ROLE OF ENTERIC GLIA IN MECHANISMS OF VISCERAL HYPERSENSITIVITY

By

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ABSTRACT

Abdominal pain is the most common gastrointestinal (GI) issue and the most debilitating feature of disorders of gut-brain interaction (DGBI) such as irritable bowel syndrome (IBS) and the inflammatory bowel diseases (IBDs). Abdominal pain is driven by mechanisms that lead to the sensitization of peripheral nerve fibers. However, very little is known about the specific mechanisms that sensitize nerve terminals in the intestine.

Nociceptors densely innervate enteric ganglia where they interact with enteric glia. Neuron-glia communication regulates gut reflexes in health and glia contribute to neuroinflammation by releasing mediators that contribute to enteric neuron death. Glial cells are key players in central pain pathways, but whether enteric glia fulfill similar functions during the development of visceral pain is unknown. We recently discovered that communication between nociceptors and enteric glia drives neuroinflammation in the intestine. Nociceptors release mediators that activate enteric glia and promote neuroinflammation by driving reactive gliosis. Enteric glia possess the ability to modulate afferent nociceptor signaling through the release of neuromodulators or through communication with other cell types like enteric neurons and immune cells. This dissertation aimed to define novel glial mechanisms that sensitize visceral afferent nerves during inflammation.

To address this question, we used in vivo and in vitro models of acute inflammation in combination with novel transgenic animal models, protein and RNA labeling, Ca2+ imaging techniques, chemogenetics, and visceromotor reflex recordings and assessed the contribution of enteric glia to nociceptive signaling in the myenteric plexus. We discovered that enteric glia sensitize TRPV1-nociceptor activity during acute inflammation, and they

do so through mechanisms that involve IL-1β and the connexin-43-dependent release of PGE2. In vivo, recordings show that colonic IL-1β shifts normal innocuous stimuli toward a noxious range through mechanisms that require glial Cx43, given that glial knock animals did not develop hypersensitivity. We further provide evidence that enteric glia modulate visceral hypersensitivity through mechanisms involving endocannabinoids. Specifically, the endocannabinoid hydrolyzing enzyme, monoacylglycerol lipase (MAGL), expressed by enteric glia, regulates neural-glial signaling in the myenteric plexus, and this effect is altered during inflammation. Visceral hypersensitivity induced in animal models of colitis is ameliorated by either the pharmacological inhibition or genetic ablation of MAGL. Our data also demonstrate that the glial-mediated mechanisms of visceral hypersensitivity during colitis are sexually dimorphic.

Our data provide new evidence for the active role of enteric glia in afferent signaling and visceral hypersensitivity. Specifically, we demonstrate that 1) glia-nociceptor signaling in the intestine is required for the sensitization of nociceptors and visceral hypersensitivity, 2) enteric glia can modulate nociceptor sensitivity directly through the release of pro-inflammatory neuromodulators like PGE2 3) or through regulation of endocannabinoid signaling via MAGL, and 4) the glial mechanisms involved in visceral hypersensitivity during acute inflammation are sex-dependent and are predominant in females. Results from these studies identify fundamental glial mechanisms that govern the activity of nociceptors in the intestine. Identifying the mechanisms contributing to the development of visceral pain will facilitate the development of new, more effective therapies for those suffering from chronic abdominal pain.

To my sister

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Chapter 1:

INTRODUCTION

OVERVIEW

The gut-brain axis modulates afferent information by integrating a series of ascending and descending pathways that include the enteric and central nervous systems, connecting the brain's emotional and cognitive centers to the intestine's peripheral functions (*1*). This connection ensures the gastrointestinal tract's homeostasis during physiological changes and adapts gastrointestinal (GI) function to the organism's overall state. Although the brain receives interoceptive information from the gut for autonomic reflex pathways, under pathological conditions such as GI diseases, this connection is disrupted, leading to abnormal perception, and heightened visceral sensitivity (*2*, *3*). Abdominal pain is a characteristic feature of GI pathology, and patients report it as the most debilitating symptom associated with the condition (*4*–*7*).

The molecular and neurobiological mechanisms that drive visceral pain in GI disorders are largely unresolved, but neural plasticity involving the sensitization of visceral afferent nerve fibers that project to the intestine is currently the most widely accepted mechanism (*1*). GI diseases often involve alterations within the enteric nervous system (ENS), which can be due to congenital causes, environmental effects, or a combination of factors (*8*, *9*). Inflammation plays a significant role in ENS neuronal damage and death (*10*–*12*), particularly in inflammatory bowel disease (IBD) (*13*) and disorders of gut-brain interaction (DGBI) such as irritable bowel syndrome (IBS) (*14*, *15*). Primary afferent neurons densely innervate the ENS (*16*) where they intermingle with enteric glia (*17*). Glial cells have recently been linked to the continuous proinflammatory environment surrounding sensory nerves that contribute to the development of chronic pain (*18*, *19*). Enteric glia release gliotransmitters that modulate gut reflexes, such as motility (*20*, *21*)

and secretions (*22*), and play important roles in neuronal activity and survival during health and disease (*11*, *20*, *23*, *24*). Additionally, enteric glia mediate intercellular signaling and neuroinflammation in the intestine (*10*, *11*) via bidirectional communication with nociceptors (*17*). Therefore, glial mechanisms likely regulate visceral hypersensitivity.

This dissertation investigates how enteric glial mechanisms modulate nociceptor activity in the gut and regulate visceral hypersensitivity with the goal of elucidating the pathophysiological mechanisms of chronic abdominal pain.

INNERVATION OF THE GI TRACT

The GI tract is a complex system that is innervated by intrinsic enteric neurons and extrinsic projections, including sympathetic and parasympathetic efferents as well as visceral afferents (**Figure 1.1**) (*25*). The relative roles of the enteric and central nervous systems differ considerably along the digestive tract, with the CNS primarily responsible for controlling movements of the striated muscle esophagus and monitoring the state of the stomach (*26*, *27*). In contrast, the ENS in the small intestine and colon contains full reflex circuits, including sensory neurons, interneurons, and motor neurons, through which muscle activity, fluid fluxes, and local blood flow are controlled (*28*). The complex interplay between the ENS, extrinsic innervation, and central control mechanisms allows the colon and rectum to regulate a wide range of digestive processes, while also integrating with higher brain centers to influence behavior and overall health.

The gut-brain axis plays a critical role in coordinating many of these gut functions, allowing communication from the gut to the brain via sensory signals transmitted by extrinsic sensory nerve fibers located within the wall of the colon or rectum. These signals

are transmitted via their axons within the spinal splanchnic and pelvic nerves to the spinal cord, where they can be processed and integrated with other sensory and motor inputs to generate appropriate behavioral responses (*29*, *30*). Additionally, the gut-brain axis involves top-down modulation of gut function by higher brain centers, such as through emotional and cognitive influences on digestive processes (*25*). The ENS forms a series of interconnected ganglia that can generate reflexive responses to sensory inputs, allowing the gut to operate largely autonomously, even in the absence of extrinsic neural input (*31*). The extrinsic innervation of the colon and rectum by sympathetic and parasympathetic fibers also plays an important role in regulating gut function, particularly in response to stress or other physiological challenges (*3*, *25*, *32*). Overall, the complex interplay between the ENS, extrinsic innervation, and central control mechanisms allows the colon and rectum to effectively regulate a wide range of digestive processes, while also integrating with higher brain centers to influence behavior and overall health via the gut-brain axis.

Chapter 1 aims to summarize the organization and function of neural innervation of the gut in health. How neuroplasticity in these networks contribute to disease and pain will be discussed in Chapter 2.

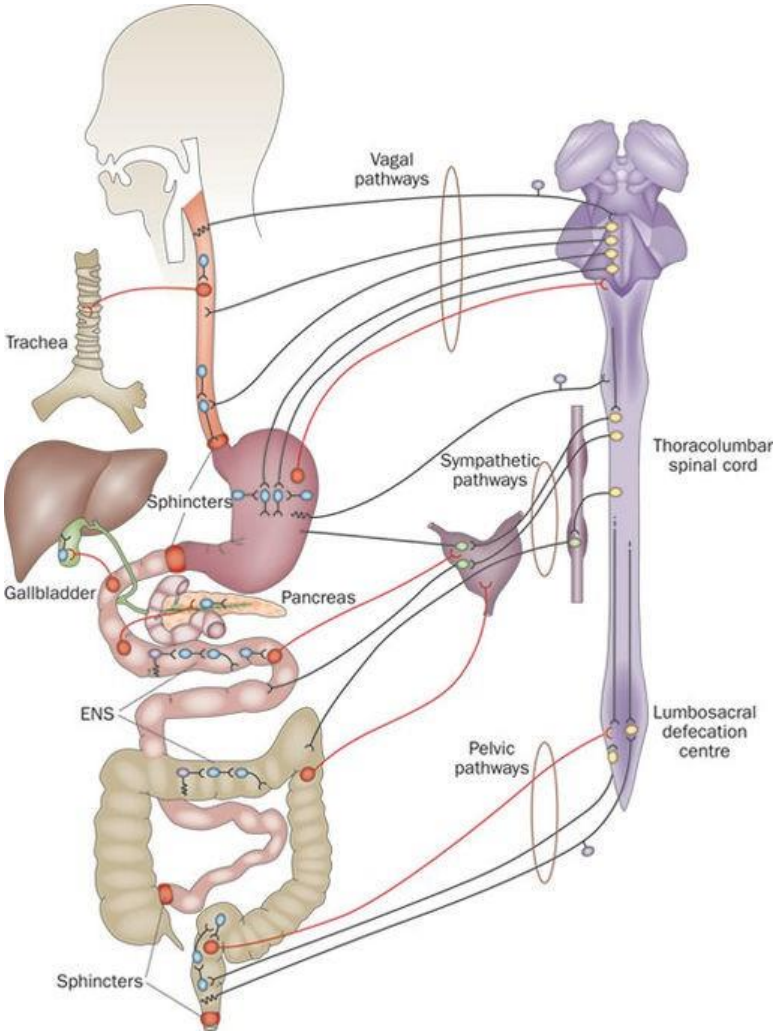

Figure 1.1: The gastrointestinal tract is innervated by extrinsic and intrinsic nerves. The GI tract has full reflex circuits of the ENS (motor neurons and interneurons in blue, sensory neurons in purple). Pathways from the gastrointestinal tract project outwards via intestinofugal neurons (red). Sensory information goes both to the ENS, through intrinsic primary afferent neurons and to the CNS through extrinsic sensory neurons (purple). Pathways from the CNS innervate the GI tract through vagal, thoracolumbar and pelvic pathways. Abbreviations: CNS, central nervous system, ENS, enteric nervous system. From Furness 2012 (*28*).

INTRINSIC INNERVATION OF THE GI TRACT

The GI system differs from other organ systems because it has an intrinsic nervous system that can mostly operate without input from the CNS (*25*, *31*). The ENS is responsible for local neural regulation of GI functions such as modulating gastric acid secretion, regulating fluid movement across the epithelium, determining motility patterns, changing local blood flow, and interacting with the gut's immune and endocrine systems (*28*, *31*). This intrinsic network comprises enteric nerve cell bodies of sensory, inter- and motor neurons grouped into ganglia and interconnected by bundles of nerve processes forming two plexuses, the myenteric plexus (Auerbach's plexus) and the submucosal plexus (Meissner's plexus; **Figure 1.2**). The ENS controls motility, mucosal secretion and absorption, mucosal growth, local blood flow and the immune function in the gut (*28*).

The myenteric plexus is a network of ganglia that contains around two-thirds of all enteric neurons and is located between the longitudinal and circular muscle layers of the gastrointestinal (GI) tract. It is responsible for controlling the smooth muscle contractions and relaxations that regulate GI motility throughout the entire GI tract. Changes to the myenteric plexus can lead to alterations in GI motility (*28*, *31*). In contrast, the submucosal plexus is situated in the submucosal space between the mucosal and circular muscle layers of the gut and controls local blood flow and luminal absorptions and secretions (*28*). This plexus is only present in the small and large intestine, with minimal innervation in the esophagus or stomach. The ganglia and interconnecting fibers in the submucosal plexus are smaller than those of the myenteric plexus, and in humans, the submucosal ganglia form two distinct but interconnected plexuses (*33*). Both plexuses consist of two types of cells: enteric neurons and enteric glial cells (**Figure 1.2**).

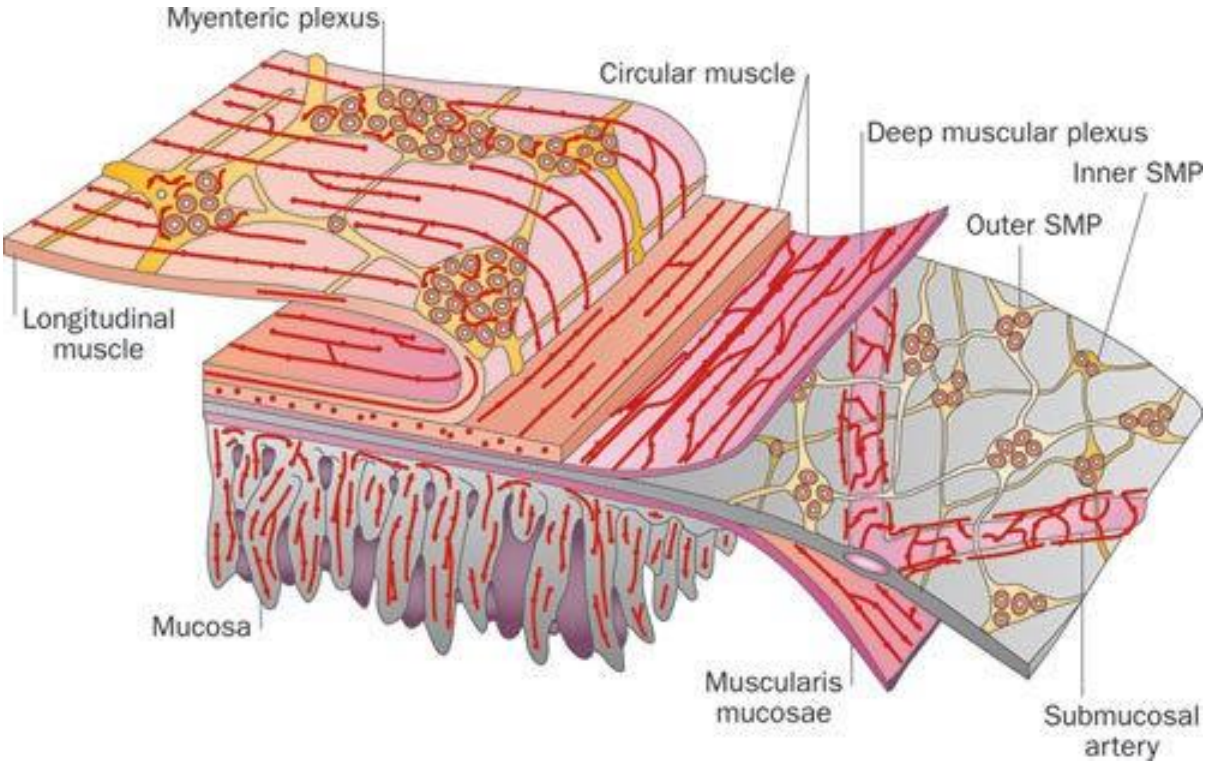

Figure 1.2: Organization of the enteric nervous system. The enteric nervous system is composed of two ganglionated plexuses, the submucosal plexus between the circular muscle and mucosal layers, and the myenteric plexus between the longitudinal and circular muscle layers. In humans, the submucosal plexus is divided into an inner and outer SMP. Figure from Furness 2012 (*28*).

Enteric neurons

The ENS is composed of three types of neurons: IPANs, interneurons, and motor neurons, each with specific functional roles during motor reflex activity (*34–36*). Enteric neurons are classified based on their morphology, electrophysiological properties, and neurochemical characteristics. Dogiel's classification system, based on neuron size and shape, is an early classification method of enteric neurons (*35*; **Figure 1.3**), with type I neurons being large multipolar neurons with long axons that form synapses with smooth muscle cells and serve as motor neurons or interneurons. In contrast, type II neurons are multi-axonal with large round or oval cell bodies and act as sensory neurons, predominantly IPANs of the submucosal and myenteric plexus. Type I neurons have short, broad, laminar dendrites and their cell bodies range in size from 13-35μm in length and 9-22μm in width (*31*, *35*). They have an excitatory or inhibitory role in the ENS reflex circuitry. Type II neurons have a major diameter ranging from 22-47μm and a minor diameter of 13-22μm and are sensory neurons (*37*, *38)*.

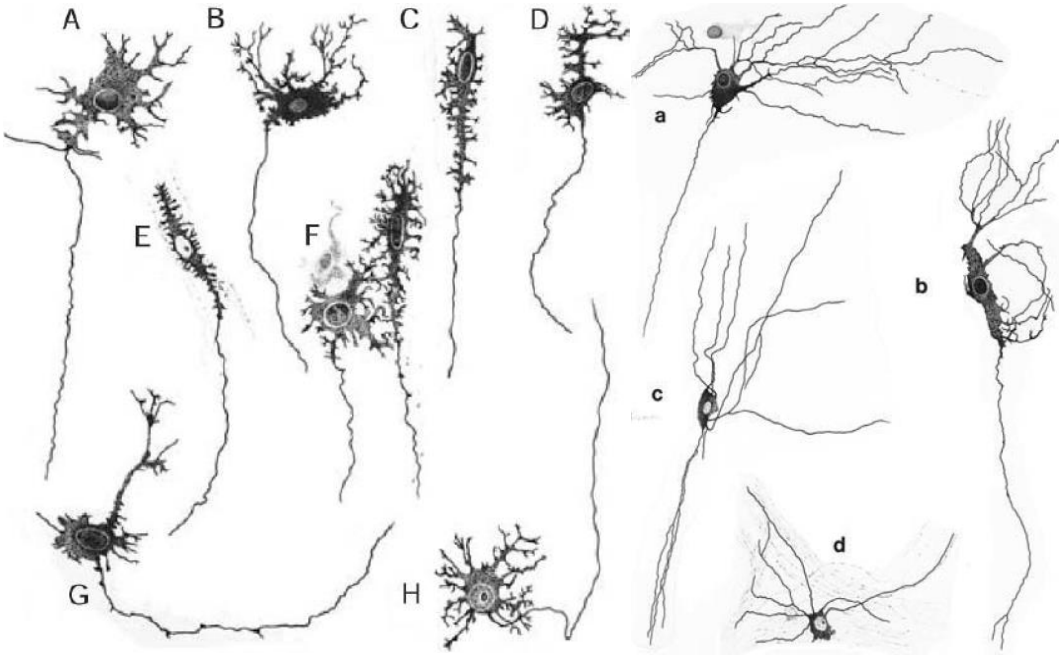

Figure 1.3: Examples of Dogiel type I and type II neurons as defined and drawn by Dogiel (1899). (**A,C,E,F,H**) neurons from the myenteric plexus of the guinea pig intestine. (**D,G**) neurons from the myenteric plexus of human small intestine. (**H**) neuron from dog gall bladder. Figure from Furness 2006 (1). (**a-b**) Neurons form the myenteric plexus of the guinea pig small intestine. (**c**) Neuron from myenteric plexus of human small intestine. (**d**) Neuron from the myenteric plexus of guinea pig large intestine. Figure from Furness 2006 (*37*).

Enteric neurons can be classified based on electrophysiological properties (*39*, *40*) and neurochemical coding (*36*, *46*). S-type neurons are fast-acting motor and interneurons with short-duration after-hyperpolarizing potentials (AHPs) lasting between 20-100ms (*39*). AH-type neurons are sensory neurons that respond to trains of stimuli with slow excitatory postsynaptic potentials (sEPSPs) and have a more significant duration of action potential than S-type neurons (*41*, *42*). AH-type neurons are limited in firing rate due to the AHP under resting conditions (*43*, *44, 45*). Enteric neurons can also be further classified based on their neurochemical coding into sensory neurons, motor neurons (excitatory or inhibitory), and interneurons (ascending or descending) (*36*, *46*). Sensory neurons primarily use acetylcholine (ACh), tachykinins (TKNs), and calcitonin gene-related peptide (CGRP) as their primary neurotransmitters. Excitatory motor neurons use ACh and substance P (SP) and project orally or locally to the circular or longitudinal smooth muscle to induce muscle contractions (*47*). Inhibitory motor neurons use nitric oxide (NO) and vasoactive intestinal peptide (VIP) as their primary neurotransmitters (*47*). Ascending and descending interneurons primarily release ACh and are immunoreactive for choline acetyltransferase (ChAT).

The enteric neurons of the myenteric plexus monitor luminal contents, epithelial distortion, and gut distension through afferent endings that innervate the mucosal and muscular compartments (*28*). The ganglia in which these neurons reside act as signal integration sites where context-appropriate, efferent reflexes are generated (**Fig 1.4**). The simplest reflex arc involves the activation of IPANs, which simultaneously recruit ascending and descending neural pathways to evoke contraction and relaxation of the gut's musculature, respectively (*48*). This results in the contraction of proximal gut

segments and the relaxation of distal gut segments, ultimately propelling the luminal contents forward. This fundamental paradigm is repeated throughout the GI tract, with ascending networks predominantly cholinergic and descending networks predominantly nitrergic, often with purinergic co-transmission. Understanding the classification of enteric neurons provides insight into the mechanisms underlying the regulation of gastrointestinal functions.

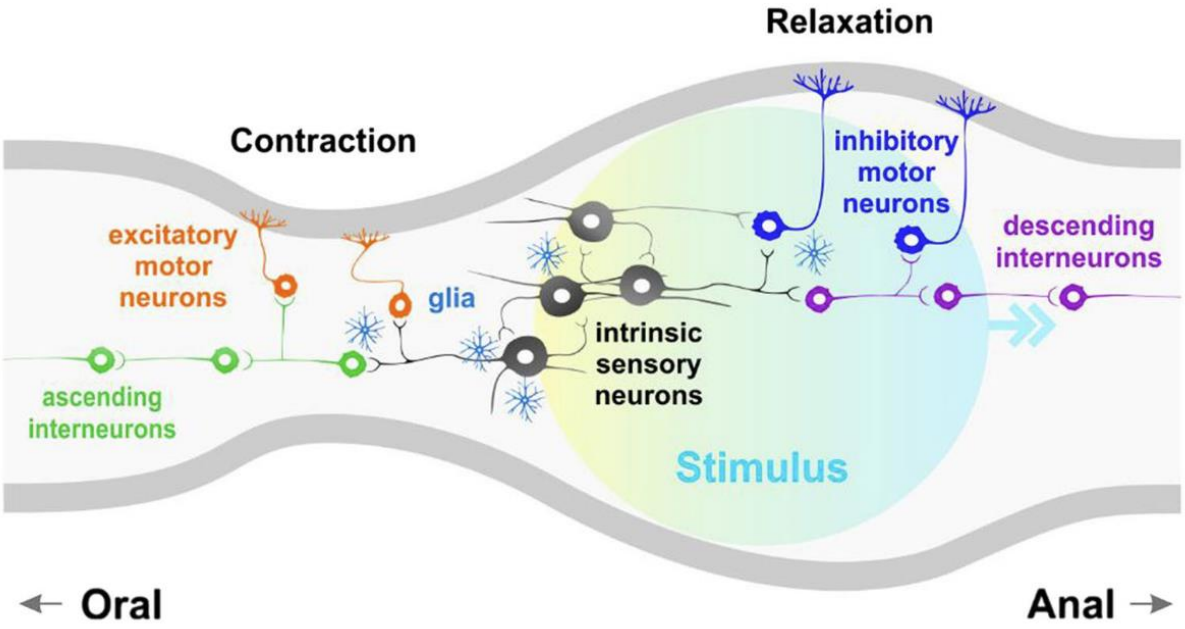

Figure 1.4: Structural arrangement of enteric neurons of the myenteric plexus. Schematic representing the enteric circuitry underlying the peristaltic reflex. Intrinsic sensory neurons synapse with ascending and descending interneurons that form chains along the length of the intestine, as well as interneurons, and excitatory and inhibitory motor neurons. Upon detection of a luminal stimulus, the activation of ascending interneurons connected to excitatory motor neurons evoke a contraction orally, while the activation of descending interneurons connected to inhibitory motor neurons elicit a relaxation anally to propel the contents along. Image from Fung & Vandenberghe 2020 (*48*).

Enteric glia

Enteric glia are a unique type of peripheral glial cells that can be found in all layers of the gut wall, surrounding enteric neurons. They are crucial for the functioning of neuronal circuits in the enteric nervous system (*49*–*54*), as they detect neuronal activity by expressing receptors for various neurotransmitters (*55*, *56*). The activation of enteric glia can also modulate neuronal circuit activity (*20*, *23*, *49*). Glia originate from neural crest precursors that colonize the intestinal tract during embryonic development (*57*) and express markers for mature glial cells like the calcium-binding protein S100β and glial fibrillary acidic protein (GFAP) (*58*, *59*). Enteric glia are a diverse group of cells that can be categorized into four subgroups based on their location and morphology (*60*–*62*; **Figure 1.5***)*. They include Type I, which are star-shaped cells with short, irregularly branched processes located within myenteric and submucosal ganglia; Type II, elongated glia found within nerve fiber bundles connecting myenteric ganglia; Type III, long-branched glia located within the mucosal layer of the gut; and Type IV or intramuscular glia, which are elongated glia associated with nerve fibers within the muscle layers of the gut (*61*).

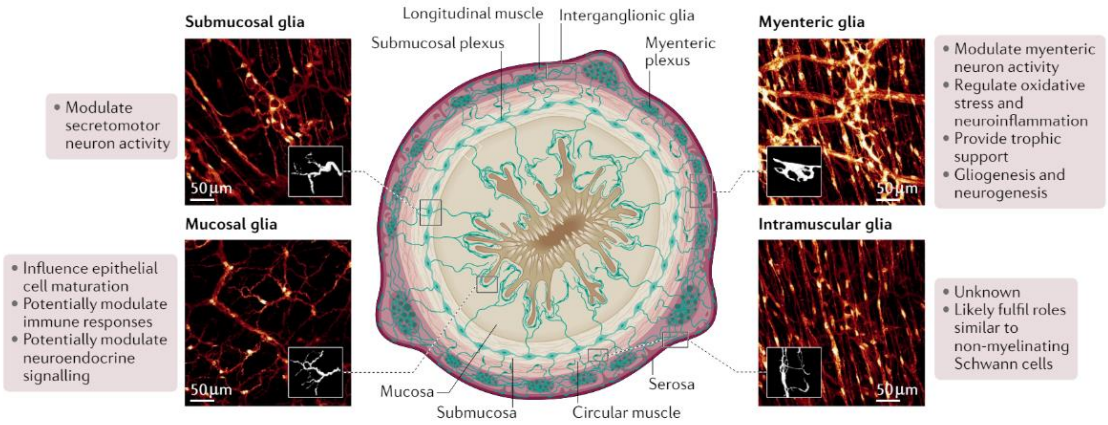

Figure 1.5: Populations of enteric glia. Enteric glia are classified into four subtypes based on their morphology. Figure from Seguella 2021 (*61*).

Like astrocytes in the central nervous system, enteric glia fill the same niche within enteric ganglia, receiving synaptic contacts from neurons and serving as the primary points of neuron-glia communication (*56;* **Figure 1.6**). However, this isn't likely true for the glia outside of the ganglia, which are more like Schwann cells (*61*). Although there are similarities between enteric glia and astrocytes, not all properties can be generalized to both cell types. Enteric glia are derived from the neural crest while astrocytes come from precursor cells in the neural tube (*63*). Moreover, enteric glia do not express certain astrocytic properties such as aldehyde dehydrogenase 1 family member L1 (Aldh1L1) (*64*) and instead express non-astrocytic molecules like SOX10 (*66*), emphasizing their unique identity as a distinct population of glia.

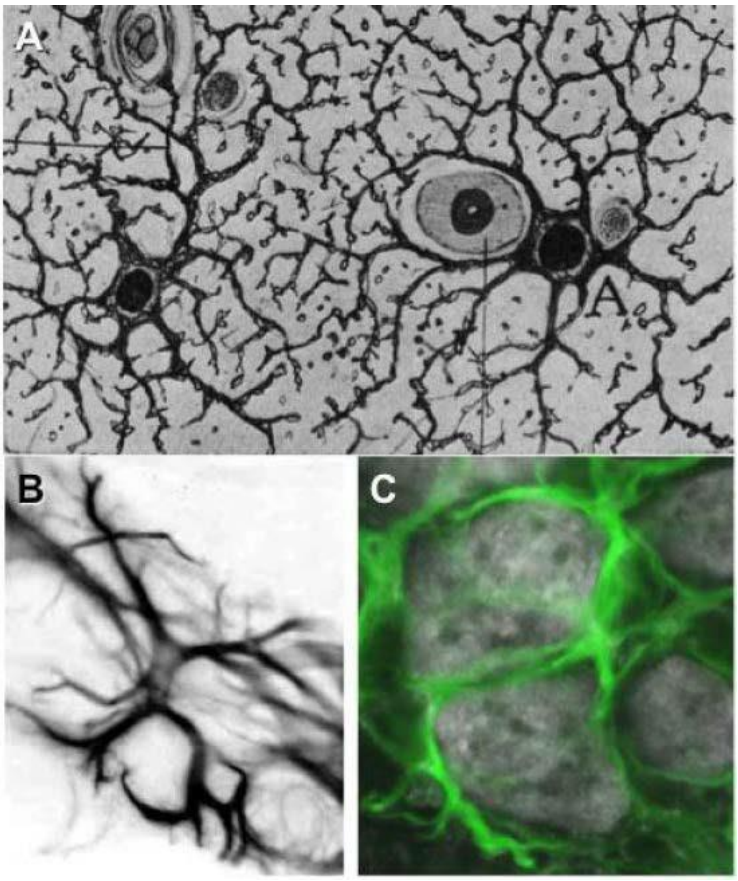

Figure 1.6: Enteric glial morphology resembles astrocyte morphology. (**A**) Drawing of astrocytes by Santiago Ramon y Cajal. (**B-C**) Representative images of enteric glial cells in the myenteric plexus of the mouse colon labeled with GFAP (black in **B** and green in **C**). Figure from Gulbransen 2014 (*56*).

Enteric glia express a variety of receptors that allow them to respond to changes in neurotransmitter concentration and to modulate neuron-to-neuron communication. They also can mediate glia-to-glia communication. Glia have receptors for neurotransmitters such as cholinergic receptors (67, 68), adrenergic receptors (69, 70), purinergic receptors (50–52), serotonin receptors (54), and others (71–73). This enables them to respond to nearby neuronal activity and be innervation targets. They can also modulate neuronal signals in the ENS, which contributes to the control of GI motility (74–76). Like astrocytes, enteric glia express connexin-43 (Cx43) hemichannels, and their activity is important for glia-to-glia signaling and mediating enteric neuron death during inflammation (11, 77, 78).

Enteric glia release compounds that can modify surrounding cells (55), such as adenosine triphosphate (ATP), NO, prostaglandin E-2 (PGE2), and neuroprotective compounds. ATP is the most well-characterized substance released by enteric glia, and its release through Cx43 hemichannels triggers intracellular Ca2+ waves from neighboring glial cells (77, 79). Attenuating glial ATP release alters GI motility and can drive neuron death during inflammation (77). Enteric glia also produce NO, which modulates epithelial ion transport and contributes to neuroinflammation during inflammation (80). PGE2 may modulate neurotransmission and contribute to disease development (71). Enteric glia secrete neuroprotective compounds, such as reduced glutathione and prostaglandin derivatives, which promote neuron health and modify function under physiological conditions (56).

Enteric glia respond to various neurotransmitters and modulators, including ATP, norepinephrine, glutamate, thrombin, serotonin, bradykinin, histamine, and endothelin

(56). The activation of glial Ca2+ responses modulates neuronal circuits that underlie colonic peristalsis (20). Enteric glia can modulate enteric neurotransmission by regulating available neurotransmitters or by releasing neuroactive substances, a process called gliotransmission (55). Enteric glia also regulate the bioavailability of neurotransmitters in the extracellular environment, prevent excitotoxic neuron death, and interact with non-neuronal cell types to modulate inflammation in the gut (20, 22, 11, 81).

Enteric glia are key mediators in GI pathophysiology (82). Glial alterations are associated with a wide range of GI disorders, and inflammation drives morphological and functional changes in enteric glia that could contribute to GI pathophysiology. The expression of glial markers such as GFAP, S100β, and SOX10 are measured to indicate glial alterations in disease (83, 84). Inflammation alters expression of glial receptors and enzymes and increases glial proliferation. Inflammation drives glial alterations that contribute to neuron death primarily through ATP release (11). Loss of glial neuroprotective functions can result in a decrease in enteric neuronal populations (81). Enteric glia can also contribute to inflammation by secreting proinflammatory cytokines and immunomodulatory signals (71, 78, 85). Additionally, enteric glia can interact with afferent nerves that innervate the intestine, which is a key mechanism that drives neuroinflammation in the intestine and contributes to GI pathophysiology (17). These mechanisms likely contribute to mechanisms of abdominal pain and will be described in further detail in Chapter 2. Overall, enteric glia play a pivotal role in modulating ENS function.

EXTRINSIC INNERVATION OF THE GI TRACT

The connection between the CNS and the GI tract is established by the extrinsic primary afferents, which provide the basis for both nonpainful and painful sensations in the gut, such as satiety and inflammation. Connections between the gut and the CNS can be classified as vagal, spinal thoracolumbar, and spinal lumbosacral and include sensory, sympathetic, and parasympathetic pathways (*25*).

Vagal innervation

The vagus nerve plays an essential role in the regulation of the GI tract, providing sensory innervation and efferent control pathways for upper digestive organs. The vagal nerve detects stimuli from the esophagus, stomach, small intestine, liver, and pancreas. Vagal sensory innervation controls various functions such as appetite and satiety, esophageal propulsion, gastric volume, contractile activity, and acid secretion, contraction of the gallbladder, and secretion of pancreatic enzymes (*26*, *27*, *86*–*88*). Vagal afferent nerve endings consist of intraganglionic laminar endings (IGLEs), intramuscular arrays (IMAs), and mucosal varicose nerve endings (*30*, *89*) (**Figure 1.7**). IGLEs are complex branching nerve endings that give rise to flat expansions within myenteric ganglia (*90*). IMAs are formed by single afferent axons that branch within the circular muscle layer to form arrays of varicose fibers that run parallel to muscle bundles (*88*). Three types of vagal mucosal afferents have been identified: gastric mucosal afferent endings, afferents supplying villi in the small intestine (villus afferents), and afferents supplying intestinal crypts (crypt afferents) (*91*). Gastric mucosal afferent endings are sensitive to low intensity stroking of the mucosa and chemical stimuli, such as acid in the lumen (*30*, *92*). Villus afferents have axons that project toward the villus tip,

where they branch extensively, and are ideally positioned to detect substances released from the epithelium, including local hormones such as cholecystokinin (CCK) and serotonin (5HT). Crypt afferents form subepithelial rings of varicose processes below the crypt-villus junction (*30*, *92*).

Vagal efferent pathways arise from the dorsal motor nucleus of the vagus. Most of these neurons are cholinergic and are involved in the regulation of gastrointestinal functions such as gastric motility and secretion of digestive enzymes (*30*, *93*, *94*). The vagal efferent pathways also regulate the gastrointestinal immune system and the release of hormones such as insulin, glucagon, and somatostatin. The vagus nerve also influences the ENS, which contains intrinsic neurons that regulate various gastrointestinal functions through inhibitory or excitatory pathways (*92*).

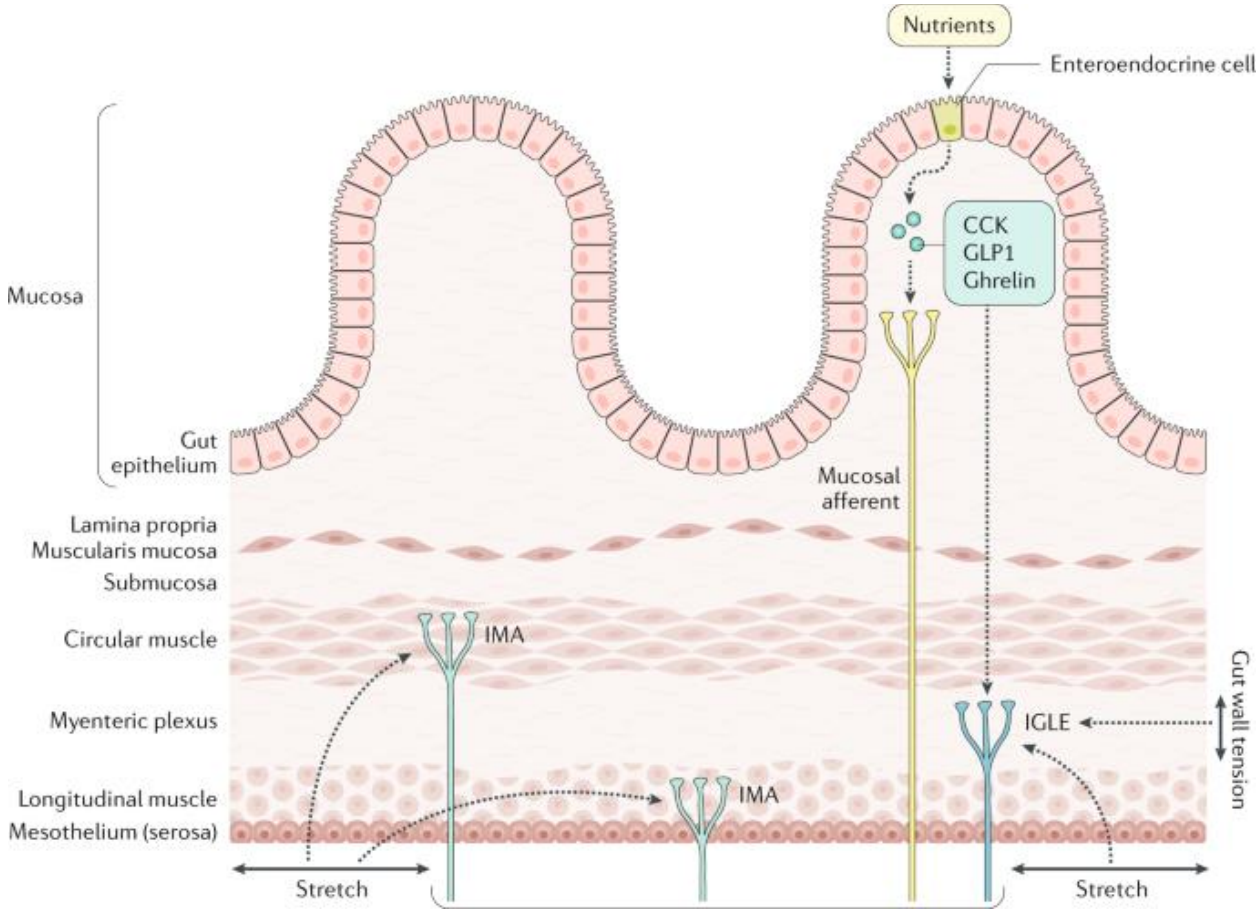

Figure 1.7: Classification and distribution of vagal afferent verve endings in the GI tract. Diagram showing three basic types of nerve terminal known as intramuscular arrays (IMAs), intraganglionic laminar endings (IGLEs), and mucosal afferent endings. Image from Waise et al 2018 (*94*).

Spinal innervation

The colon/rectum are innervated by specialized spinal sensory afferents traveling via two distinct anatomical spinal pathways: the lumbar splanchnic and sacral pelvic nerves (**Figure 1.8**) (*3*). The nerve fibers that innervate the colon are divided into two groups: splanchnic and pelvic afferents (*95*). The cell bodies of these fibers are located in different parts of the dorsal root ganglia, either in the thoracolumbar region (T10-L2) or the lumbosacral region (L5-S1). Once these afferents reach the spinal cord, they synapse with different types of neurons in the dorsal horn, including excitatory and inhibitory interneurons, as well as second-order neurons of the dorsal column, spinothalamic tract, and spinoparabrachial pathway (*96*, *97*). The spinoparabrachial pathway is mainly composed of projections from the superficial dorsal horn, which are associated with autonomic and emotional responses to painful stimuli. These projections feed into different limbic and cognitive centers, such as the amygdala, hypothalamus, and periaqueductal gray (*3*, *96*, *97*). The spinothalamic tract signal, on the other hand, is relayed through the thalamus to cortical areas responsible for sensory discrimination and localization, as well as limbic areas for the emotional component of pain. The thalamus also influences prefrontal cortex signaling, particularly in the anterior cingulate cortex, which is associated with visceral pain responses. The output from the cortical and limbic regions, in response to pain, activates the descending inhibitory circuitry within the brainstem. This circuitry causes the release of inhibitory neurotransmitters in the dorsal horn of the spinal cord to regulate autonomic output responses to pain (*30*, *95*). The axons of the afferent neurons located in the DRG are bundled into roots/nerves that contain a mix of fibers with varying excitability.

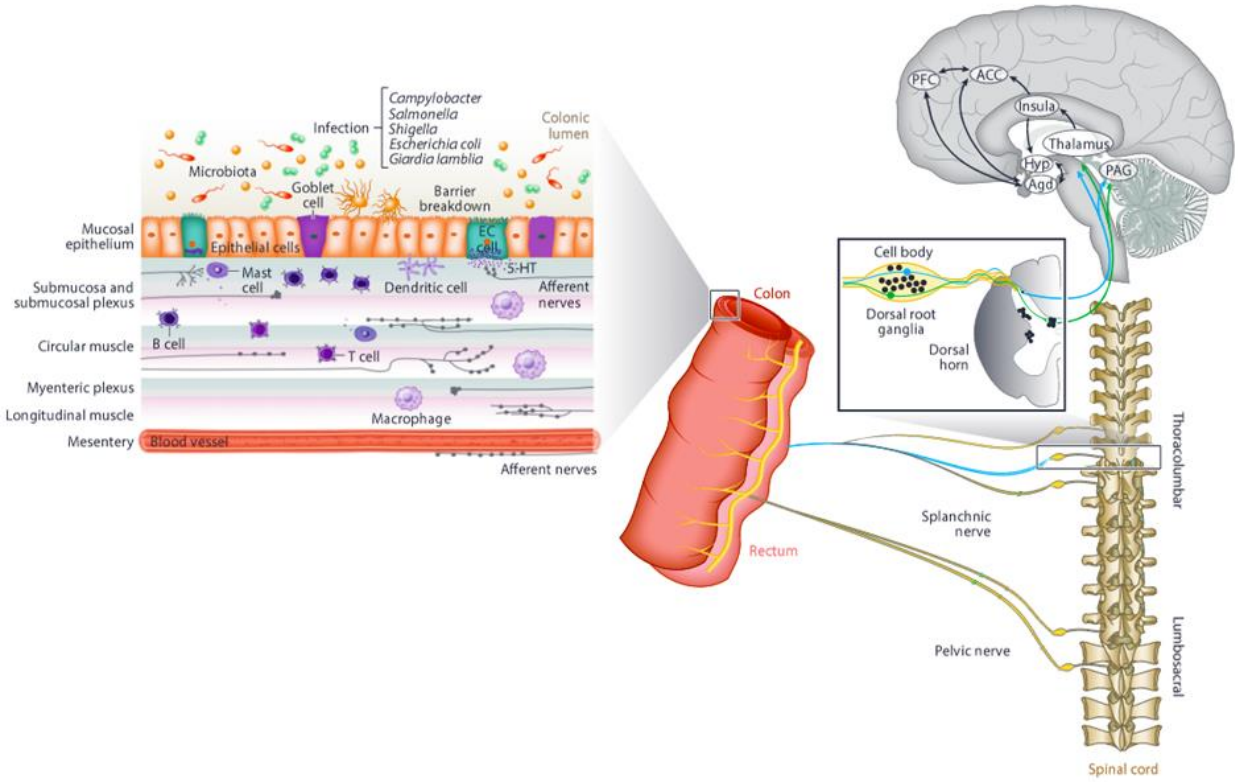

Figure 1.8: Schematic overview of the extrinsic sensory innervation of the colon/rectum. The colon and rectum are innervated by spinal afferents that follow the splanchnic and pelvic nerves. These afferents have cell bodies within the thoracolumbar and lumbosacral dorsal root ganglia, respectively. The peripheral projections of these afferents innervate the mucosa, myenteric/submucosal ganglia, and muscle; they also wrap around blood vessels within the submucosa and on the mesenteric attachment. These afferents allow the detection of the full range of mechanical and chemical stimuli. Image adapted from Grundy et al 2018 (*3*).

