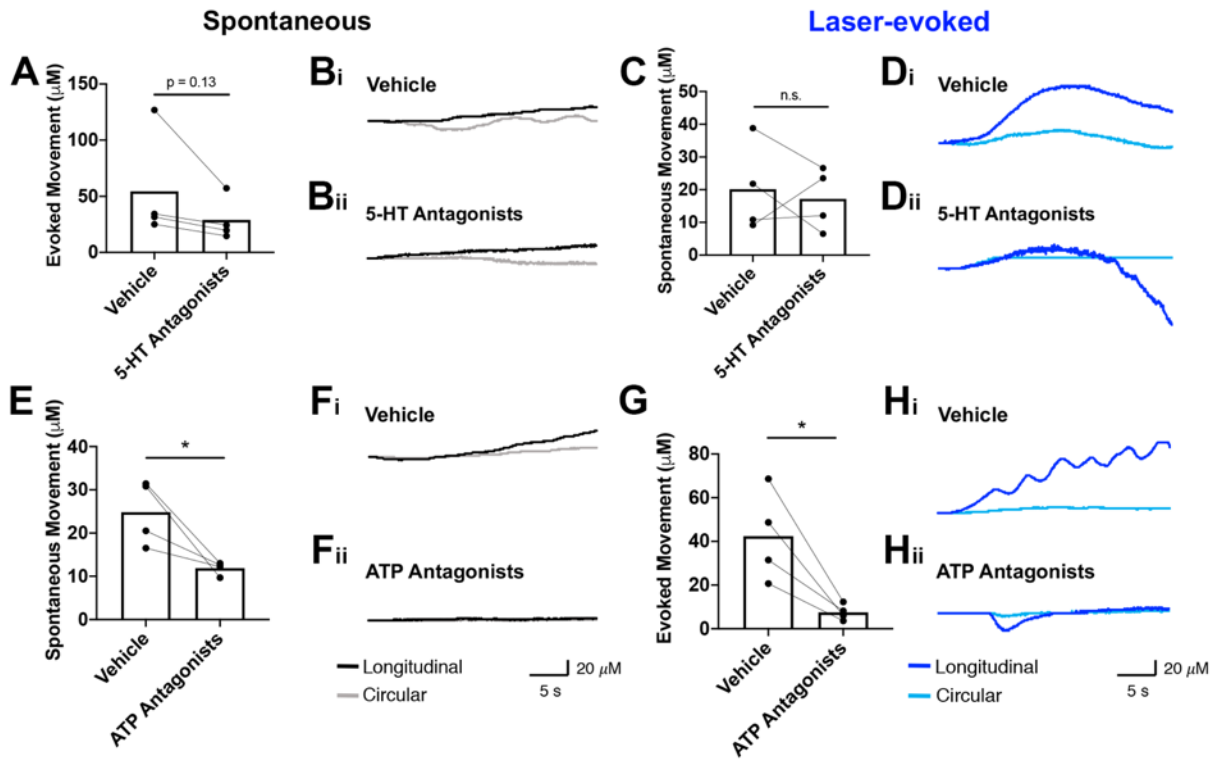


Figure 19. ATP mediates epithelium-ENS interactions

(A) Total spontaneous movement (combined x- and y-directions) did not change with the addition of 5-HT receptor antagonists (n = 4; p = 0.99; paired t-test). Example traces show similar spontaneous movement in the presence of vehicle (Bi) and 5-HT receptor antagonists (Bii). (C) The blue light-evoked movement also did not change after the addition of 5-HT receptor antagonists (p = 0.48; paired t-test). Example traces show similar evoked movement in the presence of vehicle (Di) and 5-HT receptor antagonists (Dii). (E) Spontaneous movement significantly decreased after addition of ATP receptor antagonists (p = 0.05; paired t-test). Example traces show spontaneous movement with vehicle treatment (Fi) that is decreased in the presence of ATP receptor antagonists (Fii). (G) Evoked movement was also significantly reduced with ATP receptor antagonists (p = 0.03; paired t-test). Example traces show evoked movement with vehicle treatment (Hi) that is decreased in the presence of ATP receptor antagonists (Hii).

4.3.5 Optogenetic activation of the epithelium facilitates colonic migrating motor complexes (CMMC)

Given the impact of epithelial activation on local motility in the colon, we next tested whether activation of the epithelium could influence whole-colon motility patterns. Colonic migrating motor complexes (CMMCs) are rhythmic propulsive contractions mediated by enteric neurons that normally occur every 3-5 min. Video recordings captured CMMCs at spontaneous baseline levels and with the application of blue laser to the whole colon, 1 cm from the anus (**Figure 20A**). Laser stimulation occurred every 2 min for 20 s and changes in CMMC rate were quantified in Vil-ChR2 (n = 10) and control littermate mice (n = 10). There was a significant interaction between genotype and laser in these experiments ($p = 0.02$; two-way ANOVA). Post-hoc analysis revealed that blue light activation of the epithelium increased the rate of CMMCs in Vil-ChR2 mice (baseline CMMC rate: 0.22 ± 0.02 per min vs. CMMC rate with laser: 0.32 ± 0.03 per min; $p = 0.001$; Sidak's test; **Figure 20B**). The rate of CMMCs in the control mice did not change (baseline CMMC rate: 0.25 ± 0.02 per min vs. CMMC rate with laser: 0.26 ± 0.02 per min; $p = 0.88$; Sidak's test).