These fibers include low-threshold mechanosensory fibers, higher-threshold Aβ nociceptors, and Aδ and C fibers. Aβ, Aδ, and C fibers carry peripheral sensation information to their respective soma in the DRG (*98*). Myelinated Aδ fibers have a relatively high velocity to carry acute nociceptive details such as temperature, mechanical, and chemical-induced stimuli to the DRG. Unmyelinated C fibers have a smaller diameter and slower conduction velocity. They also carry nociceptive input to the DRG but contribute to the more diffuse and profound secondary pain after an injury (*98*). DRGs also have a large population of glial cells, with each DRG having approximately eight times more glia than neurons. Specialized glial cells, known as satellite glial cells, envelop each primary sensory neuron in the DRG to create an independent functional unit. In Chapter 2, we will describe the contributions of peripheral glia to nociceptive signaling.

Spinal afferent neurons that innervate the gut are predominantly peptidergic polymodal C-fibers that are immunoreactive for CGRP, TKNs, and transient receptor potential cation channel subfamily V member 1 (TRPV1) (*99*, *100*). These afferents are classified by their location within the gut (**Figure 1.8**) and express a wide variety of pro- and antinociceptive ion channels and receptors that dictate visceral afferent sensitivity and peripheral drive to the spinal cord. Inflammation causes long-term changes in spinal afferent neurons causing neurons to become hypersensitive to GI pain sensation (*101*, *102*). How inflammation alters afferent signaling and nociception will be explored in detail in Chapter 2.

Mucosal afferents are important in detecting luminal contents. They have low activation thresholds and respond to very light stroking or compression of the mucosa

(*103*, *104*). Mucosal afferents respond to serotonin that is released from the epithelium (*105*) and are mechanically stimulated through transient receptor potential ankyrin 1 (TRPA1) channels (*104*, *105*). Similarly, muscular afferents also have low mechanical stimulation thresholds and provide necessary information for coordinated reflexes by signaling distension caused by fecal matter (*103*, *104*). These afferents also signal into the noxious range and likely contribute to nociception at high stimulus intensities. The acid-sensing ion channel 3 (ASIC3) and the transient receptor potential channel TRPV1 play key roles in the function of pelvic muscular afferents. Another population of afferent sensory neurons can be found wrapped around blood vessels in the mesentery and submucosa. In contrast to muscular/mucosal afferents, vascular afferents respond to high-threshold stimuli and a variety of inflammatory and immune mediators (*30*, *103*, *104*, *106*). Vascular afferents also respond to noxious levels of distension and serve a role in signaling mechanically induced pain (*103*, *104*, *107*, *108*). These afferent nerves are stimulated by purinoreceptor subtypes P2X, P2Y, Protease activated receptor 2 (PAR2), and TRPV1). Further, numerous ion channels including voltage-gated sodium channels (NaV1.1, NaV1.8, NaV1.9), ASIC3, TRPV4, TRPA1, TRPV1 are all integral to high-threshold afferent function (*95*). Finally, there exists a population of afferent nerves that are mechanically insensitive and are generally referred to as silent afferents (*3*, *30*). These nerves respond to inflammatory mediators (histamine, bradykinin, capsaicin, ATP) (*109*–*111*) and can become mechanically sensitized during disease states. In sensitized states, there is a decrease in the proportion of mechanically insensitive afferents but a corresponding increase in the proportion of mechanically sensitive high-threshold vascular afferents (*109*). Indicating the possible recruitment of silent nociceptors to

noxious signaling and persistent pain states. These subclasses of afferents allow detection of non-noxious physiological stimuli, including luminal events, muscle stretch during organ distension and contraction, as well as noxious mechanical (bloating, intense distension/contraction) and chemical stimuli (*112*).

SUMMARY AND AIMS OF THE DISSERTATION

Bidirectional communication between the enteric and central nervous systems is essential for homeostatic gut function (*28*). Mechanical and chemical stimuli that result from stretch or luminal content of the gut are transduced to the brain through the nociceptive nerve endings that project from the spinal cord and innervate the intestine (*30*, *32*). Inflammation can drastically alter nociceptive signaling which can result in persistent pain states. Currently, the mechanisms that drive these inflammatory pain states are incompletely understood. Recently, glial cells have been highlighted as key regulators of pain mechanisms (*18*). Enteric glia are unique peripheral glial cells that regulate GI function and have important roles in GI pathophysiology (*11*, *12*, *20*, *23*, *60*, *77*). In health, glial activation can influence enteric circuits involved in the regulation of motility (*20*) and secretion (*23*) and intracellular signaling between nociceptors and glia contributes neurogenic inflammation within the gut (*17*). The objective of this dissertation is to understand the circumstances under which enteric glial activation is triggered and the downstream consequences of various glial signal transduction pathways in nociceptive signaling. Chapter 2 discusses the potential role of enteric glia in visceral hypersensitivity and its link to abdominal pain disorders. Chapter 3 studies how communication between enteric glia and nociceptors modulates visceral hypersensitivity during inflammation. Chapter 4, studies glial endocannabinoid regulation and how it

contributes to the regulation of inflammatory pain. Together, these chapters aim to improve the understanding of glial signaling mechanisms the contribute to visceral hypersensitivity. Further, this dissertation serves to improve broader understanding of abdominal pain mechanisms.

REFERENCES

1. E. A. Mayer, K. Tillisch, The Brain-Gut Axis in Abdominal Pain Syndromes. *Annual Review of Medicine*. 62, 381–396 (2011).

2. A. Ancona, C. Petito, I. Iavarone, V. Petito, L. Galasso, A. Leonetti, L. Turchini, D. Belella, D. Ferrarrese, G. Addolorato, A. Armuzzi, A. Gasbarrini, F. Scaldaferri, The gut–brain axis in irritable bowel syndrome and inflammatory bowel disease. *Digest Liver Dis*. 53, 298–305 (2021).

3. L. Grundy, A. Erickson, S. M. Brierley, Visceral Pain. *Annu Rev Physiol*. 81, 1–24 (2018).

4. J. Keohane, C. O'Mahony, L. O'Mahony, S. O'Mahony, E. M. Quigley, F. Shanahan, Irritable Bowel Syndrome–Type Symptoms in Patients With Inflammatory Bowel Disease: A Real Association or Reflection of Occult Inflammation? *The American Journal of Gastroenterology*. 105, ajg2010156 (2010).

5. I. M. Minderhoud, B. Oldenburg, J. A. Wismeijer, G. P. Henegouwen, A. Smout, IBS-Like Symptoms in Patients with Inflammatory Bowel Disease in Remission; Relationships with Quality of Life and Coping Behavior. *Digestive Diseases and Sciences*. 49, 469–474 (2004).

6. S. J. Halpin, A. C. Ford, Prevalence of Symptoms Meeting Criteria for Irritable Bowel Syndrome in Inflammatory Bowel Disease: Systematic Review and Meta-Analysis. *The American Journal of Gastroenterology*. 107, ajg2012260 (2012).

7. W. R. Best, J. M. Becktel, J. W. Singleton, F. Kern, Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology*. 70, 439–44 (1976).

8. R. D. Giorgio, V. Stanghellini, G. Barbara, R. Corinaldesi, F. D. Ponti, M. Tonini, G. Bassotti, C. Sternini, Primary Enteric Neuropathies Underlying Gastrointestinal Motor Dysfunction. *Scand J Gastroentero*. 35, 114–122 (2000).

9. R. D. Giorgio, S. Guerrini, G. Barbara, V. Stanghellini, F. D. Ponti, R. Corinaldesi, P. L. Moses, K. A. Sharkey, G. M. Mawe, Inflammatory neuropathies of the enteric nervous system. *Gastroenterology*. 126, 1872–1883 (2004).

10. B. D. Gulbransen, M. Bashashati, S. A. Hirota, X. Gui, J. A. Roberts, J. A. MacDonald, D. A. Muruve, D. M. McKay, P. L. Beck, G. M. Mawe, R. J. Thompson, K. A. Sharkey, *Nature Medicine*, in press, doi:10.1038/nm.2679.

11. I. A. M. Brown, J. L. McClain, R. E. Watson, B. A. Patel, B. D. Gulbransen, Enteric Glia Mediate Neuron Death in Colitis Through Purinergic Pathways That Require Connexin-43 and Nitric Oxide. *Cmgh Cell Mol Gastroenterology Hepatology*. 2, 77–91 (2016).

12. M. M. Ahmadzai, J. L. McClain, C. Dharshika, L. Seguella, F. Giancola, R. D. Giorgio, B. D. Gulbransen, LPAR1 regulates enteric nervous system function through glial signaling and contributes to chronic intestinal pseudo-obstruction. *J Clin Investigation*. 132, e149464 (2022).

13. H. I. R. Magalhães, P. Castelucci, Enteric nervous system and inflammatory bowel diseases: Correlated impacts and therapeutic approaches through the P2X7 receptor. *World J Gastroentero*. 27, 7909–7924 (2021).

14. A. M. Holland, A. C. Bon-Frauches, D. Keszthelyi, V. Melotte, W. Boesmans, The enteric nervous system in gastrointestinal disease etiology. *Cell Mol Life Sci*. 78, 4713–4733 (2021).

15. F. Matheis, P. A. Muller, C. L. Graves, I. Gabanyi, Z. J. Kerner, D. Costa-Borges, T. Ahrends, P. Rosenstiel, D. Mucida, Adrenergic Signaling in Muscularis Macrophages Limits Infection-Induced Neuronal Loss. *Cell*. 180, 64-78.e16 (2020).

16. N. J. Spencer, J. Sorensen, L. Travis, L. Wiklendt, M. Costa, T. Hibberd, Imaging activation of peptidergic spinal afferent varicosities within visceral organs using novel CGRPα-mCherry reporter mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 311, G880–G894 (2016).

17. N. M. Delvalle, C. Dharshika, W. Morales-Soto, D. E. Fried, L. Gaudette, B. D. Gulbransen, Communication between enteric neurons, glia, and nociceptors underlies the effects of tachykinins on neuroinflammation. *Cellular and Molecular Gastroenterology and Hepatology*, 321–344 (2018).

18. R.-R. R. Ji, T. Berta, M. Nedergaard, Glia and pain: is chronic pain a gliopathy? *Pain*. 154 Suppl 1, S10-28 (2013).

19. R.-R. Ji, A. Chamessian, Y.-Q. Zhang, Pain regulation by non-neuronal cells and inflammation. *Science*. 354, 572–577 (2016).

20. J. L. McClain, D. E. Fried, B. D. Gulbransen, Agonist-Evoked Ca2+ Signaling in Enteric Glia Drives Neural Programs That Regulate Intestinal Motility in Mice. *Cell Mol Gastroenterology Hepatology*. 1, 631–645 (2015).

21. J. L. McClain, V. Grubišić, D. Fried, R. A. Gomez-Suarez, G. M. Leinninger, J. Sévigny, V. Parpura, B. D. Gulbransen, Ca2+ Responses in Enteric Glia Are Mediated by Connexin-43 Hemichannels and Modulate Colonic Transit in Mice. *Gastroenterology*. 146, 497-507.e1 (2014).

22. V. Grubišić, A. Verkhratsky, R. Zorec, V. Parpura, Enteric glia regulate gut motility in health and disease. *Brain Res Bull*. 136, 109–117 (2018).

23. V. Grubišić, B. D. Gulbransen, Enteric glial activity regulates secretomotor function in the mouse colon but does not acutely affect gut permeability. *The Journal of Physiology*. 595, 3409–3424 (2017).

24. J. L. McClain, B. D. Gulbransen, The acute inhibition of enteric glial metabolism with fluoroacetate alters calcium signaling, hemichannel function, and the expression of key proteins. *Journal of Neurophysiology*. 117, 365–375 (2017).

25. J. B. Furness, B. P. Callaghan, L. R. Rivera, H.-J. Cho, Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease. *Adv Exp Med Biol*. 817, 39–71 (2014).

26. A. Jean, Brain Stem Control of Swallowing: Neuronal Network and Cellular Mechanisms. *Physiol Rev*. 81, 929–969 (2001).

27. F. J. Ingelfinger, Esophageal Motility. *Physiol Rev*. 38, 533–584 (1958).

28. J. B. Furness, The enteric nervous system and neurogastroenterology. *Nat Rev Gastroentero*. 9, 286 (2012).

29. V. Prato, F. J. Taberner, J. Hockley, G. Callejo, A. Arcourt, B. Tazir, L. Hammer, P. Schad, P. A. Heppenstall, E. S. Smith, S. G. Lechner, Functional and Molecular Characterization of Mechanoinsensitive “Silent” Nociceptors. *Cell Reports*. 21, 3102–3115 (2017).

30. S. J. H. Brookes, N. J. Spencer, M. Costa, V. P. Zagorodnyuk, Extrinsic primary afferent signalling in the gut. *Nat Rev Gastroentero*. 10, 286–296 (2013).

31. K. A. Sharkey, G. M. Mawe, The enteric nervous system. *Physiol Rev* (2022), doi:10.1152/physrev.00018.2022.

32. N. J. Spencer, M. A. Kyloh, L. Travis, K. N. Dodds, Sensory nerve endings arising from single spinal afferent neurons that innervate both circular muscle and myenteric ganglia in mouse colon: colon-brain axis. *Cell Tissue Res*. 381, 25–34 (2020).

33. G. Gabella, P. Trigg, Size of neurons and glial cells in the enteric ganglia of mice, guinea-pigs, rabbits and sheep. *J Neurocytol*. 13, 49–71 (1984).

34. M. Costa, S. J. H. Brookes, G. W. Hennig, Anatomy and physiology of the enteric nervous system. *Gut*. 47, iv15 (2000).

35. J. B. Furness, Types of neurons in the enteric nervous system. *Journal of the Autonomic Nervous System*. 81, 87–96 (2000).

36. M. Costa, S. J. H. Brookes, P. A. Steeled, I. Gibbins, E. Burcher, C. J. Kandiah, Neurochemical classification of myenteric neurons in the guinea-pig ileum. *Neuroscience*. 75, 949–967 (1996).

37. Furness, JB, *The Enteric Nervous System* (Oxford: Blackwell Publishing, 2006).

38. J. C. Bornstein, R. Hendriks, J. B. Furness, D. C. Trussell, Ramifications of the axons of AH-neurons injected with the intracellular marker biocytin in the myenteric plexus of the guinea pig small intestine. *J Comp Neurol*. 314, 437–451 (1991).

39. G. D. S. Hirst, M. E. Holman, I. Spence, Two types of neurones in the myenteric plexus of duodenum in the guinea-pig. *J Physiology*. 236, 303–326 (1974).

40. V. Iyer, J. C. Bornstein, M. Costa, J. B. Furness, Y. Takahashi, T. Iwanaga, Electrophysiology of guinea-pig myenteric neurons correlated with immunoreactivity for calcium binding proteins. *J Autonom Nerv Syst*. 22, 141–150 (1988).

41. J. C. Bornstein, J. B. Furness, W. A. A. Kunze, Electrophysiological characterization of myenteric neurons: how do classification schemes relate? *J Autonom Nerv Syst*. 48, 1–15 (1994).

42. W. A. A. Kunze, J. B. Furness, J. C. Bornstein, Simultaneous intracellular recordings from enteric neurons reveal that myenteric ah neurons transmit via slow excitatory postsynaptic potentials. *Neuroscience*. 55, 685–694 (1993).

43. G. D. Hirst, S. M. Johnson, D. F. van Helden, The slow calcium-dependent potassium current in a myenteric neurone of the guinea-pig ileum. *J Physiology*. 361, 315–337 (1985).

44. G. D. S. Hirst, H. C. McKirdy, A nervous mechanism for descending inhibition in guinea-pig small intestine. *J Physiology*. 238, 129–143 (1974).

45. J. B. Furness, Encyclopedia of Neuroscience, 1122–1125 (2009).

46. A. E. Lomax, J. B. Furness, Neurochemical classification of enteric neurons in the guinea-pig distal colon. *Cell Tissue Res.* 302, 59–72 (2000).

47. H. M. Cousins, F. R. Edwards, G. D. Hirst, I. R. Wendt, Cholinergic neuromuscular transmission in the longitudinal muscle of the guinea-pig ileum. *J Physiology*. 471, 61–86 (1993).

48. C. Fung, P. V. Berghe, Functional circuits and signal processing in the enteric nervous system. *Cell Mol Life Sci*, 1–18 (2020).

49. M. J. Broadhead, P. O. Bayguinov, T. Okamoto, D. J. Heredia, T. K. Smith, Ca2+ transients in myenteric glial cells during the colonic migrating motor complex in the isolated murine large intestine. *J Physiology*. 590, 335–350 (2012).

50. B. D. Gulbransen, K. A. Sharkey, Purinergic Neuron-to-Glia Signaling in the Enteric Nervous System. *Gastroenterology*. 136, 1349–1358 (2009).

51. P. Gomes, J. Chevalier, W. Boesmans, L. Roosen, V. V. Abbeel, M. Neunlist, J. Tack, V. P. Berghe, ATP-dependent paracrine communication between enteric neurons and glia in a primary cell culture derived from embryonic mice. *Neurogastroenterology & Motility*. 21, 870-e62 (2009).

52. B. C. Kimball, M. W. Mulholland, Enteric Glia Exhibit P2U Receptors that Increase Cytosolic Calcium by a Phospholipase C-Dependent Mechanism. *Journal of Neurochemistry*. 66, 604–612 (1996).

53. W. Boesmans, M. A. Martens, N. Weltens, M. M. Hao, J. Tack, C. Cirillo, P. V. Berghe, Imaging neuron-glia interactions in the enteric nervous system. *Front Cell Neurosci*. 7, 183 (2013).

54. W. Boesmans, C. Cirillo, V. V. den Abbeel, C. V. den Haute, I. Depoortere, J. Tack, P. V. Berghe, Neurotransmitters involved in fast excitatory neurotransmission directly activate enteric glial cells. *Neurogastroenterol Motil*. 25, e151–e160 (2013).

55. V. Grubišić, B. D. Gulbransen, Enteric glia: the most alimentary of all glia. *The Journal of Physiology*. 595, 557–570 (2017).

56. B. D. Gulbransen, Enteric Glia. *Colloquium Ser Neurogl Biology Medicine Physiology Dis*. 1, 1–70 (2014).

57. T. P. Rothman, V. M. Tennyson, M. D. Gershon, Colonization of the bowel by the precursors of enteric glia: Studies of normal and congenitally aganglionic mutant mice. *J Comp Neurol*. 252, 493–506 (1986).

58. D. Cocchia, F. Michetti, G.-L. Ferri, J. M. Polak, L. Probert, P. J. Marangos, Evidence for the presence of S-100 protein in the glial component of the human enteric nervous system. *Nature*. 297, 409 (1982).

59. K. Jessen, R. Mirsky, Astrocyte-like glia in the peripheral nervous system: an immunohistochemical study of enteric glia. *Journal of Neuroscience*. 3, 2206–2218 (1983).

60. M. M. Ahmadzai, L. Seguella, B. D. Gulbransen, Circuit-specific enteric glia regulate intestinal motor neurocircuits. *P Natl Acad Sci Usa*. 118, e2025938118 (2021).

61. L. Seguella, B. D. Gulbransen, Enteric glial biology, intercellular signalling and roles in gastrointestinal disease. *Nat Rev Gastroentero*. 18, 571–587 (2021).

62. L. Seguella, J. L. McClain, G. Esposito, B. D. Gulbransen, *J Neurosci*, in press, doi:10.1523/jneurosci.2379-20.2022.

63. D. Riethmacher, E. Sonnenberg-Riethmacher, V. Brinkmann, T. Yamaai, G. R. Lewin, C. Birchmeier, Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature*. 389, 725–730 (1997).

64. W. Boesmans, N. P. Rocha, H. J. Reis, M. Holt, P. V. Berghe, The astrocyte marker Aldh1L1 does not reliably label enteric glial cells. *Neurosci Lett*. 566, 102–105 (2014).

65. V. Grubišić, B. D. Gulbransen, Astrocyte Cell Surface Antigen 2 and Other Potential Cell Surface Markers of Enteric glia in the Mouse Colon. *Asn Neuro*. 14, 17590914221083204 (2022).

66. H. M. Young, B. R. Jones, S. J. McKeown, The Projections of Early Enteric Neurons Are Influenced by the Direction of Neural Crest Cell Migration. *J Neurosci*. 22, 6005–6018 (2002).

67. G. A. Cheadle, T. W. Costantini, V. Bansal, B. P. Eliceiri, R. Coimbra, Cholinergic Signaling in the Gut: A Novel Mechanism of Barrier Protection through Activation of Enteric Glia Cells. *Surg Infect*. 15, 387–393 (2014).

68. N. M. Delvalle, D. E. Fried, G. Rivera-Lopez, L. Gaudette, B. D. Gulbransen, The cholinergic activation of enteric glia is a physiological mechanism that contributes to the regulation of gastrointestinal motility. *Am J Physiol-gastr L*. 315, G473–G483 (2018).

69. B. D. Gulbransen, J. S. Bains, K. A. Sharkey, Enteric Glia Are Targets of the Sympathetic Innervation of the Myenteric Plexus in the Guinea Pig Distal Colon. *The Journal of Neuroscience*. 30, 6801–6809 (2010).

70. Y. Nasser, W. Ho, K. A. Sharkey, Distribution of adrenergic receptors in the enteric nervous system of the guinea pig, mouse, and rat. *J Comp Neurol*. 495, 529–553 (2006).

71. M. Murakami, T. Ohta, K.-I. Otsuguro, S. Ito, Involvement of prostaglandin E2 derived from enteric glial cells in the action of bradykinin in cultured rat myenteric neurons. *Neuroscience*. 145, 642–653 (2007).

72. B. J. Segura, W. Zhang, R. A. Cowles, L. Xiao, T. R. Lin, C. Logsdon, M. W. Mulholland, Lysophosphatidic acid stimulates calcium transients in enteric glia. *Neuroscience*. 123, 687–693 (2004).

73. W. Zhang, G. Sarosi, D. Barnhart, D. I. Yule, M. W. Mulholland, Endothelin-activated calcium signaling in enteric glia derived from neonatal guinea pig. *Am J Physiol-gastr L*. 272, G1175–G1185 (1997).

74. J. de M. Coelho-Aguiar, A. C. Bon-Frauches, A. L. T. Gomes, C. P. Veríssimo, D. P. Aguiar, D. Matias, B. B. de M. Thomasi, A. S. Gomes, G. A. de C. Brito, V. Moura-Neto, The Enteric Glia: Identity and Functions. *Glia*. 63, 921–935 (2015).

75. B. D. Gulbransen, K. A. Sharkey, Novel functional roles for enteric glia in the gastrointestinal tract. *Nature Reviews Gastroenterology & Hepatology*. 9, 625 (2012).

76. M. Neunlist, M. Rolli-Derkinderen, R. Latorre, L. Landeghem, E. Coron, P. Derkinderen, R. Giorgio, Enteric Glial Cells: Recent Developments and Future Directions. *Gastroenterology*. 147, 1230–1237 (2014).

77. J. L. McClain, V. Grubišić, D. Fried, R. A. Gomez-Suarez, G. M. Leinninger, J. Sévigny, V. Parpura, B. D. Gulbransen, Ca2+ Responses in Enteric Glia Are Mediated by

Connexin-43 Hemichannels and Modulate Colonic Transit in Mice. *Gastroenterology*. 146, 497-507.e1 (2014).

78. V. Grubišić, J. L. McClain, D. E. Fried, I. Grants, P. Rajasekhar, E. Csizmadia, O. A. Ajijola, R. E. Watson, D. P. Poole, S. C. Robson, F. L. Christofi, B. D. Gulbransen, Enteric Glia Modulate Macrophage Phenotype and Visceral Sensitivity following Inflammation. *Cell Reports*. 32, 108100 (2020).

79. W. Zhang, B. J. Segura, T. R. Lin, Y. Hu, M. W. Mulholland, Intercellular calcium waves in cultured enteric glia from neonatal guinea pig. *Glia*. 42, 252–262 (2003).

80. S. J. MacEachern, B. A. Patel, D. M. McKay, K. A. Sharkey, Nitric oxide regulation of colonic epithelial ion transport: a novel role for enteric glia in the myenteric plexus. *J Physiology*. 589, 3333–3348 (2011).

81. I. A. Brown, B. D. Gulbransen, The antioxidant glutathione protects against enteric neuron death in situ, but its depletion is protective during colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 314, G39–G52 (2018).

82. K. A. Sharkey, Emerging roles for enteric glia in gastrointestinal disorders. *Journal of Clinical Investigation*. 125, 918–925 (2015).

83. G. B. von Boyen, N. Schulte, C. Pflüger, U. Spaniol, C. Hartmann, M. Steinkamp, Distribution of enteric glia and GDNF during gut inflammation. *Bmc Gastroenterol*. 11, 3 (2011).

84. A. Cornet, T. C. Savidge, J. Cabarrocas, W.-L. Deng, J.-F. Colombel, H. Lassmann, P. Desreumaux, R. S. Liblau, Enterocolitis induced by autoimmune targeting of enteric glial cells: A possible mechanism in Crohn's disease? *Proc National Acad Sci*. 98, 13306–13311 (2001).

85. S. Hernandez, W. Morales-Soto, V. Grubišić, D. Fried, B. D. Gulbransen, Pyridostigmine bromide exposure creates chronic, underlying neuroimmune disruption in the gastrointestinal tract and brain that alters responses to palmitoylethanolamide in a mouse model of Gulf War Illness. *Neuropharmacology*. 179, 108264 (2020).

86. M. L. Schubert, Gastric secretion. *Curr Opin Gastroen*. 19, 519–525 (2003).

87. A. J. Page, J. A. Slattery, C. Milte, R. Laker, T. O'Donnell, C. Dorian, S. M. Brierley, L. A. Blackshaw, Ghrelin selectively reduces mechanosensitivity of upper gastrointestinal vagal afferents. *Am J Physiol-gastr L*. 292, G1376–G1384 (2007).

88. T. L. Powley, R. J. Phillips, Vagal intramuscular array afferents form complexes with interstitial cells of Cajal in gastrointestinal smooth muscle: analogues of muscle spindle organs? *Neuroscience*. 186, 188–200 (2011).

89. K. M. Smith-Edwards, S. A. Najjar, B. S. Edwards, M. J. Howard, K. M. Albers, B. M. Davis, Extrinsic Primary Afferent Neurons Link Visceral Pain to Colon Motility Through a Spinal Reflex in Mice. *Gastroenterology*. 157, 522-536.e2 (2019).

90. F. B. Wang, T. L. Powley, Topographic inventories of vagal afferents in gastrointestinal muscle. *J. Comp. Neurol.* 421, 302–24 (2000).

91. A. J. Page, C. M. Martin, L. A. Blackshaw, Vagal Mechanoreceptors and Chemoreceptors in Mouse Stomach and Esophagus. *J Neurophysiol*. 87, 2095–2103 (2002).

92. J. B. Furness, C. Jones, K. Nurgali, N. Clerc, Intrinsic primary afferent neurons and nerve circuits within the intestine. *Prog Neurobiol*. 72, 143–164 (2004).

93. B. Bonaz, V. Sinniger, S. Pellissier, The Vagus Nerve in the Neuro-Immune Axis: Implications in the Pathology of the Gastrointestinal Tract. *Frontiers in Immunology*. 8, 1452 (2017).

94. T. M. Z. Waise, H. J. Dranse, T. K. T. Lam, The metabolic role of vagal afferent innervation. *Nat Rev Gastroentero*. 15, 625–636 (2018).

95. S. M. Brierley, T. J. Hibberd, N. J. Spencer, Spinal Afferent Innervation of the Colon and Rectum. *Front Cell Neurosci*. 12, 467 (2018).

96. S. Sikandar, A. H. Dickenson, Visceral pain: the ins and outs, the ups and downs. *Current Opinion in Supportive and Palliative Care*. 6, 17 (2012).

97. A. J. Todd, Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci*. 11, 823–836 (2010).

98. M. F. Esposito, R. Malayil, M. Hanes, T. Deer, Unique Characteristics of the Dorsal Root Ganglion as a Target for Neuromodulation. *Pain Med*. 20, S23–S30 (2019).

99. T. Grhkn, G. J. Dockray, Characterization of the peptidergic afferent innervation of the stomach in the rat, mouse and guinea-pig. *Neuroscience*. 25, 181–193 (1988).

100. L. L. Tan, J. C. Bornstein, C. R. Anderson, Distinct chemical classes of medium-sized transient receptor potential channel vanilloid 1-immunoreactive dorsal root ganglion neurons innervate the adult mouse jejunum and colon. *Neuroscience*. 156, 334–343 (2008).

101. M. J. Beyak, Visceral afferents - determinants and modulation of excitability. *Autonomic neuroscience : basic & clinical*. 153, 69–78 (2010).

102. B. Feng, J. H. La, E. S. Schwartz, G. F. Gebhart, Irritable Bowel Syndrome: Methods, Mechanisms, and Pathophysiology. Neural and neuro-immune mechanisms of visceral hypersensitivity in irritable bowel syndrome. *Am J Physiol-gastr L*. 302, G1085–G1098 (2012).

103. S. M. Brierley, R. C. W. Jones, G. F. Gebhart, L. A. Blackshaw, Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. *Gastroenterology*. 127, 166–178 (2004).

104. P. A. Hughes, S. M. Brierley, C. M. Martin, S. J. H. Brookes, D. R. Linden, L. A. Blackshaw, Post-inflammatory colonic afferent sensitisation: different subtypes, different pathways and different time courses. *Gut*. 58, 1333 (2009).

105. N. W. Bellono, J. R. Bayrer, D. B. Leitch, J. Castro, C. Zhang, T. A. O'Donnell, S. M. Brierley, H. A. Ingraham, D. Julius, Enterochromaffin Cells Are Gut Chemosensors that Couple to Sensory Neural Pathways. *Cell*. 170, 185-198.e16 (2017).

106. M. Sadeghi, A. Erickson, J. Castro, A. Deiteren, A. M. Harrington, L. Grundy, D. J. Adams, S. M. Brierley, Contribution of membrane receptor signalling to chronic visceral pain. *Int J Biochem Cell Biology*. 98, 10–23 (2018).

107. S. M. Brierley, P. A. Hughes, A. J. Page, K. Y. Kwan, C. M. Martin, T. A. O'Donnell, N. J. Cooper, A. M. Harrington, B. Adam, T. Liebregts, G. Holtmann, D. P. Corey, G. Y. Rychkov, L. A. Blackshaw, The Ion Channel TRPA1 Is Required for Normal Mechanosensation and Is Modulated by Algesic Stimuli. *Gastroenterology*. 137, 2084-2095.e3 (2009).

108. S. M. Brierley, A. J. Page, P. A. Hughes, B. Adam, T. Liebregts, N. J. Cooper, G. Holtmann, W. Liedtke, L. A. Blackshaw, Selective Role for TRPV4 Ion Channels in Visceral Sensory Pathways. *Gastroenterology*. 134, 2059–2069 (2008).

109. B. Feng, G. F. Gebhart, Characterization of silent afferents in the pelvic and splanchnic innervations of the mouse colorectum. *Am J Physiol-gastr L*. 300, G170–G180 (2011).

110. B. Feng, J. La, E. S. Schwartz, T. Tanaka, T. P. McMurray, G. F. Gebhart, Long-term sensitization of mechanosensitive and -insensitive afferents in mice with persistent colorectal hypersensitivity. *Am J Physiol-gastr L*. 302, G676–G683 (2012).

111. S. M. Brierley, R. Carter, W. Jones, L. Xu, D. R. Robinson, G. A. Hicks, G. F. Gebhart, L. A. Blackshaw, Differential chemosensory function and receptor expression of splanchnic and pelvic colonic afferents in mice. *J Physiology*. 567, 267–281 (2005).

112. S. M. Brierley, D. R. Linden, Neuroplasticity and dysfunction after gastrointestinal inflammation. *Nat Rev Gastroentero*. 11, 611–627 (2014).

Chapter 2:

ENTERIC GLIA: A NEW PLAYER IN ABDOMINAL PAIN

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ABSTRACT

Chronic abdominal pain is the most common gastrointestinal issue and contributes to the pathophysiology of functional bowel disorders and inflammatory bowel disease. Current theories suggest that neuronal plasticity and broad alterations along the brain-gut axis contribute to the development of chronic abdominal pain, but the specific mechanisms involved in chronic abdominal pain remain incompletely understood. Accumulating evidence implicates glial cells in the development and maintenance of chronic pain. Astrocytes and microglia in the central nervous system and satellite glia in dorsal root ganglia contribute to chronic pain states through reactive gliosis, the modification of glial networks, and the synthesis and release of neuromodulators. In addition, new data suggest that enteric glia, a unique type of peripheral glia found within the enteric nervous system, have the potential to modify visceral perception through interactions with neurons and immune cells. Understanding these emerging roles of enteric glia is important to fully understand the mechanisms that drive chronic pain and to identify novel therapeutic targets. In this review, we discuss enteric glial cell signaling mechanisms that have the potential to influence chronic abdominal pain.

ABDOMINAL PAIN DISORDERS

Abdominal pain is the most common gastrointestinal (GI) issue and results in over 15 million office visits per year (*1*). Chronic abdominal pain affects at least 10-15% of the general population and is a characteristic feature of functional bowel disorders such as IBS, IBDs, and is a growing problem associated with chronic opioid use (*2*–*4*). IBD, such as Crohn's disease and ulcerative colitis, is a chronic inflammatory disorder of the gastrointestinal tract that affects about 0.5% of the population in the Western world. Although the cause of IBD is not fully understood, it is believed to occur in individuals with genetic susceptibility who have an exaggerated immune response to the gut microbiota. Symptoms of IBD include abdominal pain, diarrhea, and gastrointestinal bleeding. IBD has significant economic costs, with the United States alone spending over $6 billion annually. Acute abdominal pain is associated with inflammatory flare-ups in IBD, but a large subset of patients that suffer from IBD eventually develop chronic abdominal pain even after achieving clinical remission. This condition, called comorbid irritable bowel syndrome (loosely termed “IBS-IBD”), is present in up to 46-59% of Crohn’s disease patients and 36-38% of ulcerative colitis patients in remission (*5*–*7*). Abdominal pain linked to these disorders is common in both adults and pediatric patients and is a major contributing factor to their low quality of life and high morbidity. As a result, the US Food and Drug Administration now requires the use of abdominal pain as a patient-reported outcome to assess the efficacy of new therapies for Crohn’s disease (*4*).

IBS is a chronic functional gastrointestinal disorder characterized by abdominal pain and changes in bowel habits. IBS is diagnosed based on the Rome IV criteria and does not involve overt inflammation-induced pathology to the intestine. IBS affects over

11% of the global population, with females being more likely to be affected (*8*). The causes of IBS are complex and may involve stress and immune responses (*9*). IBS patients have a reduced quality of life and experience additional clinical symptoms, including stool irregularities, as well as mental health comorbidities such as anxiety and depression. The direct and indirect costs of IBS in the United States are approximately $30 billion per year. Besides being an important well-known trigger for IBD, inflammation has also been found to contribute to the development of IBS. Acute gastroenteritis caused by pathogens such as *Escherichia coli, Campylobacter, Giardia lamblia,* and *Salmonella* is highly associated with the development of IBS, a condition known as post-infectious IBS (*10*, *11*).

The mechanisms that drive the development of chronic abdominal pain remain largely unresolved and this presents a major barrier to progress in the development of new therapies. Current therapies for IBD, for example, primarily focus on controlling inflammation with amino salicylates, corticosteroids, immunomodulators, and biological agents. While these agents clearly benefit the treatment of active inflammation, they do not address abdominal pain (*12*). Similarly, only 24% of IBS patients report complete relief of abdominal pain after motility-related symptom treatment. Leading drugs such as antidepressants (neuromodulators), antispasmodics, and opioids (*13*) have significant adverse effects in the CNS and on gut functions. Opioids are the current frontline therapy for chronic abdominal pain, but chronic opioid use has serious complications and produces a condition called narcotic bowel syndrome that is characterized by abdominal pain (*14*). This is a serious and growing issue that requires a more sophisticated

understanding of the causal mechanisms to permit the development of more effective therapies.

Current theories suggest that neuronal plasticity involving the sensitization of visceral afferent sensory nerve fibers and broad alterations to the brain-gut axis contribute to the development of chronic abdominal pain (*15*, *16*). Much of this theory is based on evidence demonstrating changes in neuronal sensitivity, firing patterns, and network activity in the periphery, brain, and spinal cord (*17*–*19*).

KNOWN MECHANISMS THAT CONTRIBUTE TO ABDOMINAL PAIN

Visceral hypersensitivity refers to an increased perception of stimuli arising from the viscera and is a major mechanism that contributes to abdominal pain in both IBS and IBD (*20*). This involves alterations along the brain-gut axis; a network of afferent and efferent neural pathways that link cognitive, emotional, and autonomic centers in the brain to neuroendocrine centers, the ENS, and the immune system. The sensitization of visceral afferent nerve fibers is driven by neuroplasticity and leads to a skewed perception of sensory stimuli. Nociceptive information is transduced by the peripheral axon terminals of primary afferent neurons whose cell bodies reside in dorsal root ganglia (DRG). These “nociceptors” are the axon terminals of polymodal c-fibers that transduce a variety of potentially noxious stimuli (mechanical, chemical, and thermal) to the brain. The sensitivity of nociceptors is one of the most important factors that gates the transmission of noxious information and alterations to nociceptor sensitivity in GI disorders can lead to an increased perception of pain (hyperalgesia) or the painful perception to innocuous stimuli (allodynia).

Immune activation and neuroplasticity are two key mechanisms involved in the generation of chronic abdominal pain. Nociceptor sensitivity is profoundly altered by inflammatory mediators (*21*). Active inflammation such as an IBD flare-up or acute gastroenteritis involves the release of proinflammatory mediators that alter nociceptor sensitivity. Inflammatory mediators including adenosine triphosphate (ATP) (*22*), histamine (*23*), interleukin-1b (IL-1β) (*24*), proteases (*25*), and bradykinins (*26*) cause pain by interacting with receptors on nociceptors. Other inflammatory mediators as interleukin-6 (IL-6) (*27*) and tumor-necrosis factor alpha (TNF-α) (*28*) lead to the sensitization of nociceptors through indirect mechanisms. Nociceptors, themselves, also contribute to local inflammation (termed “neuroinflammation”) though axon reflexes that produce inflammatory mediators including substance P (SP), neurokinin A (NKA), ATP, and calcitonin-gene related peptide (CGRP).

Neuroimmune activation following a psychological stressor or peripheral inflammation results in neuroplastic changes along nerve afferents that contribute to visceral hyperalgesia (*29*, *30*). Neuroimmune modulators drive neuroplasticity through mechanisms that include the downregulation of glutamate transporters and the subsequent upregulation of synaptic glutamate and the ionotropic glutamate receptor, N-methyl-D-aspartate (NMDA), signaling (*31*, *32*). Neuroplastic changes in the expression and function of transient receptor potential vanilloid receptor type-1 (TRPV1) channels present on nociceptors innervating the intestinal mucosa are also implicated in visceral hypersensitivity in IBS (*33*). Increased levels of TRPV1 are present in patients with idiopathic rectal hypersensitivity (*34*) and an increased density of TRPV1 immunoreactive

nerve fibers is preset in the colonic mucosa of IBS patients, correlating with the degree of abdominal pain experienced (*35*).

Alterations to emotional circuits of the brain, primarily those associated with stress and anxiety, contribute to visceral hypersensitivity in patients with IBS (*36*). Various stressors, both chronic and acute, regulate visceral pain responses in animal models (*37*, *38*) and are associated with an increase in symptom severity in functional (*39*) and inflammatory GI disorders (*40*, *41*). Brain regions associated with visceral pain, emotional arousal, and attention are consistently activated in neuroimaging studies of IBS patients (*42*). Further, alterations to gray matter density in areas associated with corticolimbic inhibition, stress, and arousal circuits indicate altered activity in the brains of IBS patients (*43*). The activation of these regions contributes to acute and chronic stress-induced hyperalgesia in the colon, in part, by stimulating the release of factors such as corticotropin releasing factor (CRF) (*44*, *45*).

GLIAL CELLS IN ABDOMINAL PAIN PATHWAYS

A plethora of emerging studies link nerve fiber sensitization with alterations to glial cells. Increased glial activity is characteristic of all forms of pain and glia contribute to the transition from acute to chronic pain through interactions with neurons that alter neurotransmission (*46*, *47*). For example, increases in astrocyte and microglial cell markers are present in animal models of acute pain inflammatory pain, and neuropathic pain (*48*–*51*). Structural changes and altered signaling in peripheral glial cells, such as satellite glia in DRG, are implicated in abdominal pain pathways(*52*). Furthermore, enteric glial cells, a unique class of peripheral glia found within the ENS, have bi-directional interactions with neurons in the gut that impact both gut reflexes in health and

neuroinflammation (*53*–*58*). Therefore, an emerging theme is that glia present at all sites along ascending and descending pathways that transduce and modify nociceptive information deriving from the intestine have the potential to broadly influence visceral perception (**Figure 2.1**).

Most of our understanding of glia in pain transmission stems from studies of glia at the first synapse in ascending pain pathways in the dorsal horn of the spinal cord. Microglia and astrocytes are the major glial cell types implicated in altering pain transmission at this level. Microglial activation in the spinal cord occurs in response to injury or inflammation and is reflected by increased cell number and altered gene expression profiles (*46*, *51*, *59*, *60*). Likewise, spinal astrocytes become activated and undergo reactive gliosis in animal models of neuropathic pain and increased expression of glial fibrillary acidic protein (GFAP), one marker of reactive astrocytes, mirrors the intensity of pain hypersensitivity (*51*, *60*, *61*). The inhibition of astrocyte activity results in a significant attenuation of inflammatory hyperalgesia, suggesting that astrocytic activation is at least partly necessary for the development of pain (*60*). Evidence from studies using in vivo optogenetics suggest that the selective activation of spinal astrocytes is sufficient to increase pain hypersensitivity (*62*). There is still much controversy surrounding glial optogenetic data, since several studies suggest that the activation of astrocytes driven by channel rhodopsin is an effect of cell swelling and is not driven by normal calcium signaling pathways (*63*). Alternative methods of glial-specific activation that more closely reflect physiological mechanisms, such as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), should be implemented to further validate these findings.

Glial activity in the spinal cord contributes to neuroplasticity, in part, by stimulating the release of glial-derived substances termed “gliotransmitters”. Active glial cells produce several proinflammatory mediators that contribute to the activation and sensitization of nerve fibers to central sensitization in the dorsal horn (*15*). For example, TNF-α produced by spinal microglia and astrocytes contributes to hyperalgesia and allodynia in pre-clinical pain models (*64*, *65*). TNF-α released by astrocytes and microglia also contributes to central sensitization and neuroplasticity through mechanisms that involve the downregulation of glutamate transporters and subsequently, glutamate excitotoxicity (*32*). Other key gliotransmitters such as purines activate nerve fibers directly (*22*) and in animal models of chronic pain, astrocytes increase the release of ATP and the production of pro-algesic mediators such as glutamate, cytokines, and chemokines (*15*).

Glial mechanisms similar to those occurring in the spinal cord may also affect processing in the brain. Although less is known regarding the contributions of glia in the brain to pain conditions, increased astrocyte activity is observed in the nucleus of the solitary tract following colonic inflammation (*66*). Likewise, microglial activation within the thalamus occurs following nociceptive spinal cord injury (*67*). Alterations to glia in the brain are likely associated with the emotional experience of pain and can be correlated with mood disorders such as depression and anxiety (*68*).

Multiple populations of peripheral glial cells actively contribute to modifying pain transmission through effects on nociceptors. For example, satellite glia play crucial roles in visceral sensitization. Satellite glia are located in peripheral sensory ganglia where they envelop neuronal cell bodies (*69*). Satellite glia undergo cell division and upregulate their production of GFAP following inflammation or injury to peripheral nerves and express and

release pro-inflammatory cytokines including TNF-α and IL-1β. In addition, satellite glia increase gap-junction-mediated coupling following nerve injury and inflammation (*52*). These changes to satellite glia are linked to gastrointestinal pain and are also implicated in chronic abdominal pain in IBS.

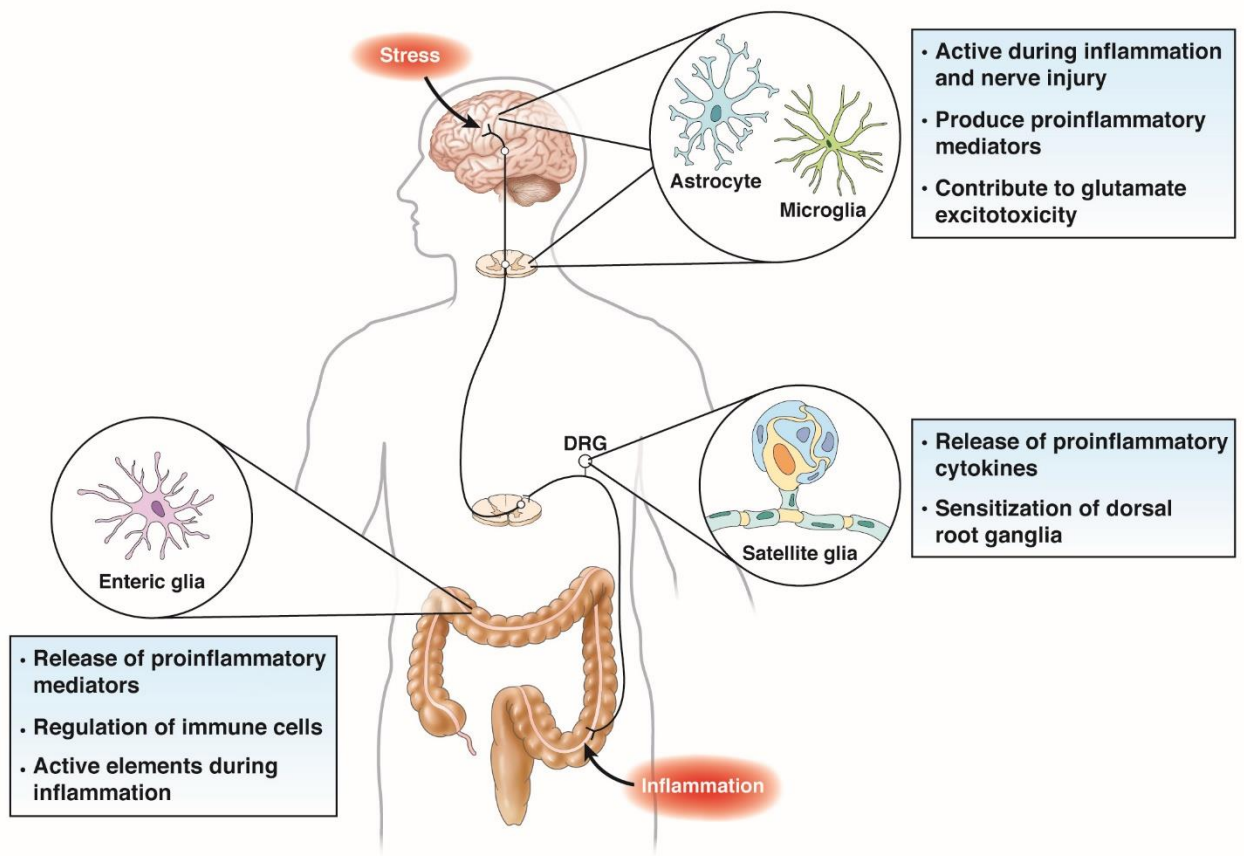

Figure 2.1: Major populations of glia that contribute to chronic pain. Astrocytes and microglia are present in the central nervous system and primarily affect chronic pain pathways in the brain and in the spinal cord. Satellite glia are peripheral glial cells located in the dorsal root ganglia where they contribute the sensitization of dorsal root ganglion cells. Enteric glial cells are located within the intestine and form part of the enteric nervous system. Enteric glia are located adjacent to the nerve endings of primary afferent fibers and intercellular interactions between enteric glia and neurons have the potential to influence chronic pain.

ENTERIC GLIA IN ABDOMINAL PAIN

Enteric glial cells are a unique class of peripheral glia that are associated with neurons in the ENS. Enteric glia play instrumental roles in gut reflexes that regulate motility and secretion, influence the epithelial barrier, and modulate neuroinflammation (*54*, *70*–*73*). Yet how enteric glia might influence visceral hypersensitivity and nociceptor sensitization is still relatively unknown.

Enteric glia, particularly those associated with neurons in the myenteric plexus, share some morphological and functional properties with astrocytes that might suggest similar roles in pain transmission. For example, enteric glia express molecular markers such as GFAP (*74*) and S100β (*75*), provide trophic and protective support to enteric neurons (*76*, *77*), exhibit activity encoded by intracellular calcium signaling in response to neurotransmitters, and release gliotransmitters through membrane channels composed of connexin-43 (Cx-43) (*57*). Connexins are a major structural component of gap junctions and hemichannels in glia and connexin-mediated glial mechanisms play a crucial role in development of chronic neuropathic pain (*78*). Bi-directional communication between enteric neurons and glia mediated by the Cx-43 dependent release of enteric gliotransmitters regulates gut reflexes that control motility and secretions (*57*, *58*, *79*, *80*) and the Cx-43 dependent release of gliotransmitters such as ATP during neuroinflammation drives neurodegeneration through the activation of neuronal P2X7 receptors (*56*, *81*). Recent studies demonstrate that disruption of enteric glia function with fluorocitrate is sufficient prevent visceral hyperalgesia in preclinical models of colitis (*82*). Enteric glia are intimately associated with nociceptors in the intestine and the activation of TRPV1+ sensory neurons within the myenteric plexus elicits glial activity and

gliotransmitter release through similar mechanisms (*83*). Whether gliotransmitters subsequently have direct or indirect effects on nociceptor activity or sensitivity is still unknown, but this remains an active area of research.

Enteric glia are targets of stress, which is a primary component in the development of visceral hypersensitivity. Several forms of stress, such as chronic stress and early life stress alter glial signaling and contribute to pain sensitivity in IBS (*36*). The contribution of enteric glia to stress induced hyperalgesia is still undefined. However, early life stress causes structural changes in enteric glial cells that are correlated with inefficient gastric motility (*84*). Further, stress activates sympathetic pathways in the intestine that activate enteric glia via purinergic transmission (*85*).

Enteric glia contribute to intestinal inflammatory and immune responses (*86*) and interact with, and express, cytokines and immunoregulatory signals that contribute to altered nociceptive signaling (*86*–*88*). Altered immune responses are key components in the development of chronic pain and mediators that sensitize nociceptors such as NKA (*83*), SP (*83*), proteases (*89*), and TRPV4 (*90*) agonists all elicit responses in enteric glia. This raises the possibility that enteric glia contribute to nociceptor sensitization caused by these mediators. In support, perturbing enteric glial metabolism with reduces visceromotor responses (VMR) in mice (*91*).

Together, these multiple lines of evidence suggest that crosstalk between enteric glia and nociceptors contributes to mechanisms that drive visceral hypersensitivity (**Figure 2.2**). The precise mechanisms by which enteric glia influence nociceptors are still being discovered. Yet new data that we will discuss in the following sections are offering

promising leads that could identify novel molecular targets for new abdominal pain therapies.

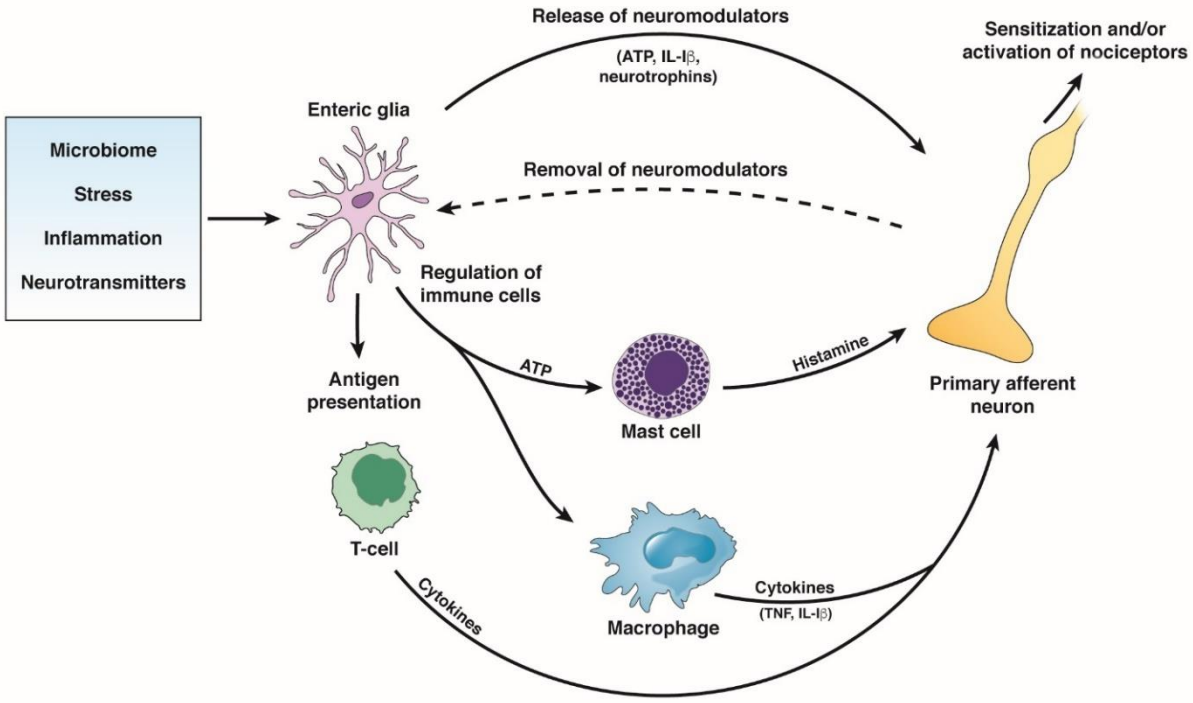

Figure 2.2: Overview of potential enteric glial cell mechanisms that contribute to the sensitization of nociceptors. Highlighted are pathways that directly and indirectly sensitize nociceptors. Enteric glial activity can be influenced by a number of factors, such as inflammation, stress, neurotransmission and/or the gut microbiota. Enteric glia release neuromodulators that act on primary afferent neurons by binding to receptors on nociceptor nerve endings or by sensitizing nociceptors. Enteric glia possess ectoenzymes that remove neuromodulators from the extracellular space. Enteric glia also have the potential to indirectly modulate nociceptive neurons by activating immune cells either through the release of immune cell regulators or by acting as antigen-presenting cells.

DIRECT MODULATION OF NOCICEPTORS BY ENTERIC GLIA

Enteric glia play active roles in neurotransmission in the intestine by generating and regulating the availability of neurotransmitters. Many of the transmitter systems regulated by enteric glia are involved in the activation and sensitization of nociceptors. For example, enteric glia are a primary source of ATP, which contributes to cell-to-cell communication in both health and disease and acts on P2X receptors expressed by nociceptors (*22*, *56*, *92*, *93*). Purines and purinergic signaling from glial cells are altered during inflammation and are strongly implicated in the process of visceral hypersensitivity and abdominal pain (*94*, *95*). Glial cells, including enteric glia, also possess ecto-enzymes such as NTPDase2 that are responsible for the degradation of ATP and other transmitters (*56*, *96*, *97*). Astrocytic NTPDase2 expression is altered during inflammation and could contribute to the sensitization of sensory neurons by increasing purinergic signaling (*97*). The similarities between enteric glia and astrocytes suggest that a similar behavior could be observed in enteric glia. Glial ATP release is triggered by the excitation of enteric glial cells, intracellular calcium signaling, and the subsequent opening of Cx-43 hemichannels (*56*), which is a molecular mechanism highly implicated in the development of nociceptive pain and inflammatory pain (*56*, *78*, *98*). In spinal astrocytes, Cx-43 expression is highly elevated following spinal cord injury and the inhibition of Cx-43 reduces allodynia and hyperalgesia (*78*, *98*, *99*). A direct link between Cx-43 on enteric glial cells and nociceptors sensitization has yet to emerge. However, due to the implications of astroglial Cx-43 in chronic pain and the analogous nature of enteric glia, it is likely that enteric Cx-43 is readily involved in the mechanisms of abdominal pain. In support, we recently

showed that bi-directional communication between nociceptors and enteric glia involves glial Cx-43 (*83*).

Pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α play major roles in the development of intestinal pathology and are known contributors to the development and maintenance of inflammatory and neuropathic pain (*15*, *46*). These cytokines are increased during IBS and IBD90,91. Astrocytes, microglia, and satellite glia produce proinflammatory cytokines such as IL-1β during inflammation and nerve injury (*100–105*). Similarly, enteric glia produce, and respond to cytokines including IL-6 and IL-1β (*87*, *88*, *106*). Experimental studies support a proinflammatory, pronociceptive role for IL-6 and the upregulation of IL-6 correlates with the development of hyperalgesia and allodynia in animal models (*107–109*). Blocking IL-6 production and activity reduces or alleviates nociceptor sensitization (*110*, *111*). Similarly, IL-1β has pronociceptive effects and treatment with IL-1β in vivo causes increased VMR to colorectal distension in animal models of inflammatory pain (*112*).

Enteric glia also interact with TNF-α signaling pathways and TNF-α is heavily implicated in the development of visceral hypersensitivity. Microglia and satellite glia increase their expression of TNF-α during nerve injury and inflammation (*92*, *93*, *113*) and the excitation of DRG neurons in mouse colitis models stimulates TNF-α production (*93*). The exogenous application of TNF-α increases DRG neuron activity and visceral pain responses in TNBS-colitis, which is reversed by the administration of anti-TNF-α (*93*). Evidence regarding the production of TNF-α by enteric glia is still controversial (*86*). However, TNF-α does alter enteric glial cell behavior and exposure to TNF-α induces increased GFAP expression and calcium signaling in enteric glia (*77*, *114*). It is possible

that these effects are mediated through modifications to Cx-43 hemichannels based on the known effects of TNF-α in spinal cord injury (*115*, *116*).

Neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) are also associated with neuropathic pain and inflammatory processes109. Enteric glia are a significant source, and target of neurotrophins in the intestine and alterations in the expression of neurotrophins and their receptors are associated with changes in enteric glial activity (*117*, *118*). The upregulation of BDNF expression by DRG neurons contributes to visceral pain (*117*) and enteric glia in the colon of IBS patients highly express the BDNF receptor TrkB81 (*119*). In addition, recent data suggest that BDNF induced colonic hypersensitivity involves reactive enteric glia because visceral pain responses and GFAP expression were reduced in BDNF knockout mice81. Similarly, NGF secretion by cultured enteric glia and mRNA levels of NGF and its receptor TrkA are increased in the presence of LPS, TNFa and IL1b (*120*). Given the multiple biologic roles of neurotrophins in the maintenance of neuronal survival and function (*121*), changes in glial neurotrophin expression and/or signaling could contribute to the neuroplasticity involved in visceral hypersensitivity.

The gut microbiome has emerged as an essential component of the brain-gut axis and alterations in the composition of the microbiome are observed in a number of gastrointestinal and pain conditions (*122*). Altered fecal microbiota in IBS patients may even predict severity (*123*). The microbiota produce of a range of neuroactive compounds such as the neurotransmitters GABA, serotonin, norepinephrine, and dopamine (*124*) and other bacterial products such as LPS (*125*) and formyl peptides (*126*). This suggests that

the gut microbiome could influence pain pathways, but mechanistic links between the gut microbiome, sensory nerves, and alterations in the brain-gut axis are still relatively undefined. New data suggest that some effects of the microbiome could be exerted through effects on enteric glial function and development (*127*). The generation of the mucosal enteric glial cell network parallels the maturation of the gut microbiome (*128*, *129*) and the impaired mucosal glial population in germ free mice can be restored by introducing a normal gut microbiome (*129*). The LPS receptor, TLR4 is located on enteric glial cells and is increased in mice following exposure to stress (*38*, *130*). VMR in maternally separated mice can be suppressed with oxytocin, which also causes a downregulation of enteric glial cell activity and TLR4 expression. This is indicative of a TLR4-dependent mechanism of enteric glial cells in modulating visceral pain responses (*130*–*132*). The gut microbiome also produces several gliomodulators such as proteases that directly act on enteric glial cells and are implicated in the pathophysiology of visceral hypersensitivity (*8*, *89*, *124*). However, the significance of interactions between the microbiome and glia in the generation of visceral pain is still unknown and will require additional mechanistic studies to unravel.

INDIRECT MODULATION OF NOCICEPTORS BY ENTERIC GLIA

Inflammation is a key component in the development of chronic pain (*15*, *102*) and abnormalities in immune responses are considered one of the primary causes leading to the development of IBS (*133*). Approximately 10% of all cases of IBS are associated with a previous inflammatory insult such as a bacterial infection (*133*). Immune cells play an important role an important role in the sensitization of sensory nerve fibers during inflammation and contribute to the development of both acute and chronic pain (*15*). Their

active role in pain is primarily due to the release of proinflammatory mediators that modulate ion channel expression in primary afferent fibers such as TRPV1 receptors (*134–137*). Histamine, for example, activates nerve fibers and contributes to neurogenic inflammation and pain (*138*). Blocking H1 histamine receptors expressed by primary sensory afferent neurons in mice reduces pain responses after cutaneous injection of formalin to the paw (*139*).

Recently, the concept that both central and peripheral glial cells function as immunoregulatory cells has emerged (*131*). Glial cell-mast cell communication is critical for the development of inflammation (*140*, *141*). Histamine clearance is an essential process for avoiding excessive histaminergic neuronal activation. Astrocytes play a prominent role in histamine clearance via histamine N-methyl transferase (HNMT), a histamine metabolizing enzyme (*142*). Astrocyte activity is modulated via histamine receptors on their cell membrane (*115*) and microglial activation through histamine results in the subsequent release of inflammatory mediators (*116*). Enteric glia exhibit the potential to function through similar pathways since their activity is also increased in the presence of histamine (*143*).

Current evidence supports the notion that enteric glia modify immune responses by the secretion of cytokines and other chemical transmitters and/or by acting as antigen presenting cells. Enteric glia also modulate adaptive immune responses and have immunosuppressive effects on T cells in vitro (*144*). Glial proinflammatory mediators including S100B, ATP, and nitric oxide increase oxidative stress in the local environment and contribute to neuronal cell death and the release of apoptotic signaling that triggers immune responses (*56*, *114*, *145*, *146*). Enteric glia are also capable of expressing major

histocompatibility complex II (MHCII) molecules and ligands necessary for T cell signaling (*73*, *145*, *147*, *148*). Mouse enteric glia also express major MHCII in response to pathogenic microorganisms which functions to modulate the activation of T-lymphocyte subsets involved in tolerance through mechanisms associated with autophagy (*73*). Recent data show that enteric glia promote muscularis macrophages to adopt a pro-inflammatory phenotype through mechanisms that involve Cx43 and M- CSF release during chronic colitis in mice (*72*). Several types of immune cells are involved in inflammatory pain, particularly in the persistence of hyperalgesia (*149*). Immune cell activity can also contribute to neuropathic pain because of damage to peripheral nerves or to the CNS (*15*). Together these data show that enteric glia can modulate immune cells, which in turn can influence nociception.

ROLE FOR ENTERIC GLIA IN OPIOID INDUCED HYPERALGESIA

Current treatments targeting chronic pain are limited and often involve the use of opioids (*150*). However, opioid use has serious side effects that include persistent constipation and antinociceptive tolerance that limit clinical efficacy. Opioid-induced hyperalgesia can result from chronic opioid use and produce an IBS-like syndrome termed “narcotic bowel syndrome” (*151*, *152*). Narcotic bowel syndrome is a subset of opioid bowel dysfunction characterized by recurring abdominal pain that worsens with the continued or escalating use of narcotics (*153*).

Glial cells play a role in modulating opioid behavior (*153*–*155*). Chronic opioid treatment causes glial release of nitric oxide, prostaglandins, excitatory amino acids, and growth factors. It also causes changes in purinergic signaling and increases the release of glial mediators that enhance the release of pain transmission (*153*). Opiates modulate

central glial activity by directly binding to the glial (μ)-opioid receptor causing the release of proinflammatory cytokines or indirectly through the release of dynorphins (*154*). Chronic exposure to opioids leads to robust astrocyte and microglia reactions and induces hyperalgesia (*155*). Acute opioid treatment also produces marked satellite glia reaction in DRG (*156*). The bulk of these effects result from altered glial purinergic signaling. Chronic opioid treatment leads to the upregulation of P2X4 and P2X7 receptors in spinal microglia and contributes to opioid tolerance (*156*–*158*). Similar effects are observed in enteric glial cells. Long-term morphine treatment enhances purinergic activity in enteric glia that subsequently leads to barrier dysfunction and constipation through mechanisms that require glial Cx-43 activity (*132*). Opioid exposure upregulates proinflammatory cytokine release and contributes to morphine tolerance (*159*). Chronic morphine exposure causes the release of TNF-α, IL-1β, and IL-6 from glial cells, all of which are known contributors to chronic pain (*160*). Chronic opioid exposure induces glial responses through TLR4 and reinforces the rewarding effects of opioids. Blocking TLR4 signaling attenuates the development of analgesic tolerance and hyperalgesia (*161*, *162*). Enteric glia express TLR receptors and TLR4 deficient mice fail to develop opioid induced bowel disfunctions (*163*). Although these findings implicate enteric glial cells as playing a major role in morphine induced bowel disfunction and opioid tolerance, more in-depth studies are needed to clearly define the role of glia in opioid induced hyperalgesia.

CONCLUSIONS

New therapies are desperately needed to combat the common clinical issue of chronic abdominal pain. However, their development is limited primarily by the incomplete understanding of mechanisms that drive the development and maintenance visceral pain. Recent discoveries suggest that significant new insight into these mechanisms could be gained by focusing on glial cells. Glia are intertwined with mechanisms that produce nociceptive hyperalgesia and allodynia in pathological conditions. Specifically, glia are responsive to neurotransmitters in pain circuits and produce, secrete, and regulate modulators that activate and sensitize nociceptors. Moreover, glial cells actively contribute to processes involved in neuroplasticity. The precise mechanisms whereby peripheral glia contribute to neuroplasticity in pain pathologies is still a developing area, but current discoveries suggest that these mechanisms are involved in the transition from acute to chronic pain.

The contributions of glial cells to pain hypersensitivity are not centralized, but in fact require the involvement of several glial cell types along the brain-gut axis. There is substantial evidence regarding enteric glia suggests that they are involved in visceral hypersensitivity and chronic pain. Enteric glia are primed for both immune and stress responses. Increasing evidence supports their role in nociception, as they can influence nerve fiber activity either directly by releasing pronociceptive modulators or indirectly by interacting with the immune system. Their reactivity is also linked with altered ion channel signaling on nociceptor nerve endings. In addition to stress and inflammatory visceral hypersensitivity, enteric glia also show promise in playing a key role in the mechanisms of opioid induced hyperalgesia and narcotic bowel syndrome.

Enteric glia signaling involving Cx-43 is currently the most well described glial mechanism that contributes to neuroinflammation and intercellular communication (*56*–*58*). However, the therapeutic potential of this target is untested. Existing drugs that modulate Cx-43 are poorly selective and interact with connexin hemichannels and gap junctions in multiple tissues and cell types, which could potentially compromise normal gut function. This suggests that targeting mechanisms up- or downstream of glial Cx-43 may prove more effective. In support, a clinical trial targeting downstream neuronal mechanisms with the P2X7 receptor antagonist AZD9056 showed significant promise in the treatment of visceral pain in CD patients (*164*). Other potential targets on enteric glia to modulate pain are less defined but targeting enteric glial IL-1b and IL-6 signaling could be promising since both contribute to pain pathologies (*108*, *111*, *112*). Similarly, modulating glial PAR and neurotrophin signaling may also serve to regulate glial activity and potentially reduce visceral hypersensitivity (*165*, *166*).

Further studies should focus on elucidating the role of enteric glial cells in chronic abdominal pain, since these cell types are essential in maintaining intestinal homeostasis and are highly active elements in GI disorders. In general, glia demonstrate great promise in filling the existing gaps in knowledge with regards to chronic abdominal pain. However, it is still necessary to define the specific mechanisms involved in these processes.

REFERENCES

1. A. F. Peery, S. D. Crockett, A. S. Barritt, E. S. Dellon, S. Eluri, L. M. Gangarosa, E. T. Jensen, J. L. Lund, S. Pasricha, T. Runge, M. Schmidt, N. J. Shaheen, R. S. Sandler, Burden of Gastrointestinal, Liver, and Pancreatic Diseases in the United States. *Gastroenterology*. 149, 1731-1741.e3 (2015).

2. K. E. Mansfield, J. Sim, J. L. Jordan, K. P. Jordan, A systematic review and meta-analysis of the prevalence of chronic widespread pain in the general population. *PAIN*. 157, 55 (2016).

3. G. F. Longstreth, G. W. Thompson, W. D. Chey, L. A. Houghton, F. Mearin, R. C. Spiller, Functional Bowel Disorders. *Gastroenterology*. 130, 1480–1491 (2006).

4. M. Regueiro, J. B. Greer, E. Szigethy, Etiology and Treatment of Pain and Psychosocial Issues in Patients With Inflammatory Bowel Diseases. *Gastroenterology*. 152, 430-439.e4 (2017).

5. J. Keohane, C. O'Mahony, L. O'Mahony, S. O'Mahony, E. M. Quigley, F. Shanahan, Irritable Bowel Syndrome–Type Symptoms in Patients With Inflammatory Bowel Disease: A Real Association or Reflection of Occult Inflammation? *The American Journal of Gastroenterology*. 105, ajg2010156 (2010).

6. I. M. Minderhoud, B. Oldenburg, J. A. Wismeijer, G. P. Henegouwen, A. Smout, IBS-Like Symptoms in Patients with Inflammatory Bowel Disease in Remission; Relationships with Quality of Life and Coping Behavior. *Digestive Diseases and Sciences*. 49, 469–474 (2004).

7. S. J. Halpin, A. C. Ford, Prevalence of Symptoms Meeting Criteria for Irritable Bowel Syndrome in Inflammatory Bowel Disease: Systematic Review and Meta-Analysis. *The American Journal of Gastroenterology*. 107, ajg2012260 (2012).

8. H. Ceuleers, H. Spaendonk, N. Hanning, J. Heirbaut, A.-M. Lambeir, J. Joossens, K. Augustyns, J. G. Man, I. Meester, B. Y. Winter, Visceral hypersensitivity in inflammatory bowel diseases and irritable bowel syndrome: The role of proteases. *World Journal of Gastroenterology*. 22, 10275 (2016).

9. C. Carco, W. Young, R. B. Gearry, N. J. Talley, W. C. McNabb, N. C. Roy, Increasing Evidence That Irritable Bowel Syndrome and Functional Gastrointestinal Disorders Have a Microbial Pathogenesis. *Front Cell Infect Mi*. 10, 468 (2020).

10. J. K. Marshall, M. Thabane, A. X. Garg, W. F. Clark, P. Moayyedi, S. M. Collins, W. H. S. Investigators, Eight year prognosis of postinfectious irritable bowel syndrome following waterborne bacterial dysentery. *Gut*. 59, 605 (2010).

11. R. Spiller, K. Garsed, Postinfectious Irritable Bowel Syndrome. *Gastroenterology*. 136, 1979–1988 (2009).

12. J.-F. Colombel, U. Mahadevan, Inflammatory Bowel Disease 2017: Innovations and Changing Paradigms. *Gastroenterology*. 152, 309–312 (2017).

13. J. BouSaba, W. Sannaa, M. Camilleri, Pain in irritable bowel syndrome: Does anything really help? *Neurogastroenterol Motil*. 34, e14305 (2022).

14. M. Camilleri, A. Lembo, D. A. Katzka, Opioids in Gastroenterology: Treating Adverse Effects and Creating Therapeutic Benefits. *Clinical Gastroenterology and Hepatology*. 15, 1338–1349 (2017).

15. R.-R. Ji, A. Chamessian, Y.-Q. Zhang, Pain regulation by non-neuronal cells and inflammation. *Science*. 354, 572–577 (2016).

16. E. A. Mayer, K. Tillisch, The Brain-Gut Axis in Abdominal Pain Syndromes. *Annual Review of Medicine*. 62, 381–396 (2011).

17. M. S. Gold, G. F. Gebhart, Nociceptor sensitization in pain pathogenesis. *Nature Medicine*. 16, 1248 (2010).

18. F. Cervero, J. Laird, Understanding the signaling and transmission of visceral nociceptive events. *Journal of Neurobiology*. 61, 45–54 (2004).

19. A. I. Basbaum, D. M. Bautista, G. Scherrer, D. Julius, Cellular and Molecular Mechanisms of Pain. *Cell*. 139, 267–284 (2009).

20. M. Simrén, H. Törnblom, O. S. Palsson, M. A. van Tilburg, L. Oudenhove, J. Tack, W. E. Whitehead, Visceral hypersensitivity is associated with GI symptom severity in functional GI disorders: consistent findings from five different patient cohorts. *Gut*. 67, 255–262 (2018).

21. C. Stein, H. Machelska, Modulation of Peripheral Sensory Neurons by the Immune System: Implications for Pain Therapy. *Pharmacological Reviews*. 63, 860–881 (2011).

22. S. P. Cook, E. W. McCleskey, Cell damage excites nociceptors through release of cytosolic ATP. *Pain*. 95, 41–47 (2002).

23. M. M. Wouters, D. Balemans, S. Wanrooy, J. Dooley, V. Cibert-Goton, Y. A. Alpizar, E. E. Valdez-Morales, Y. Nasser, P. P. Veldhoven, W. Vanbrabant, S. der Merwe, R. Mols, B. Ghesquière, C. Cirillo, I. Kortekaas, P. Carmeliet, W. E. Peetermans, S. Vermeire, P. Rutgeerts, P. Augustijns, P. W. Hellings, A. Belmans, S. Vanner, D. C. Bulmer, K. Talavera, P. Berghe, A. Liston, G. E. Boeckxstaens, Histamine Receptor H1–Mediated Sensitization of TRPV1 Mediates Visceral Hypersensitivity and Symptoms in Patients With Irritable Bowel Syndrome. *Gastroenterology*. 150, 875-887.e9 (2016).

24. A. M. Binshtok, H. Wang, K. Zimmermann, F. Amaya, D. Vardeh, L. Shi, G. J. Brenner, R.-R. Ji, B. P. Bean, C. J. Woolf, T. A. Samad, Nociceptors Are Interleukin-1β Sensors. *The Journal of Neuroscience*. 28, 14062–14073 (2008).

25. S. Amadesi, G. S. Cottrell, L. Divino, K. Chapman, E. F. Grady, F. Bautista, R. Karanjia, C. Barajas-Lopez, S. Vanner, N. Vergnolle, N. W. Bunnett, Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cε- and A-dependent mechanisms in rats and mice. *The Journal of Physiology*. 575, 555–571 (2006).

26. M. Bandell, G. M. Story, S. Hwang, V. Viswanath, S. R. Eid, M. J. Petrus, T. J. Earley, A. Patapoutian, Noxious Cold Ion Channel TRPA1 Is Activated by Pungent Compounds and Bradykinin. *Neuron*. 41, 849–857 (2004).

27. O. Obreja, W. Biasio, M. Andratsch, K. Lips, P. Rathee, A. Ludwig, S. Rose-John, M. Kress, Fast modulation of heat-activated ionic current by proinflammatory interleukin 6 in rat sensory neurons. *Brain*. 128, 1634–1641 (2005).

28. J. Czeschik, T. Hagenacker, M. Schäfers, D. Büsselberg, TNF-α differentially modulates ion channels of nociceptive neurons. *Neuroscience Letters*. 434, 293–298 (2008).

29. F. Azpiroz, M. Bouin, M. Camilleri, E. Mayer, P. Poitras, J. Serra, R. Spiller, Mechanisms of hypersensitivity in IBS and functional disorders. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*. 19, 62–88 (2007).

30. J. N. Sengupta, Sensory Nerves. *Handbook of experimental pharmacology*. 194, 31–74 (2009).

31. J. Li, J. A. McRoberts, H. S. Ennes, M. Trevisani, P. Nicoletti, Y. Mittal, E. A. Mayer, Experimental colitis modulates the functional properties of NMDA receptors in dorsal root ganglia neurons. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 291, G219–G228 (2006).

32. B. Sung, G. Lim, J. Mao, Altered Expression and Uptake Activity of Spinal Glutamate Transporters after Nerve Injury Contribute to the Pathogenesis of Neuropathic Pain in Rats. *Journal of Neuroscience*. 23, 2899–2910 (2003).

33. C. R. Jones, L. Xu, G. Gebhart, The Mechanosensitivity of Mouse Colon Afferent Fibers and Their Sensitization by Inflammatory Mediators Require Transient Receptor Potential Vanilloid 1 and Acid-Sensing Ion Channel 3. *The Journal of Neuroscience*. 25, 10981–10989 (2005).

34. C. Chan, P. Facer, J. Davis, G. Smith, J. Egerton, C. Bountra, N. Williams, P. Anand, Sensory fibres expressing capsaicin receptor TRPV1 in patients with rectal hypersensitivity and faecal urgency. *The Lancet*. 361, 385–391 (2003).

35. A. Akbar, Y. Yiangou, P. Facer, J. Walters, P. Anand, S. Ghosh, Increased capsaicin receptor TRPV1-expressing sensory fibres in irritable bowel syndrome and their correlation with abdominal pain. *Gut*. 57, 923–929 (2008).

36. M. Larauche, A. Mulak, Y. Taché, Stress-Related Alterations of Visceral Sensation: Animal Models for Irritable Bowel Syndrome Study. *Journal of Neurogastroenterology and Motility*. 17, 213–234 (2011).

37. T.-H. Ren, J. Wu, D. Yew, E. Ziea, L. Lao, W.-K. Leung, B. Berman, P.-J. Hu, J. J. Sung, Effects of neonatal maternal separation on neurochemical and sensory response to colonic distension in a rat model of irritable bowel syndrome. *American journal of physiology. Gastrointestinal and liver physiology*. 292, G849-56 (2006).

38. M. Tramullas, B. C. Finger, R. D. Moloney, A. V. Golubeva, G. Moloney, T. G. Dinan, J. F. Cryan, Toll-Like Receptor 4 Regulates Chronic Stress-Induced Visceral Pain in Mice. *Biological Psychiatry*. 76, 340–348 (2014).

39. S. Elsenbruch, C. Rosenberger, U. Bingel, M. Forsting, M. Schedlowski, E. R. Gizewski, Patients With Irritable Bowel Syndrome Have Altered Emotional Modulation of Neural Responses to Visceral Stimuli. *Gastroenterology*. 139, 1310-1319.e4 (2010).

40. A. Drewes, J. Frkjær, E. Larsen, H. Reddy, L. Arendt-Nielsen, H. Gregersen, Pain and mechanical properties of the rectum in patients with active ulcerative colitis. *Inflammatory Bowel Diseases*. 12, 294 (2006).

41. K. Bielefeldt, B. Davis, D. G. Binion, Pain and inflammatory bowel disease. *Inflammatory bowel diseases*. 15, 778–88 (2009).

42. K. Tillisch, E. A. Mayer, J. S. Labus, Quantitative Meta-analysis Identifies Brain Regions Activated During Rectal Distension in Irritable Bowel Syndrome. *Gastroenterology*. 140, 91–100 (2011).

43. D. A. Seminowicz, J. S. Labus, J. A. Bueller, K. Tillisch, B. D. Naliboff, C. M. Bushnell, E. A. Mayer, Regional Gray Matter Density Changes in Brains of Patients With Irritable Bowel Syndrome. *Gastroenterology*. 139, 48-57.e2 (2010).

44. M. Larauche, S. Bradesi, M. Million, P. McLean, Y. Taché, E. A. Mayer, J. A. McRoberts, Corticotropin-releasing factor type 1 receptors mediate the visceral hyperalgesia induced by repeated psychological stress in rats. *American journal of physiology. Gastrointestinal and liver physiology*. 294, G1033-40 (2008).

45. J.-H. La, Peripheral corticotropin releasing hormone mediates post-inflammatory visceral hypersensitivity in rats. *World Journal of Gastroenterology*. 14, 731 (2008).

46. R.-R. R. Ji, T. Berta, M. Nedergaard, Glia and pain: is chronic pain a gliopathy? *Pain*. 154 Suppl 1, S10-28 (2013).

47. B. E. Cairns, L. Arendt-Nielsen, P. Sacerdote, Perspectives in Pain Research 2014: Neuroinflammation and glial cell activation: The cause of transition from acute to chronic pain? *Scandinavian Journal of Pain*. 6, 3–6 (2015).

48. S. L. Stuesse, T. Crisp, D. L. McBurney, J. B. Schechter, J. A. Lovell, W. L. R. Cruce, Neuropathic pain in aged rats: behavioral responses and astrocytic activation. *Experimental Brain Research*. 137, 219–227 (2001).

49. D. E. Coyle, Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behavior. *Glia*. 23, 75–83 (1998).

50. S. M. Sweitzer, R. W. Colburn, M. Rutkowski, J. A. DeLeo, Acute peripheral inflammation induces moderate glial activation and spinal IL-1β expression that correlates with pain behavior in the rat1Published on the World Wide Web on 17 March 1999.1. *Brain Research*. 829, 209–221 (1999).

51. F. Y. Tanga, V. Raghavendra, J. A. DeLeo, Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochemistry International*. 45, 397–407 (2004).

52. M. Hanani, Role of satellite glial cells in gastrointestinal pain. *Frontiers in Cellular Neuroscience*. 9, 412 (2015).

53. I. A. Brown, B. D. Gulbransen, The antioxidant glutathione protects against enteric neuron death in situ, but its depletion is protective during colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 314, G39–G52 (2018).

54. V. Grubišić, B. D. Gulbransen, Enteric glia: the most alimentary of all glia. *The Journal of Physiology*. 595, 557–570 (2017).

55. J. L. McClain, D. E. Fried, B. D. Gulbransen, Agonist-Evoked Ca2+ Signaling in Enteric Glia Drives Neural Programs That Regulate Intestinal Motility in Mice. *Cell Mol Gastroenterology Hepatology*. 1, 631–645 (2015).

56. I. A. M. Brown, J. L. McClain, R. E. Watson, B. A. Patel, B. D. Gulbransen, Enteric Glia Mediate Neuron Death in Colitis Through Purinergic Pathways That Require Connexin-43 and Nitric Oxide. *Cmgh Cell Mol Gastroenterology Hepatology*. 2, 77–91 (2016).

57. J. L. McClain, V. Grubišić, D. Fried, R. A. Gomez-Suarez, G. M. Leinninger, J. Sévigny, V. Parpura, B. D. Gulbransen, Ca2+ Responses in Enteric Glia Are Mediated by Connexin-43 Hemichannels and Modulate Colonic Transit in Mice. *Gastroenterology*. 146, 497-507.e1 (2014).

58. V. Grubišić, B. D. Gulbransen, Enteric glial activity regulates secretomotor function in the mouse colon but does not acutely affect gut permeability. *The Journal of Physiology*. 595, 3409–3424 (2017).

59. R.-D. Gosselin, M. R. Suter, R.-R. Ji, I. Decosterd, Glial Cells and Chronic Pain. *The Neuroscientist*. 16, 519–531 (2010).

60. W. Guo, H. Wang, M. Watanabe, K. Shimizu, S. Zou, S. C. LaGraize, F. Wei, R. Dubner, K. Ren, Glial–Cytokine–Neuronal Interactions Underlying the Mechanisms of Persistent Pain. *The Journal of Neuroscience*. 27, 6006–6018 (2007).

61. A. Romero-Sandoval, N. Chai, N. Nutile-McMenemy, J. A. DeLeo, A comparison of spinal Iba1 and GFAP expression in rodent models of acute and chronic pain. *Brain Research*. 1219, 116–126 (2008).

62. Y. Nam, J.-H. Kim, J.-H. Kim, M. Jha, J. Jung, M.-G. Lee, I.-S. Choi, I.-S. Jang, D. Lim, S.-H. Hwang, H.-J. Cho, K. Suk, Reversible Induction of Pain Hypersensitivity following Optogenetic Stimulation of Spinal Astrocytes. *Cell Reports*. 17, 3049–3061 (2016).

63. W.-H. Cho, E. Barcelon, S. Lee, Optogenetic Glia Manipulation: Possibilities and Future Prospects. *Experimental Neurobiology*. 25, 197–204 (2016).

64. Y. Zhong, L.-J. Zhou, W.-J. Ren, W.-J. Xin, Y.-Y. Li, T. Zhang, X.-G. Liu, The direction of synaptic plasticity mediated by C-fibers in spinal dorsal horn is decided by Src-family kinases in microglia: The role of tumor necrosis factor-α. *Brain, Behavior, and Immunity*. 24, 874–880 (2010).

65. Y. Gao, L. Zhang, J. R. Glia, Spinal injection of TNF-α-activated astrocytes produces persistent pain symptom mechanical allodynia by releasing monocyte chemoattractant protein-1 (2010).

66. Y.-N. Sun, J.-Y. Luo, Z.-R. Rao, L. Lan, L. Duan, GFAP and Fos immunoreactivity in lumbo-sacral spinal cord and medulla oblongata after chronic colonic inflammation in rats. *World journal of gastroenterology*. 11, 4827–32 (2005).

67. P. Zhao, S. G. Waxman, B. C. Hains, Modulation of Thalamic Nociceptive Processing after Spinal Cord Injury through Remote Activation of Thalamic Microglia by Cysteine–Cysteine Chemokine Ligand 21. *The Journal of Neuroscience*. 27, 8893–8902 (2007).

68. G. Rajkowska, J. Miguel-Hidalgo, Gliogenesis and glial pathology in depression. *CNS & neurological disorders drug targets*. 6, 219–33 (2007).

69. M. Hanani, Satellite glial cells in sympathetic and parasympathetic ganglia: In search of function. *Brain Research Reviews*. 64, 304–327 (2010).

70. B. D. Gulbransen, Do nerves make bowels irritable? *American journal of physiology. Gastrointestinal and liver physiology* (2018), doi:10.1152/ajpgi.00129.2018.