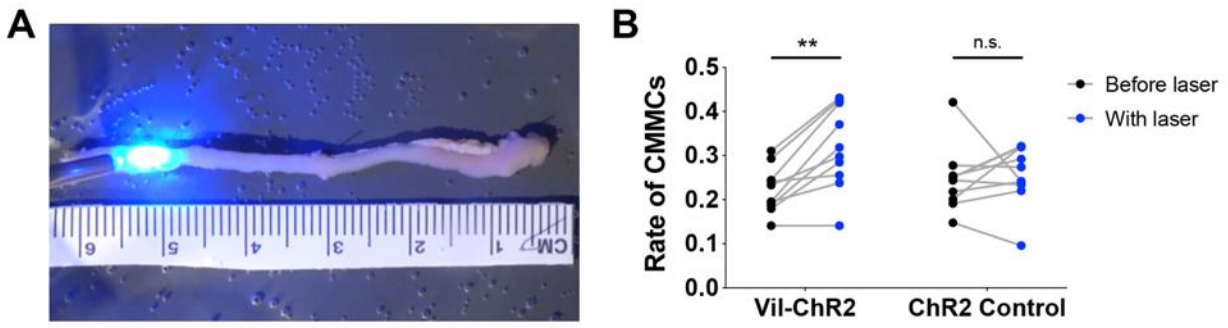


Figure 20. Optogenetic activation of the epithelium facilitates CMMCs

(A) Video recordings captured CMMCs at spontaneous baseline levels and with the application of blue laser to the whole colon, 1 cm from the anus. Changes in CMMC rate were quantified in Vil-ChR2 (n = 10) and control littermate mice (n = 10). (B) There was a significant interaction between genotype and laser in these experiments (p = 0.02; two-way ANOVA). Blue light activation of the epithelium increased the rate of CMMCs in Vil-ChR2 mice (p = 0.001; Sidak's test). The rate of CMMCs in the control mice did not change (p = 0.88; Sidak's test).

4.4 Discussion

The goal of this study was to investigate the role of the epithelium in colon motility. We employed optogenetic stimulation of epithelial cells coupled with optical measurement of neuronal activity, establishing this study as the first to interrogate ENS circuits using all optical techniques. The ability to selectively activate epithelial cells and measure activity in the colon allowed an examination of how the epithelium communicates with myenteric neurons to influence colon motility. Given that epithelial cells can release neurotransmitters, we predicted that optogenetic activation of colon epithelial cells would evoke measurable responses in myenteric neurons. Results indicate that epithelial activation initiated Ca^{2+} activity in myenteric neurons in the distal colon and produced a functional outcome, evidenced by local colon motility. Our studies also

suggest that ATP, and possibly 5-HT, underlie this local epithelial-evoked movement. In full-length, closed colon preparations, epithelial activation in the distal colon increased the rate of CMMCs initiated in the proximal colon, indicating that the colon epithelium has potential to influence large-scale coordination of ENS activity.

Studies have suggested that colon epithelial cells have reciprocal interactions with myenteric neurons (Walsh & Zemper, 2019). However, it has been a challenge to record activity from myenteric neurons and simultaneously activate the epithelium. For example, electrophysiological recording in the myenteric plexus *in situ* requires removal of the epithelium and submucosal layer (Bertrand, 2003). Recent studies have taken advantage of genetically encoded calcium indicators (GECI) to measure neuronal activity in the ENS in intact preparations (Hennig et al., 2015; Koussoulas, Swaminathan, Fung, Bornstein, & Foong, 2018; Li et al., 2019; Swaminathan, Hill-Yardin, Bornstein, & Foong, 2019). Although GECIs do not provide the same single-spike resolution as intracellular recordings, they act as a proxy for neuronal activity that enables monitoring of activity in peripheral neurons *in situ* (Akerboom et al., 2013; T. W. Chen et al., 2013; Smith-Edwards, DeBerry, Saloman, Davis, & Woodbury, 2016; Tian, Hires, & Looger, 2012). Green fluorescent GECIs, such as GCaMP, are more commonly used to measure neuronal activity but could not be used in this study because the blue excitation light for these GECIs also activates ChR2. Red-shifted GECIs require a green excitation light, enabling us to image the myenteric plexus while using blue light to activate ChR2-expressing epithelial cells. Unfortunately, red-shifted GECIs, including jRCaMP1b and jRGECO1a used here, are not as sensitive to Ca^{2+} compared to GCaMP6s (Dana et al., 2016), but are sufficient to report spontaneous activity and evoked responses in many myenteric neurons, albeit at lower numbers (Smith-Edwards et al., 2019). Therefore, it is likely that the neural activation observed in response

to laser stimulation of the epithelium is an underrepresentation. Future studies should determine whether the combination of red-shifted ChR2 and GCaMP6s (the reverse of what was used here) is more useful for investigating intercellular connectivity.

Our imaging parameters enabled measurement of neural activity in addition to localized colon tissue movement, which we found was reliably evoked with blue light stimulation of the epithelium. As previous studies have shown, these motility changes are likely a result of epithelial cells signaling to IPANs that have nerve terminals in the mucosa and cell bodies in the myenteric plexus (Costa et al., 2000) and form synaptic connections with interneurons and motor neurons (Smolilo et al., 2020). Compared to direct electrical stimulation of ENS circuits, the latency of neural activation and movement evoked by laser stimulation was longer. This longer latency is likely explained by the release of neuroactive chemicals from the epithelium which then have to reach IPAN nerve terminals before engaging the sensory-motor reflex. This compares to our previous studies where ChR2-mediated activation of the colon epithelium initiated action potential (AP) firing in extrinsic primary afferent neurons (ExPANs). The latency between epithelial stimulation and AP firing (mean 15 s) was significantly longer than the latency to AP firing when the ExPANs were directly activated (mean 0.34 s) (Chapter 1).

We sought to determine the neuroactive chemicals mediating epithelial-myenteric neuron communication. Previous studies have suggested that 5-HT released by enterochromaffin (EC) cells in the colon has a critical role in peristalsis and generation of CMMCs (Heredia et al., 2009; Heredia et al., 2013; Jin, Foxx-Orenstein, & Grider, 1999). Other studies indicate that epithelial-released ATP acts on IPANs in the colon (Bertrand, 2003; Bertrand & Bornstein, 2002; Burnstock, 2014). We used antagonist cocktails for both 5-HT and ATP, blocking the receptors for these neurotransmitters present on enteric neurons. Blocking ATP receptors almost completely

abolished the local epithelial-evoked colon movement and while blocking 5-HT receptors also reduced the evoked movement, there was only a trend toward significance. This result was surprising, given that studies have long suggested that release of 5-HT from the mucosa initates sensory-motor reflexes in the colon to produce motility (Grider et al., 1996). However, the laser-evoked movement was not robust to begin with in experiments using 5-HT receptor blockers, possibly explaining the lack of significant effect. It is also possible that addition of the 5-HT antagonists to the bath prevented epithelial-evoked colon motility patterns that were not captured in our imaging studies, which only recorded local movements in a 30 s timeframe. The release of 5-HT may be more important in regulating CMMCs, which occur on the order of minutes, than in mediating these localized movements occurring only seconds after epithelial stimulation.

The ability of ATP antagonists to block the fast, local contractions suggests that ATP has more rapid effects in activating IPAN nerve terminals. Studies have shown that IPANs display low levels of activity even in the absence of applied stimuli and these activity levels are diminished without the mucosa intact (Kunze, Bertrand, Furness, & Bornstein, 1997). This observation combined with the present study suggests that there is ongoing purinergic signaling from the epithelium to surrounding IPANs, which may contribute to spontaneous movements of the colon. A major caveat to these interpretations is that ATP signaling also occurs between ENS neurons in descending pathways (Burnstock, 2014) and in neuron-glia communication (Gulbransen & Sharkey, 2009), so the use of these antagonists may have blocked colon motility independent of epithelial signaling.

This study demonstrates the potential for using optical techniques to interrogate intercellular communication between neuronal and non-neuronal cell types in the colon. Optogenetic activation of colon epithelial cells revealed their role in ENS neuron activation and

colon motility, but we were not able to parse out the effects of different epithelial cell types in this study, as ChR2 is ubiquitously expression in the intestinal epithelium under the villin promoter. Future studies will target specific activation to enterochromaffin cells as well as other subpopulations of EECs, as the neurotransmitters released from these cells may have the most direct input to surrounding neurons. Other non-neuronal cell types that are involved in ENS function include enteric glia, immune cells, and interstitial cells of Cajal (Furness, 2012). Optical techniques can be employed to better understand how these different cell types work together to maintain colon health and homeostasis.

5.0 Discussion

The experiments in this dissertation were inspired by the intriguing idea that neurons are not solely responsible for conveying sensory information from peripheral tissues. Non-neuronal cells of target tissues also have a significant role in sensory signaling, as studies from the skin and bladder epithelium have shown (Baumbauer et al., 2015; Maksimovic et al., 2014; Pang et al., 2015; Winder et al., 2014). In the colon, epithelial lining cells express many of the membrane receptors involved in detection of mechanical and chemical stimuli found in sensory neurons, suggesting a direct role in monitoring the contents of the gut lumen (see **Figure 1**). As they also release neuroactivators such as ATP, 5-HT and glutamate, they have long been implicated as modulators of neural activity but inconclusively shown to act in a direct manner. My work has addressed this challenging question. Chapter 1 details how optogenetic activation of the colon epithelium initiates action potential firing in extrinsic primary afferent neurons (ExPANs), which send sensory information from the colon to neurons in the central nervous system. Visceromotor response (VMR) experiments showed that this activation results in nociceptive responses *in vivo*. In Chapter 2, I have shown that optogenetic inhibition of the colon epithelium attenuated these nociceptive responses and that inhibition of epithelial cells is effective in reducing inflammation-induced visceral hypersensitivity.