71. J. L. McClain, V. Grubišić, D. Fried, R. A. Gomez-Suarez, G. M. Leinninger, J. Sévigny, V. Parpura, B. D. Gulbransen, Ca2+ Responses in Enteric Glia Are Mediated by Connexin-43 Hemichannels and Modulate Colonic Transit in Mice. *Gastroenterology*. 146, 497-507.e1 (2014).

72. V. Grubišić, J. L. McClain, D. E. Fried, I. Grants, P. Rajasekhar, E. Csizmadia, O. A. Ajijola, R. E. Watson, D. P. Poole, S. C. Robson, F. L. Christofi, B. D. Gulbransen, Enteric Glia Modulate Macrophage Phenotype and Visceral Sensitivity following Inflammation. *Cell Reports*. 32, 108100 (2020).

73. A. K. Chow, V. Grubišić, B. D. Gulbransen, Enteric Glia Regulate Lymphocyte Activation via Autophagy-Mediated MHC-II Expression. *Cell Mol Gastroenterology Hepatology*. 12, 1215–1237 (2021).

74. K. Jessen, R. Mirsky, Astrocyte-like glia in the peripheral nervous system: an immunohistochemical study of enteric glia. *Journal of Neuroscience*. 3, 2206–2218 (1983).

75. D. Cocchia, F. Michetti, G.-L. Ferri, J. M. Polak, L. Probert, P. J. Marangos, Evidence for the presence of S-100 protein in the glial component of the human enteric nervous system. *Nature*. 297, 409 (1982).

76. B. D. Gulbransen, K. A. Sharkey, Novel functional roles for enteric glia in the gastrointestinal tract. *Nature Reviews Gastroenterology & Hepatology*. 9, 625 (2012).

77. G. von Boyen, M. Steinkamp, M. Reinshagen, K.-H. Schäfer, G. Adler, J. Kirsch, Proinflammatory cytokines increase glial fibrillary acidic protein expression in enteric glia. *Gut*. 53, 222–228 (2004).

78. M. J. Chen, B. Kress, X. Han, K. Moll, W. Peng, R. Ji, M. Nedergaard, Astrocytic CX43 hemichannels and gap junctions play a crucial role in development of chronic neuropathic pain following spinal cord injury. *Glia*. 60, 1660–1670 (2012).

79. D. E. Fried, R. E. Watson, S. C. Robson, B. D. Gulbransen, Ammonia modifies enteric neuromuscular transmission through glial γ-aminobutyric acid signaling. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 313, G570–G580 (2017).

80. J. L. McClain, D. E. Fried, B. D. Gulbransen, Agonist-Evoked Ca2+ Signaling in Enteric Glia Drives Neural Programs That Regulate Intestinal Motility in Mice. *Cell Mol Gastroenterology Hepatology*. 1, 631–645 (2015).

81. B. D. Gulbransen, M. Bashashati, S. A. Hirota, X. Gui, J. A. Roberts, J. A. MacDonald, D. A. Muruve, D. M. McKay, P. L. Beck, G. M. Mawe, R. J. Thompson, K. A. Sharkey, *Nature Medicine*, in press, doi:10.1038/nm.2679.

82. E. Lucarini, L. Seguella, M. Vincenzi, C. Parisio, L. Micheli, A. Toti, C. Corpetti, A. D. Re, S. Squillace, D. Maftei, R. Lattanzi, C. Ghelardini, L. D. C. Mannelli, G. Esposito, Role of Enteric Glia as Bridging Element between Gut Inflammation and Visceral Pain Consolidation during Acute Colitis in Rats. *Biomed*. 9, 1671 (2021).

83. N. M. Delvalle, C. Dharshika, W. Morales-Soto, D. E. Fried, L. Gaudette, B. D. Gulbransen, Communication between enteric neurons, glia, and nociceptors underlies the effects of tachykinins on neuroinflammation. *Cellular and Molecular Gastroenterology and Hepatology*, 321–344 (2018).

84. Y. Fujikawa, K. Tominaga, F. Tanaka, T. Tanigawa, T. Watanabe, Y. Fujiwara, T. Arakawa, Enteric glial cells are associated with stress-induced colonic hyper-contraction in maternally separated rats. *Neurogastroenterology & Motility*. 27, 1010–1023 (2015).

85. B. D. Gulbransen, J. S. Bains, K. A. Sharkey, Enteric Glia Are Targets of the Sympathetic Innervation of the Myenteric Plexus in the Guinea Pig Distal Colon. *The Journal of Neuroscience*. 30, 6801–6809 (2010).

86. J. Cabarrocas, T. C. Savidge, R. S. Liblau, Role of enteric glial cells in inflammatory bowel disease. *Glia*. 41, 81–93 (2003).

87. E. Tjwa, J. M. Bradley, C. M. Keenan, A. Kroese, K. A. Sharkey, Interleukin-1β activates specific populations of enteric neurons and enteric glia in the guinea pig ileum and colon. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 285, G1268–G1276 (2003).

88. A. Rühl, S. Franzke, S. Collins, W. Stremmel, Interleukin-6 expression and regulation in rat enteric glial cells. *American journal of physiology. Gastrointestinal and liver physiology*. 280, G1163-71 (2001).

89. R. Garrido, B. Segura, W. Zhang, M. Mulholland, Presence of functionally active protease-activated receptors 1 and 2 in myenteric glia. *Journal of Neurochemistry*. 83, 556–564 (2002).

90. N. Cenac, T. Bautzova, P. Faouder, N. A. Veldhuis, D. P. Poole, C. Rolland, J. Bertrand, W. Liedtke, M. Dubourdeau, J. Bertrand-Michel, L. Zecchi, V. Stanghellini, N. W. Bunnett, G. Barbara, N. Vergnolle, Quantification and Potential Functions of Endogenous Agonists of Transient Receptor Potential Channels in Patients With Irritable Bowel Syndrome. *Gastroenterology*. 149, 433-444.e7 (2015).

91. P. Wang, C. Du, F.-X. Chen, C.-Q. Li, Y.-B. Yu, T. Han, S. Akhtar, X.-L. Zuo, X.-D. Tan, Y.-Q. Li, BDNF contributes to IBS-like colonic hypersensitivity via activating the enteroglia-nerve unit. *Scientific Reports*. 6, 20320 (2016).

92. T. Berta, C.-K. Park, Z.-Z. Xu, R.-G. Xie, T. Liu, N. Lü, Y.-C. Liu, R.-R. Ji, Extracellular caspase-6 drives murine inflammatory pain via microglial TNF-α secretion. *The Journal of clinical investigation*. 124 (2014).

93. D. Song, Y. Li, D. Tang, L. Huang, Y. Yuan, Neuron-glial communication mediated by TNF-α and glial activation in dorsal root ganglia in visceral inflammatory hypersensitivity. *American journal of physiology. Gastrointestinal and liver physiology*. 306, G788-95 (2014).

94. S. Liang, C. Xu, G. Li, Y. Gao, P2X receptors and modulation of pain transmission: Focus on effects of drugs and compounds used in traditional Chinese medicine. *Neurochemistry International*. 57, 705–712 (2010).

95. A. Deiteren, L. van der Linden, A. de Wit, H. Ceuleers, R. Buckinx, J.-P. Timmermans, T. G. Moreels, P. A. Pelckmans, J. G. Man, B. Y. Winter, P2X3 Receptors Mediate Visceral Hypersensitivity during Acute Chemically-Induced Colitis and in the Post-Inflammatory Phase via Different Mechanisms of Sensitization. *PLOS ONE*. 10, e0123810 (2015).

96. E. G. Lavoie, B. D. Gulbransen, M. Martín-Satué, E. Aliagas, K. A. Sharkey, J. Sévigny, Ectonucleotidases in the digestive system: focus on NTPDase3 localization. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 300, G608–G620 (2011).

97. D. Brisevac, A. Bajic, I. Bjelobaba, M. Milosevic, M. Stojiljkovic, C. Beyer, T. Clarner, M. Kipp, N. Nedeljkovic, Expression of Ecto-Nucleoside Triphosphate Diphosphohydrolase1-3 (NTPDase1–3) by Cortical Astrocytes After Exposure to Pro-inflammatory Factors In Vitro. *Journal of Molecular Neuroscience*. 51, 871–879 (2013).

98. R. S. Tonkin, C. Bowles, C. J. Perera, B. A. Keating, P. Makker, S. S. Duffy, J. G. Lees, C. Tran, A. S. Don, T. Fath, L. Liu, S. J. O'Carroll, L. Nicholson, C. R. Green, C. Gorrie, G. Moalem-Taylor, Attenuation of mechanical pain hypersensitivity by treatment with Peptide5, a connexin-43 mimetic peptide, involves inhibition of NLRP3 inflammasome in nerve-injured mice. *Experimental Neurology*. 300, 1–12 (2018).

99. S.-R. Choi, D.-H. Roh, S.-Y. Yoon, S.-G. Kwon, H.-S. Choi, H.-J. Han, A. J. Beitz, J.-H. Lee, Astrocyte sigma-1 receptors modulate connexin 43 expression leading to the induction of below-level mechanical allodynia in spinal cord injured mice. *Neuropharmacology*. 111, 34–46 (2016).

100. F. A. White, H. Jung, R. J. Miller, Chemokines and the pathophysiology of neuropathic pain. *Proceedings of the National Academy of Sciences*. 104, 20151–20158 (2007).

101. A. K. Clark, A. A. Staniland, F. Marchand, T. K. Kaan, S. B. McMahon, M. Malcangio, P2X7-Dependent Release of Interleukin-1β and Nociception in the Spinal Cord following Lipopolysaccharide. *The Journal of Neuroscience*. 30, 573–582 (2010).

102. J.-M. Zhang, J. An, Cytokines, Inflammation, and Pain. *International Anesthesiology Clinics*. 45, 27 (2007).

103. Y.-R. Wen, M. R. Suter, R.-R. Ji, G.-C. Yeh, Y.-S. Wu, K.-C. Wang, T. Kohno, W.-Z. Sun, C.-C. Wang, Activation of p38 mitogen-activated protein kinase in spinal microglia contributes to incision-induced mechanical allodynia. *Anesthesiology*. 110, 155–65 (2009).

104. M. Takeda, M. Takahashi, S. Matsumoto, Contribution of the activation of satellite glia in sensory ganglia to pathological pain. *Neuroscience & Biobehavioral Reviews*. 33, 784–792 (2009).

105. M. Bashashati, M. Moradi, I. Sarosiek, Interleukin-6 in irritable bowel syndrome: A systematic review and meta-analysis of IL-6 (-G174C) and circulating IL-6 levels. *Cytokine*. 99, 132–138 (2017).

106. B. Takac, S. Mihaljević, M. Stefanić, L. Glavas-Obrovac, A. Kibel, M. Samardzija, Importance of interleukin 6 in pathogenesis of inflammatory bowel disease. *Collegium antropologicum*. 38, 659–64 (2014).

107. J. DeLeo, R. Colburn, M. Nichols, A. Malhotra, Interleukin-6-mediated hyperalgesia/allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*. 16, 695–700 (1996).

108. K. Okamoto, D. P. Martin, J. D. Schmelzer, Y. Mitsui, P. A. Low, Pro- and Anti-inflammatory Cytokine Gene Expression in Rat Sciatic Nerve Chronic Constriction Injury Model of Neuropathic Pain. *Experimental Neurology*. 169, 386–391 (2001).

109. P. Murphy, M. Ramer, L. Borthwick, J. Gauldie, P. Richardson, M. Bisby, Endogenous interleukin-6 contributes to hypersensitivity to cutaneous stimuli and changes in neuropeptides associated with chronic nerve constriction in mice. *European Journal of Neuroscience*. 11, 2243–2253 (1999).

110. S. H. Ferreira, B. B. Lorenzetti, S. Poole, Bradykinin initiates cytokine-mediated inflammatory hyperalgesia. *British Journal of Pharmacology*. 110, 1227–1231 (1993).

111. X.-J. Xu, J.-X. Hao, S. Andell-Jonsson, V. Poli, T. Bartfai, Z. Wiesenfeld-Hallin, NOCICEPTIVE RESPONSES IN INTERLEUKIN-6-DEFICIENT MICE TO PERIPHERAL INFLAMMATION AND PERIPHERAL NERVE SECTION. *Cytokine*. 9, 1028–1033 (1997).

112. T. Nozu, S. Miyagishi, R. Nozu, K. Takakusaki, T. Okumura, Repeated water avoidance stress induces visceral hypersensitivity: Role of interleukin-1, interleukin-6, and peripheral corticotropin-releasing factor. *Journal of Gastroenterology and Hepatology*. 32, 1958–1965 (2017).

113. Y. Liu, L.-J. Zhou, J. Wang, D. Li, W.-J. Ren, J. Peng, X. Wei, T. Xu, W.-J. Xin, R.-P. Pang, Y.-Y. Li, Z.-H. Qin, M. Murugan, M. P. Mattson, L.-J. Wu, X.-G. Liu, TNF-α Differentially Regulates Synaptic Plasticity in the Hippocampus and Spinal Cord by Microglia-Dependent Mechanisms after Peripheral Nerve Injury. *The Journal of Neuroscience*. 37, 871–881 (2016).

114. A. Liñán-Rico, F. Turco, F. Ochoa-Cortes, A. Harzman, B. J. Needleman, R. Arsenescu, M. Abdel-Rasoul, P. Fadda, I. Grants, E. Whitaker, R. Cuomo, F. L. Christofi, Molecular Signaling and Dysfunction of the Human Reactive Enteric Glial Cell Phenotype: Implications for GI Infection, IBD, POI, Neurological, Motility, and GI Disorders. *Inflammatory Bowel Diseases*. 22, 1812 (2016).

115. A. Kárpáti, T. Yoshikawa, T. Nakamura, T. Iida, T. Matsuzawa, H. Kitano, R. Harada, K. Yanai, Histamine elicits glutamate release from cultured astrocytes. *Journal of Pharmacological Sciences* (2018), doi:10.1016/j.jphs.2018.05.002.

116. J. Zhu, C. Qu, X. Lu, S. Zhang, Activation of Microglia by Histamine and Substance P. *Cellular Physiology and Biochemistry*. 34, 768–780 (2014).

117. C.-M. Xia, M. A. Gulick, S. J. Yu, J. R. Grider, K. S. Murthy, J. F. Kuemmerle, H. I. Akbarali, L.-Y. Qiao, Up-regulation of brain-derived neurotrophic factor in primary afferent pathway regulates colon-to-bladder cross-sensitization in rat. *Journal of Neuroinflammation*. 9, 30 (2012).

118. W. Liu, W. Yue, R. Wu, Effects of diabetes on expression of glial fibrillary acidic protein and neurotrophins in rat colon. *Autonomic Neuroscience*. 154, 79–83 (2010).

119. M. B. Levanti, I. Esteban, E. Ciriaco, P. Pérez-Piñera, R. Cabo, O. García-Suarez, B. Pardo, I. Silos-Santiago, J. Cobo, J. A. Vega, Enteric glial cells express full-length TrkB and depend on TrkB expression for normal development. *Neuroscience Letters*. 454, 16–21 (2009).

120. V. G. Boyen, M. Steinkamp, M. Reinshagen, K. -H. Schäfer, G. Adler, J. Kirsch, Nerve Growth Factor Secretion in Cultured Enteric Glia Cells is Modulated by Proinflammatory Cytokines. *Journal of Neuroendocrinology*. 18, 820–825 (2006).

121. D. Siniscalco, C. Giordano, F. Rossi, S. Maione, V. de Novellis, Role of Neurotrophins in Neuropathic Pain. *Current Neuropharmacology*. 9, 523–529 (2011).

122. M. Chichlowski, C. Rudolph, Visceral Pain and Gastrointestinal Microbiome. *Journal of Neurogastroenterology and Motility*. 21, 172–181 (2015).

123. E. Quigley, The Gut-Brain Axis and the Microbiome: Clues to Pathophysiology and Opportunities for Novel Management Strategies in Irritable Bowel Syndrome (IBS). *Journal of Clinical Medicine*. 7, 6 (2018).

124. R. D. Moloney, A. C. Johnson, S. M. O'Mahony, T. G. Dinan, B. Meerveld, J. F. Cryan, Stress and the Microbiota–Gut–Brain Axis in Visceral Pain: Relevance to Irritable Bowel Syndrome. *CNS Neuroscience & Therapeutics*. 22, 102–117 (2016).

125. F. Ochoa-Cortes, T. Ramos-Lomas, M. Miranda-Morales, I. Spreadbury, C. Ibeakanma, C. Barajas-Lopez, S. Vanner, Bacterial cell products signal to mouse colonic nociceptive dorsal root ganglia neurons. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 299, G723–G732 (2010).

126. E. Husebye, P. Hellström, T. Midtvedt, Intestinal microflora stimulates myoelectric activity of rat small intestine by promoting cyclic initiation and aboral propagation of migrating myoelectric complex. *Digestive diseases and sciences*. 39, 946–56 (1994).

127. P. S. Kabouridis, R. Lasrado, S. McCallum, S. H. Chng, H. J. Snippert, H. Clevers, S. Pettersson, V. Pachnis, The gut microbiota keeps enteric glial cells on the move; prospective roles of the gut epithelium and immune system. *Gut microbes*. 6, 398–403 (2015).

128. J. K. Nicholson, E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia, S. Pettersson, Host-Gut Microbiota Metabolic Interactions. *Science*. 336 (2012), doi:10.1126/science.1223813.

129. P. S. Kabouridis, R. Lasrado, S. McCallum, S. Chng, H. J. Snippert, H. Clevers, S. Pettersson, V. Pachnis, Microbiota Controls the Homeostasis of Glial Cells in the Gut Lamina Propria. *Neuron*. 85, 289–295 (2015).

130. S. Xu, B. Qin, A. Shi, J. Zhao, X. Guo, L. Dong, Oxytocin inhibited stress induced visceral hypersensitivity, enteric glial cells activation, and release of proinflammatory cytokines in maternal separated rats. *European Journal of Pharmacology*. 818, 578–584 (2018).

131. A. K. Chow, B. D. Gulbransen, Potential roles of enteric glia in bridging neuroimmune communication in the gut. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 312, G145–G152 (2017).

132. S. Bhave, A. Gade, M. Kang, K. F. Hauser, W. L. Dewey, H. I. Akbarali, Connexin–purinergic signaling in enteric glia mediates the prolonged effect of morphine on constipation. *The FASEB Journal*. 31, 2649–2660 (2017).

133. G. Barbara, C. Cremon, R. Giorgio, G. Dothel, L. Zecchi, L. Bellacosa, G. Carini, V. Stanghellini, R. Corinaldesi, Mechanisms underlying visceral hypersensitivity in irritable bowel syndrome. *Current gastroenterology reports*. 13, 308–15 (2011).

134. S. Franchi, P. Sacerdote, A. Panerai, The prokineticin system: an interface between neural inflammation and pain. *Neurological Sciences*. 38, 27–30 (2017).

135. K. Lis, T. Grygorowicz, A. Cudna, D. E. Szymkowski, E. Bałkowiec-Iskra, Inhibition of TNF reduces mechanical orofacial hyperalgesia induced by Complete Freund's Adjuvant by a TRPV1-dependent mechanism in mice. *Pharmacological Reports*. 69, 1380–1385 (2017).

136. D. Utsumi, K. Matsumoto, T. Tsukahara, K. Amagase, M. Tominaga, S. Kato, Transient receptor potential vanilloid 1 and transient receptor potential ankyrin 1 contribute to the progression of colonic inflammation in dextran sulfate sodium-induced colitis in mice: Links to calcitonin gene-related peptide and substance P. *Journal of pharmacological sciences* (2018), doi:10.1016/j.jphs.2017.12.012.

137. N. J. Spencer, E. I. Magnúsdóttir, J. E. Jakobsson, G. Kestell, B. N. Chen, D. Morris, S. J. Brookes, M. C. Lagerström, CGRPα within the Trpv1-Cre population contributes to visceral nociception. *American journal of physiology. Gastrointestinal and liver physiology*. 314, G188–G200 (2018).

138. A. Rosa, R. Fantozzi, The role of histamine in neurogenic inflammation. *British Journal of Pharmacology*. 170, 38–45 (2013).

139. J. Mobarakeh, A. Torkaman-Boutorabi, A. Rahimi, S. Ghasri, R. Nezhad, A. Hamzely, B. Sima, K. Takahashi, K. Nunoki, K. Yanai, Interaction of histamine and calcitonin gene-related peptide in the formalininduced pain perception in rats. *Biomedical Research*. 32, 195–201 (2011).

140. S. D. Skaper, L. Facci, M. Zusso, P. Giusti, Neuroinflammation, Mast Cells, and Glia: Dangerous Liaisons. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry*. 23, 478–498 (2017).

141. S. D. Skaper, L. Facci, P. Giusti, Mast cells, glia and neuroinflammation: partners in crime? *Immunology*. 141, 314–327 (2014).

142. T. Yoshikawa, F. Naganuma, T. Iida, T. Nakamura, R. Harada, A. S. Mohsen, A. Kasajima, H. Sasano, K. Yanai, Molecular mechanism of histamine clearance by primary human astrocytes. *Glia*. 61, 905–916 (2013).

143. B. C. Kimball, M. W. Mulholland, Enteric Glia Exhibit P2U Receptors that Increase Cytosolic Calcium by a Phospholipase C-Dependent Mechanism. *Journal of Neurochemistry*. 66, 604–612 (1996).

144. L. Kermarrec, T. Durand, M. Neunlist, P. Naveilhan, I. Neveu, Enteric glial cells have specific immunosuppressive properties. *J Neuroimmunol*. 295, 79–83 (2016).

145. F. Turco, G. Sarnelli, C. Cirillo, I. Palumbo, F. D. Giorgi, A. D'Alessandro, M. Cammarota, M. Giuliano, R. Cuomo, Enteroglial-derived S100B protein integrates bacteria-induced Toll-like receptor signalling in human enteric glial cells. *Gut*. 63, 105–115 (2013).

146. S. J. MacEachern, B. A. Patel, C. M. Keenan, M. Dicay, K. Chapman, D.-M. McCafferty, T. C. Savidge, P. L. Beck, W. K. MacNaughton, K. A. Sharkey, Inhibiting Inducible Nitric Oxide Synthase in Enteric Glia Restores Electrogenic Ion Transport in Mice With Colitis. *Gastroenterology*. 149, 445-455.e3 (2015).

147. K. Geboes, Histological classification of chronic gastritis: An iconoclastic view. *Gastroenterology*. 103, 1117–1118 (1992).

148. A. B. M. da Silveira, E. C. de Oliveira, S. G. Neto, A. O. Luquetti, R. T. Fujiwara, R. C. Oliveira, A. Brehmer, Enteroglial cells act as antigen-presenting cells in chagasic megacolon. *Hum Pathol*. 42, 522–532 (2011).

149. S. Ibiza, B. García-Cassani, H. Ribeiro, T. Carvalho, L. Almeida, R. Marques, A. M. Misic, C. Bartow-McKenney, D. M. Larson, W. J. Pavan, G. Eberl, E. A. Grice, H. Veiga-Fernandes, Glial-cell-derived neuroregulators control type 3 innate lymphoid cells and gut defence. *Nature*. 535, 440 (2016).

150. K. M. Dunn, K. W. Saunders, C. M. Rutter, C. J. Banta-Green, J. O. Merrill, M. D. Sullivan, C. M. Weisner, M. J. Silverberg, C. I. Campbell, B. M. Psaty, M. Korff, Opioid prescriptions for chronic pain and overdose: a cohort study. *Annals of internal medicine*. 152, 85–92 (2010).

151. L. F. Chu, M. S. Angst, D. Clark, Opioid-induced hyperalgesia in humans: molecular mechanisms and clinical considerations. *The Clinical journal of pain*. 24, 479–96 (2008).

152. D. M. Grunkemeier, J. E. Cassara, C. B. Dalton, D. A. Drossman, The narcotic bowel syndrome: clinical features, pathophysiology, and management. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 5, 1126–39; quiz 1121–2 (2007).

153. L. R. Watkins, M. R. Hutchinson, I. N. Johnston, S. F. Maier, Glia: novel counter-regulators of opioid analgesia. *Trends in neurosciences*. 28, 661–9 (2005).

154. A. K. Rattan, G. A. Tejwani, Effect of chronic treatment with morphine, midazolam and both together on dynorphin(1–13) levels in the rat. *Brain Research*. 754, 239–244 (1997).

155. J. R. Pain, Targeting microglial purinergic signaling to improve morphine analgesia (2010).

156. T. Berta, T. Liu, Y. Liu, X. Z. Molecular …, Acute morphine activates satellite glial cells and up-regulates IL-1β in dorsal root ganglia in mice via matrix metalloprotease-9 (2012).

157. R. Horvath, D. J. of Neuroscience, Morphine enhances microglial migration through modulation of P2X4 receptor signaling (2009), doi:10.1523/JNEUROSCI.4595-08.2009.

158. Z. Zhou, X. Peng, S. Hao, D. Fink, M. M. therapy, HSV-mediated transfer of interleukin-10 reduces inflammatory pain through modulation of membrane tumor necrosis factor α in spinal cord microglia (2008).

159. M. R. Hutchinson, B. D. Coats, S. S. Lewis, Y. Zhang, D. B. Sprunger, N. Rezvani, E. M. Baker, B. M. Jekich, J. L. Wieseler, A. A. Somogyi, D. Martin, S. Poole, C. M. Judd, S. F. Maier, L. R. Watkins, Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. *Brain, Behavior, and Immunity*. 22, 1178–1189 (2008).

160. F. Wei, W. Guo, S. Zou, R. K. of Neuroscience, Supraspinal glial–neuronal interactions contribute to descending pain facilitation (2008), doi:10.1523/JNEUROSCI.3593-08.2008.

161. M. R. Hutchinson, Y. Zhang, M. Shridhar, J. H. Evans, M. M. Buchanan, T. X. Zhao, P. F. Slivka, B. D. Coats, N. Rezvani, J. Wieseler, T. S. Hughes, K. E. Landgraf, S. Chan, S. Fong, S. Phipps, J. J. Falke, L. A. Leinwand, S. F. Maier, H. Yin, K. C. Rice, L. R. Watkins, Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain, Behavior, and Immunity*. 24, 83–95 (2010).

162. L. Nicotra, L. C. Loram, L. R. Watkins, M. R. Hutchinson, Toll-like receptors in chronic pain. *Experimental Neurology*. 234, 316–329 (2012).

163. E. A. Beckett, V. Staikopoulos, M. R. Hutchinson, Differential effect of morphine on gastrointestinal transit, colonic contractions and nerve-evoked relaxations in Toll-Like Receptor deficient mice. *Scientific reports*. 8, 5923 (2018).

164. A. Eser, J.-F. Colombel, P. Rutgeerts, S. Vermeire, H. Vogelsang, M. Braddock, T. Persson, W. Reinisch, Safety and Efficacy of an Oral Inhibitor of the Purinergic Receptor P2X7 in Adult Patients with Moderately to Severely Active Crohn's Disease: A Randomized Placebo-controlled, Double-blind, Phase IIa Study. *Inflammatory Bowel Diseases*. 21, 2247 (2015).

165. G. Esposito, E. Capoccia, F. Turco, I. Palumbo, J. Lu, A. Steardo, R. Cuomo, G. Sarnelli, L. Steardo, Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR-α activation. *Gut*. 63, 1300–1312 (2014).

166. L.-Y. Qiao, Neurotrophin signaling and visceral hypersensitivity. *Frontiers in Biology*. 9, 216–224 (2014).

Chapter 3:

ENTERIC GLIA REGULATE VISCERAL HYPERSENSITIVITY DURING INFLAMMATION THROUGH INTERCELLULAR SIGNALING WITH GUT NOCICEPTORS

ABSTRACT

Abdominal pain in irritable bowel syndrome and inflammatory bowel disease is thought to be driven by processes that sensitize sensory nerves innervating the gut. How sensory nerves become sensitized is not clear, but their terminals in the gut are surrounded by enteric glia. Here, we tested the hypothesis that intercellular enteric glia-to-nociceptor signaling contributes to visceral hypersensitivity during inflammation. In vivo and in vitro models of acute inflammation were used in combination with protein and RNA labeling, and cellular assays of activity and mediator release. Mechanisms of interaction between glia and nociceptors were studied using *Trpv1*$^{Cre;GCaMP5gtdT}$*;GFAP-hM3Dq* mice, in which glial activity is controlled by chemogenetics while simultaneously recording nociceptor activity using calcium imaging. Mice lacking glial connexin-43 were used in combination with visceromotor reflex recordings to disrupt glial intercellular signaling and study its impact on visceral sensitivity. Acute colitis induces a transient increase in proinflammatory cytokines including IL-1β, which is produced in part by glia and facilitates glial connexin-43 function. Provoking glial activity under these conditions changes the normal benign influence of glia on nociceptors to one where glia have a sensitizing effect on gut-innervating nociceptors. The mechanisms responsible for glial-driven visceral hypersensitivity involve an upregulation of glial COX-2 and an increase in stimulated glial PGE2 release which acts on nociceptor EP4 receptors. In vivo recordings show that colonic IL-1β shifts normal innocuous stimuli toward a noxious range through mechanisms that require glial connexin-43. Enteric glia sensitize gut nociceptors during inflammation. Cell-specific therapies targeting the glial mechanisms identified here could benefit treatments for visceral pain.

INTRODUCTION

Abdominal pain is the most common gastrointestinal issue and a major feature of irritable bowel syndrome (IBS) and inflammatory bowel disease (IBS) (*1*, *2*). Treating pain remains difficult due to the poor understanding of the underlying mechanisms. The most prevalent theory describing the etiology of abdominal pain is that acute inflammation promotes visceral hypersensitivity (*3*), which contributes to abdominal pain by facilitating nociceptive signaling along the gut-brain axis (*4*). Visceral hypersensitivity can be caused by changes at multiple points along the ascending and descending neural pathways that transduce nociceptive information from the periphery; however, changes in the sensitivity of nerve fibers that innervate the intestine is considered a primary cause and an attractive therapeutic target (*5*). Although these conditions are distinct clinical entities, there is significant overlap in symptom profiles of these patients. For example, approximately 40% of individuals with IBD who are in remission display symptoms that match the diagnostic criteria for IBS (*6*). Moreover, post-infectious IBS diagnosis account for a considerable subset of cases (*7*–*9*), and it is closely linked to the development of IBS following acute gastroenteritis caused by pathogens like Escherichia coli and Salmonella (*10*, *11*). Given these observations, it is likely that mechanisms associated with inflammation and nociceptive signaling is pivotal to the transition from acute to chronic pain and can be broadly applied in treatment strategies for both IBD's and functional bowel disorders.

Nociceptive information in the gut is transduced by the peripheral axon terminals of primary afferent neurons that express the transient receptor potential cation channel subfamily V member 1 (TRPV1) and are sensitized during acute inflammation (*12*, *13*). Nociceptor sensitization can lead to enhanced sensations of painful stimuli (hyperalgesia)

and/or painful sensations in response to normally innocuous stimuli (allodynia). Mechanisms responsible for these phenomena are thought to involve mediators released by immune cells such as mast cells that act on afferent nerve terminals (*14*). However, this theory has been questioned because mast cell numbers are inconsistent in IBS (*15*) and is at odds with the anatomical arrangement of nerve fibers and mast cells in the gut wall (*16*). Mast cells are predominantly associated with mucosal afferents, which comprise only 10% of afferent terminals in the colon (*17*, *18*). Sensitization of mucosal afferents could contribute to hyperalgesia but would not explain allodynia, which involves the sensitization of high threshold nerves that densely innervate the myenteric plexus and muscularis where mast cells are rare (*19*). Therefore, additional mechanisms must be active at the level of the myenteric plexus that contribute visceral hypersensitivity.

Nociceptors innervating the myenteric plexus are surrounded by enteric glia (*2*, *20*). Communication between enteric glia, neurons, and immune cells has emerged as an important mechanism that modulates neural-controlled gut functions (*21*, *22*) as well as neuroimmune interactions and neuroinflammatory responses (*23*, *24*). Enteric neuroinflammation is driven by processes that involve nociceptor-to-enteric glia signaling (*2*), but whether glia have reciprocal effects on nociceptive nerve sensitivity is unclear. Related populations of glia in the central nervous system have well defined roles in promoting synaptic plasticity (*25*) and sensitization of central pain pathways (*26*). Interactome gene analysis data suggest that enteric glia could have similar roles in the gut because glial genes upregulated during inflammation are linked to downstream pain-promoting mechanisms in colon-projecting nociceptors (*27*). While enteric glia are known to contribute to chronic visceral hypersensitivity through interactions with immune cells

(*24*), how they might directly influence nociceptive nerve terminal sensitivity remains unknown.

The goal of this study was to test the hypothesis that enteric glia contribute to visceral hypersensitivity by regulating the sensitivity of nociceptive nerve terminals innervating the intestine. To this end, we developed new tools to measure visceral nociceptor activity in real time in the intestine and used glial-specific perturbations to test the impact of glial signaling on nociceptor sensitivity in contexts of health and inflammation. Our results uncover a completely novel mechanism whereby enteric glia sensitize visceral afferent nerve terminals in the myenteric plexus in a sex-dependent manner. The molecular mechanisms underlying glial-mediated nociceptor sensitization identified here address a critical gap in understanding mechanisms that lead to visceral pain and highlight enteric glia as a potential target for cell-specific therapies.

MATERIALS AND METHODS

Animals

All work involving animals was conducted in accordance with the National Institutes of Health (NIH) *Guide for Care and Use of Laboratory Animals* and was approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University (MSU). An equal number of 14-16 weeks male and female mice with *C57BL/6* background were used for all experiments unless otherwise stated. All mice were maintained on a 12h:12h light-dark cycle in a temperature-controlled environment with access to food (Diet Number 2919; Envigo, Indianapolis, IN) and water ad libitum.

Transgenic mice expressing the genetically encoded calcium indicator GCaMP5g in sensory neurons (*Trpv1*$^{Cre;GCaMP5g\text{-}tdT}$) were bred in-house and were generated by crossing *B6.129-Trpv1*$^{tm1[cre]Bbm/J}$ mice [TRPV1Cre (017769; RRID: IMSR_JAX:017769; Jackson Laboratory)] with *Polr2a*$^{tm1(CAG\text{-}GcaMP5g,\text{-}tdTomato)Tvrd}$ (RRID: IMSR_ JAX:024477) mice (*2*). These mice were crossed with *GFAP-hM3Dq* (MGI:6148045; gifted by Dr. Ken McCarthy; University of North Carolina at Chapel Hill) (*21*) and were bred for experiments as heterozygotes to produce *Trpv1*$^{Cre;GcaMP5gtdT}$*;GFAP-hM3Dq* mice. Mice with a tamoxifen-sensitive deletion of the gene encoding Cx43 (*Gja1*) in enteric glia (*Sox10*^{creERT2}*;Cx43*$^{f/f}$) were also bred in-house and generated as previously described (*28*) by crossing *Sox10*^{CreERT2} (MGI:5910373; gifted by Dr. Vassilis Pachnis, The Francis Crick Institute, London, UK) mice with *Gja1*^{tm1Dlg} mice [Cx43f/f (008039; RRID: IMSR_JAX:008039; Jackson Laboratory)]. Cre^{ERT2} activity was induced in by feeding the animals with chow containing tamoxifen citrate (400mg/kg, TD.14084; Envigo) for 2 weeks followed by 1 week of normal chow before use. Double- and Triple-transgenic

mice were maintained as hemizygous for Cre (*Trpv1*^{Cre} *or Sox10*^{CreERT2}) and homozygous for the floxed allele (*Polr2a*$^{tm1(CAG-GcaMP5g,-tdTomato)Tvrd}$ or *Cx43*$^{f/f}$). Genotyping was performed by the Research Technology Support Facility Genomics Core at MSU and Transnetyx (Cordova, TN).

Acute colitis model

Colitis was induced in male and female mice under isoflurane anesthesia by an enema of 0.1 mL of a solution containing 5.5 mg of di-nitro benzenesulfonic acid (DNBS; 150959, MP Biomedicals) dissolved in 50% ethanol (*29*). Control animals received 0.9% saline enema. Mice were monitored closely and weighed daily and sacrificed at 6 hours, 48 hours or 3 weeks following DNBS.

Multiplex Cytokine Assay

Whole colon samples were collected from control and DNBS treated mice at 6 hours, 48 hours, and 3 weeks. Tissue was processed for multiplex immunoassay as previously described (*30*). Colonic samples were homogenized in a Tris-buffered saline Tween buffer solution to extract protein. After, homogenates were centrifuged at 10,000*g* for 10 minutes at 4°C. Cytokine/chemokine levels were evaluated using bead-based multiplex immunoassays (Eve Technologies, Calgary, AB, Canada).

Glial permeability assay

Glial Cx43 hemichannel activity was measured by ethidium bromide (EtBr) dye uptake as previously described (*31*, *32*). Live whole-mount longitudinal muscle-myenteric plexus (LMMP) preparations were dissected from from the distal colon in 35mm^2 sylgard-coated dishes and treated with an enzyme mix containing 1 U/mL Dispase (17105-041, Gibco) and 150 U/mL of Collagenase Type II (17101-015, Gibco) for 15 minutes at room

temperature. Preparations were incubated for 30 minutes at 37°C in 5% CO_2/95% air in either DMEM/F-12 (11039047, Thermofisher) media only (Control) or interleukin 1-β (IL1-β, 10ng/mL; 401-ML-005, R&D Systems), interleukin-6 (IL-6, 10ng/mL; 406-ML-005, R&D Systems), interferon-γ (IFN-γ, 10ng/mL, BMS326, Invitrogen), tumor necrosis factor-α (TNFα, 10ng/mL; 315-01A, PeproTech), interleukin-17 (IL-17, 10ng/mL; ab9567, Abcam) in the presence or absence of mimetic peptide antagonists for Cx43 (43Gap26, 100 μM; 62644, Anaspec, Inc.) or pannexin-1 (10Panx, 300 μM; 61911, Anaspec Inc.), and the pore blocker gadolinium (Gd, 25 μM; G7532, Sigma). After, the mixture was removed and preparations were incubated with ethidium bromide (EtBr; 5 μM; 15585-011, Invitrogen) in the presence or absence of the purinergic agonist ADP (100 μM; A5285, Sigma) for 10 minutes at 37°C. Extracellular EtBr was removed by three washes in fresh DMEM. EtBr fluorescence was immediately recorded using an upright Olympus BX51WI fixed-stage microscope fitted with a 40X water-immersion objective (LUMPlan N, 0.8 n.a.) and a Lambda DG-4 Plus xenon light source (Sutter). Ganglia were identified based on morphological features defined in previous work and acquired using MetaMorph Software (Molecular Devices).

Immunohistochemistry

Preparations of mouse colonic myenteric plexus prepared from segments of intestine preserved in Zamboni's fixative or 4% PFA overnight at 4°C and were processed for immunohistochemistry as previously described (*33*). LMMP whole-mounts were rinsed 3 times for 10 minutes each with 0.1% Triton X-100 in PBS (PBSTriton) followed by a 45-minute incubation in blocking solution (4% normal goat or normal donkey serum, 0.4% Triton X-100, and 1% bovine serum albumin). Preparations were incubated with primary

antibodies overnight at room temperature and secondary antibodies for 2 hours at room temperature before mounting (**Table 3.1**).

Fluorescence in situ hybridization (RNAscope)

LMMP tissue preparations from distal colon were collected from control and DNBS mice 48h after treatment. Samples were generated following the fixation and dissection procedures described above. RNAscope was performed using the Advanced Cell diagnostics (ACD) RNAscope 2.5 HD HD Assay-RED reported elsewhere (*34*). Tissues were dehydrated and subsequently rehydrated by a serial ethanol gradient (25%, 50%, 75%, 100% in PBS with 0.1% Triton X-100) prior to H2O2 treatment. Tissues were then digested with Protease III for 45 minutes and incubated with a probe for *Ptgs2* (**Table 3.1**). Tissues were washed 3 times for 5 minutes between each step with PBS (before probe incubation) or with RNAscope™ wash buffer (between amplification steps). Labeling was confirmed with species-specific positive and negative controls. Immunohistochemistry was performed following completion of the RNAscope protocol as described above.

Imaging

Immunohistochemistry and RNAscope fluorescent labeling was evaluated using the 20 or 40x objective (0.75 numerical aperture, Plan Fluor; Nikon) of an upright epifluorescence microscope (Nikon Eclipse Ni) with a Retiga 2000R camera (Qimaging) controlled by Qcapture Pro 7.0 (Qimaging) software. Representative images were acquired through the 60x-oil immersion objective (PlanApochromat, 1.42 numerical aperture) of an inverted Fluoview FV1000 confocal microscope (Olympus) or Zeiss LSM

880 NLO confocal system (Zeiss) using Zen Black software and a 20x objective (0.8 numerical aperture, Plan ApoChromat; Zeiss).

Glial PGE2 release

PGE2 was measured in supernatants collected from cultures of primary enteric glia. Enteric glia were isolated and cultured using a modification of protocols previously described (*24*). Whole colons were cut in small 2-3 cm segments and rod-dissected in DMEM-F12 to remove the myenteric plexus and longitudinal muscle layers and were digested in 2.5 mL of HBSS/HEPES buffer (14064, Gibco) containing Liberase TH (12.5 µg/mL; 5401119001, Sigma) and Dnase I (100 Kuntz units/ml; D5025-15KU, Sigma) for 30 minutes at 37°C. Cell suspensions were spun down (5 min at 1000x*g* and 4°C) and resuspended in 2 ml of complete medium made up of DMEM/Nutrient Mixture F-12 (11330-032, Thermofisher) containing phenol red supplemented with L-glutamine and HEPES (H3375, Sigma), penicillin and streptomycin (100 U/ml and 100µg/ml; 15140122, Gibco), and 10% fetal bovine serum (FBS, Denville Scientific, Inc.). Cells were plated in 24 well plates on coverslips coated with poly D-Lysine (100µg/ml, A-003-E, Sigma). Cell suspensions were initially seeded in the complete medium and plates were placed in an incubator (37C, 95% O_2 / 5% CO_2). New media composed of DMEM/Nutrient Mixture F-12 containing phenol red supplemented with L-glutamine and HEPES and substituted with antibiotics (penicillin 100 U/mL and streptomycin 100 mg/mL), GIBCO N-2 Supplement (0.2%; 17502048), GIBCO G-5 Supplement (0.2%; 17503012) and mouse NGF-β (0.05%; GFM11-20, Cells GS) was exchanged every 2 days. Experiments were performed once cells reached 50%–80% confluency (1-2 weeks). Cells were treated with IL-1β (10ng/mL) or media for 1 hour and stimulated with ADP (100µM) for 10 minutes. In

some cases, Cx43 activity was blocked with 43Gap26 (100 μM) for 10 minutes prior to stimulation. After, supernatants were collected and cells were fixed in 4% PFA for 30 minutes. PGE2 release was quantified using PGE2 ELISA kit (KGE004B, R&D Systems). Culture purity was evaluated with IHC for GFAP, peripherin, and α-smooth muscle actin (**Table S1**) and considered for analysis if above a 90% ratio of glia. All experimental groups were run in duplicate and averaged for statistical analysis.