These experiments were also inspired by growing interest in the gut-brain axis. Not only are disorders of the gut such as IBS and IBD prevalent, but there is increasing evidence that gut-brain interactions have a role in "non-GI" diseases. These include depression, autism, diabetes, and Parkinson's, suggesting that colon health has an important role for whole-body homeostasis (Bessac et al., 2018; Chalazonitis & Rao, 2018; Dinan & Cryan, 2017). To understand these gut-

brain connections, it is critical to mechanistically define how the gut detects sensory stimuli, transmits these signals to the ENS and CNS and in so doing, maintains proper gut motility. The epithelium is at the forefront of this sensory cascade, as it interacts with all luminal contents, which includes the microorganisms comprising the gut microbiome (Dinan & Cryan, 2017). Towards understanding this complex interaction, Chapter 3 describes studies that show that activation of the epithelium can lead to activation of ENS neurons and in so doing, initiate colon motility.

5.1 Role of the Colon Epithelium in Visceral Nociception

Studies of Chapter 1 showed that optogenetic activation of the colon epithelium in villin-ChR2 mice engages neural circuitry involved in conveying sensory information from the colon to the central nervous system. Studies performed in an *ex vivo* colon-nerve preparation show that ChR2-mediated activation of the epithelium initiates action potential (AP) firing in colon ExPANs. This ExPAN activity had a functional result, as epithelial stimulation also resulted in visceral nociceptive responses *in vivo*. The neuronal responses to light stimulation of epithelial cells could be blocked with ATP (purinergic) receptor antagonists, in most ExPANs that were interrogated.

ExPAN responses to epithelial activation were similar to those elicited by mechanical stimulation (probing, brushing, and stretching of the colon tissue). They were also comparable to blue light-mediated responses when ChR2 was expressed in the ExPANs themselves (under the TRPV1 promoter). However, there was a latency between ChR2-mediated epithelial activation and ExPAN firing, ranging from 0.03 – 60 s, suggesting that epithelial cells could communicate with ExPANs via several mechanisms. Transmission from epithelial cells may be diffuse and have slow and indirect action on colon ExPAN terminals. Long latencies may be explained by the

location of the ExPAN terminal, i.e., ExPANS with nerve terminals in the serosal layer (distant from the mucosa) may have a longer latency to epithelial-induced firing than those with terminals in the submucosal layer. Anatomical locations of functionally classified ExPANs (mucosal, muscular, muscular/mucosal, serosal) have not been totally defined (Brierley et al., 2018). Thus, it is difficult at this time to define mechanistic links between epithelial cells and AP firing in specific ExPAN types. Intermediary cell types, such as intrinsic neurons and immune cells, may also have a role where neurotransmitters released from the epithelium may act on ExPANs, which could explain the long latencies.

On the other hand, some epithelial-induced ExPAN responses may have been mediated through a more direct connection. Studies show that communication from enteroendocrine cells (EECs) in the epithelium to colon ExPANs may be achieved through direct synaptic transmission (Kaelberer et al., 2018), which may have been reflected in our studies, particularly when ExPAN latency to firing was in the millisecond time range. EECs in the colon are rare, comprising only 1% of the total epithelial cell population (Bertrand, 2009). Thus, it is possible that the varied ExPAN firing latencies seen in our studies were a reflection of the composition of epithelial cells in a given receptive field. Shorter latencies may have been due to the percentage of EECs in the blue light-activated field, whereas longer latencies were due to fields with enriched enterocytes, which likely have a modulatory role in ExPAN activity than a direct role. This variability was also reflected in the behavioral experiments, where optogenetic activation of the epithelium did not always elicit a VMR, e.g., the response rate in Vil-ChR2 mice was 78%, compared to nearly 100% in TRPV1-ChR2 mice.

The longer latencies in most cases of epithelial-induced ExPAN firing also suggested involvement of a chemical mediator. We found that addition of ATP receptor antagonists to the

colon-nerve prep blocked ExPAN responses to epithelial stimulation in most cases. This finding supports the purinergic hypothesis proposed in previous studies, in which intestinal epithelial cells release endogenous ATP in response to distension stimuli in the colon, which then acts on P2X and P2Y receptors on ExPAN terminals (Burnstock, 1999, 2001, 2013; Wynn et al., 2004; Wynn et al., 2003). These studies used non-specific activation of epithelial cells, however, since colon distension activates all mechanosensitive cells outside of the epithelium as well. Our study enabled activation of epithelial cells in the absence of any mechanical or chemical stimuli, confirming the ability of epithelial-derived ATP to activate ExPANs.

To summarize Chapter 2, activating the epithelium alone, without addition of mechanical stimuli, initiated ExPAN firing in an ATP-dependent manner. These findings suggest that epithelial cells have a significant influence on the activity of colon ExPANs and resulting nociceptive signaling. With evidence of a direct role in pain signaling, it is also possible that the epithelium contributes to the ExPAN sensitization involved in visceral hypersensitivity. In Chapter 3, I hypothesized that inhibition of the colon epithelium would attenuate nociceptive signaling and inflammatory hypersensitivity.