Ca^{2+} imaging

Live whole-mounts of colon myenteric plexus from either *$Trpv1^{Cre;GCaMP5g\text{-}tdT}$ or $Trpv1^{Cre;GCaMP5gtdT}$;GFAP-hM3Dq* mice were prepared for Ca^{2+} imaging as previously described (*21*). Whole-mount circular muscle-myenteric plexus (CMMP) preparations were microdissected from mouse colons were continuously superfused with fresh, prewarmed (37°C) Kreb's buffer consisting of (in mM): 121 NaCl, 5.9 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 10 HEPES, 21.2 NaHCO3, 1 pyruvic acid, and 8 glucose (pH adjusted to 7.4 with NaOH) at a flow rate of 2-3 mL min-1. Preparations were treated with IL-1β (10 μg/mL) and either 43Gap26 (100 μM) or L-161,982 (50μM; 10011565, Cayman Chemical) to disrupt the release of glial mediators through Cx43 and inhibit EP4 receptors on nociceptors. CNO (10 μM; National Institute on Drug Abuse Drug Supply Program at the National institute of Health), ADP (100μM), and capsaicin (3 μM; 360376, Sigma) were applied for 30 seconds to activate glia and TRPV1 positive nociceptors, respectively. hM3Dq receptor expression in glia was validated by with Fluo-4 AM. Fluo-4 was loaded in the dark with Pluronic F-127 and 200mmol/L probenecid (P36400, Invitrogen) in DMEM/F-12. Images were acquired every second through 40x water-immersion objective (LUMPlanFI, 0.8 numerical aperture) of an upright Olympus BX51WI fixed stage

microscope (Olympus, Tokyo, Japan) using NIS-Elements software (v.4.5; Nikon, Tokyo, Japan) and an Andor Zyla sCMOS camera (Oxford Instruments, Abingdon, United Kingdom). To obtain higher spatial and temporal resolution of Ca^{2+} responses during validation of our genetic models, representative videos were obtained using a Nikon A1R HD25 confocal spinning-disk microscope (Nikon, Tokyo, Japan). Whole-mounts were superfused with Krebs buffer, (37°C) at 2–3 mL/min. To obtain higher spatial and temporal resolution of Ca^{2+} responses during validation of our genetic models, representative videos were obtained using a Nikon A1R HD25 confocal spinning-disk microscope (Nikon, Tokyo, Japan). In these cases, tissues were imaged through a 20x Nikon objective lens (CFI Apochromat LWD Lambda20xC WI, 0.95 numerical aperture). Confocal images were captured using a Nikon DS-Ri2 digital camera (Nikon, Tokyo, Japan) and recorded using NIS-Elements C software (Nikon).

In vivo assessment of visceral sensitivity

Visceral sensitivity was measured as previously described by non-invasive assessment of visceromotor responses (VMRs) to colorectal distensions (CRDs) (*24*, *35*). Intracolonic pressure was measured by a miniaturized pressure transducer catheter (SPR-524, Mikro-Tip catheter, Millar Instruments) equipped with a custom-made plastic balloon. Mice were trained to restrainers for 3 hours one day prior to the experiment. Mice received an enema of 0.9% saline or IL-1β and following 3 hours were briefly anesthetized with isoflurane while the pressure transducer-balloon was inserted into the colorectum. Mice were then acclimated to restraints for 30 minutes before starting the CRD procedure. Graded phasic distensions (20, 40, 60, and 80 mmHg, 2 times each, 20 second duration, 3-minute interstimulus interval) were delivered to the balloon by a barostat (G&J

Electronics) and VMR recordings were acquired using LabChart 8 (AD instruments). VMR recordings were performed as repeated measures experiments where control recordings were performed one week prior to conducting experiments with IL-1β.

Analysis and Statistics

Sample size was determined using a priori power analysis (G*Power 3.1) assuming 80% power with a desired P value of 0.05 and anticipating medium effect sizes (f = 1.0) based on previous experience and similar studies described in the literature. Whole animals were considered as individual n's for immunoassay, immunohistochemistry, *in situ* hybridization, and *in vivo* analysis. Averaged ganglionic cellular responses from multiple mice represented n's for Ca^{2+} imaging and dye uptake experiments. Immunolabeling data were analyzed in FIJI (National Institutes of Health) by calculating arbitrary mean fluorescence values per ganglionic area. Dye uptake experiments considered average glial fluorescence values per ganglion and each experiment was normalized to the control mean of the day. Ca^{2+} imaging traces represent the average change in fluorescence over time of the mean number of individual afferents per preparation. Individual nerve fibers were identified by tdTomato expression and morphology (*36*) and mean fluorescence was quantified using SparkAn (Dr. Adrian Bonev, University of Vermont) or NIS-Elements Analysis software. Ca^{2+} recordings were motion corrected using the ND Processing function in NIS-Elements software. Raw VMR traces were processed by running the SmoothSec and root mean square functions in LabChart 8 software to filter phasic colonic contractions (*24*). Following filtering the integral from the response at each distension pressure and the baseline mean from 20 seconds prior to the distension were obtained. Responses were considered significant if

they were at least 2 SDV above the baseline mean. Data were analyzed using Students' t-test, one-way or two-way ANOVA using Graphpad 9 software (Prism) and are presented as mean ± standard error of the mean (SEM).

Chemicals and Reagents

Chemicals and reagents were purchased from Millipore Sigma (Burlington, MA) unless otherwise stated.

RESULTS

Enteric glia contribute to the inflammatory milieu that drives visceral hypersensitivity

Acute inflammation produces visceral hypersensitivity in mice and humans (*3*) by disrupting the balance between pro- and anti-inflammatory mediators (*37*) that can both sensitize and/or activate visceral nociceptors (*3*, *24*). The sensitizing effects of proinflammatory mediators on nociceptors is often studied in the preclinical mouse DNBS colitis model based on its translational relevance and reproducibility (*38*). However, the inflammatory profile in this model is not well characterized. To determine which pro-inflammatory mediators are present during nociceptor sensitization, we performed a comprehensive multiplex immune assay on whole colon samples from male and female mice across the time course of DNBS colitis (**Figure 3.1A**). Several key pro-inflammatory cytokines were elevated in the colon at 6 hours (early inflammation) and 48 hours (peak inflammation), most of which returned to baseline levels during the resolution phase of colitis (3 weeks post DNBS treatment). Notably, levels of key cytokines implicated in the pathogenesis of GI disorders (*39*) and pain hypersensitivity (*25*) were increased in both males and females at 6 and 48 hours after DNBS treatment (**Figure 3.1B**). Concentrations of interleukin 1-β (IL-1β) and interleukin-6 (IL-6) increase drastically at 6h post-treatment, both showing levels over 100 times higher than controls in males and females. Tumon necrosis factor-α (TNF-α) levels increased over 70-fold at 48 hours in females and a 17-fold increase was observed in males although it did not reach statistical significance. Similarly, interleukin-17 (IL-17) was increased in males at 6 hours and females at 48 hours. Surprisingly, interferon-γ (IFN-γ) decreased in males at 6 hours and no change was observed in females. Analysis of published bulk RNA-sequencing data

from enteric glia at peak colitis (*2*) showed that pathways associated with these cytokines were significantly upregulated in enteric glia (**Figure 3.1C**). Notably, production of IL-1β and IL-6 are upregulated in enteric glia during DNBS, as well as associated receptors. Interestingly, enteric glia upregulate their expression of the non-canonical IL-1 receptor type 2 which binds IL-1β in order to inhibit the transduction of its signaling. This could suggest that enteric glia posses self-regulatory mechanisms to protect against the effects of the cytokine. IL-1β production contributes to visceral hypersensitivity (*3*, *25*) by directly modulating nociceptive nerves. Enteric glia are a potential source of IL-1β surrounding nociceptors. In support, immunolabeling data showed a robust 45% increase in IL-1β protein expression by myenteric glia during DNBS colitis (**Figure 3.1D-E**).

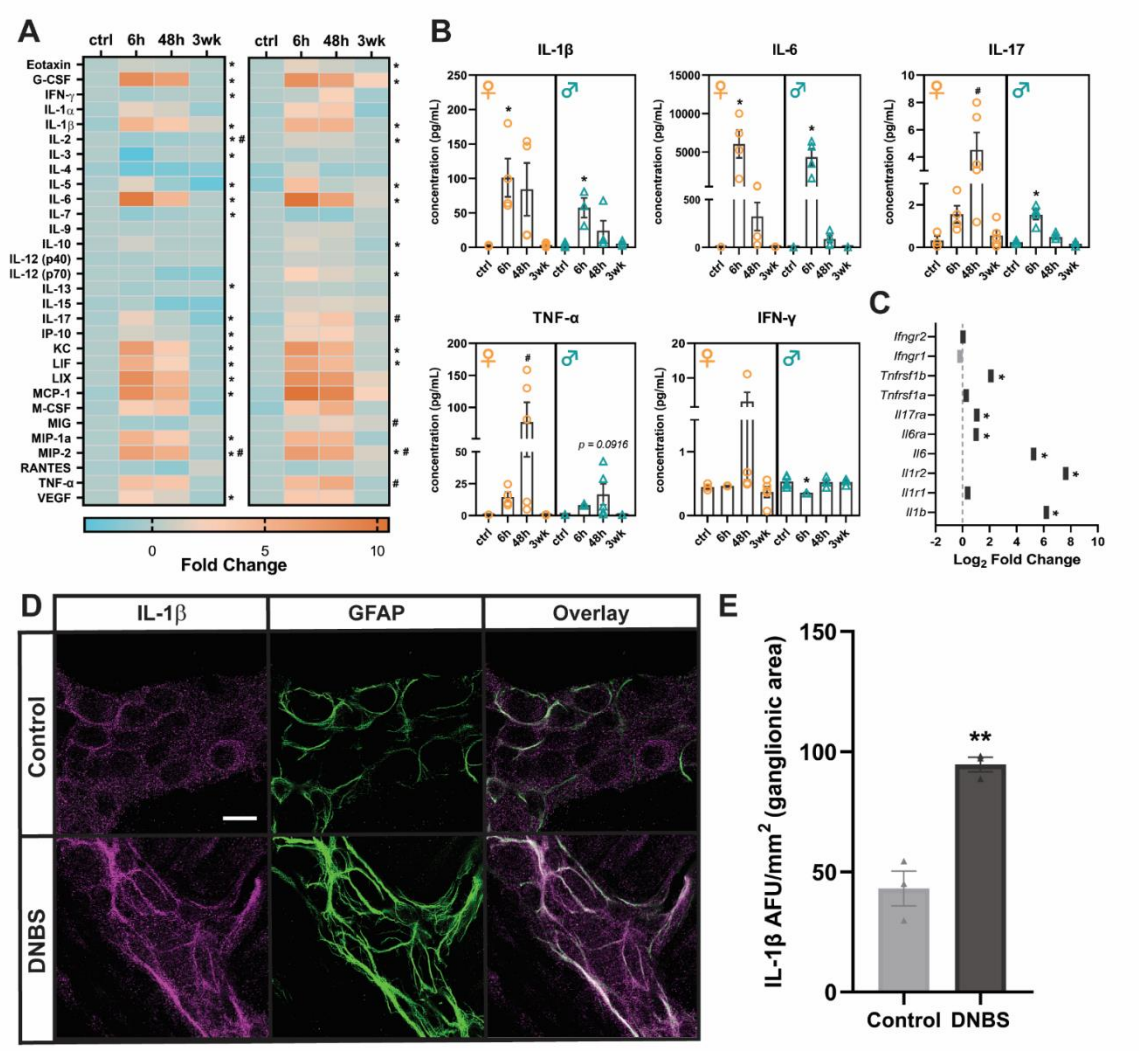

Figure 3.1: IL-1β expression is increased in enteric glia following inflammation. (A) Heat maps showing immune profile of DNBS colitis time course in male and female mice represented as fold change. (B) Concentrations in pg/mL of select cytokines from male (blue) and female (orange) mice at baseline or following DNBS colitis at 6h, 48h, and 3wk (One-way ANOVA followed by a Dunnett's multiple comparisons test, $p<0.05$, '*'

Figure 3.1: (cont'd)

significant at 6h, '#' significant at 48h, n=3-5 mice). (C) Bulk RNA sequencing data sourced from Delvalle et al. presented as Log_2 fold change, showing that enteric glia differentially regulate cytokine and receptor expression following DNBS colitis (differential expression analysis (DESeq2), p-adjusted<0.1, n=3 mice). (D) Representative confocal images showing overlay of double-label immunohistochemistry of GFAP and IL-1β within the myenteric plexus of colon from mice treated with saline (control) or DNBS and quantification (E). Significant differences were determined by student's t-test (*$p < 0.05$, n = 3 mice) (scale = 20μm).

Proinflammatory cytokines facilitate glial Cx43 hemichannel gating

In the CNS, proinflammatory cytokines contribute to chronic pain through effects on glial connexin-43 (Cx43) hemichannels (*26*). Enteric glia express Cx43 hemichannels and Cx43-mediated signaling regulates intercellular communication (*22*, *31*) and underlying GI functions (*40*) yet how proinflammatory mediators affect enteric glial Cx43 activity is not understood. Cellular uptake of the intercalating agent ethidium bromide (EtBr) is a selective method to measure Cx43 hemichannel opening in glia (*41*). We used this assay to study how proinflammatory cytokines produced during DNBS colitis alter glial Cx43. We initially tested the singular effects of proinflammatory cytokines on Cx43-mediated dye uptake. Glial Cx43 hemichannel opening was increased in the presence of proinflammatory cytokines to varied extents in both male (**Figure 3.2A**) and female (**Figure 3.2B**) mice. Incubation with IL-17 had no impact on dye uptake whereas IL-1β, IL-6, IFN-γ, and TNF-α induced a robust increase in glial EtBr fluorescence when compared to controls. Therefore, cytokines upregulated during active inflammation increase Cx43 hemichannel activity in otherwise unstimulated enteric glia. Given that these cytokines similarly impacted Cx43 hemichannels in males and females, the following experiments include pooled data from both sexes.

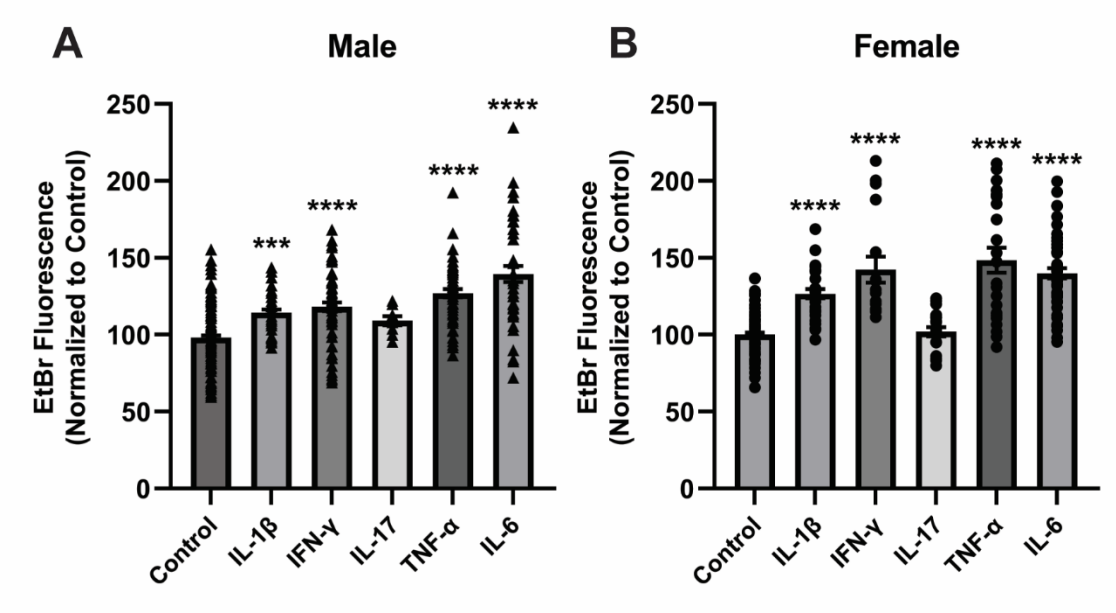

Figure 3.2: Impact of pro-inflammatory cytokines on glial dye uptake. Quantification of Cx43-mediated EtBr dye uptake in unstimulated enteric glia within the myenteric plexus. Preparations from both male (A) or female (B) mice were stimulated with either IL-6, IL-17, TNF-α or IFN-γ. Significant differences were determined by one-way ANOVA followed by post-hoc analysis with a Dunnett's multiple comparisons test (***$p < 0.001$, ****$p < 0.0001$) (n = 10-15 ganglia from multiple mice per treatment group).

Cx43-dependent EtBr uptake in enteric glia is increased following purinergic stimulation with the $P2Y_1$ agonist ADP, which is an active mechanism during colitis (*31*, *40*). Thus, we investigated how proinflammatory cytokines affect Cx43 opening in enteric glia following purinergic activation (**Figure 3.3A**). Treatment with ADP increased EtBr uptake by approximately 30%, confirming that this paradigm was efficient at activating enteric glia and stimulating Cx43 hemichannel opening. TNF-α (**Figure 3.10D**), IFN-γ (**Figure 3.10C**), and IL-17 (**Figure 3.10B**) had no effect on EtBr dye uptake in stimulated glia. In contrast, IL-1β (**Figure 3.3Bi-C**) and IL-6 (**Figure 3.10A**) potentiated dye uptake in stimulated glia by approximately 24 and 50%, respectively. IL-1β and IL-6 mediated effects were dependent on Cx43 as their actions were diminished by blocking Cx43 hemichannels with the mimetic peptide 43Gap26 (**Figure 3.3Bii-C & Figure 3.10A**). IL-1β-mediated potentiation of dye uptake was absent in the presence of the pore-channel blocker Gd, which rules out potential channel independent effects of IL-1β on Cx43 function. Likewise, repeating these experiments in glia from mice with a targeted deletion of glial Cx43 (*Sox10^{CreERT2};Cx43$^{f/f}$*) (*28*) showed that IL-1β is unable to potentiate EtBr dye uptake in baseline and stimulated glia in the absence of Cx43 (**Figure 3.3D**). Dye uptake was independent of pannexin-1 channels expressed by enteric neurons (*33*) and unaffected by the mimetic peptide 10Panx (**Figure 3.3C**). Together these data show that IL-1β sensitizes mechanisms involved in the release of neuroactive substances from glia.

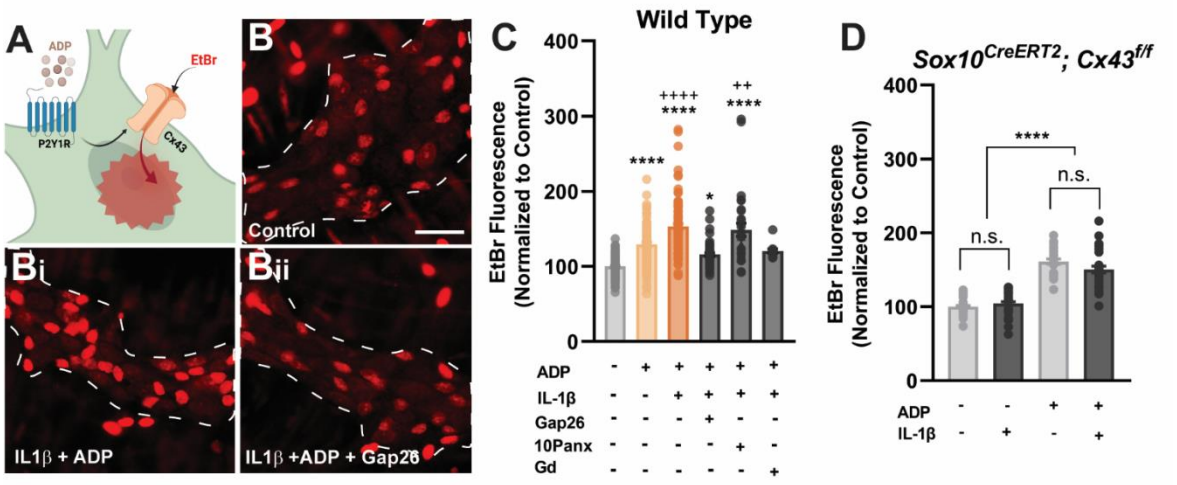

Figure 3.3: Effects of IL-1β on dye uptake through Cx43 hemichannels in ADP-stimulated enteric glia. (A) Schematic diagram illustrating experimental paradigm. (B-Bii) Representative images and (C) quantification of Cx43-mediated EtBr dye uptake in enteric glia within the myenteric plexus with buffer or ADP in the presence or absence of IL-1β, the mimetic peptides 43Gap26 and 10Panx, and gadolinium. (D) Quantification of dye uptake in in *Sox10^{cre}ER^{T2}::Cx43$^{f/f}$* mice. Significant differences were determined by Ordinary one-way ANOVA followed by a Tukey's post-hoc test. (*p<0.05, ****p<0.0001 to control, ++p<0.01, ++++p<0.0001 to ADP) (n = 10-25 ganglia from multiple mice per treatment group) (scale=20μM).

Enteric glia sensitize nociceptors through Cx43-dependent mechanisms in proinflammatory conditions

Glial Cx43 signaling is active during the acute phase of colitis when the sensitization of nociceptors occurs (*2*, *11*, *26*). Enteric glia display a tight anatomical and functional relationship with TRPV1+ nociceptors (*2*) (**Figure 3.4A**), suggesting that glial activation could affect nociceptor activity. To test how glial signaling influences nociceptor sensitivity, we conducted Ca^{2+} imaging in *Trpv1*$^{Cre;GCaMP5g\text{-}tdT}$ mice. This line conditionally expresses the genetically encoded Ca^{2+} indicator GCaMP5g in TRPV1+ nerves to specifically study the activity of nociceptive nerve terminals in the gut wall itself (**Figure 3.4B,C**). While most GCaMP and tdTomato expression is confined to extrinsic nerves in this line, some myenteric neurons also display reporter expression (**Figure 3.11A**). However, enteric neurons expressing tdTomato and GCaMP were unresponsive to capsaicin, supporting previous data showing that enteric neurons do not express functional TRPV1 in adult animals (*42*) (**Figure 3.11B**). Therefore, nerve fiber Ca^{2+} responses observed in this line primarily reflect responses in extrinsic nociceptors that innervate the myenteric plexus.

Nociceptor sensitivity was assessed by measuring Ca^{2+} responses evoked by the TRPV1 agonist capsaicin. Effects of glial activation were studied by stimulating glia with the $P2Y_1$ agonist ADP and by blocking downstream glial Cx43 hemichannel-mediated transmitter release with the mimetic peptide 43Gap26 (**Figure 3.4B**). TRPV1+ nerve fibers exhibited robust responses to capsaicin in control preparations and stimulating glia with ADP did not activate nerve fibers or alter their responses to capsaicin under basal conditions (**Figure 3.4E**). However, ADP increased nociceptor responses to capsaicin in

the presence of IL-1β by ~60% (**Figure 3.4D, F**). This effect required glial activation by ADP because treatment with IL-1β alone had no effect on capsaicin responses in nociceptors (**Figure 3.4F**). Nociceptor sensitization by ADP also required glial Cx43 and was blocked in the presence of 43Gap26 (**Figure 3.4F**). Therefore, purinergic activation of glia sensitizes nociceptive nerves in the intestine through Cx43-dependent signaling during inflammation.

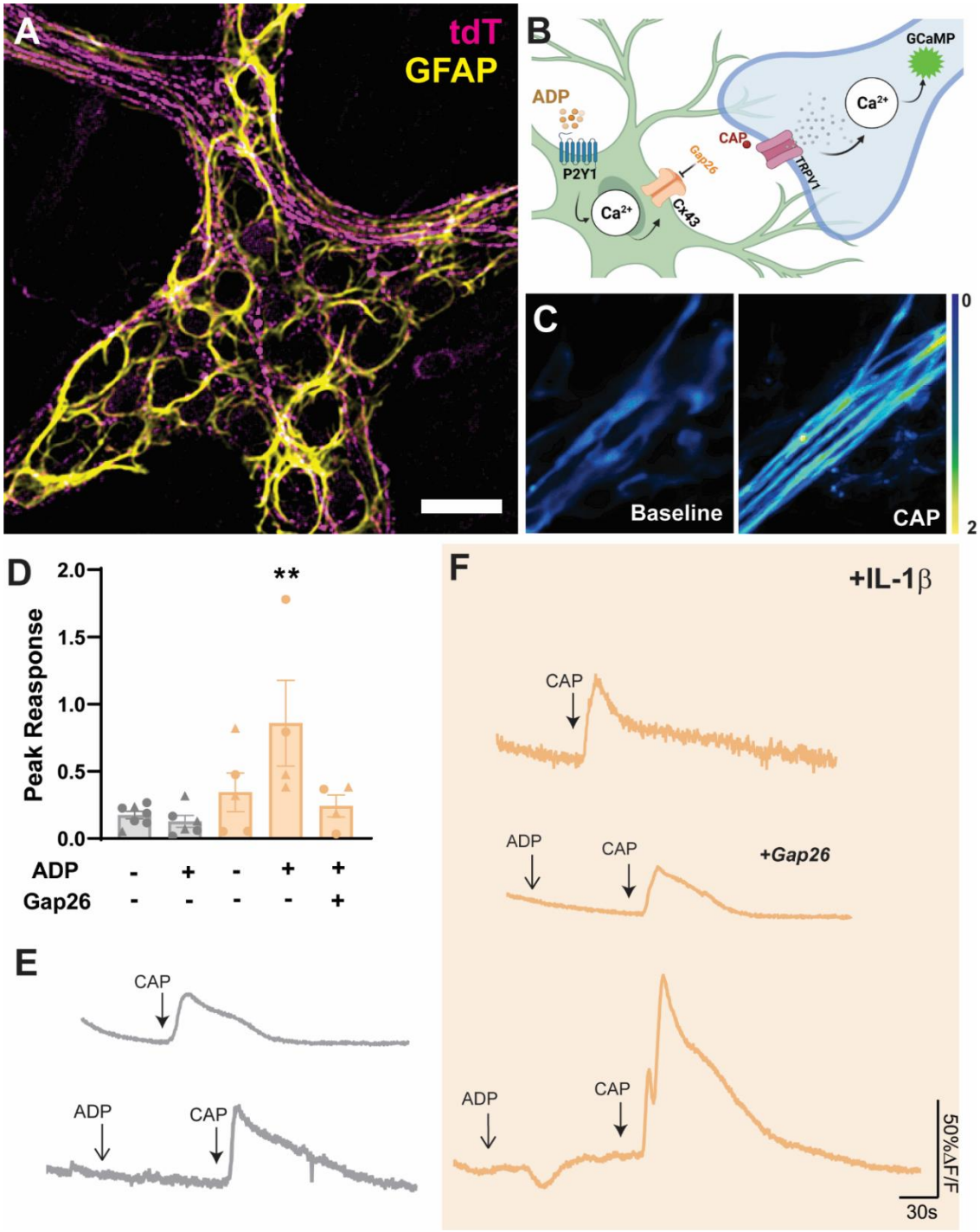

Figure 3.4: Enteric glia sensitize TRPV1+ nociceptors through Cx43-dependent mechanisms involving IL-1β signaling. (A) Representative confocal image in *TRPV1Cre;GCaMP5g-tdT* mice showing

Figure 3.4: (cont'd)

enteric glia labeled with GFAP (yellow) and TRPV1+ nociceptors (magenta) labeled with tdTomato. (B) Schematic of the experimental design and (C) representative images depicting baseline and Ca^{2+} responses provoked by capsaicin (CAP) in nerve fibers. (E-F) Representative traces and (D) quantification of peak fluorescence values of control (gray) and IL-1β treated preparations (orange). Representative traces reported as ΔF/F over time. Enteric glia were stimulated with ADP (open arrow) and nociceptors were stimulated with capsaicin (filled arrow). Significant differences were determined by Ordinary one-way ANOVA followed by a Dunnett's post-hoc test (**$p<0.01$ to control; n = 5-7 ganglia from multiple mice per treatment group) (scale=20μM). Individual values were represented as triangles for males and circles for females.

PGE2 is a glial-derived mediator that sensitizes TRPV1+ nociceptors

Our Ca^{2+} imaging studies in *Trpv1*$^{Cre;GCaMP5g\text{-}tdT}$ mice show that enteric glia sensitize TRPV1+ nociceptors through Cx43-dependent mechanisms. Cx43 controls glial intercellular communication by regulating the production of mediators such as prostaglandin E2 (PGE2) (*43*). PGE2 production is elevated during inflammation and facilitates nociceptive transmission through actions on EP4 receptors expressed by visceral nociceptors (*44*). Thus, we speculated that glial PGE2 release mediates the sensitizing effects of glial activation on TRPV1+ nociceptors.

Analysis of glial RNA sequencing data shows that enteric glia express several genes associated with PGE2 synthesis and regulation (*2*). Notably, there is a 6-fold increase in COX-2 gene (*Ptgs2*) expression in enteric glia during peak colitis in mice (**Figure 3.5A**). To confirm these data, we performed *in-situ* hybridization for *Ptgs2* from control and inflamed mice. *Ptgs2* RNA was increased when compared to control samples at 48 hours following DNBS treatment (**Figure 3.5B**). Increased *Ptgs2* expression colocalized with Sox10+ glia and not with peripherin+ neurons within myenteric ganglia, indicating that enteric glia are the main cellular site of PGE2 production in myenteric ganglia during acute inflammation. To provide direct evidence of glial PGE2 release, we quantified PGE2 release from primary cultures of enteric glia (**Figure 3.6**). Stimulating enteric glial cultures with ADP increased PGE2 release in the presence of IL-1β (**Figure 3.6A**). This effect required glial Cx43 hemichannel opening and was blocked by 43Gap26. Interestingly, 43Gap26 also decreased basal PGE2 levels when compared to unstimulated controls. Therefore, COX-2 activity in enteric glia and their subsequent

PGE2 production has the potential to promote enteric neuroplasticity during inflammation (*45*).

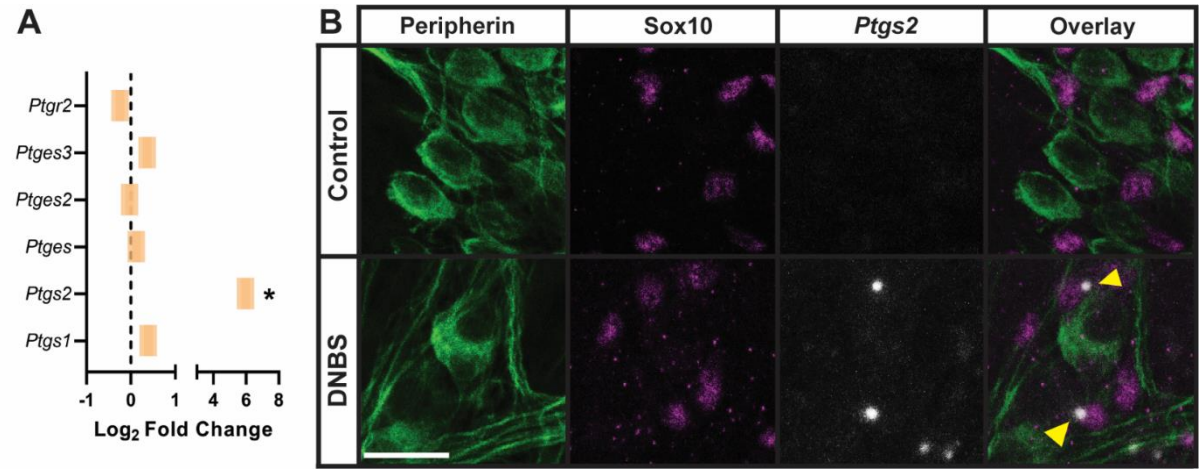

Figure 3.5: COX-2 expression is upregulated in enteric glia during colitis. (A) RNAseq data from Ribo-tag mice treated with either saline or DNBS represented as Log_2 fold change (differential expression analysis (DESeq2), $p<0.1$, $n=3$ mice). (B) Representative confocal images of myenteric ganglia from either control (top panels) or DNBS (bottom panels) treated mice labeling *Ptgs2* RNA (gray) with RNAscope followed by IHC labeling neurons with peripherin (green) and glia with Sox10 (magenta) (scale = 20μM).

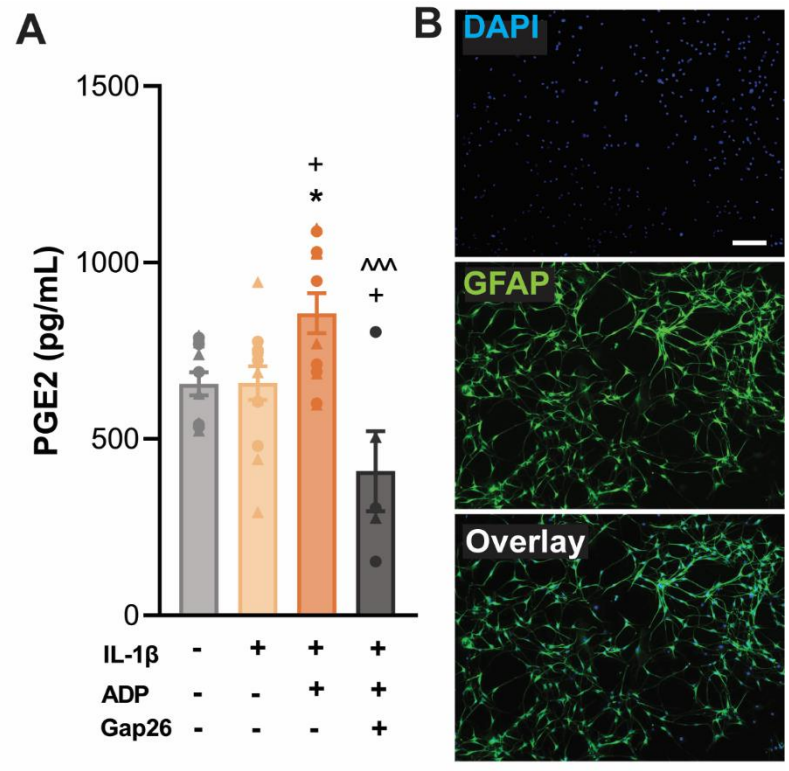

Figure 3.6: Stimulated enteric glia release PGE2 in the presence of IL1β. (A) Quantification of PGE2 release from enteric glia in cell culture supernatants treated with IL-1β and stimulated with ADP in the presence of 43Gap26. (B) Representative images of primary enteric glia cultures labeled with GFAP. Significant differences were determined by Ordinary one-way ANOVA followed by a Tukey's post-hoc test (*$p<0.05$ to control, $^{+}p<0.05$ to IL-1β only, ^^^$p<0.001$; n = 5-12 mice) (scale=50μM). Individual values are represented as triangles for males and circles for females.

PGE2 is a ubiquitous molecule that is released by multiple cell types including mast cells, which also modulate visceral hypersensitivity (*14*). Mast cells are mainly present at the level of the mucosa and are rare at the levels of the myenteric plexus and muscularis where high threshold afferent terminals are located (*17*, *19*). To determine relative potential contributions of mast cells vs glia, we quantified mast cells at the level of the myenteric plexus in mast cell reporter (*MCPT5*$^{Cre;GCaMP5g\text{-}tdT}$) mice (*46*) and assessed glial numbers by Sox10 immunolabeling within myenteric ganglia of WT mice (**Figure 3.7**). Average mast cell density was 29,121 (± 3,118) mast cells per cm^2 in the distal myenteric plexus, which was significantly lower than the density of Sox10+ glia in the same region (61,032 ± 3,580 cells per cm^2) (**Figure 3.7A**). These counts show that glia far outnumber mast cells in regions surrounding afferent terminals by a factor of at least 2:1. Higher glial abundance and closer apposition to nerve fibers in the myenteric plexus suggests that the glia contribution to PGE2 production may play a dominant role in nociceptor sensitization within the myenteric plexus.

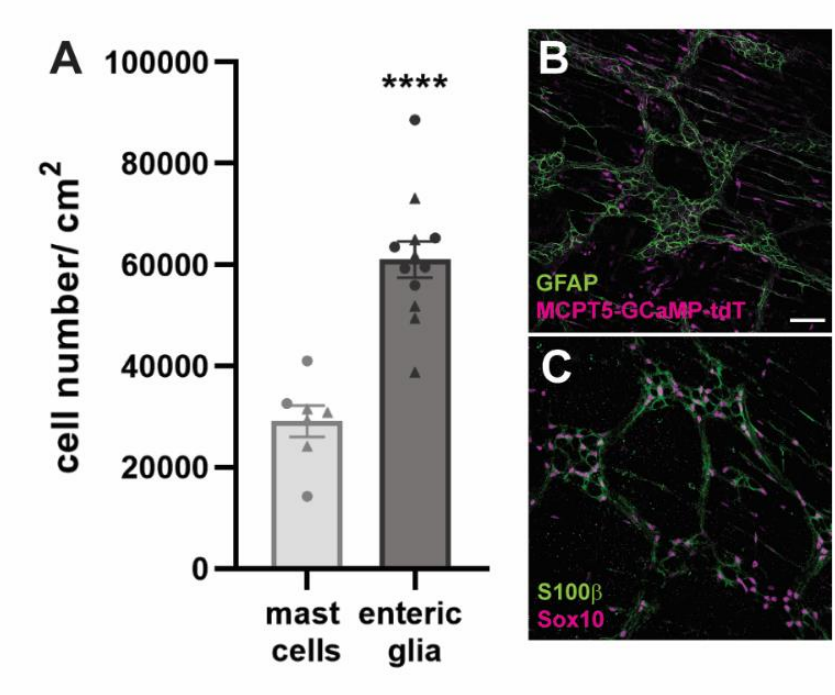

Figure 3.7: Relative abundance of enteric glia and mast cells at the level of the myenteric plexus. (A) Quantification and representative confocal images of glial (C) and mast cell (B) numbers within the myenteric plexus. Mast cells were quantified in *MCPT5-GCaMP-tdT* mice and were identified by tdTomato labeling. GFAP immunolabeling was used to identify myenteric ganglia. Enteric glia were identified and quantified via immunohistochemistry with S100β and Sox10 in wild type mice. Individual values represented as triangles for males and circles for females. Data were evaluated with a Student's t-test (****$p < 0.0001$, n = 7-12 mice) (scale = 50 μm).

Our Ca^{2+} imaging data show that purinergic activation of enteric glia modulates nociceptive signaling through Cx43-dependent mechanisms. However, it is possible that $P2Y_1$ receptor expression by afferent (*47*) and enteric neurons (*48*) and immune cells (*49*) could complicate our interpretations. To circumvent this issue, we developed a dual chemogenetic and optogenetic reporter mouse model, *Trpv1*$^{Cre;GCaMP5gtdT}$*;GFAP-hM3Dq*, to permit selective glial activation while imaging nociceptive nerve terminal activity in the gut with GCaMP. Consistent with our prior work characterizing the *GFAP::hM3Dq* glial chemogenetic line (*21*, *50*), immunolabeling confirmed that hM3Dq receptor expression is confined to glial processes and cell bodies co-labeled with GFAP (**Figure 3.8.A**). Further, increases in glia fluorescence were observed when stimulated with CNO using the Ca^{2+} indicator dye Fluo-4 (**Figure 3.12**).

Our initial studies with this model were directed at determining whether specifically activating glia without the potential confounds of agonists on surrounding cells would produce similar effects on nociceptor sensitivity (**Figure 3.8B**). As was the case with ADP, activation of enteric glia with CNO did not induce Ca^{2+} responses in TRPV1+ nociceptors and did not alter capsaicin responses under baseline conditions (**Figure 3.8D**). However, in the presence of IL-1β, activating enteric glia with CNO resulted in a clear potentiation of nociceptor capsaicin responses (**Figure 3.8C,E**). Therefore, glial activation alone is sufficient to sensitize nociceptive afferents during inflammation. Similar to our findings using ADP as a glial agonist, blocking Cx43 with 43Gap26 inhibited the sensitization of nociceptors elicited by glial activation with CNO (**Figure 3.8C,E**). Likewise, blocking nociceptor EP4 receptors with the selective antagonist L-161,982 inhibited the sensitization of nociceptors induced by glial activity (**Figure 3.8C,E**). Therefore, glial

activity driven by chemogenetic Gq GPCRs sensitizes TRPV1+ nociceptors in the context of inflammation through glial Cx43-dependent PGE2 release, which acts on nociceptor EP4 receptors.

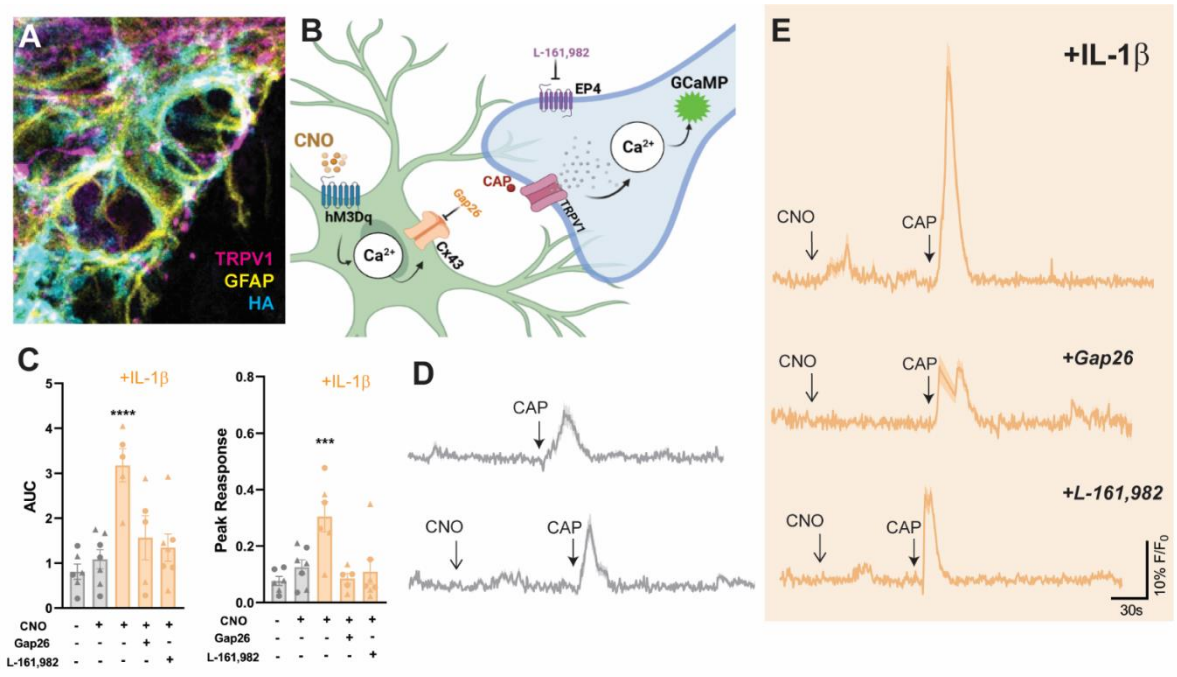

Figure 3.8: Enteric glia sensitize nociceptors via the Cx43-dependent release of PGE2. (A) Representative confocal image showing co-localization of the HA (cyan) tagged hM3Dq receptor on enteric glia labeled with GFAP (yellow) and TRPV1+ nerve fibers (magenta) labeled with tdTomato in *GFAP-HM3Dq;TRPV1Cre;GCaMP5g-tdT* mice. (B) Schematic of the experimental design. (C) Quantification and (D-E) representative traces of peak fluorescence values and AUC of control (gray) and IL-1β treated preparations (orange). Representative traces were reported as ΔF/F over time. Enteric glia were stimulated with CNO (filled arrow) and nociceptors were stimulated with capsaicin (CAP, open arrow). Significant differences were determined by Ordinary one-way ANOVA followed by a Dunnett's post-hoc test (***p<0.001, ****p<0.0001 to control; n = 5-7 ganglia from multiple mice per treatment group) (scale=20μM). Individual values are represented as triangles for males and circles for females.

Visceral hypersensitivity driven by IL-1β requires glial Cx43-mediated signaling in vivo

To test whether local glial-mediated sensitization contributes to broader pain behaviors *in vivo*, we assessed VMRs to CRDs in mice via non-invasive pressure recordings (*24*, *35*, *51*). This technique has been validated in multiple prior studies and eliminates the potential confounding factor of the surgery required for EMG recordings which can sensitize nociceptors and change enteric glial phenotype (*24*, *35*). Female mice exhibited larger VMRs in response to a normally non-noxious distension pressure of 20mmHg following an enema of IL-1β (**Figure 3.9C**); however, IL-1β had no effect on VMRs elicited in response to non-noxious or noxious distension pressures in males (**Figure 3.9A**). This suggests that females are more sensitive to developing allodynia following acute inflammation driven by IL-1β and supports the concept that sensitization of high threshold nerves innervating the myenteric plexus are responsible for the effect. In line with our cellular imaging studies, baseline visceral sensitivity did not require glial Cx43 signaling and was not different between controls and animals lacking glial Cx43 (*Sox10*^{CreERT2}*;Cx43*$^{f/f}$) (**Figure 3.9B,D**). In contrast, ablating glial Cx43 abolished the sensitizing effect of IL-1β on VMRs in females without changing responses in males (**Figure 3.9D**). No significant changes in colonic compliance were observed between strains or IL-1β and control animals (**Figure 3.13**). Therefore, glial intercellular communication mediated by Cx43 contributes to visceral hypersensitivity during inflammation in a sex dependent manner.

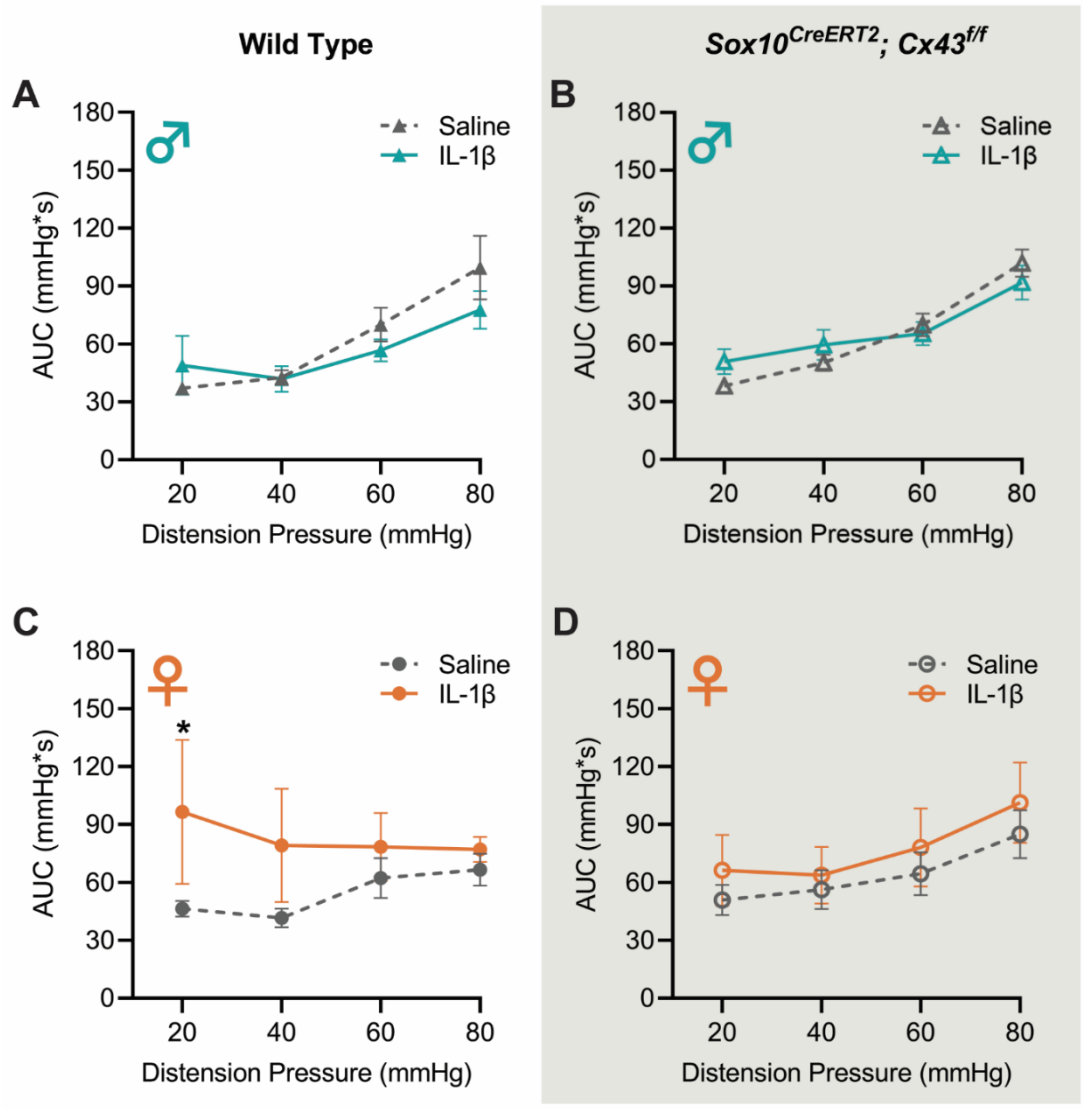

Figure 3.9: Enteric glia impact afferent nerves and integrated reflexes through Cx43-dependent mechanism. Traces showing VMR responses to CRD in mice shown as the area under the curve (AUC) for each distension pressure. Data show VMR responses in both male (blue) and female (orange) mice in wild type (A,C) or *Sox10^{cre}ER^{T2}::Cx43$^{f/f}$* (B,D) mice following treatment of saline/IL1β enema. AUC values for each group were analyzed via repeated measures two-way ANOVA followed by a Bonferroni's post hoc analysis ($p < 0.05$, n = 6-9 mice).

DISCUSSION

Treatments for visceral pain are limited by an unsatisfactory understanding of the causal mechanisms. Here, we show for the first time that enteric glia sensitize nociceptive nerve terminals that innervate the intestine through novel intercellular signaling mechanisms. Much of the prior work in this area has approached mechanisms of visceral hypersensitivity by studying responses in dorsal root ganglion (DRG) neurons or spinal cord circuits, which are distant to the primary events taking place in the intestine that sensitize sensory transduction. In this study, we circumvented this issue by developing a GCaMP reporter model that allows studying afferent nerve terminals in the intestine. By combining the nociceptor GCaMP model with glial chemogenetics, we were able to determine that normal glial signaling does not impact nociceptor sensitivity in health but plays a major role in sensitizing afferents during inflammation. The mechanisms responsible for glial-driven visceral hypersensitivity involve facilitating effects of proinflammatory cytokines on glial Cx43 hemichannels, an upregulation of glial COX-2, and an increase in stimulated glial PGE2 release which acts on nociceptor EP4 receptors. These novel glial mechanisms offer new insight into the primary mechanisms that promote visceral pain at the initial site of transduction.

Glia play a major role in modulating ascending information in pain pathways. For example, processes mediated by astrocytes and microglia produce synaptic changes that facilitate the flow of afferent information in the dorsal horn of the spinal cord (*25*, *52*). Increased glial activity and gliotransmitter release in the CNS are characteristic of all forms of pain and glia contribute to the transition from acute to chronic pain through interactions with neurons that create long-lasting changes in neurotransmission. Here,

we show that enteric glia share these properties at the most distant peripheral terminals of afferent neurons innervating the gut. Enteric glia, particularly those associated with neurons in the myenteric plexus, share some morphological and functional properties with astrocytes that might suggest similar roles in pain transmission. This is supported by prior RNAseq data showing that enteric glia upregulate genes associated with sensory transduction during inflammation (*2*) and that these glial genes are linked with downstream mechanisms in colon-projecting DRG neurons (*27*). In this study, we provide functional evidence for direct interactions between enteric glia and nociceptors and show that these mechanisms sensitize afferent transduction. Therefore, an emerging theme is that glia present at all sites along ascending and descending pathways that transduce and modify nociceptive information deriving from the intestine have the potential to broadly influence visceral perception.

Enteric glia are sufficient to sensitize TRPV1+ nociceptors under proinflammatory conditions as reflected by increased nociceptor GCaMP5g responses to capsaicin following glial activation with either ADP or CNO. Given this result, it was surprising that activating enteric glia under basal conditions did not change TRPV1+ responses to capsaicin. One interpretation of this difference could be that enteric glia are normally under an inhibitory influence that is released during inflammation. This possibility has been proposed for the nitrergic system, which is impaired following gut inflammation (*40*, *53*). Alternatively, the gain of glial function observed during inflammation could reflect a protective response that prevents further damage. These possibilities are not exclusive and likely occur in tandem to mediate homeostatic functions of glia. However, the former seems most likely given the observed upregulation of proinflammatory genes, increased

secretory capacity through Cx43, and acute effects on nerve fibers (*24*, *54*). Another interesting finding was that ADP alone did not induce responses in nociceptors. This was unexpected since DRG neurons express purinergic receptors (*47*). However, these studies have centered at the level of the cell body which may express a different cohort of receptors than nerve endings.

Our results show that enteric glia possess the necessary machinery to produce and release PGE2 during inflammation. This conclusion is supported by prior cell culture evidence showing glia PGE2 production under proinflammatory settings (*43*). PGE2 is an important mediator of pain that can sensitize TRPV1+ nociceptors(*44*). PGE2 also plays an important role in enteric neuroplasticity and is necessary for producing changes in enteric neuron excitability and dysmotility in models of colitis (*45*). Our data suggest that many of these changes to both intrinsic and extrinsic neurons during inflammation could be attributed to glial PGE2 production. Other cell types do contribute to the available pool of PGE2 during inflammation and mast cell-derived PGE2 is considered particularly important in visceral hypersensitivity (*14*). However, mast cell numbers in IBS are inconsistent(*15*), decrease in frequency from the cecum to the rectum (*16*), and where present, are mainly associated with mucosal afferents (*17*, *18*). Mucosal afferents comprise about 10% of colon-innervating afferent terminals and display low activation thresholds, which if further sensitized could contribute to hyperalgesia but would not cause a major shift in visceral sensitivity. Most spinal afferents innervate deeper layers such as the circular muscle (25%) and myenteric ganglia (26%) (*18*). Myenteric afferent terminals exhibit high activation thresholds and sensitizing mechanisms that decrease their activation thresholds would cause a major shift in sensory signaling associated with

allodynia (*17*). Glia surround these nerve terminals and outnumber mast cells at this level of the gut wall. Therefore, glia and mast cells likely have complementary compartmentalized roles in visceral hypersensitivity in which glia play a dominant role in sensitizing high threshold afferents at the level of the myenteric plexus (*55*).

An important observation of this study is that the effects of IL-1β and glial Cx43-dependent signaling on visceral pain are sex-dependent. IL-1β increased visceral sensitivity in female mice in a glial Cx43-dependent manner. Surprisingly, IL-1β had no effect on *in vivo* measures of visceral sensitivity in males and ablating glial Cx43 also had no effect. Sexual dimorphism in pain mechanisms is well known (*56*, *57*) and the nature of the pain-producing insult impacts males and females differentially. The sex differences observed here may be due to greater proinflammatory responses in females than males. This conclusion is supported by our cytokine expression analysis in which females exhibited greater increases in most cytokines evaluated including IL-1β. Hence, similar glial mechanisms may be active in both sexes, but males may require a higher concentration of cytokine to induce hypersensitivity. This could explain why sex differences were not observed in ex vivo studies when cells were exposed to equivalent cytokine concentrations. Another possibility is that female mice are more sensitive to PGE2. Recent single-cell sequencing data from DRG show that prostaglandin signaling is upregulated in female mice (*57*). Further, PGE2 produces greater effects on mechanical allodynia in females when compared to males. This suggests that PGE2 pain thresholds are lower in females, which could explain the observed differences in our VMR data.

New therapies are desperately needed to combat the common clinical issue of abdominal pain. Our study identifies enteric glial signaling as a novel mechanism that promotes visceral hypersensitivity at the initial site of sensory transduction. Inflammation-induced changes in enteric glia increase their potential to modulate surrounding cells such as intrinsic and extrinsic neurons through effects on glial transmitter synthesis and release mechanisms. Our data show that perturbing these glial mechanisms limits visceral sensitization. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most frequently used analgesics that suppress inflammatory pain by inhibiting COX-1 and/or COX-2 and reducing the production of prostanoids (*44*). However, products of COX-2 activity, such as PGE2, are involved in diverse physiological processes and current drugs have serious side effects on gastrointestinal, renal, and cardiovascular systems (*58*). Therefore, cell-specific therapies targeting glial mechanisms could avoid these side effects and represent an important step forward in limiting visceral pain at its source in the intestine.

REFERENCES

1. A. F. Peery, S. D. Crockett, A. S. Barritt, E. S. Dellon, S. Eluri, L. M. Gangarosa, E. T. Jensen, J. L. Lund, S. Pasricha, T. Runge, M. Schmidt, N. J. Shaheen, R. S. Sandler, Burden of Gastrointestinal, Liver, and Pancreatic Diseases in the United States. *Gastroenterology*. 149, 1731-1741.e3 (2015).

2. N. M. Delvalle, C. Dharshika, W. Morales-Soto, D. E. Fried, L. Gaudette, B. D. Gulbransen, Communication between enteric neurons, glia, and nociceptors underlies the effects of tachykinins on neuroinflammation. *Cellular and Molecular Gastroenterology and Hepatology*, 321–344 (2018).

3. C. Stein, H. Machelska, Modulation of Peripheral Sensory Neurons by the Immune System: Implications for Pain Therapy. *Pharmacological Reviews*. 63, 860–881 (2011).

4. M. Simrén, H. Törnblom, O. S. Palsson, M. A. van Tilburg, L. Oudenhove, J. Tack, W. E. Whitehead, Visceral hypersensitivity is associated with GI symptom severity in functional GI disorders: consistent findings from five different patient cohorts. *Gut*. 67, 255–262 (2018).

5. E. A. Mayer, K. Tillisch, The Brain-Gut Axis in Abdominal Pain Syndromes. *Annual Review of Medicine*. 62, 381–396 (2011).

6. I. M. Minderhoud, B. Oldenburg, J. A. Wismeijer, G. P. Henegouwen, A. Smout, IBS-Like Symptoms in Patients with Inflammatory Bowel Disease in Remission; Relationships with Quality of Life and Coping Behavior. *Digestive Diseases and Sciences*. 49, 469–474 (2004).

7. C. Carco, W. Young, R. B. Gearry, N. J. Talley, W. C. McNabb, N. C. Roy, Increasing Evidence That Irritable Bowel Syndrome and Functional Gastrointestinal Disorders Have a Microbial Pathogenesis. *Front Cell Infect Mi*. 10, 468 (2020).

8. M. El-Salhy, Irritable bowel syndrome: diagnosis and pathogenesis. *World journal of gastroenterology*. 18, 5151–63 (2012).

9. A. Villani, M. Lemire, M. Thabane, A. Belisle, G. Geneau, A. X. Garg, W. F. Clark, P. Moayyedi, S. M. Collins, D. Franchimont, J. K. Marshall, Genetic Risk Factors for Post-Infectious Irritable Bowel Syndrome Following a Waterborne Outbreak of Gastroenteritis. *Gastroenterology*. 138, 1502–1513 (2010).

10. P. Hughes, Brierley, L. Blackshaw, Post-inflammatory modification of colonic afferent mechanosensitivity. *Clinical and Experimental Pharmacology and Physiology*. 36, 1034–1040 (2009).

11. D. Balemans, S. Mondelaers, V. Cibert-Goton, N. Stakenborg, J. Aguilera-Lizarraga, J. Dooley, A. Liston, D. Bulmer, V. P. Berghe, G. Boeckxstaens, M. Wouters, Evidence for long-term sensitization of the bowel in patients with post-infectious-IBS. *Scientific reports*. 7, 13606 (2017).

12. M. Kyloh, S. Nicholas, V. P. Zagorodnyuk, S. J. Brookes, N. J. Spencer, Identification of the Visceral Pain Pathway Activated by Noxious Colorectal Distension in Mice. *Frontiers in Neuroscience*. 5, 16 (2011).

13. N. Esquerre, L. Basso, M. Defaye, F. A. Vicentini, N. Cluny, D. Bihan, S. A. Hirota, A. Schick, H. B. Jijon, I. A. Lewis, M. B. Geuking, K. A. Sharkey, C. Altier, Y. Nasser, Colitis-Induced Microbial Perturbation Promotes Postinflammatory Visceral Hypersensitivity. *Cell Mol Gastroenterology Hepatology*. 10, 225–244 (2020).

14. G. Grabauskas, X. Wu, J. Gao, J.-Y. Li, D. K. Turgeon, C. Owyang, Prostaglandin E2, Produced by Mast Cells in Colon Tissues from Patients with Irritable Bowel Syndrome, Contributes to Visceral Hypersensitivity in Mice. *Gastroenterology* (2020), doi:10.1053/j.gastro.2020.02.022.

15. M. Bashashati, S. Moossavi, C. Cremon, M. R. Barbaro, S. Moraveji, G. Talmon, N. Rezaei, P. A. Hughes, Z. X. Bian, C. H. Choi, O. Y. Lee, M. Coëffier, L. Chang, L. Ohman, M. J. Schmulson, R. W. McCallum, M. Simren, K. A. Sharkey, G. Barbara, Colonic immune cells in irritable bowel syndrome: A systematic review and meta-analysis. *Neurogastroenterol Motil*. 30, e13192 (2018).

16. W. L. Hasler, G. Grabauskas, P. Singh, C. Owyang, Mast cell mediation of visceral sensation and permeability in irritable bowel syndrome. *Neurogastroenterol Motil*. 34, e14339 (2022).

17. L. Grundy, A. Erickson, S. M. Brierley, Visceral Pain. *Annu Rev Physiol*. 81, 1–24 (2018).

18. N. J. Spencer, M. Kyloh, M. Duffield, Identification of Different Types of Spinal Afferent Nerve Endings That Encode Noxious and Innocuous Stimuli in the Large Intestine Using a Novel Anterograde Tracing Technique. *Plos One*. 9, e112466 (2014).

19. M. Schemann, M. Camilleri, Functions and Imaging of Mast Cell and Neural Axis of the Gut. *Gastroenterology*. 144, 698-704.e4 (2013).

20. W. Morales-Soto, B. D. Gulbransen, Enteric glia: a new player in abdominal pain. *Cell Mol Gastroenterology Hepatology*. 7, 433–445 (2018).

21. J. L. McClain, D. E. Fried, B. D. Gulbransen, Agonist-Evoked Ca2+ Signaling in Enteric Glia Drives Neural Programs That Regulate Intestinal Motility in Mice. *Cell Mol Gastroenterology Hepatology*. 1, 631–645 (2015).

22. V. Grubišić, A. Verkhratsky, R. Zorec, V. Parpura, Enteric glia regulate gut motility in health and disease. *Brain Res Bull*. 136, 109–117 (2018).

23. A. K. Chow, V. Grubišić, B. D. Gulbransen, Enteric Glia Regulate Lymphocyte Activation via Autophagy-Mediated MHC-II Expression. *Cell Mol Gastroenterology Hepatology*. 12, 1215–1237 (2021).

24. V. Grubišić, J. L. McClain, D. E. Fried, I. Grants, P. Rajasekhar, E. Csizmadia, O. A. Ajijola, R. E. Watson, D. P. Poole, S. C. Robson, F. L. Christofi, B. D. Gulbransen, Enteric Glia Modulate Macrophage Phenotype and Visceral Sensitivity following Inflammation. *Cell Reports*. 32, 108100 (2020).

25. R.-R. Ji, A. Chamessian, Y.-Q. Zhang, Pain regulation by non-neuronal cells and inflammation. *Science*. 354, 572–577 (2016).

26. M. J. Chen, B. Kress, X. Han, K. Moll, W. Peng, R. Ji, M. Nedergaard, Astrocytic CX43 hemichannels and gap junctions play a crucial role in development of chronic neuropathic pain following spinal cord injury. *Glia*. 60, 1660–1670 (2012).

27. A. Wangzhou, C. Paige, S. V. Neerukonda, D. K. Naik, M. Kume, E. T. David, G. Dussor, P. R. Ray, T. J. Price, A ligand-receptor interactome platform for discovery of pain mechanisms and therapeutic targets. *Sci Signal*. 14 (2021), doi:10.1126/scisignal.abe1648.

28. V. Grubišić, B. D. Gulbransen, Enteric glial activity regulates secretomotor function in the mouse colon but does not acutely affect gut permeability. *The Journal of Physiology*. 595, 3409–3424 (2017).

29. B. D. Gulbransen, M. Bashashati, S. A. Hirota, X. Gui, J. A. Roberts, J. A. MacDonald, D. A. Muruve, D. M. McKay, P. L. Beck, G. M. Mawe, Activation of neuronal P2X7 receptor–pannexin-1 mediates death of enteric neurons during colitis. *Nature medicine*. 18 (2012).

30. S. Hernandez, D. E. Fried, V. Grubišić, J. L. McClain, B. D. Gulbransen, *Faseb J*, in press, doi:10.1096/fj.201802572r.

31. J. L. McClain, B. D. Gulbransen, The acute inhibition of enteric glial metabolism with fluoroacetate alters calcium signaling, hemichannel function, and the expression of key proteins. *Journal of Neurophysiology*. 117, 365–375 (2017).

32. J. A. Orellana, D. E. Hernández, P. Ezan, V. Velarde, M. V. L. Bennett, C. Giaume, J. C. Sáez, Hypoxia in high glucose followed by reoxygenation in normal glucose reduces the viability of cortical astrocytes through increased permeability of connexin 43 hemichannels. *Glia*. 58, 329–343 (2010).

33. B. D. Gulbransen, M. Bashashati, S. A. Hirota, X. Gui, J. A. Roberts, J. A. MacDonald, D. A. Muruve, D. M. McKay, P. L. Beck, G. M. Mawe, R. J. Thompson, K. A. Sharkey, *Nature Medicine*, in press, doi:10.1038/nm.2679.

34. M. M. Ahmadzai, J. L. McClain, C. Dharshika, L. Seguella, F. Giancola, R. D. Giorgio, B. D. Gulbransen, LPAR1 regulates enteric nervous system function through glial signaling and contributes to chronic intestinal pseudo-obstruction. *J Clin Investigation*. 132, e149464 (2022).

35. M. Larauche, G. Gourcerol, L. Wang, K. Pambukchian, S. Brunnhuber, D. W. Adelson, J. Rivier, M. Million, Y. Taché, Cortagine, a CRF1 agonist, induces stresslike alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. *Am J Physiology Gastrointest Liver Physiology*. 297, G215-27 (2009).

36. N. J. Spencer, E. I. Magnúsdóttir, J. E. Jakobsson, G. Kestell, B. N. Chen, D. Morris, S. J. Brookes, M. C. Lagerström, CGRPα within the Trpv1-Cre population contributes to visceral nociception. *American journal of physiology. Gastrointestinal and liver physiology*. 314, G188–G200 (2018).

37. M. F. Neurath, Cytokines in inflammatory bowel disease. *Nat Rev Immunol*. 14, 329–342 (2014).

38. A. C. Johnson, A. D. Farmer, T. J. Ness, B. G.-V. Meerveld, Critical evaluation of animal models of visceral pain for therapeutics development: A focus on irritable bowel syndrome. *Neurogastroenterol Motil Official J European Gastrointest Motil Soc*. 32, e13776 (2019).

39. A. M'Koma, O. Korolkova, J. Myers, S. Pellom, L. Wang, A. M'Koma, *Clinical Medicine Insights: Gastroenterology*, in press, doi:10.4137/cgast.s20612.

40. I. A. M. Brown, J. L. McClain, R. E. Watson, B. A. Patel, B. D. Gulbransen, Enteric Glia Mediate Neuron Death in Colitis Through Purinergic Pathways That Require Connexin-43 and Nitric Oxide. *Cmgh Cell Mol Gastroenterology Hepatology*. 2, 77–91 (2016).

41. J. A. Orellana, P. J. Sáez, K. F. Shoji, K. A. Schalper, N. Palacios–Prado, V. Velarde, C. Giaume, M. Bennett, J. C. Sáez, Modulation of Brain Hemichannels and Gap Junction Channels by Pro-Inflammatory Agents and Their Possible Role in Neurodegeneration. *Antioxidants & Redox Signaling*. 11, 369–399 (2009).

42. N. Y. Lai, M. A. Musser, F. A. Pinho-Ribeiro, P. Baral, A. Jacobson, P. Ma, D. E. Potts, Z. Chen, D. Paik, S. Soualhi, Y. Yan, A. Misra, K. Goldstein, V. N. Lagomarsino, A. Nordstrom, K. N. Sivanathan, A. Wallrapp, V. K. Kuchroo, R. Nowarski, M. N. Starnbach, H. Shi, N. K. Surana, D. An, C. Wu, J. R. Huh, M. Rao, I. M. Chiu, Gut-Innervating Nociceptor Neurons Regulate Peyer's Patch Microfold Cells and SFB Levels to Mediate Salmonella Host Defense. *Cell*. 180, 33-49.e22 (2020).

43. S. Valès, G. Bacola, M. Biraud, M. Touvron, A. Bessard, F. Geraldo, K. A. Dougherty, S. Lashani, C. Bossard, M. Flamant, E. Duchalais, S. Marionneau-Lambot, T. Oullier, L. Oliver, M. Neunlist, F. M. Vallette, L. V. Landeghem, Tumor cells hijack enteric glia to activate colon cancer stem cells and stimulate tumorigenesis. *Ebiomedicine* (2019), doi:10.1016/j.ebiom.2019.09.045.

44. A. Kawabata, Prostaglandin E2 and Pain—An Update. *Biol Pharm Bull*. 34, 1170–1173 (2011).

45. D. R. Linden, K. A. Sharkey, W. Ho, G. M. Mawe, Cyclooxygenase-2 contributes to dysmotility and enhanced excitability of myenteric AH neurones in the inflamed guinea pig distal colon. *J Physiology*. 557, 191–205 (2004).

46. J. L. McClain, E. A. Mazzotta, N. Maradiaga, N. Duque-Wilckens, I. Grants, A. J. Robison, F. L. Christofi, A. J. Moeser, B. D. Gulbransen, Histamine-dependent interactions between mast cells, glia, and neurons are altered following early-life adversity in mice and humans. *Am J Physiol-gastr L*. 319, G655–G668 (2020).

47. J. R. Hockley, M. M. Tranter, C. McGuire, G. Boundouki, V. Cibert-Goton, M. A. Thaha, A. L. Blackshaw, G. J. Michael, M. D. Baker, C. H. Knowles, W. J. Winchester, D. C. Bulmer, P2Y Receptors Sensitize Mouse and Human Colonic Nociceptors. *The Journal of Neuroscience*. 36, 2364–2376 (2016).

48. J. D. Wood, The enteric purinergic P2Y1 receptor. *Curr Opin Pharmacol*. 6, 564–570 (2006).

49. Z.-G. Gao, Y. Ding, K. A. Jacobson, P2Y13 receptor is responsible for ADP-mediated degranulation in RBL-2H3 rat mast cells. *Pharmacol Res*. 62, 500–505 (2010).

50. M. M. Ahmadzai, L. Seguella, B. D. Gulbransen, Circuit-specific enteric glia regulate intestinal motor neurocircuits. *P Natl Acad Sci Usa*. 118, e2025938118 (2021).

51. M. Larauche, J. Erchegyi, C. Miller, M. S. Sim, J. Rivier, D. Behan, Y. Taché, Peripheral CRF-R1/CRF-R2 antagonist, astressin C, induces a long-lasting blockade of acute stress-related visceral pain in male and female rats. *Peptides*. 157, 170881 (2022).

52. M. Defaye, N. S. Abdullah, M. Iftinca, A. Hassan, F. Agosti, Z. Zhang, M. Cumenal, G. W. Zamponi, C. Altier, Gut-innervating TRPV1+ Neurons Drive Chronic Visceral Pain via Microglial P2Y12 Receptor. *Cell Mol Gastroenterology Hepatology*. 13, 977–999 (2022).

53. T. K. Smith, S. D. Koh, A model of the enteric neural circuitry underlying the generation of rhythmic motor patterns in the colon: the role of serotonin. *Am J Physiol-gastr L*. 312, G1–G14 (2017).

54. N. Froger, J. A. Orellana, C.-F. Calvo, E. Amigou, M. G. Kozoriz, C. C. Naus, J. C. Sáez, C. Giaume, Inhibition of cytokine-induced connexin43 hemichannel activity in astrocytes is neuroprotective. *Molecular and Cellular Neuroscience*. 45, 37–46 (2010).

55. J. Aguilera-Lizarraga, H. Hussein, G. E. Boeckxstaens, Immune activation in irritable bowel syndrome: what is the evidence? *Nat Rev Immunol*, 1–13 (2022).

56. D. K. Prusator, B. G.-V. Meerveld, Sex-related differences in pain behaviors following three early life stress paradigms. *Biol Sex Differ*. 7, 29 (2016).

57. D. Tavares-Ferreira, P. R. Ray, I. Sankaranarayanan, G. L. Mejia, A. Wangzhou, S. Shiers, R. Uttarkar, S. Megat, P. Barragan-Iglesias, G. Dussor, A. N. Akopian, T. J. Price,

Sex Differences in Nociceptor Translatomes Contribute to Divergent Prostaglandin Signaling in Male and Female Mice. *Biol Psychiat*. 91, 129–140 (2022).

58. C. Patrono, Cardiovascular effects of cyclooxygenase-2 inhibitors: a mechanistic and clinical perspective. *Brit J Clin Pharmaco*. 82, 957–964 (2016).

APPENDIX

SUPPLEMENTAL MATERIALS

Table 3.1. RNAscope probes and IHC antibodies				
RNAscope Probes				
Probe Target	***Vendor***	***Catalog Number***		***Channel***
Ptgs2	ACD Bio-Techne	316621		C1
Immunohistochemistry (IHC) Antibodies				
Primary antibodies				
Target	***Vendor***	***Catalog Number***	***Dilution***	***RRID***
Chicken anti-GFAP	Abcam	ab4674	1:1000	AB_304558
Mouse anti-Peripherin	Santa Cruz	sc-377093	1:100	
Rabbit anti-IL-1β	Abcam	ab9722	1:500	AB_308765
Goat anti-tdTomato	Sicgen	AB8181-200	1:1000	
Rabbit anti-dsRed	Takara/Living Colors	632496	1:1000	AB_10013483
Rabbit anti-Sox10	Millipore	HPA068898	1:200	AB_2686054
Mouse anti-α smooth muscle actin	Abcam	ab7817	1:1000	AB_262054
Rabbit anti- HA (C29F4)	Cell Signaling	3724	1:500	AB_1549585
Rabbit anti- S100β	Abcam	ab52642	1:200	AB_882426
Secondary antibodies				
Donkey anti-chicken Alexa Fluor 488	Jackson Laboratories	703-545-155	1:400	AB_2340375
Donkey anti-chicken Cy5	Jackson Laboratories	703-175-155	1:400	AB_2340365
Donkey anti-mouse Alexa Fluor 488	Jackson Laboratories	715-545-150	1:400	AB_2340846
Donkey anti-rabbit Alexa Fluor 594	Jackson Laboratories	711-585-152	1:400	AB_2340621
Donkey anti-goat Alexa Fluor 594	Jackson Laboratories	705-585-147	1:400	AB_2340433
Donkey anti-rabbit Alexa Fluor 647	Jackson Laboratories	711-605-152	1:400	AB_2492288
Donkey anti-mouse Alexa Fluor 594	Jackson Laboratories	715-585-150	1:400	AB_2340854
Donkey anti-rabbit Alexa Fluor 488	Jackson Laboratories	711-545-152	1:400	AB_2313584

Table 3.1: Table of RNAscope probes and antibodies used in chapter 3.

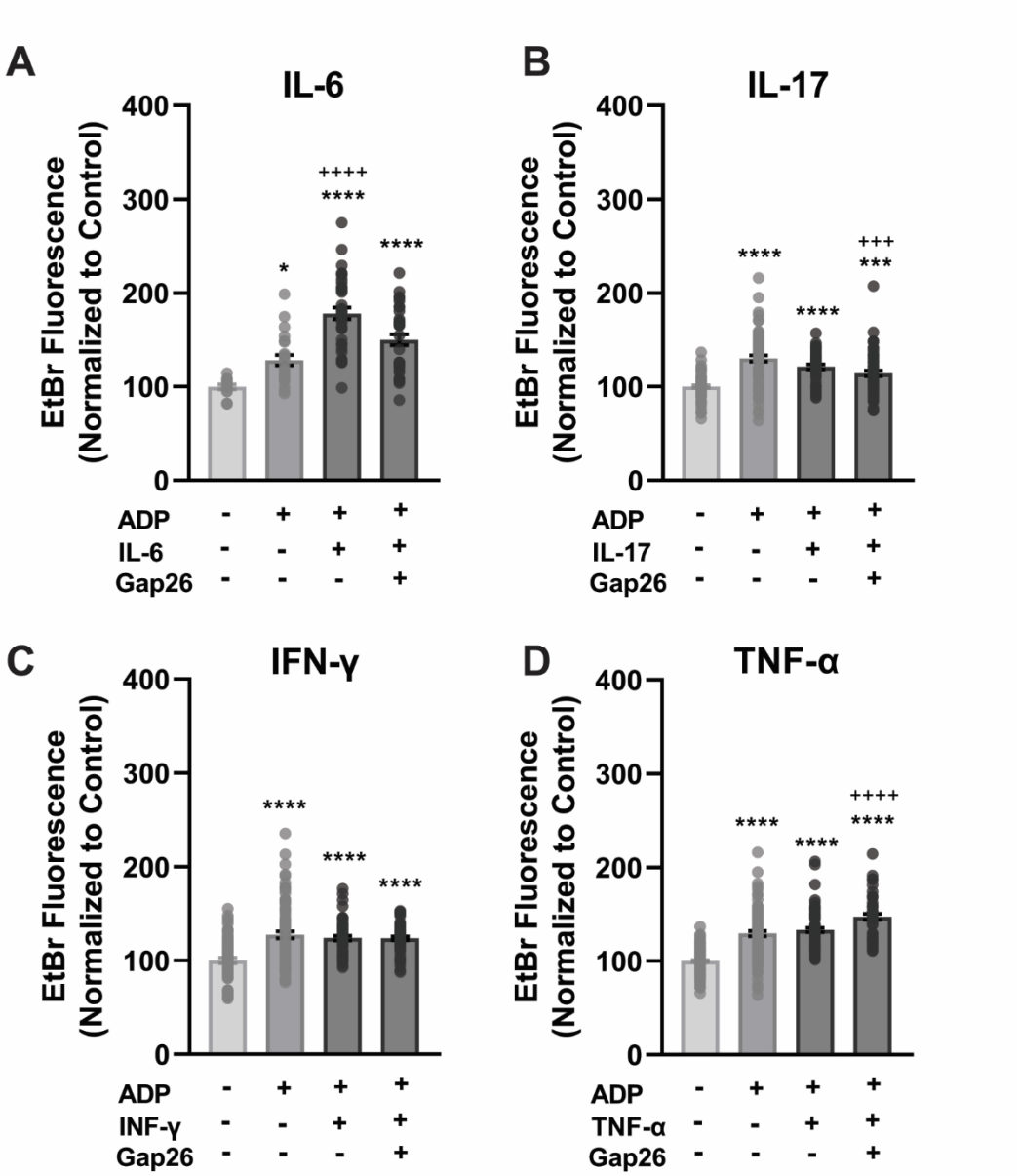

Figure 3.10: Impact of pro-inflammatory cytokines on Cx43 hemichannel-mediated dye uptake by enteric glia. Quantification of the effects of pro-inflammatory cytokines IL-6 (A), IL-17 (B), TNF-α (D) or IFN-γ (C) on EtBr dye uptake in enteric glia stimulated with ADP in the presence or absence of the mimetic peptide 43Gap26. Significant differences were determined by one-way ANOVA followed by post-hoc analysis with a Tukey's multiple comparisons test (*p <0.05, ***p = 0.0005, ****p < 0.0001 to control,

Figure 3.10: (cont'd)

+++p = 0.001, ++++p < 0.0001 to ADP) (n = 10-15 ganglia from multiple mice per treatment group).

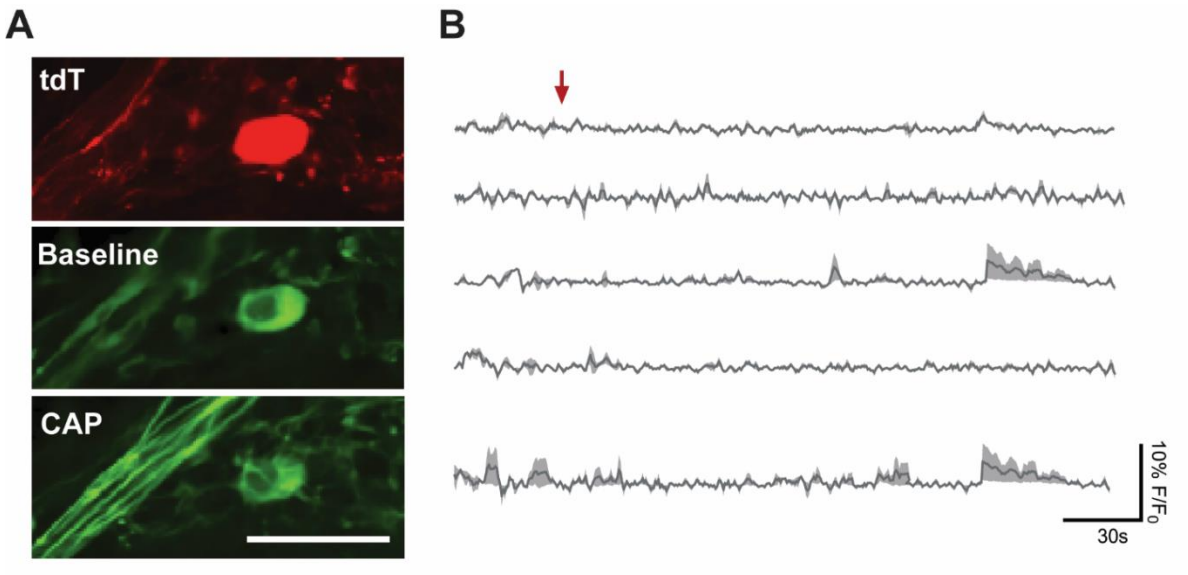

Figure 3.11: Rare enteric neurons that express tdTomato in Trpv1$^{Cre;GCaMP5g\text{-}tdT}$ and Trpv1$^{Cre;GCaMP5g\text{-}tdT}$;GFAP-hM3Dq mice do not express functional TRPV1. Representative images (A) and traces (B) of tdTomato+ cell bodies in the myenteric plexus following stimulation with capsaicin (arrow indicates point of stimulation by capsaicin) (n = 6 independent preparations from multiple mice). Note that capsaicin causes a pronounced increase in GCaMP fluorescence in extrinsic nerve fibers traveling through the ganglion but has no effect on enteric neuron GCaMP fluorescence (A).

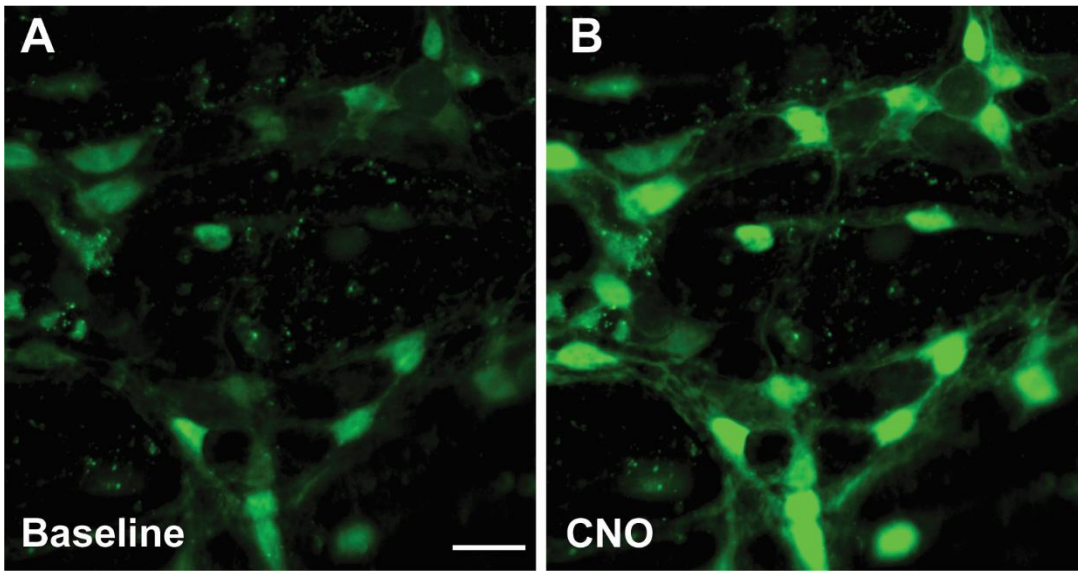

Figure 3.12: Enteric glia express functional hM3Dq receptors in Trpv1$^{Cre;GCaMP5g\text{-}tdT}$;GFAP-hM3Dq mice. Representative images from myenteric glia in whole mount preparations isolated from *Trpv1$^{Cre;GCaMP5g\text{-}tdT}$;GFAP-hM3Dq* mice loaded with Fluo-4 at baseline (A) and following stimulation with CNO (B, n = 3 mice). In these experiments, enteric glia were identified based on morphology and cell body Fluo-4 fluorescence and are easily differentiated from GCaMP-expressing nerve fibers which also express tdTomato (scale = 20 μm).