In studies of Chapter 3, I used the villin gene promoter to target the inhibitory yellow light-activated archaerhodopsin (Arch) protein to colon epithelial cells. I used an established VMR protocol to assess colon sensitivity to a noxious distension stimulus (60 mmHg balloon distension) with and without yellow light illumination of the colon lumen. Arch-mediated inhibition of colon epithelial cells diminished visceral nociceptive responses in healthy mice. Yellow light was effective in approximately 50% of distension + laser trials, and in the trials that produced an effect, light-mediated inhibition of the epithelium diminished VMRs to distension by over 60%. With directly inhibition of ExPANs (in TRPV1-Arch animals), yellow light was effective in reducing

the VMR by nearly 85%. Of note, TRPV1-Arch animals tended to show more consistent responses to yellow light (VMR was inhibited in an average of 75% of trials) compared to the epithelium. The inconsistent efficacy of inhibiting the epithelium further suggests a modulatory role for the epithelium in nociceptive signaling.

After testing the effects of yellow light-mediated epithelial inhibition in healthy mice, I then investigated the effects of epithelial and ExPAN inhibition in the dextran sulfate sodium (DSS) model of inflammation, which is commonly used in mouse studies to mimic inflammatory bowel disease. DSS induced marked visceral hypersensitivity, which was effectively reduced by light-mediated inhibition of the epithelium. This inhibition was not as effective in the inflamed colon however; in naïve mice, Arch-mediated epithelial inhibition reduced the VMR by 80%, whereas in DSS-treated mice, it reduced the VMR by 50%. Arch-mediated inhibition of ExPANs in the DSS-treated mice was also effective at reducing hypersensitivity, to a similar extent as epithelial inhibition. The ability of epithelial inhibition to reduce nociceptive responses and visceral hypersensitivity suggest the epithelial lining is an important component of nociceptive signaling pathways.

5.1.1 Experimental Limitations

The optogenetic techniques employed in the described studies enabled specific activation and inhibition of the colon epithelium and colon ExPANs. It has historically been difficult to investigate the epithelial contribution to neuronal activity due to the intimate association of nerve terminals and the epithelium. For example, addition of chemical or mechanical stimulation to the colon stimultaneously activates the epithelium and nerves, making the relative contribution of each cell type undecipherable. Light-mediated activation and inhibition of the colon epithelium was

enabled by opsin gene expression under regulation of the mouse villin gene promoter. In this system ChR2 or Arch is expressed in all colon epithelial cell types. These diverse cell types likely have different roles in sensory signaling. The most obvious example is that EECs are electrically excitable and can form direct synaptic connections with surrounding neurons. In contrast, while enterocytes can release ATP and other molecules relevant to sensory signaling, they likely do so in a slower manner, having more of a modulatory influence on surrounding neurons. Use of the villin-Cre driver to target ChR2 and Arch did not allow a discrimination of which epithelial subpopulation mediated the effects of light stimulation. Another consideration is that the TRPV1-cre driver used to express ChR2 and Arch in colon ExPANs is not entirely specific to TRPV1-expressing neurons in the adult mouse. TRPV1 is transiently expressed in many developing neurons and becomes restricted to a smaller population in the adult mouse. Use of TRPV1-cre therefore targets neurons outside of the adult TRPV1 population (Cavanaugh, Chesler, Braz, et al., 2011; Hjerling-Leffler, Alqatari, Ernfors, & Koltzenburg, 2007). This is likely a minor issue however, since the vast majority of colon ExPANs in the adult mouse express TRPV1 (S. A. Malin et al., 2009).

There are additional experimental limitations concerning the use of optogenetics. One consideration is that ChR2 is a large protein and it is not completely understood how expression of the protein itself can affect normal cellular processes. It is also unclear how light activation of opsin expressing cells can affect their function; for example, it is possible that cells inhibited via Arch activation could produce an exaggerated rebound response after removal of yellow light, meaning some results could be caused by excitation, rather than inhibition of those cells (Bernard, 2020). Lastly, there could be unexpected consequences of light stimulation itself, such rises in temperature, which could affect cell functions (e.g., enzymatic reactions) (Tyssowski & Gray,

2019). Although we did not detect any broad temperature changes in colon tissue, there could have been smaller, more localized changes that can affect cellular functions.

Another experimental limitation is the use of optogenetics in non-neuronal cells. Colon epithelial cells, with the exception of EECs, are not electrically excitable, making it difficult to determine the effect of opsin activation on their endogenous signaling. For example, it has been determined that ChR2 activation in neurons depolarizes the membrane and causes activation of voltage gated Na^+ channels and action potential firing. Although ChR2-mediated membrane depolarization does occur in non-neuronal cells like keratinocytes and colon epithelium, the effects of this depolarization have not been defined (Baumbauer et al., 2015; Moehring et al., 2018). Based on our pharmacological studies, ATP is a possible factor released in response to ChR2-mediated depolarization. However, numerous attempts to obtain consistent measure of ATP in colon cells activated by light stimuli have been exceptionally difficult. Similarly, opsin-mediated inhibition is likely not the same in neuronal and non-neuronal cells. For example, Arch expression in neurons enables membrane hyperpolarization and prevents AP firing (Chow et al., 2010). The effect of hyperpolarization in an epithelial cell very likely has a different effect than Arch-mediated inhibition of neurons.

A final experimental consideration is the variability of the VMR experimental assay, where measures obtained using simultaneous balloon distension and yellow light stimulation proved to be inconsistent (Chapter 2). A likely reason for this inconsistency is the presence of luminal contents that could block or reduce the power of blue or yellow light reaching the epithelium. This illustrates an issue that will need to be addressed if opsins are to be a therapeutic possibility for treatment of diseases of the gut.

5.1.2 Future Directions

Data discussed in the previous sections support the hypothesis that the combination of all epithelial cell types is necessary for normal sensation in the colon, and also highlight the need for further studies. To determine the contribution of each cell type to sensory signaling, it will be imperative to develop cell type-specific manipulation paradigms applicable to colon physiology, for instance via optogenetics and chemogenetics. The most obvious cellular targets that should be interrogated are EECs, since they can form synapses with surrounding neurons and are electrically excitable (Alcaino et al., 2018; F. Wang et al., 2017). Enterochromaffin cells (ECs) are one type of EEC that show evidence of these synapses. Importantly, they express the receptors TRPA1 and Piezo2, which are critical in mechanosensation and pain signaling (Alcaino et al., 2018; Bellono et al., 2017). This receptor expression profile and connectivity with surrounding colon ExPANs may indicate that ECs have a more salient role in sensory signaling than other cell types. As such, it will be important to determine which functional classes of ExPANs most frequently synapse with EECs. Additionally, towards the goal of determining which colon epithelial cell types mediate visceral pain signaling, comprehensive studies are needed to identify all sensory receptors expressed in each cell type, similar to the single-cell RNAseq analysis of small intestine epithelium (Haber et al., 2017).

Studies in Chapter 1 demonstrated the role of ATP in colon epithelial-neuronal signaling, but purinergic receptor antagonists did not completely block the epithelial-induced ExPAN firing, indicating involvement of other neurotransmitters. Additionally, these studies employed antagonist cocktails for P2X and P2Y receptors expressed on ExPANs, falling short of defining purinergic receptors most critical to epithelial-ExPAN signaling. Future studies should examine the role of other epithelial-released molecules, such as neuropeptides, in modulation of colon

ExPAN activity. Functional analysis of specific colon ExPAN receptors is also needed to determine which receptors are important in epithelial-neural signaling. An obvious candidate is 5-HT, as studies have begun to show with specificity that 5-HT released from ECs can act on 5-HT_3 receptors expressed on ExPANs (Bellono et al., 2017). However, these studies did not include a behavioral assay to confirm the role of EC-ExPAN interactions in visceral nociception.

Lastly, different optogenetic techniques should be employed to circumvent the issue of luminal contents in the colon blocking laser access to target cells. One tool that should be tested is a wireless cuff that can locally deliver laser stimuli by wrapping around the outside of the organ (Mickle & Gereau, 2018; Samineni et al., 2017). This local laser modulation of sensory signaling could be effective since it is apparent from colon motility studies that light stimulation can penetrate the colon wall and reach the epithelium (Hibberd, Feng, et al., 2018). If optogenetics actually become a viable therapeutic option, light will be most likely be delivered via small electrodes rather than a long fiber optic. Thus, it is important to begin validation of these wireless cuffs as tools for regulation of colon function.

5.2 Role of the Colon Epithelium in Gut Motility

After demonstrating that the colon epithelium initiates activity in ExPANs, I sought to examine the role of the epithelium in intrinsic sensory signaling. Intrinsic primary afferent neurons (IPANs) of the ENS terminate in the mucosal layer and are responsible for initiating ENS motor reflexes in response to luminal stimuli (Furness, Robbins, et al., 2004). Although these nerve endings are closely associated with the epithelium (see **Figure 2**), it is unclear how essential colon epithelial cells are in conveying sensory information to initiate these reflexes. In the experiments

in Chapter 4, I employed optogenetic techniques to enable ChR2-mediated activation of colon epithelial cells and monitored myenteric neuronal activity using genetically encoded Ca^{2+} indicators. Given their ability to release neurotransmitters, I predicted that optogenetic activation of colon epithelial cells would result in measurable responses in myenteric neurons.

Results showed that colon epithelial cells induce neuronally-mediated changes in gut motility, most likely through purinergic signaling. Epithelial stimulation initiated Ca^{2+} activity in myenteric neurons in the distal colon, which was strong enough to produce a functional outcome, evidenced by local colon motility. My studies suggest that ATP, and possibly 5-HT, underlie the local epithelial-evoked movement we observed. Additionally, in full-length, closed colon preparations, epithelial activation in the distal colon increased the rate of rhythmic colonic migrating motor complexes (CMMCs) initiated in the proximal colon, indicating that the colon epithelium has the potential to influence large-scale coordination of ENS activity.