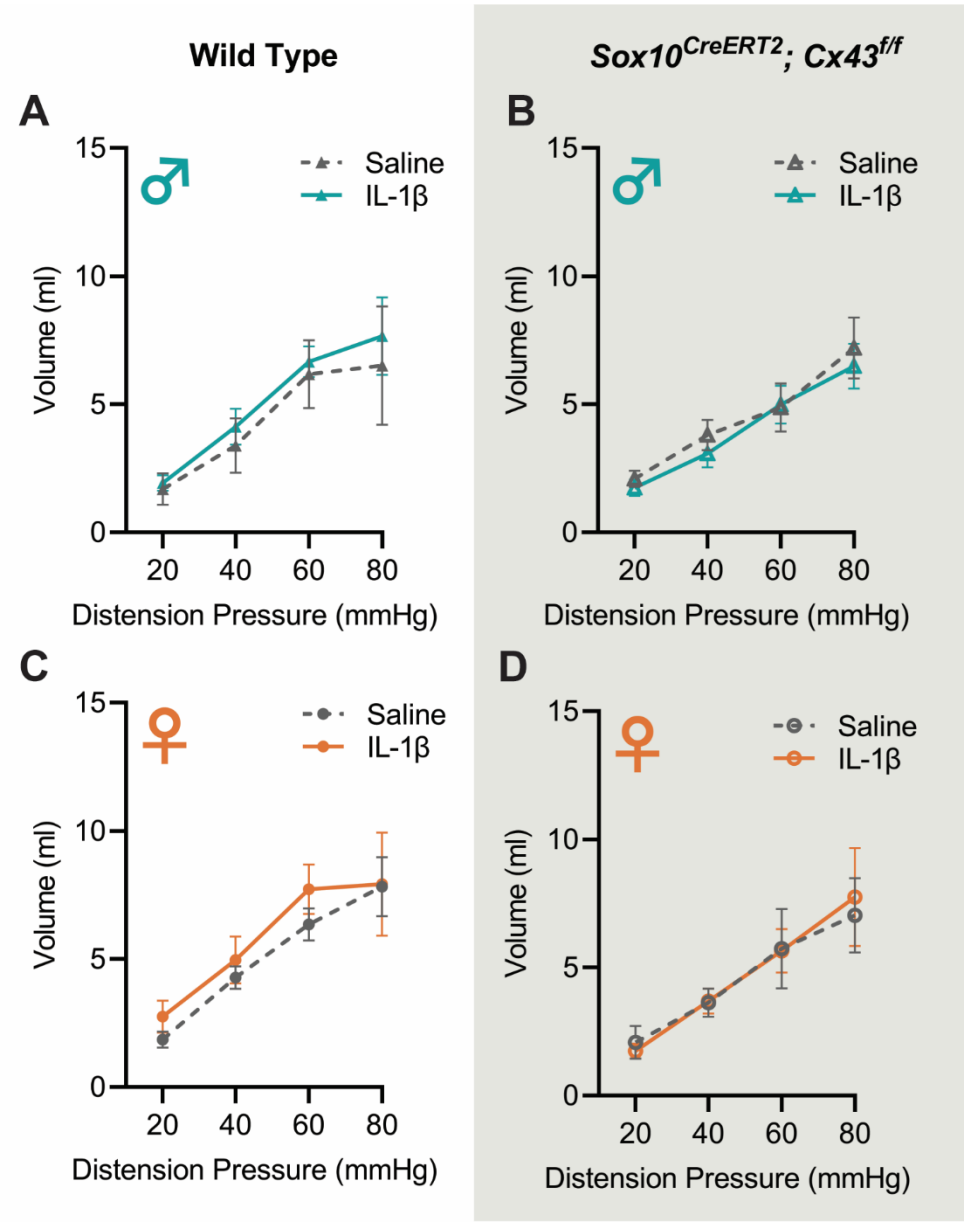

Figure 3.13: Colonic compliance is unchanged by ablating glial Cx43 or by intraluminal IL-1b. Traces showing compliance curves for visceromotor responses (VMR) to CRD in mice. Curves were obtained by plotting the mean balloon volume from each step against the corresponding distension pressure. Data show both male (blue) and female (orange) wild type (A,C) or *Sox10^{CreERT2};Cx43$^{f/f}$* (B,D) mice following enemas

Figure 3.13: (cont'd)

of saline or IL-1β. Curves for each group were analyzed via repeated measures two-way ANOVA followed by a Bonferroni's post hoc analysis ($p < 0.05$, n = 6-9 mice).

Chapter 4:

ENDOCANNABINOIDS MODULATE VISCERAL HYPERSENSITIVITY FOLLOWING INFLAMMATION THROUGH GLIA-DEPENDENT MECHANISMS

ABSTRACT

Visceral hypersensitivity is thought to be driven by inflammatory processes that sensitize the sensory nerves that innervate the gut. Enteric glia are active elements during inflammation and likely contribute to the sensitizing environment that leads to the sensitization of nociceptors. The endocannabinoid system is known to modulate inflammation and visceral hypersensitivity. However, the exact mechanisms involved in these processes in the intestine and whether enteric glia are an important component in these mechanisms has yet to be described. Enteric glia highly express Monoacylglycerol lipase (MAGL) the hydrolyzing enzyme responsible the production of 2-arachidonoyl glycerol (2-AG). 2-AG is readily described as an anti-inflammatory and analgesic compound. Thus, we hypothesize that that increasing endogenous levels of 2-AG through the inhibition of glial MAGL would improve the development of visceral hyperalgesia associated with inflammatory colitis. We used RNA labeling and Ca^{2+} with the *Wnt1*$^{Cre;GCaMP5g\text{-}tdT}$*;GFAP::hM3Dq* to understand the contribution of endocannabinoid system components on neural-glial signaling through the use of chemogenetics to stimulate enteric glia and the pharmacological inhibition of MAGL, cannabinoid receptor 1 (CB1), and cannabinoid receptor 2 (CB2). The di-nitro-benzene sulfonic acid (DNBS) model of colitis was used in combination with the *Trpv1*$^{Cre;GCaMP5gtdT}$ model to study the how inflammation altered nociceptor signaling in the intestine. Mice lacking glial MAGL were used in combination with visceromotor reflex recordings to increase endogenous levels of 2-AG and study its impact on visceral sensitivity. Our studies show that endocannabinoid signaling within the myenteric plexus is differentially regulated between male and female mice. Inhibition of MAGL under baseline conditions induces a decrease

in glial signaling within the myenteric ganglia of the distal colon, however this effect is lost in females and flipped in males following colitis. Similarly, DNBS colitis increases TRPV1+ nociceptor Ca^{2+} signaling and density in both males and females in vitro and increases visceral hypersensitivity in vivo in females. Pharmacological inhibition and genetic ablation of glial MAGL reverses these effects in females but has no effect in males. This study identifies sexual-dimorphic glial specific mechanisms involving endocannabinoid signaling ad inflammation.

INTRODUCTION

Visceral hypersensitivity is currently the most widely accepted mechanism responsible for abdominal pain and is a result of the sensitization of the nociceptive afferents that project from the spinal cord to the intestine (*1*). Visceral hypersensitivity is a hallmark of Inflammatory Bowel Diseases (IBDs) (*2*, *3*) and disorders of gut-brain interaction like irritable bowel syndrome (IBS) (*4*) and is considered the most debilitating symptom associated with these conditions. Mediators released during active inflammation can directly activate and sensitize colon-innervating afferents resulting in visceral hypersensitivity (*5*–*10*). Frontline drugs such as antidepressants (neuromodulators), antispasmodics, and opioids (*11*) have significant adverse effects on the CNS and on gut functions. Opioids, which are often used to control chronic visceral pain, have serious GI complications and chronic use produces a condition called narcotic bowel syndrome that paradoxically worsens pain symptoms (*12*). Controlling inflammation with aminosalicylates, corticosteroids, immunomodulators, and biological agents clearly benefits the treatment of active inflammation, but these therapies do not address abdominal pain (*13*). Addressing the mechanisms that induce changes in nociceptive signals that are transmitted from the abdomen to the spinal cord is necessary to develop effective approaches for abdominal pain treatment.

The endocannabinoid system primarily consists of cannabinoid receptors 1 (CB1) and 2 (CB2), their endogenous agonists, anandamide (AEA) and 2-2-arachidonoyl glycerol (2-AG), and the hydrolyzing enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (*14*–*16*). These components are ubiquitously expressed throughout the body and are important in regulating numerous physiological functions

and have a significant role in inflammation and disease (*16*). CB1 and CB2 receptors are densely expressed on spinal dorsal horn, the dorsal root ganglia, the peripheral terminals of primary afferent neurons and within the pain descending pathway (*17*). CB1 receptors are also co-expressed along with transient receptor potential cation channel subfamily V member 1 (TRPV1) and play an important role in regulating nociceptive signaling and visceral hypersensitivity (*18*–*20*). Both CB1 and CB2 receptors elicit analgesic effects under basal conditions and during inflammation-induced hyperalgesia (*21*, *22*). For this reason, cannabinoids are an attractive treatment for abdominal pain in IBD and IBS. Cannabinoid receptor agonists show activity in preclinical models of visceral and neuropathic pain (*21*, *23*–*25*) and endocannabinoid-like dietary supplements alleviate abdominal pain and discomfort in clinical trials with IBS patients (*26*). However, the clinical development of these candidates is limited because of the psychoactive side effects from a lack of receptor selectivity or loss of activity from partial CB2 agonism. To avoid potential adverse/non-specific effects from 'exocannabinoids', the focus for drug development has begun to consider regulating endocannabinoids through the selective inhibition of MAGL and FAAH (*27*, *28*). Particularly, increased levels of 2-AG through the inhibition of MAGL has shown great promise in alleviating inflammatory pain with minimal off-target effects (*29*–*31*).

Enteric glia are closely associated with nociceptive nerves within the intestine (*32*) and are known to contribute to visceral hypersensitivity during inflammation (*33*, *34*). However, the precise mechanisms that involve enteric glia in nociceptor sensitization are not well described. Very little work has focused on understanding the role of enteric glia in endocannabinoid signaling pathways (*14*). Existing evidence from other glial

populations such as astrocytes and microglia in the brain, show strong support for glial involvement in endocannabinoid signaling and regulation (*35*–*38*). Furthermore, enteric glia express, and modulate inflammation through, peroxisome proliferator-activated receptor alpha (PPARα) which are non-canonical endocannabinoid receptors and bind AEA (*14*, *39*, *40*). Single-cell (*41*) and bulk (*32*) RNA sequencing from enteric glia show that they readily express MAGL RNA suggesting that they play an important role in regulating endocannabinoids in the intestine. Astrocytes and neurons in the central nervous system regulate 2-AG signaling (*42*) and studies suggest that astrocytic MAGL function is predominant in inflammatory contexts (*38*). Recent, interactome analysis of enteric glial gene profiles during acute DNBS colitis and colon projecting DRG neurons identified MAGL as a potential mechanism whereby enteric glia could influence nociceptor sensitivity (*43*). Thus, we hypothesized that disrupting MAGL function in enteric glia would prevent the development of inflammatory visceral hypersensitivity.

The objective of this study was to understand the role of endocannabinoids in glia signaling in the myenteric plexus and how these mechanisms contribute to nociceptive signaling during inflammation. We incorporated imaging techniques in situ and in vivo measurements of hypersensitivity to study the impact of inflammation and glial MAGL on nociceptive signaling. Our findings reveal a brand-new process in which enteric glia sensitize visceral afferent nerve terminals in the myenteric plexus in a sex-dependent manner. These mechanisms fill a significant knowledge gap and highlight enteric glia as a target for therapies aimed at treating abdominal pain disorders.

MATERIALS AND METHODS

Animals

All work involving animals was conducted in accordance with the National Institutes of Health (NIH) *Guide for Care and Use of Laboratory Animals* and was approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University (MSU). An equal number of 12-14 weeks male and female mice with *C57BL/6* background were used for all experiments unless otherwise stated. All mice were maintained on a 12h:12h light-dark cycle in a temperature-controlled environment with access to food (Diet Number 2919; Envigo, Indianapolis, IN) and water ad libitum.

Transgenic mice expressing the genetically encoded calcium indicator GCaMP5g in sensory neurons (*Trpv1*$^{Cre;GCaMP5g\text{-}tdT}$) and enteric neurons and glia (*Wnt1*$^{Cre;GCaMP5g\text{-}tdT}$) were bred in-house and were generated by crossing *B6.129-Trpv1*$^{tm1[cre]Bbm/J}$ mice [TRPV1Cre (017769; RRID: IMSR_JAX:017769; Jackson Laboratory)] (*32*) and *B6.Cg-E2f1*$^{Tg(Wnt1\text{-}cre)2Sor/J}$ mice [The Jackson Laboratory, stock no. 022501; RRID: IMSR_JAX:022501] (*44*) with *Polr2a*$^{tm1(CAG\text{-}GcaMP5g,\text{-}tdTomato)Tvrd}$ (RRID: IMSR_ JAX:024477) mice, respectively. *Wnt1*^{Cre}*;GCaMP5g-tdT* mice were crossed with *GFAP-hM3Dq* (MGI:6148045; gifted by Dr. Ken McCarthy; University of North Carolina at Chapel Hill) (*45*) and were bred for experiments as heterozygotes to produce *Wnt1*$^{Cre;GCaMP5g\text{-}tdT}$*;GFAP::hM3Dq* (*44*, *46*) mice. Mice with a tamoxifen-sensitive deletion of the gene encoding MAGL (*Mgll*) in enteric glia (*Sox10*^{creERT2}*;Mgll*$^{f/f}$) were also bred in-house and generated as previously described by crossing *Sox10*^{CreERT2} (MGI:5910373; gifted by Dr. Vassilis Pachnis, The Francis Crick Institute, London, UK) mice with *MAGL*^{loxP} mice(MGI: 5883232; gifted y Dr. Benjamin Cravatt, The Scripps Research Institute, La Jolla, CA).

Cre^{ERT2} activity was induced by injecting mice with an IP of Tamoxifen (100mg/kg; T5648, Sigma) dissolved in a 1:9 solution of ethanol and sunflower oil respectively. Double- and Triple-transgenic mice were maintained as hemizygous for Cre (*Trpv1*^{Cre} *or Sox10*^{CreERT2}) and homozygous for the floxed allele (*Polr2a*$^{tm1(CAG-GcaMP5g,-tdTomato)Tvrd}$ or *Mgll*$^{f/f}$). Genotyping was performed by the Research Technology Support Facility Genomics Core at MSU and Transnetyx (Cordova, TN).

Inflammation model

Colitis was induced in male and female mice under isoflurane anesthesia by an enema of 0.1 mL of a solution containing 0.2mg/g of di-nitro benzenesulfonic acid (DNBS; 556971, Sigma) dissolved in 30% ethanol (*47*). Control animals received 0.9% saline enema as previously described (*48*, *49*). Mice were monitored closely and weighed daily for one week and used for experiments 1 or 3 weeks following DNBS. In a subset of experiments, received an IP injection of the potent MAGL inhibitor, JZL184 (10mg/kg), dissolved in a 1:1:9 solution of DMSO, Tween-20, and 0.9% saline or vehicle solution. Animals were injected every other day 48 hours following DNBS treatment until collection at day 7.

Chemicals and Reagents

Chemicals and reagents were purchased from Millipore Sigma (Burlington, MA) unless otherwise stated.

Immunohistochemistry

Preparations of mouse colonic myenteric plexus prepared from segments of intestine from healthy and DNBS treated mice preserved in 4% PFA overnight at 4°C and were processed for immunohistochemistry as previously described (*48*). LMMP whole-

mounts were rinsed 3 times for 10 minutes each with 0.1% Triton X-100 in PBS (PBSTriton) followed by a 45-minute incubation in blocking solution (4% normal goat or normal donkey serum, 0.4% Triton X-100, and 1% bovine serum albumin). Preparations were incubated with primary antibodies overnight at room temperature and secondary antibodies for 2 hours at room temperature before mounting (**Table 4.1**).

Fluorescence in situ hybridization (RNAscope)

LMMP tissue preparations from distal colon were collected from control and DNBS mice. Samples were generated following the fixation and dissection procedures described above. RNAscope was performed using the Advanced Cell Diagnostics (ACD) RNAscope 2.5 HD HD Assay-RED reported elsewhere (*50*). Tissues were dehydrated and subsequently rehydrated by a serial ethanol gradient (25%, 50%, 75%, 100% in PBS with 0.1% Triton X-100) prior to H2O2 treatment. Tissues were then digested with Protease III for 45 minutes and incubated with a probe for *Mgll*, *Cnr1*, and *Cnr2* (**Table 4.1**). Tissues were washed 3 times for 5 minutes between each step with PBS (before probe incubation) or with RNAscope™ wash buffer (between amplification steps). Labeling was confirmed with species-specific positive and negative controls. Immunohistochemistry was performed following completion of the RNAscope protocol as described above.

Table 1. RNAscope probes and IHC antibodies				
RNAscope Probes				
Probe Target	***Vendor***	***Catalog Number***		***Channel***
Mgll	ACD Bio-Techne	478831		C1
Cnr1	ACD Bio-Techne			C1
Cnr2	ACD Bio-Techne			C1
Immunohistochemistry (IHC) Antibodies				
Primary antibodies				
Target	***Vendor***	***Catalog Number***	***Dilution***	***RRID***
Mouse anti-Peripherin	Santa Cruz	sc-377093	1:100	
Rabbit anti-Sox10	Millipore	HPA068898	1:200	AB_2686054
Secondary antibodies				
Donkey anti-mouse Alexa Fluor 488	Jackson Laboratories	715-545-150	1:400	AB_2340846
Donkey anti-rabbit Alexa Fluor 647	Jackson Laboratories	711-605-152	1:400	AB_2492288

Table 4.1: Lists the antibodies and RNAscope probes used in chapter 4.

Imaging

Immunohistochemistry and RNAscope fluorescent labeling was evaluated using the 20 or 40x objective (0.75 numerical aperture, Plan Fluor; Nikon) of an upright epifluorescence microscope (Nikon Eclipse Ni) with a Retiga 2000R camera (Qimaging) controlled by Qcapture Pro 7.0 (Qimaging) software. Representative images were acquired through the 60x-oil immersion objective (PlanApochromat, 1.42 numerical aperture) of an inverted Fluoview FV1000 confocal microscope (Olympus) or Zeiss LSM 880 NLO confocal system (Zeiss) using Zen Black software and a 20x objective (0.8 numerical aperture, Plan ApoChromat; Zeiss).

Ca^{2+} imaging

Live whole-mounts of colon myenteric plexus from either *$Trpv1^{Cre;GCaMP5g-tdT}$ or $Wnt1^{Cre;GCaMP5g-tdT}$* mice were prepared for Ca^{2+} imaging as previously described (*45*). Whole-mount circular muscle-myenteric plexus (CMMP) preparations were microdissected from mouse colons were continuously superfused with fresh, prewarmed (37°C) Kreb's buffer consisting of (in mM): 121 NaCl, 5.9 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 10 HEPES, 21.2 NaHCO3, 1 pyruvic acid, and 8 glucose (pH adjusted to 7.4 with NaOH) at a flow rate of 2-3 mL min-1. Preparations were treated with JZL184 (1 μM), SR-141716A (500nM), and SR-144528 (1μM) to either disrupt endocannabinoid signaling by inhibiting MAGL activity (*51*, *52*) and blocking CBR1 (*53*) and CBR2 (*54*), respectively. CNO (100 μM; National Institute on Drug Abuse Drug Supply Program at the National institute of Health) and capsaicin (3 μM; 360376, Sigma) were used to activate glia and TRPV1-positive nociceptors, respectively. Drugs were either superfused in the bath with Krebs buffer for 30 seconds or locally applied using filamented borosilicate glass

microelectrodes that were backfilled with drug-containing Krebs buffer and puffed onto the surface of ganglia. Images were acquired every 0.5 seconds through 20x or 40x water-immersion objective (LUMPlanFI, 1.0 or 0.8 numerical aperture) of an upright Olympus BX51WI fixed stage microscope (Olympus, Tokyo, Japan) using NIS-Elements software (v.4.5; Nikon, Tokyo, Japan) or MetaMorph Software (Molecular Devices, Sunnyvale, CA) and an Andor Zyla or Neo sCMOS camera (Oxford Instruments, Abingdon, United Kingdom). Whole-mounts were superfused with Krebs buffer, (37°C) at 2–3 mL/min.

In vivo assessment of visceral sensitivity

Visceral sensitivity was measured as previously described by non-invasive assessment of visceromotor responses (VMRs) to colorectal distensions (CRDs) (*33*, *55*). Intracolonic pressure was measured by a miniaturized pressure transducer catheter (SPR-524, Mikro-Tip catheter, Millar Instruments) equipped with a custom-made plastic balloon. Mice were acclimated to restraints for 30 minutes before starting the CRD procedure. Graded phasic distensions (20, 40, 60, and 80 mmHg, 2 times each, 20 second duration, 3-minute interstimulus interval) were delivered to the balloon by a barostat (G&J Electronics) and VMR recordings were acquired using LabChart 8 (AD instruments). VMR's were recorded following either 1 or 3 weeks after receiving an enema of 0.9% saline or DNBS (0.2 mg/g in 30% ethanol).

Analysis and Statistics

Sample size was determined using a priori power analysis (G*Power 3.1) assuming 80% power with a desired P value of 0.05 and anticipating medium effect sizes (f = 1.0) based on previous experience and similar studies described in the literature.

Whole animals were considered as individual n's for immunohistochemistry, *in situ* hybridization, and *in vivo* analysis. Immunolabeling data were analyzed in FIJI (National Institutes of Health) by calculating arbitrary mean fluorescence or average cell count per ganglionic area. For Ca^{2+} imaging experiments individual nerve fibers (*56*) and enteric neurons and glia (*44*) were identified by tdTomato expression and morphology and Ca^{2+} imaging traces represent the average change in fluorescence over time of the mean number of individual afferents per preparation or total cell number. Mean fluorescence was quantified using FIJI (National Institutes of Health) or the Mesmerize (Dr. Kushal Kolar, University of Bergen, Norway (*57*) software. Images were pre-processed in FIJI by performing a background subtraction and implementing the StackReg plugin to correct background fluorescence and movement. Mean fluorescence (ΔF/F) 32-bit images were obtained by dividing Ca^{2+} imaging recordings by the baseline average of the 10 frames before the stimulus. A value of 1 was added to every pixel of the recording to avoid infinity values resulting from dividing by zero. 32-bit images were imported to Mesmerize and analyzed for Peak Detection, frequency of response, and curve clustering. Raw VMR traces were processed by running the SmoothSec and root mean square functions in LabChart 8 software to filter phasic colonic contractions (*33*). Following filtering the integral from the response at each distension pressure and the baseline mean from 20 seconds prior to the distension were obtained. Responses were considered significant if they were at least 2 SDV above the baseline mean. Data were analyzed using Students' t-test, one-way or two-way ANOVA using Graphpad 9 software (Prism) and are presented as mean ± standard error of the mean (SEM).

RESULTS

Enteric neurons and glia express RNA for multiple components of the endocannabinoid signaling pathway within the myenteric plexus.

CB1 and CB2 receptors are present in the enteric nervous system (ENS) and play important roles in the regulation of gastrointestinal (GI) functions (*16*). CB1 receptors are found mainly in the myenteric plexus, while CB2 receptors are found primarily in immune cells in the gut mucosa (*16*, *58*, *59*). Recent large sequencing analyses have provided further insight into the distribution of genes encoding receptors and enzymes associated with endocannabinoid signaling within the intestine. Single-cell sequencing performed on enteric neurons from mouse colon (*60*) show that enteric neurons are enriched in RNA for multiple components along the cannabinoid signaling pathway. Particularly, enteric neurons express RNA for CB1 (*Cnr1*) receptors and both endocannabinoid hydrolyzing enzymes MAGL (*Mgll*) and FAAH (*Faah*) (**Figure 4.1A**). CB2 (*Cnr2*) RNA is minimally expressed within enteric neurons which agrees with what we currently understand about endocannabinoids in the nervous system. These sequencing data suggest that CB1 and FAAH RNA is increased in females, though this did not reach statistical significance. Similarly, enteric glia also demonstrate expression levels of endocannabinoid signaling components (**Figure 4.1B**). Bulk RNA sequencing from colonic mouse glial show that enteric glia express RNA for CB1 and CB2 receptors and both MAGL and FAAH. These findings were surprising since existing immunolabeling data show limited expression of cannabinoid receptors on enteric glia (*14*, *61–63*). Concurrent with sequencing data for enteric neurons, baseline measurements for enteric glia indicate a significant increase in RNA expression levels for CB1, CB2, and MAGL in female mice. We confirmed

sequencing data using RNAscope (**Figure 4.1E-G**). **Figure 4.1E** shows *Magll* RNAscope labeling in females is increased at baseline when compared to males in both enteric glia (labeled with Sox10) and enteric neurons (labeled with peripherin). *Cnr1* receptor labeling (**Figure 4.1F**) is clearly expressed in enteric neurons in both male and female mice. *Cnr1* labeling is also observed within a small number of enteric glia. Interestingly, *Cnr2* labeling (**Figure 4.1G**) is observed at baseline within the ganglia although at a much lower degree than RNA for CB1 receptors. We've shown RNA expression for MAGL and cannabinoid receptors within neuronal and glial cell bodies in the intestine and that expression levels vary between males and females.

The endocannabinoid components we identified within myenteric ganglia are effective mediators of inflammatory responses and are associated with the development of pain (*28*, *64*, *65*). The sensitizing effects of proinflammatory mediators on nociceptors are often studied in the preclinical mouse DNBS colitis model based on its translational relevance and reproducibility (*66*). Previous studies show that DNBS-induced colitis in mice induces lasting visceral hypersensitivity in mice and that these effects are prevented by modulating endocannabinoid signaling (*67*) Thus, we investigated the impact of inflammation on the expression levels of cannabinoid receptors and endocannabinoid hydrolyzing enzymes. Analysis of published bulk RNA-sequencing data from enteric glia at peak colitis (*32*) showed that pathways associated with these receptors and enzymes were significantly upregulated during colitis (**Figure 4.1C**). MAGL expression levels in enteric glia were significantly decreased following inflammation in both male and female mice via RNAscope (**Figure 4.1E**). In contrast, *Cnr2* RNA expression increases in males and females following colitis (**Figure 4.1G**). This increase in labeling is primarily observed

outside of the ganglia, however sparse labeling is also seen in glia and neurons within myenteric ganglia. These changes are similar to those observed in humans with IBD (*68*). Notably, CB1 receptor levels remain largely unchanged following colitis (**Figure 4.1F**). This agrees with previous work that shows CB1 receptor mRNA levels are unaffected during inflammation (*69*). These data suggest that following colitis there is an increase in endocannabinoid function within the enteric nervous system via the increase of 2-AG availability which could have an important impact on enteric neuroplasticity and nociceptor signaling.

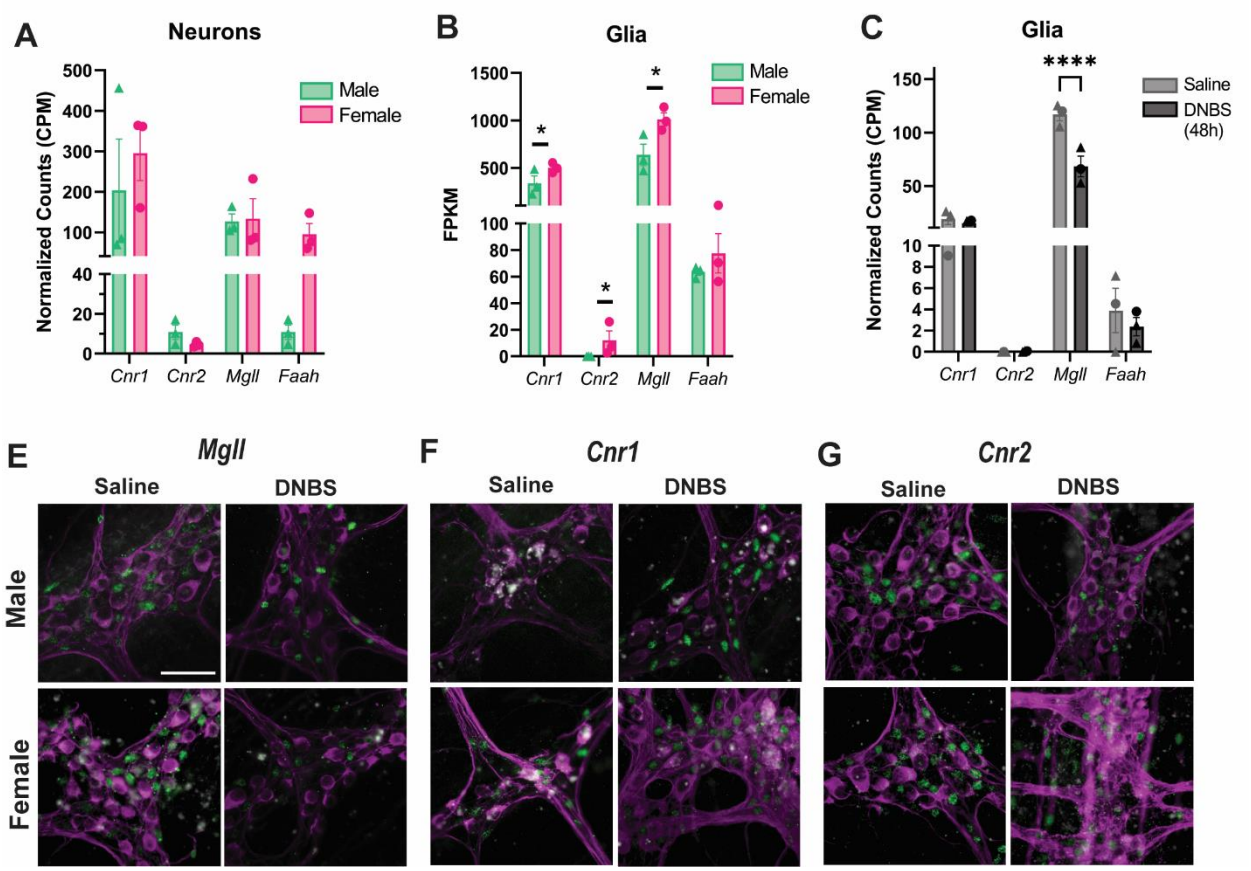

Figure 4.1: Endocannabinoid receptors and regulating enzymes are expressed by enteric glia and altered during colitis. (A) Single-cell RNA sequencing data obtained from May-Zhang et all (*60*) presented as normalized counts per million (CPM) demonstrating expression of cannabinoid receptors and regulating enzymes in male and female mouse colon. (B) Expression of cannabinoid receptors and regulating enzymes from enteric glia bulk RNA sequencing derived from male and female mouse colon (unpublished). Data are presented as fragments per kilobase of transcript per million mapped reads (FPKM). (C) Bulk RNA sequencing data sourced from Delvalle et al. (*32*) presented as CPM, showing that enteric glia differentially regulate *Mgll* enzyme

Figure 4.1: (cont'd)

expression following DNBS colitis (student t-test *p<0.05, ****p<0.0001, n=3 mice). Individual values are represented as triangles for males and circles for females.

Enteric neuron and glia Ca2+ signaling is differentially modulated by endocannabinoids in the intestine.

Next, we studied how endocannabinoids modulate signaling with the ENS and whether this occurs in a sex-dependent manner. To this end, we used the triple-transgenic *Wnt1^{Cre};GCaMP5g-tdT;GFAP::hM3Dq* (*44*) mice in which the chemogenetic actuator hM3Dq is expressed by enteric glia and GCaMP5g-tdT is expressed by both neurons and glia. This allowed us to selectively activate enteric glia with CNO and study the impact of cannabinoids in neuro-glia signaling. Our experimental approach for these experiments incorporated local drug application puffed directly onto single ganglia. This method allowed us to increase the output of data obtained by several thousand individual cells across treatment groups. To maximize the readout of the data obtained from these studies we utilized the Mesmerize (*57*) program which incorporates toolkits optimized for preprocessing, signal extraction, dataset organization, downstream analysis, and visualizations of large-scale calcium imaging data. We generated ΔF/F plots for each individual cell and utilized the ButterWorth and Peak Detect functions to identify maximum Ca^{2+} responses and the number and shape of each cell response. Furthermore, we were able to use these peak features to perform clustering analysis and study changes in the overall signaling dynamics of both neurons and glia (**Figure 4.2**).

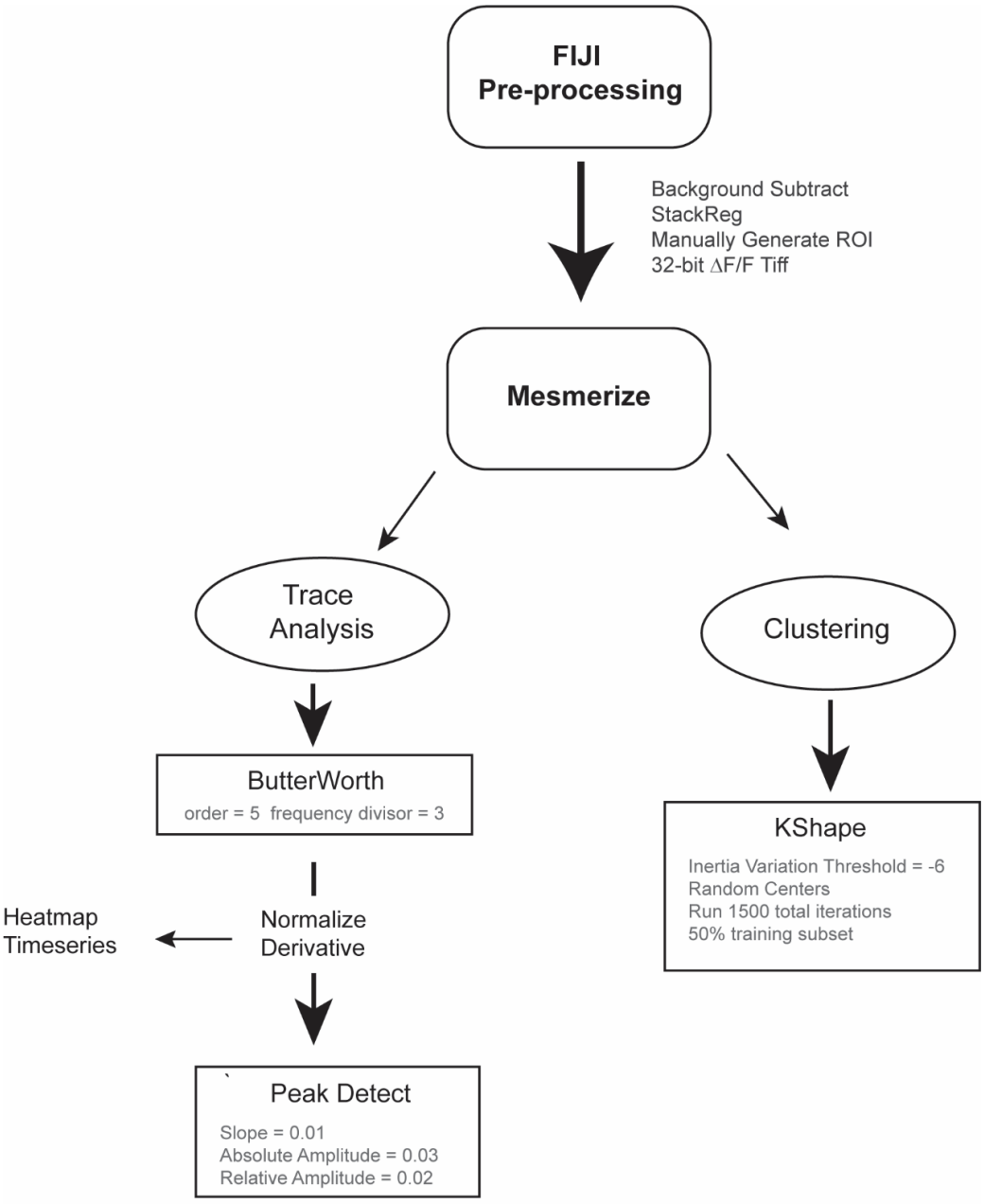

Figure 4.2: Schematic representing protocol for Ca^{2+} imaging data utilizing Mesmerize. Image depicts workflow and parameters used for the normalization of data, peak detection from traces, and curve clustering.

In agreement with prior work (*44*, *46*), glial responses to CNO were significantly different between males and females (**Figure 4.3A, D*i,* F*i***). Female glial responses to CNO are ~2 times higher for the area under the curve (**Figure 4.3B**) and possess a 1.5 times higher firing rate (**Figure 4.3C**). Increased levels of 2-AG attenuate intestinal motility and slowdown gut transit (*70*). Thus, we hypothesized that increasing 2-AG levels by inhibiting hydrolysis through MAGL would reduce Ca^{2+} activity in the myenteric plexus. Suppressing MAGL activity in situ with the potent inhibitor JZL184 reduced overall glial responses to CNO. Peak Ca^{2+} responses to CNO were significantly decreased by 17% in males and 21% in females (**Figure 4.3A, D*ii*, F*ii***). Similarly, the area under the curve for responses following JZL184 treatment and the number of responses per minute of recording also showed a significant decline in males by 53 and 65% respectively (**Figure 4.3B-C**). Female mice also showed a drop in the area under the curve by 14% following treatment but did not show any changes in frequency. In contrast to the effects induced by inhibition of MAGL, blocking both CB1 (**Figure 4.3D*iii***) and CB2 (**Figure 4.3D*iv***) receptors with specific antagonists (SR141716A and SR144528, respectively) significantly increased overall glial Ca^{2+} responses in male mice. These effects were expected since activation of these receptors are generally associated with inhibitory effects in the gut (*61*, *62*). Max responses were increased by ~30% following antagonism of cannabinoid receptors (**Figure 4.3A**). Area under the curve (**Figure 4.3B**) was increased by 174% after blocking CB1 receptors and 136% after blocking CB2 receptors. Frequency of responses (**Figure 4.3C**) were also increased by 106% and 75% after blocking endocannabinoid receptors 1 and 2 respectively. Interestingly, the overall increase observed in Ca^{2+} responses after blocking CB1 and CB2 receptors in males

resulted in responses similar to those observed in females at baseline. Surprisingly, inhibition of CB1 receptors in females had opposing effects to those observed in male mice (**Figure 4.3E*iii***). Blocking CB1 receptors in female mice reduced overall calcium responses which was observed by a 13% decrease in the peak response (**Figure 4.3A**) and a 10% decrease in the area under the curve (**Figure 4.3B**). Interestingly, inhibition of CB2 receptor signaling had no impact on glial Ca^{2+} responses in female mice (**Figure 4.3A-C, E*iv***). This was unexpected since our *Cnr2* expression data suggested that CB2 receptor levels were higher in female mice.

Our Ca^{2+} imaging data led us to speculate that altering endocannabinoid signaling could change not only the magnitude of cell responses but also the characteristics of Ca^{2+} dynamics. To study these changes, we performed clustering analysis using the KShape function in the Mesmerize software (**Figure 4.2**). Our results from these studies identified five distinct curve clusters from both male and female mice, whose distribution was altered following pharmacological modulation of endocannabinoid signaling (**Figure 4.3F-G**). The clusters identified largely differ in shape, frequency, and amplitude between male and female mice. Curves from male control animals are predominantly those characterized within cluster 4 (61%, **Figure 4.3F*v***) and cluster 1 (28%, **Figure 4.3F*ii***), which are distinguished by curves that have peak responses with about a two-fold increase from baseline and have a longer duration. Curves with shapes in cluster 0 are characterized by having the highest peaks, but shorter durations are within 5% (**Figure 4.3F*i***) of the total distribution of responses in control males. Clusters 2 (low responding, **Figure 4.3F*iii***) and 3 (non-responding, **Figure 4.3F*iv***) each account for 3% of response types in male mice. These typical responses are significantly altered following inhibition

of MAGL (**Figure 4.3F*vi***) which instead show a reduced percentage of clusters 1 (20%) and 4 (54%) and an increase in cluster 3 (9%) and cluster 0 (15%). The change in the distribution of these curves can explain the effects of JZL184 on peak, area under the curve, and frequency given that the distribution of curves shifts to include a higher percent of non-responding cells and those with a shorter duration of response. Blocking CB1 receptors in male colon preparations drastically shifts the curve distribution toward cluster 1 which results in about 50% of cells within this cluster. Treatment with SR141716A also dramatically reduces the number of non-responding cells (cluster 3) which account for <1% of the total distribution of curves. Similarly, disrupting CB2 receptor signaling changes the distribution of curves to be primarily composed of cluster 1 (51%) and does not show responses within clusters 2 and 3. These differences can again explain the changes observed in peak, the area under the curve, and frequency given that the distributions for these groups are primarily composed of responses characterized by high amplitudes and longer durations whilst eliminating low responding cell types.

Glia Ca^{2+} responses from female mice were also distributed into 5 different clusters. Baseline responses from female mice were distributed within 3 main clusters- cluster 1 (31%, **Figure 4.3G*ii***), cluster 3 (24%, **Figure 4.3G*iv***), and cluster 4 (46%, **Figure 4.3G*v***). Following inhibition of MAGL reduces the presence of cluster 1 (28%), cluster 3 (22%), and cluster 4 (34%) within this group and introduces the presence of cluster 0 (5%) and cluster 2 (10%). These additional clusters have significantly lower peak responses from the clusters present in control which can explain the reduced responses observed in peak and amplitude of responses. Inhibition of CB1 receptors also introduced cluster 0 (5%) and cluster 2 (6%). Treatment with SR141716A showed an increase in the

distribution of cluster 1 (43%) and a decrease in cluster 4 (38%), and cluster 3 (6%) when compared to control animals. The significant decrease in cluster 3 and 4 which show the highest amplitude explain the reduced responses observed in the peak and area under the curve. Blocking CB2 receptors in female mice did not induce any major changes in glia Ca^{2+} responses to CNO. However, differences were observed in our clustering analysis (**Figure 4.3G*vi***). Much like inhibition of CB1, blocking CB2 receptors also introduces cluster 0 (3%) and cluster 2 (7%) to the distribution, showed a reduction in cluster 1 (21%) and 4 (41%) and an increase in cluster 3 (27%). Although this seems to follow a similar trend to what was observed in the CB1-inhibited group, the changes in distribution were not sufficient to have an impact on the maximum response, amplitude, and frequency of the Ca^{2+} responses.

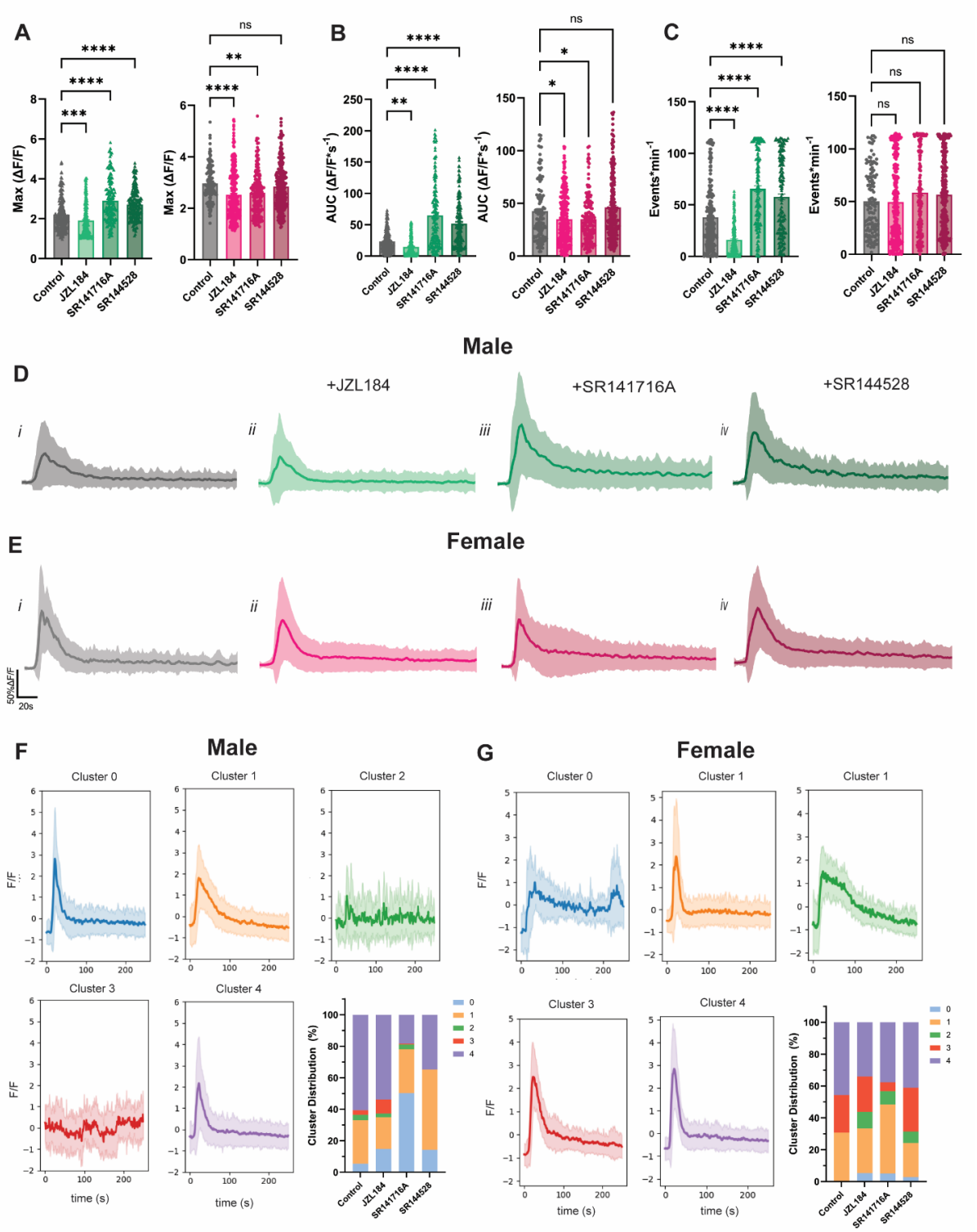

Figure 4.3: Glial Ca^{2+} signaling is modulated by endocannabinoid signaling in the gut.