These epithelial-ENS communication studies displayed several parallels to the discoveries from Chapter 1. Importantly, there was a latency between epithelial activation and myenteric neuronal responses, likely explained by the release of neuroactive chemicals from the epithelium which then have to reach IPAN nerve terminals. The latency to response ranged from 2 – 16 s and the average latency was 6.5 s. As expected, this latency was longer than localized electrical stimulation of the colon (average 0.13 s). The latency between epithelial activation and colon movement was slightly longer (average 7.7 s), indicating that myenteric neuron activation preceded colon movement. This suggests that the epithelium is directly involved in initiating sensory-motor reflexes in the ENS. In the epithelial-ExPAN studies in Chapter 1, the ExPANs also displayed a latency to response after epithelial activation, averaging around 15 s. Although a different technique was used to record ExPAN activation (extracellular teased-fiber recordings), it

is still evident that these average latencies were longer than myenteric activation. This could indicate that epithelial-ExPAN communication relies on intermediate cell types; it is possible that ExPAN responses are due to local changes in motility induced by the epithelial-initiated sensory-motor response. In Chapter 1, the functional consequence of ExPAN activation was the nociceptive responses observed in VMR experiments. The latency to VMR in response to blue light illumination of the colon lumen was actually shorter than the average latency to ExPAN firing (average of only 8 s). However, these experiments involved a wider distribution of laser stimulation, which may have simultaneously activated more ExPANs than the localized stimulation in the *ex vivo* prep, thus leading to a quicker response.

The studies in Chapters 1 and 3 indicate that epithelial-induced activation of the ENS usually occurs before ExPAN activation, suggesting that changes in gut motility can impact extrinsic sensory signaling from the colon to the CNS. However, ExPAN activation has reciprocal effects on the ENS. Another study from our lab showed that ExPAN activation initiates a spinal cord reflex where parasympathetic neurons are triggered to act on myenteric neurons and produce colon contractions (Smith-Edwards et al., 2019). This circuit demonstrates how visceral pain and colon motility are linked, which is important to understand since many disorders like IBS and IBS feature visceral pain as well as disordered colon motility. Taken together, these studies reveal the complexity of interactions between the colon epithelium and the intrinsic and extrinsic innervation of the colon.

5.2.1 Experimental Limitations

The use of optical techniques and imaging (ChR2 opsins and GECIs) in Chapter 4 enabled simultaneous activation of the colon epithelium and monitoring of neurons in the myenteric plexus.

It was previously a challenge to record activity of ENS neurons *in situ* during epithelial activation because electrophysiological recording in the myenteric plexus requires removal of the epithelium and submucosal layer (Bertrand, 2003). Although these optical tools provide unprecedented control of specific cell activation, there are a number of caveats, some of which were mentioned in 5.1.1 And as previously mentioned, the use of the villin promoter resulted in expression of ChR2 in all epithelial cell types, making it impossible for us to know which cell types were exerting the described actions on the ENS neurons.

Myenteric neuron activity was recorded using red-shifted GECIs and, as it is well-known, Ca^{2+} is only a proxy for neuronal activity. GECIs do not provide the same single-spike resolution as intracellular recordings, although they provide more accessible means to monitor entire populations of neurons *in situ* (Akerboom et al., 2013; T. W. Chen et al., 2013; Smith-Edwards et al., 2016; Tian et al., 2012). Green-fluorescent GECIs like GCaMP are more commonly used to measure neuronal activity but could not be used in this study because the blue excitation for green GECIs also activates ChR2. Red-shifted GECIs require a green excitation light, enabling us to image the myenteric plexus while using blue light to activate ChR2-expressing epithelial cells. Unfortunately, red-shifted GECIs, including the jRCaMP1b and jRGECO1a variants used here, are not as sensitive to Ca^{2+} compared to GCaMP6s (Dana et al., 2016). However their sensitivity is are sufficient to report spontaneous activity and evoked responses in many myenteric neurons, albeit at lower numbers (Smith-Edwards et al., 2019). With this caveat, it is likely that the neural activation observed in response to laser stimulation of the epithelium is an underrepresentation.

In addition to the limitations presented by the optical tools used in Chapter 4, the pharmacological methods used could be refined. Cocktails of ATP and 5-HT receptor antagonists were used to determine whether these neurotransmitters mediated the epithelial-myenteric neuron

communication. Although ATP receptor antagonists were able to block epithelial-induced local contractions, a major caveat is that ATP signaling also occurs between ENS neurons in descending pathways (Burnstock, 2014) and in neuron-glia communication (Gulbransen & Sharkey, 2009), so the use of these antagonists may have blocked colon motility independent of epithelial signaling.

5.2.2 Future Directions

Future experiments should employ optogenetic and/or chemogenetic techniques to define which specific epithelial cell types contribute to myenteric neuron activity and ENS reflexes. More defined optical techniques should also be used to specify which populations of myenteric neurons (IPANs, interneurons, or motor neurons) are being monitored in Ca^{2+} imaging studies. EC cells should be targeted first, as they are the main producer of 5-HT in the gut and it is hotly debated whether endogenous release from these cells is essential to colon motility. My results using 5-HT antagonists to block epithelial-induced local contractions were inconclusive and this may be due to the possibility that 5-HT may be more important in regulating CMMCs, which occur on the order of minutes, than in mediating localized colon contractions occurring only seconds after epithelial stimulation. Therefore, 5-HT antagonists should be utilized in whole-colon motility studies where ChR2-mediated activation of the distal colon epithelium. Further, more specific pharmacology should be used for ATP and 5-HT signaling to expand upon my results using antagonist cocktails.

Optical techniques for interrogating ENS circuits should continue to be developed. In these studies, red-shifted GECIs were expressed in ENS neurons to enable simultaneous blue light activation of ChR2, but these Ca^{2+} indicators are not as efficient as GCaMP6 (Smith-Edwards et al., 2019). GCaMP6 enabled imaging of robust spontaneous activity in the myenteric plexus,

whereas RCaMP and RGECO detected spontaneous activity in a much lower percentage of myenteric neurons. Therefore, it should be determined whether the combination of red-shifted ChR2 and GCaMP6s (the reverse of what is used in Chapter 4) is more useful for investigating intercellular activity. Electrophysiological studies should be performed in myenteric neurons to define how GCaMP6 and red-shifted GECIs approximate neuron AP firing. Additionally, optical tools can be used to measure interactions between other cells types and ENS neurons. Other non-neuronal cell types that are involved in ENS function include enteric glia, immune cells, and interstitial cells of Cajal (Furness, 2012; Smith-Edwards et al., 2019). Optical techniques can be employed to better understand how these different cell types work together to maintain colon health and homeostasis.

5.3 Colon Epithelial-Neuronal Signaling in Pathological Conditions

Acute and chronic inflammation likely affect epithelial-neuronal signaling in the colon, and more studies are required to examine how alterations at this interface affect disease processes and symptoms. One possibility is that, like neurons, epithelial cells become hypersensitive with inflammation, thus amplifying the sensory signaling pathways. This could occur via inflammation-induced changes in voltage-sensitive channels that regulate electrically excitable epithelial cell types (such as ECs) and/or via changes in proteins that regulate exocytosis/release of neuroactive substances. Another important consideration is whether these changes persist after turnover of the epithelium. Evidence has shown that the colon epithelium, including EECs, is replenished every 5 days (Tsubouchi & Leblond, 1979). However, a more recent study showed that a subpopulation of

EECs could persist for 60 days (Bohorquez et al., 2015). Studies of the bladder epithelium have shown that injury can evoke long-term changes in epithelial signaling properties (Gupta et al., 2009). It will be interesting to see whether this is true for different subtypes of colon epithelial cells. Some research shows that inflammation can affect the abundance and neurotransmitter content of EECs in the colon after inflammation, but more thorough analyses are required to assess changes at the cellular level. Using GCaMP imaging I have begun to assess changes in spontaneous Ca^{2+} activity induced by colon inflammation. Interestingly, significant increases in spontaneous Ca^{2+} activity are observed in the epithelium 1 week after DSS treatment. Future studies will assess evoked Ca^{2+} activity and how inflammation impacts specific epithelial cell types.

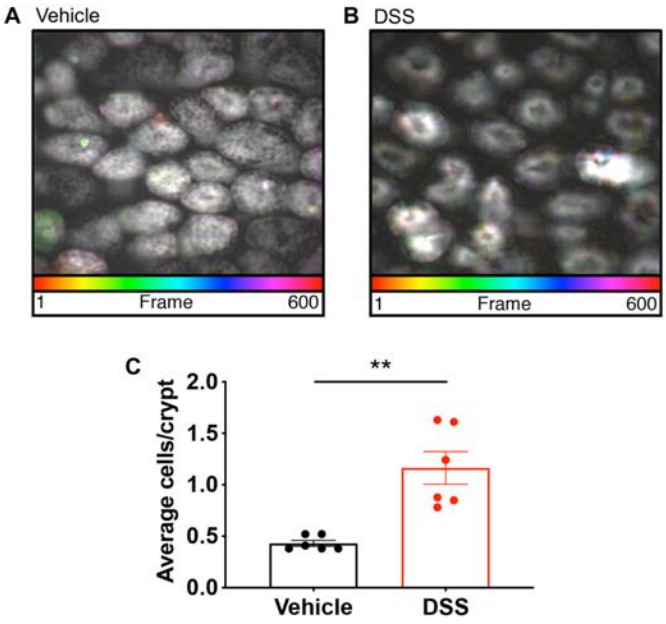


Figure 21. Spontaneous Ca2+ activity in the colon epithelium increases after DSS inflammation.

GCaMP6 was expressed in under the villin promoter to enable Ca^{2+} imaging of the colon epithelium. Mice were given vehicle or DSS treatment and preliminary studies show that spontaneous Ca^{2+} activity in vehicle-treated mice (A) was lower than DSS-treated mice (B), 7 days after DSS treatment. C) Spontaneous Ca^{2+} activity was quantified as active cells per crypt and significant increases were observed after DSS treatment.

In future studies of epithelial-neuronal communication, multiple animal models that elicit the hallmarks of colon diseases with different etiology should be employed (e.g., DSS or trinitrobenzene sulfonic acid (IBD), zymosan or repeated stress (IBS), and parasite infection models) to assess the full range of epithelial responses to different disease challenges. A thorough analysis of epithelial changes will require multimodal strategies including calcium imaging and electrophysiology techniques, high resolution anatomical analysis (e.g., 3D electron microscopic reconstruction) as well as neurotransmitter release assays. This comprehensive approach is required to fully understand the complexity of epithelial-nerve interactions. Considering the impact of epithelial-neuronal communication in nociceptive signaling and gut motility, studying these interactions will provide valuable insight into why visceral pain is often co-morbid with dysmotility.

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