Figure 4.3: (cont'd)

Summary data for the maximum response (A), area under the curve (B), and peak frequency (C) from glial Ca^{2+} responses to CNO in male (green) and female (magenta) *Wnt1^{Cre};GCaMP5g-tdT* mice following either MAGL inhibition with JZL184 (1μM) or antagonism of CB1 or CB2 receptors with SR141716A (500nM) and SR144528 (1μM) respectively. Representative traces for male (D) and female (E) mice are reported as ΔF/F over time ±SD. Representative traces and quantification of curve clusters for male (F) and female (G) mice following Mesmerize KShape analysis. Five distinct clusters were identified from each sex and their distribution was shifted from control following drug treatment. Significant differences were determined by Ordinary one-way ANOVA followed by a Dunnett's post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 to control; n = 127-306 individual glial cells from multiple mice per treatment group).

Like enteric glia, enteric neuron sequencing data show that they express components of the endocannabinoid signaling pathway, including MAGL, CB1, and CB2, although to differing extents. To how endocannabinoids modulate neuronal signaling in the gut, we again used the triple-transgenic *Wnt1^{Cre};GCaMP5g-tdT;GFAP::hM3Dq* (*44*) which also expresses GCaMP5g in enteric neurons and stimulated neurons by local application of high KCl (140mM). Similar to our observation in glia, female peak neuronal responses to KCl were significantly higher than those observed in male mice. In fact, maximum responses in female neurons were approximately 25% higher than those observed in males (**Figure 4.4A, D*i*, F*i***). In contrast, baseline levels for the area under the curve (**Figure 4.4B**) and the number of events (**Figure 4.4C**) were no different between sexes.

Inhibition of MAGL did not affect peak Ca^{2+} responses (**Figure 4.4A**) or the area under the curve (**Figure 4.4C**) in male mice. However, it significantly reduced the number of recorded Ca^{2+} events by 34% (**Figure 4.4C, D*ii***). Similarly, female mice demonstrated a significant decrease in all signaling properties following MAGL inhibition (**Figure 4.4E*ii***). Maximum neuron responses decrease by 28% (**Figure 4.4A**), the area under the curve decreased (**Figure 4.4B**) by 58%, and frequency decreased by 32% (**Figure 4.4C**). The data show that increases in endocannabinoid levels through the inhibition of MAGL attenuates neuronal signaling similar to what was observed in enteric glia. Inhibition of CB1 receptors did not have a major impact in neuronal signaling in male mice (**Figure 4.4A-D*iii***). This was unexpected given that we observed high levels of *Cnr1* expression in enteric neurons. In contrast, inhibition of CB2 receptors induced significant increases in the area under the curve (170%, **Figure 4.4B, B*iv***) and maximum calcium response in

enteric neurons (98%, **Figure 4.4C**). Blocking CB1 (**Figure 4.4E*iii***) and CB2 (**Figure 4.4E*iv***) receptors in female mice significantly reduced neuronal responses to KCl. Antagonism with SR141716A reduced peak neuronal responses by 20% (**Figure 4.4A**), area under the curve by 58% (**Figure 4.4B**), and frequency by 36% (**Figure 4.4C**). Similarly, inhibition of CB2 with SR141716A reduced peak neuronal responses by 11% (**Figure 4.4A**), area under the curve by 43% (**Figure 4.4B**), and frequency by 40% (**Figure 4.4C**).

Additionally, we performed clustering on analysis and identified three distinct enteric neuron response clusters in both male and female mice (**Figure 4.4F-G**). Typical cluster shapes in baseline male responses are predominantly found within cluster 2 (**Figure 4.4F*iii***) which accounts for about 85% of total baseline responses. Baseline responses can also be found distributed between cluster 0 (6%, **Figure 4.4F*i***) and cluster 1 (9%, **Figure 4.4F*ii***). A similar distribution can be seen within the MAGL inhibitor group (**Figure 4.4F*iv***), which likely explains why no major differences were observed in neuron responses to KCl after treatment with JZL184. Blocking CB2 receptors induced a significant change in the distribution of curves from enteric neurons and appears to become evenly distributed between cluster 1 (52%) and cluster 2 (47%) and decreases cluster 0 to account for <1% of responses. This shift in response shape can likely explain the increases in peak neuronal Ca^{2+} responses to KCl following treatment with SR144528. Surprisingly, inhibition of CB1 receptors drastically shifts the curve cluster distribution from cluster 2 (17%) to cluster 1 (80%). Similar to the changes observed in male mice, pharmacological modulation of the endocannabinoid system significantly alters Ca^{2+} response curve distribution in female mice (**Figure 4.4G**). Baseline responses

in female mice are distributed within the three clusters identified. Cluster 0 (**Figure 4.4G***i*) and cluster 2 (**Figure 4.4G***iii*) account for 39 and 44% of all baseline responses in females and cluster 1 (**Figure 4.4G***ii*) accounts for 17%. This distribution is shifted following treatment the MAGL inhibitor JZL184 showing an increase in responses associated to cluster 2 accounting for 70% of the curve distribution for this treatment group. Similarly, blocking CB1 and CB2 receptors also resulted in a drastic shift in curves within cluster 2 accounting for 90 and 88% of the total distribution for each treatment group respectively. The similar shift in clustering within each treatment group is reflected in the overall change observed in neuronal Ca^{2+} responses to KCl in female mice.

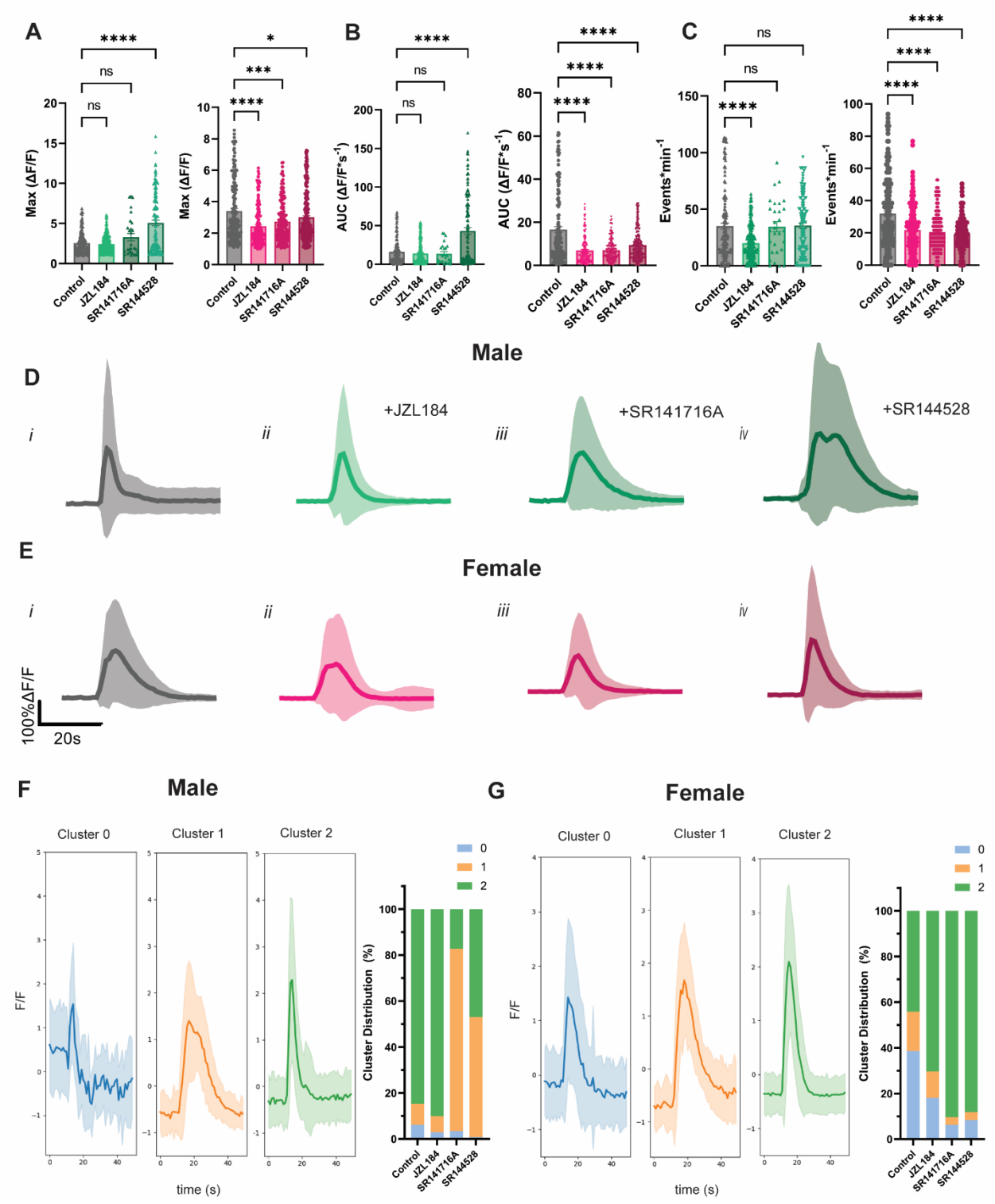

Figure 4.4: Neuronal signaling is modulated by endocannabinoid signaling in the gut.

Figure 4.4: (cont'd)

Summary data for the maximum response (A), area under the curve (B), and peak frequency (C) from enteric neuron responses to high potassium (140mM KCl) in male (green) and female (magenta) *Wnt1^{Cre};GCaMP5g-tdT* mice following either MAGL inhibition with JZL184 (1μM) or antagonism of CB1 or CB2 receptors with SR141716A (500nM) and SR144528 (1μM) respectively. Representative traces for male (D) and female (E) mice are reported as ΔF/F over time ±SD. Representative traces and quantification of curve clusters for male (F) and female (G) mice following Mesmerize KShape analysis. Three distinct clusters were identified from each sex and their distribution was shifted from control following drug treatment. Significant differences were determined by Ordinary one-way ANOVA followed by a Dunnett's post-hoc test (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ to control; n = 67-285 individual glial cells from multiple mice per treatment group).

Colitis impacts endocannabinoid mechanisms in the intestine altering enteric neurons and glia Ca2+ signaling.

Our Ca^{2+} imaging findings show that neuro-glial signaling is modulated by endocannabinoid signaling in physiological conditions. Endocannabinoid levels are significantly altered during inflammation (*64*, *68*, *71*, *72*) and increased levels of 2-AG are protective against the effects of inflammation (*28*, *64*, *65*). Thus, we investigated the impact of inflammation on endocannabinoid signaling in enteric glia. We were initially interested in understanding how DNBS-induced colitis was altering glia Ca^{2+} responses. Interestingly, glial responses to CNO appeared unchanged in male mice and decreased in female mice following DNBS treatment. In fact, glia responses in females were reduced by 22% (**Figure 4.5A**, saline control means are represented as a checkered line on the Y axis) which shows female enteric glia displaying Ca^{2+} responses similar to those observed in male control animals. Following inhibition of MAGL male mice showed an increase in peak Ca^{2+} responses (**Figure 4.5A, D*ii***) but no difference was detected in the area under the curve and frequency of the response (**Figure 4.5C**). This was surprising, as we anticipated the increase of endocannabinoids, like 2-AG, would have an inhibitory effect on glial Ca^{2+} signaling. Blocking CB1 receptors also resulted in an increase in overall glial responses (**Figure 4.5D*iii***). Maximum Ca^{2+} responses increased by 13.5% in males when compared to control animals (**Figure 4.5A**). Similarly, the area under the curve (**Figure 4.5B**) and the number of Ca^{2+} responses (**Figure 4.5C**) also increased by 72 and 36% respectively. Antagonism of CB2 also showed increased responses by 7% (**Figure 4.5A, D*iv***). Inhibiting MAGL activity during colitis had no significant impact on Ca^{2+} responses from glia in female mice when compared to untreated controls (**Figure**

4.5A-C; E*ii*). Blocking CB1 receptors (**Figure 4.5E*iii***) in female mice following colitis increased the area under the curve (89.5%, **Figure 4.5B**) and the number of Ca^{2+} events per minute (37.4%, **Figure 4.5C**). Peak responses were not affected by the antagonism of CB1 receptors in inflamed females (**Figure 4.5A**). In contrast, CB2 receptors showed a significant decrease in maximum Ca^{2+} responses in female mice following colitis (**Figure 4.5A, E*iv,***). This was unexpected as previous studies have shown that agonists for CB2 are known to reduce inflammation and attenuate reactive gliosis (*73*, *74*).

Modulation of endocannabinoids during colitis induced significant alterations in the distribution of Ca^{2+} curves in both male and female mice (**Figure 4.5F-G**). DNBS control male animals showed response curves to be primarily within cluster 0 (29%, **Figure 4.5F*i***) and cluster 1 (51%, **Figure 4.5F*ii***) with response curves also allocated within cluster 4 (18%, **Figure 4.5F*v***) and cluster 2 (2%, **Figure 4.5F*iii***) and 3 (<1%, **Figure 4.5F*iv***). Inhibition of MAGL in males following colitis shifts the curve distribution towards cluster 0 (42%) and increases the Ca^{2+} responses attributed to cluster 2 (6%). Blocking CB1 And CB2 receptors dramatically shifts the distribution of responses in favor of Cluster 1 (72%, 65%, **Figure 4.5F*vi***). Some differences were also observed within cluster distributions for females following inhibition of MAGL. DNBS control females showed a predominance of response expression within cluster 0 (40%, **Figure 4.5G*i***) and cluster 3 (42%, **Figure 4.5G*iv***). In contrast, following inhibition of MAGL with JZL184, there was an increase in responses within cluster 1 (8%, **Figure 4.5G*ii***) and cluster 2 (21%, **Figure 4.5G*iii***). Blocking CB1 receptors induced an increase in responses within cluster 1 (26%) cluster 2 (11%), and cluster 4 (11%, **Figure 4.5G*v***), when compared to control animals. Finally, antagonism of CB2 receptors induced a significant shift in the distribution of Ca^{2+}

responses towards those observed within cluster 0 (878%, **Figure 4.5G*vi***). The changes in Ca^{2+} curve distribution show that inflammation induces significant alterations to Ca^{2+} responses in both male and female enteric glia and that these dynamics can be altered with endocannabinoids. Furthermore, the presence and changes in the distribution of these clusters suggest that there exist subpopulations of enteric glia within myenteric ganglia that are differentially impacted by inflammation and endocannabinoids in a sexually dimorphic manner.

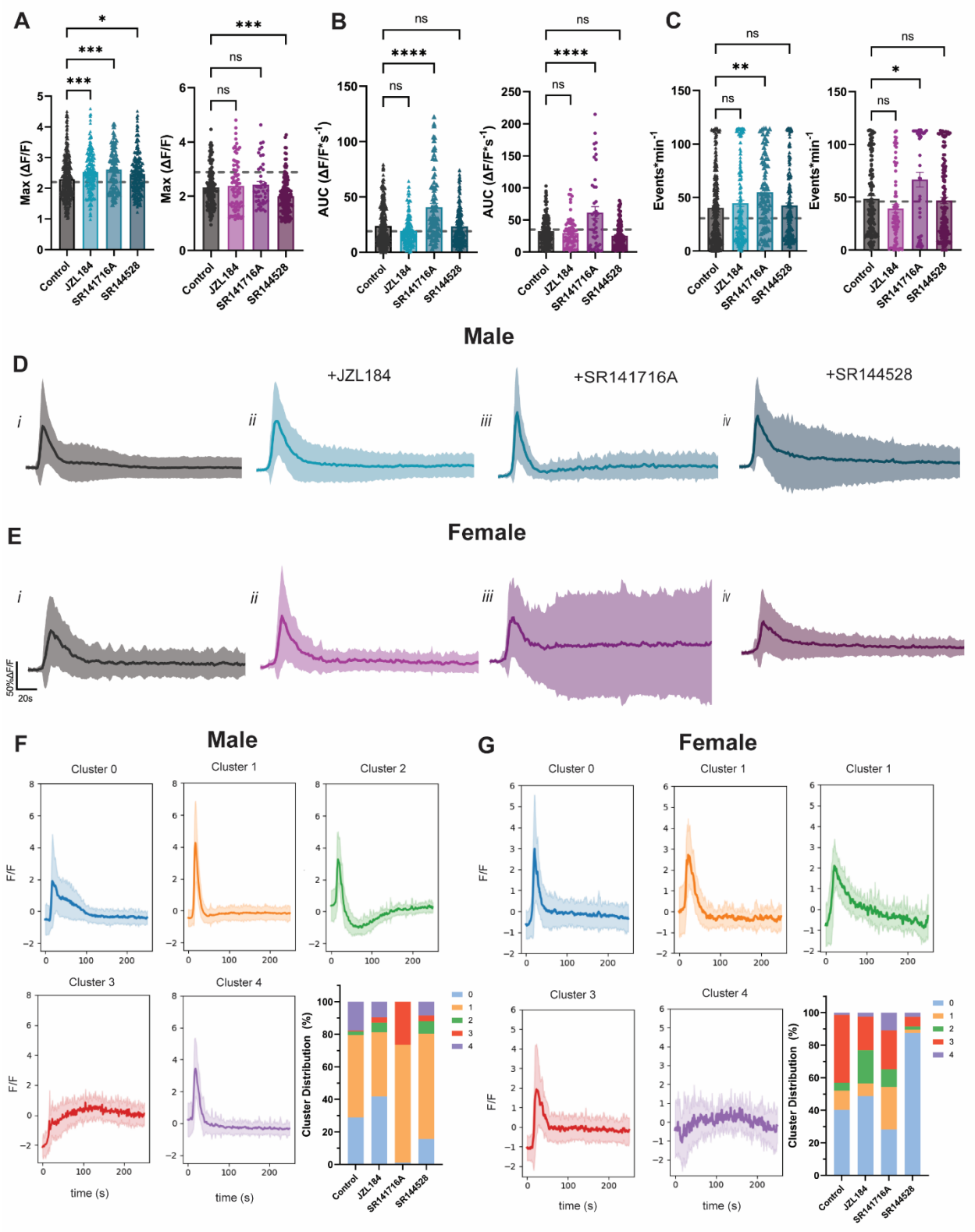

Figure 4.5: Colitis alters glial endocannabinoid regulation.

Figure 4.5: (cont'd)

Summary data for the maximum response (A), area under the curve (B), and peak frequency (C) from glial Ca^{2+} responses to CNO in male (blue) and female (purple) *Wnt1^{Cre};GCaMP5g-tdT* mice 1 week after DNBS treatment following either MAGL inhibition with JZL184 (1μM) or antagonism of CB1 or CB2 receptors with SR141716A (500nM) and SR144528 (1μM) respectively. Checkered line represents responses from untreated control animals. Representative traces for male (D) and female (E) mice are reported as ΔF/F over time ±SD. Representative traces and quantification of curve clusters for male (F) and female (G) mice following Mesmerize KShape analysis. Five distinct clusters were identified from each sex and their distribution was shifted from control following drug treatment. Significant differences were determined by Ordinary one-way ANOVA followed by a Dunnett's post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 to control; n = 164-358 individual glial cells from multiple mice per treatment group).

We then studied the impact of inflammation on neuronal endocannabinoid signaling. We found that following DNBS colitis Ca^{2+} responses in enteric neurons are significantly increased in male animals when compared to saline controls (**Figure 4.6A-C, D*i*,** saline controls appear as a checkered line on the y-axis). In contrast, female responses from enteric neurons are significantly decreased during colitis (**Figure 4.6A-C, E*i***). Inhibition of MAGL (**Figure 4.6D*ii***) in male animals induces a significant increase in peak calcium responses (12%, **Figure 4.6A**) and frequency (24.5%, **Figure 4.6C**). Blocking CB1 receptors (**Figure 4.6D*iii***) also increases peak neuronal Ca^{2+} responses to high KCl (51%, **Figure 4.6A**) and the area under curve (44%, **Figure 4.6B**). In contrast, blocking CB2 receptors during inflammation (**Figure 4.6D*iv***) resulted in reduced peak Ca^{2+} responses (54%, **Figure 4.6A**), area under the curve (77%, **Figure 4.6B**) and frequency (30%, **Figure 4.6C**) following inflammation. Contrary to its effects in males, treatment with the MAGL inhibitor JZL184 decreased Ca^{2+} responses when compared to controls in females. Peak responses in neurons were decreased by 17% (**Figure 4.6A**), area under the curve by 12.1% (**Figure 4.6B**), and frequency by 24.5% (**Figure 4.6C**). Although these did not reach statistical significance, the effect is appreciated in the average response recorded for each cell (**Figure 4.6E*ii***). Blocking CB1 (**Figure 4.6E*iii***) and CB2 receptors (**Figure 4.6E*iv***) also significantly increased overall Ca^{2+} responses in female enteric neurons following inflammation. Max responses from enteric neurons were increased by 53 and 59% (**Figure 4.6A**), area under the curve was increased by 139 and 214.5% (**Figure 4.6B**), and the number of Ca^{2+} response per minute (**Figure 4.6C**) were increased by 73 and 43%.

Much like our findings in enteric glia, following colitis Ca^{2+} responses can be clustered into different groups according to the shape of the response (**Figure 4.6F-G)**. Control neuronal clusters in males are primarily distributed within cluster 1 (69%, **Figure 4.6F*ii***), with clusters 0 (**Figure 4.6F*i***) and 2 (**Figure 4.*6Fiii***) accounting for the other 30 and 1%. Animals treated with the inhibitor for MAGL have a similar distribution to controls, only showing a slight increase in the distribution within cluster 0 (32%). Blocking CB1 and CB2 receptors shows a dramatic increase in the distribution within cluster 1 (87% and 84% respectively). Neurons from female mice treated with DNBS colitis show a slight shift in cluster distribution between treatment groups (**Figure 4.6G*iv***). Control animals show a primary distribution within cluster 0 (24%, **Figure 4.6G*i***) and cluster 1 (69%, ***Figure 4.6Gii***). Following MAGL inhibition we observe an increase in the expression within cluster 2 (15%). When blocking CB1 receptors an increase in the distribution of cluster 1 was determined (76%), whereas antagonism with CB2 induced an increase in the expression of cluster 0 (30%). Surprisingly, these differences within groups were very slight when compared to the clustering changes observed for enteric glia Ca^{2+} responses. Suggesting that endocannabinoid signaling may have a greater impact of Ca^{2+} dynamics in glia following inflammation. Notably, there does not appear to be a significant difference in cluster types between male and female mice which can be observed more prominently within glial responses.

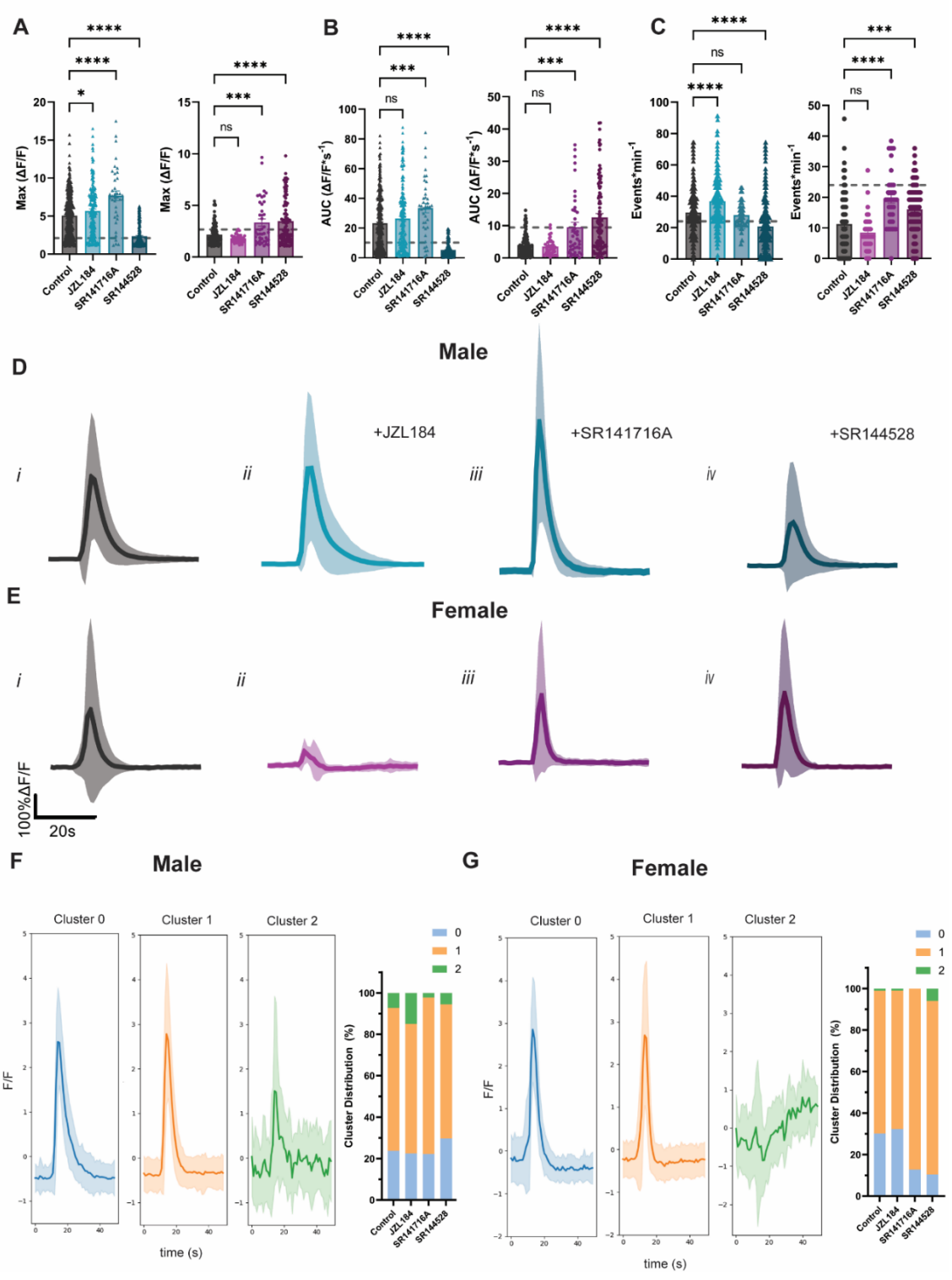

Figure 4.6: Impact of colitis on neuronal cannabinoid signaling in the enteric nervous system. Neuronal signaling is modulated by endocannabinoid signaling in the

Figure 4.6: (cont'd)

gut. Summary data for the maximum response (A), area under the curve (B), and peak frequency (C) from enteric neuron responses to high potassium (140mM KCl) in male (blue) and female (purple) *Wnt1^{Cre};GCaMP5g-tdT* mice following either MAGL inhibition with JZL184 (1μM) or antagonism of CB1 or CB2 receptors with SR141716A (500nM) and SR144528 (1μM) respectively. Representative traces for male (D) and female (E) mice are reported as ΔF/F over time ±SD. The checkered line represents responses from untreated control animals. Representative traces and quantification of curve clusters for male (F) and female (G) mice following Mesmerize KShape analysis. Three distinct clusters were identified from each sex and their distribution was shifted from control following drug treatment. Significant differences were determined by Ordinary one-way ANOVA followed by a Dunnett's post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 to control; n = 119-285 individual glial cells from multiple mice per treatment group).

Colitis induces sensitization of nociceptors in the myenteric plexus which is modulated by MAGL activity.

As highlighted previously, MAGL is mainly responsible for the degradation of the endocannabinoid, 2-AG (*30*) and pharmacological inhibition of MAGL improves histological colon alterations and decreased proinflammatory cytokine expression in models of colitis (*64*, *65*, *71*). Nociceptors become sensitized during the active phase of inflammation (*75*) which likely induces lasting changes in nociceptor signaling. We studied how MAGL inhibition affects changes in nociceptor sensitivity using Ca^{2+} imaging in *Trpv1*$^{Cre;GCaMP5g\text{-}tdT}$ mice following colitis. Treatment with DNBS colitis had a significant effect on TRPV1+ nociceptor expression and a clear increase in nociceptor innervation can be observed in both male (**Figure 4.7B**) and female (**Figure 4.7F**) mice when compared to controls (**Figure 4.7A, E**). These differences appeared both as an increase in the overall number of nociceptors expressed (**Figure 4.7Q, S**) and tdTomato fluorescence (**Figure 4.7U, W**) per area. The total number of TRPV1+ nerves in male mice was increased by 70% when compared to controls (**Figure 4.7Q**) and total fluorescence was increased by 270% (**Figure 4.7U**). Although these results did not achieve statistical significance a strong trend can be appreciated. Similarly, females show a significant increase in the overall number of TRPV1+ nerve fibers following inflammation (62%) when compared to control preparations (**Figure 4.7S**). Interestingly, tdTomato fluorescence levels did not appear to differ between the control and DNBS group (**Figure 4.7W**). These findings, fit with existing studies that show increased TRPV1 receptor mRNA (*76*) and TRPV1 nerve fiber density (*77*, *78*) in patients with IBS and IBD. Along with increases in nerve fiber density, we also found that TRPV1+ Ca^{2+} responses to

capsaicin are increased following colitis in males (**Figure 4.7A-B*ii***) and females (**Figure 4.7E-F*ii***). The area under the curve was significantly increased in female mice by 2.6-fold compared to control animals (**Figure 4.7X**) and increased in male animals by 1.9-fold, although they did not reach statistical significance (**Figure 4.7V**). A prominent trend was also observed in peak Ca^{2+} responses in females (**Figure 4.7T**) but was not observed in males (**Figure 4.7R**). Notably, following colitis we can also observe prominent GCaMP responses in very thin nerve processes that typically do not show responses under basal conditions (labeled with white arrows). This effect is observed in both males and females and suggests the recruitment of silent nociceptors following inflammation (*79*, *80*). Taken together these data show that colitis induces sensitized responses in nociceptors and that this effect last after the resolution of inflammation.

Increased levels of 2-AG via the inhibition of MAGL improve increased pain sensitivity in animal models of inflammatory pain (*27*, *28*, *31*). Furthermore, 2-AG is a direct agonist for TRPV1 receptors and induces changes in TRPV1 signaling (65-67). Given, the potent effects of MAGL inhibition on neuro-glial signaling both in baseline and inflamed conditions, we were interested in studying the impact of MAGL modulation of 2-AG on nociceptive signaling. Thus, we studied Ca^{2+} signaling on nociceptors in control and DNBS-treated animals that were treated with the MAGL inhibitor, JZL184 (10mg/kg), or a control vehicle solution. Treatment with JZL184 did not significantly impact nerve density (**Figure 4.7Q, S**) or fluorescence (**Figure 4.7U, W**) in control animals (**Figure 44.7C, G**). However, in DNBS females, nerve density remained unchanged (**Figure 4.7H**) when compared to control animals unlike DNBS-treated animals that did not receive the MAGL inhibitor. After treatment with JZL184, tdTomato fluorescence (**Figure 4.7U**) and

nerve fiber density (**Figure 4.7Q**) appeared similar following treatment with DNBS in male animals (**Figure 4.7D**). Surprisingly, tdTomato fluorescence was significantly increased in females that received the treatment following inflammation (**Figure 4.7W, H**). Treatment with JZL184, also impacted Ca^{2+} responses from nociceptors following inflammation. In female animals, blocking MAGL activity ameliorates the effects of DNBS on nociceptor Ca^{2+} responses. Peak Ca^{2+} responses (**Figure 4.7T**) and area under the curve (**Figure 4.7X**) were significantly decreased by 39% and 86% following JZL184 treatment in DNBS females (**Figure 4.7H*i*-H*ii***). Surprisingly, male animals treated with JZL184 had a trend towards increased peak Ca^{2+} responses (**Figure 4.7R**) and area under the curve (**Figure 4.7V**). Following inhibition MAGL we observe a loss of responding silent nociceptors. These data suggest that MAGL has the potential to modulate visceral hypersensitivity and it does so in a sex-dependent manner.

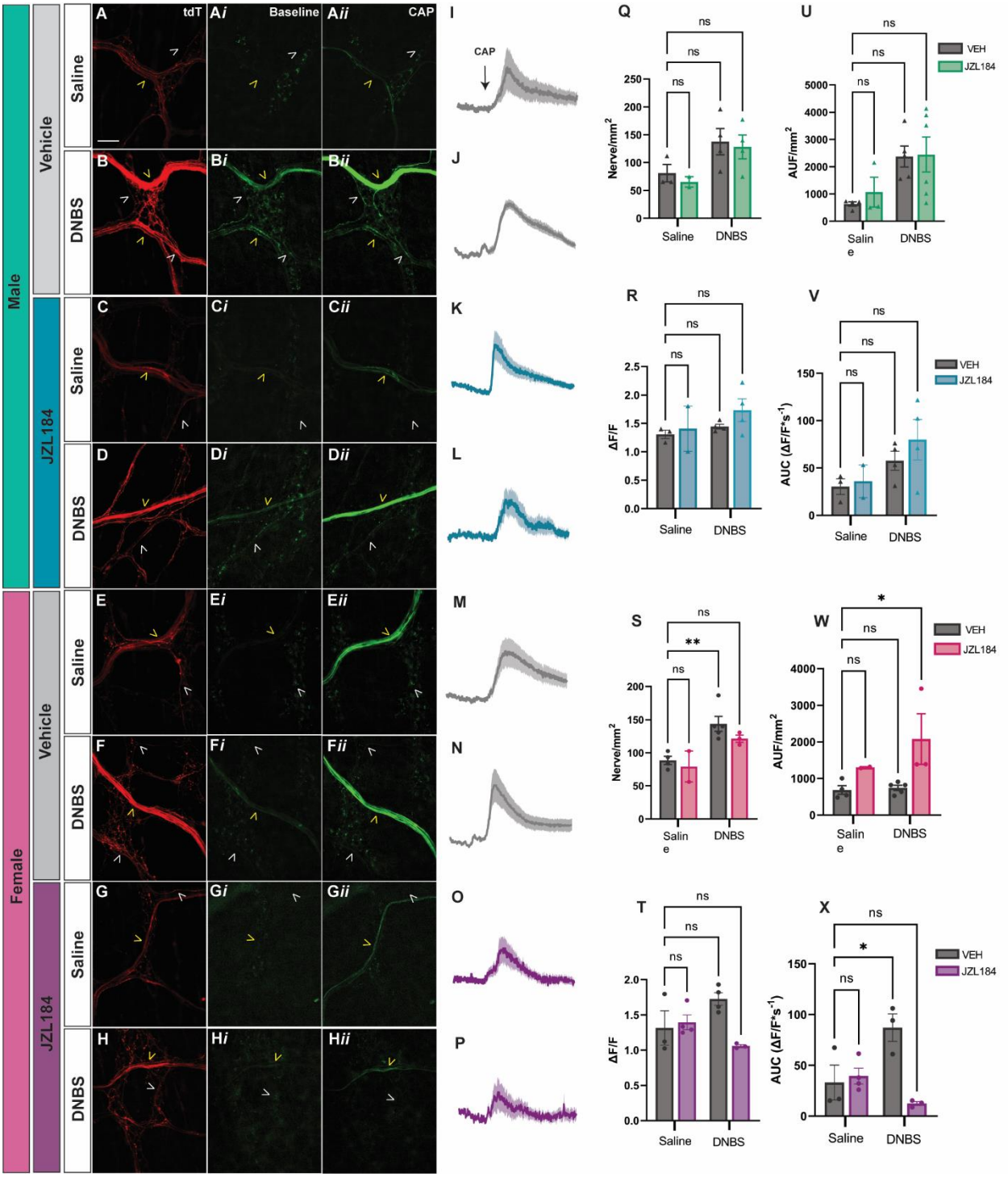

Figure 4.7: Nociceptive signaling is altered during colitis. (A-H*ii*) Representative images from male and female *TRPV1*$^{Cre;GCaMP5g\text{-}tdT}$ showing TRPV1(+) nociceptors within myenteric ganglia that express tdTomato and baseline and GCaMP responses to

Figure 4.7: (cont'd)

capsaicin (CAP) from control and DNBS colitis mice that were treated with JZL184 or vehicle. Yellow arrows indicate typically responding ociceptors and white arrows depict nociceptors that are recruited following DNBS treatment. (I-P) Show representative traces of nociceptor Ca^{2+} responses following stimulation with CAP. Representative traces reported as ΔF/F over time. Quantification of TRPV1+ nerve distribution and mean fluorescence per area in male (Q-U) and female (S-W). Mean fluorescence values are reported as arbitrary fluorescence units (AFU). Quantification of peak Ca^{2+} responses and area under the curve (AUC) of GCaMP responses to capsaicin in male (R-V) and female (T-X) *TRPV1*$^{Cre;GCaMP5g\text{-}tdT}$ mice. Significant differences were determined by Ordinary one-way ANOVA followed by a Dunnett's post-hoc test (*$p<0.05$ to control; n = 3-5) (scale=60μM)

Glial MAGL impacts reflexive pain behaviors following colitis

To determine whether the protective effects of MAGL on TRPV1+ nociceptors were glia specific, we assessed visceral hypersensitivity *in vivo* by measuring VMRs to CRDs (**Figure 4.8**) in mice via non-invasive pressure recordings (*33*, *55*, *81*) in WT and *Sox10^{CreERT2};Mgll$^{f/f}$* following colitis. This technique has been validated in multiple prior studies and eliminates the potential confounding factor of the surgery required for EMG recordings which can sensitize nociceptors and change enteric glial phenotype (*33*, *55*). In agreement with our findings from Ca^{2+} imaging experiments, female WT mice showed increased VMR responses to CRD after 1 week of DNBS treatment (**Figure 4.8C**). Furthermore, female visceral hypersensitivity was sustained until 3 weeks after DNBS treatment. Colitis effects on VMRs in female mice were lost in the *Sox10^{CreERT2};Mgll$^{f/f}$* mouse model (**Figure 4.8D**). This data supports our findings from Ca^{2+} imaging experiments that show that the inhibition of MAGL ameliorates the sensitization of nociceptors during inflammation and that these effects are primarily mediated by enteric glia. Surprisingly, DNBS does not have an effect on VMR responses in male WT animals (**Figure 4.8A**). MAGL glial knock out male mice show increased responses to VMR in saline treated animals (**Figure 4.8B**). This sensitivity is lost 1 week after treatment with DNBS. At 3 weeks post DNBS this effect is no longer observed and male knock out VMR levels are similar to baseline. Therefore, inflammation induced visceral hypersensitivity and enteric glia modulation via endocannabinoid signaling function through sex dependent mechanisms.

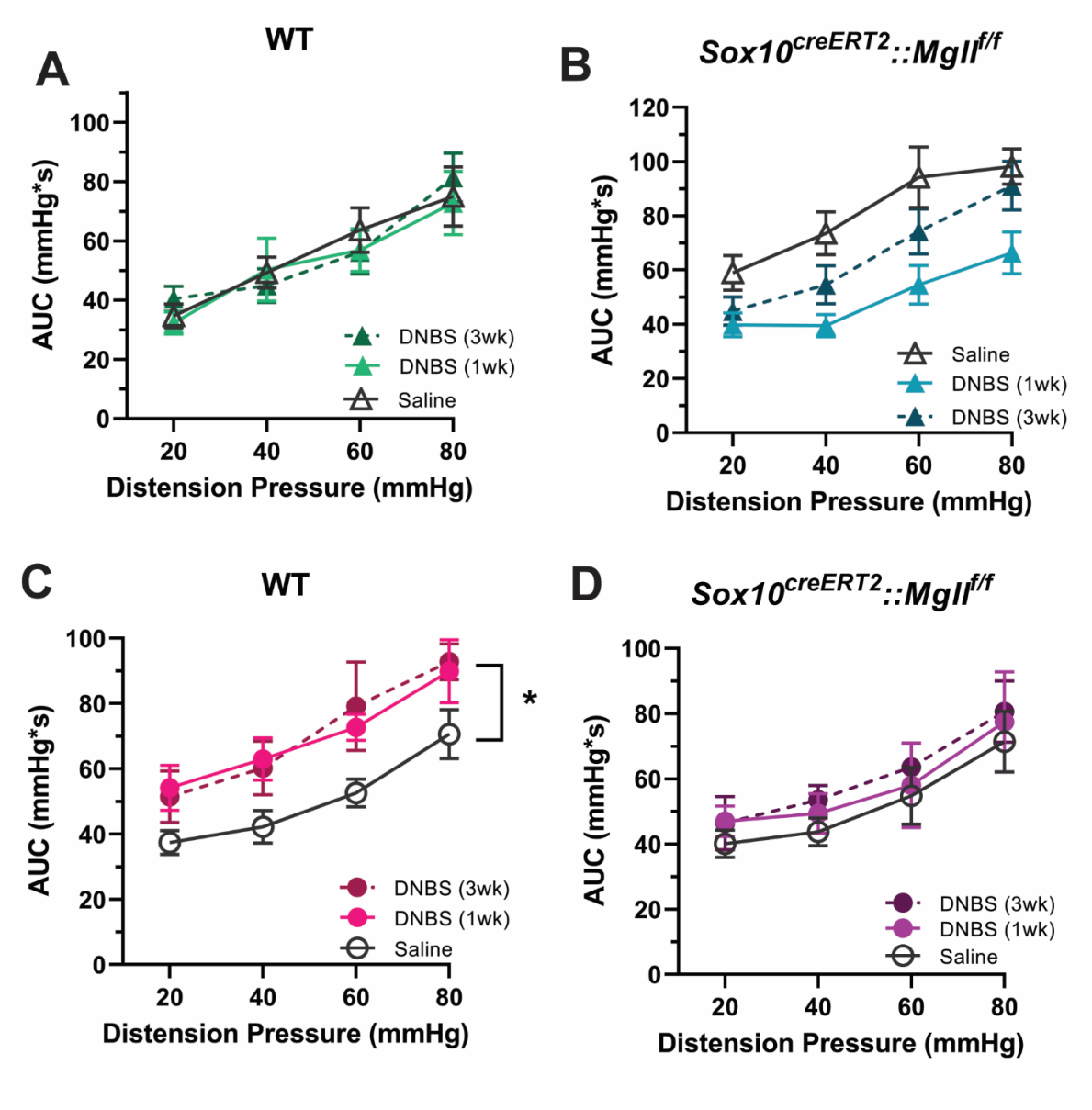

Figure 4.8: Enteric glia regulate afferent nerves and integrated reflexes through the regulations of endocannabinoids via MAGL. Traces showing VMR responses to CRD in mice shown as the area under the curve (AUC) for each distension pressure. Data show VMR responses in both male (green, blue) and female (pink, purple) mice in wild

Figure 4.8: (cont'd)

type (A,C) or *Sox10^{cre}ER^{T2}::Mgll$^{f/f}$* (B,D) mice following enema of saline or DNBS. AUC values for each group were analyzed via two-way ANOVA ($p < 0.05$, n = 8-14 mice).

DISCUSSION

The endocannabinoid system is a promising target for the regulation of pain and inflammation (*10*, *14–16*, *82*). Here, we demonstrate that endocannabinoid signaling is a major component in enteric neural-glial mechanisms and the development of visceral hypersensitivity. Very little work exists to support the role of enteric glia in endocannabinoid pain modulation (*14*, *58*). Studies within the CNS show that central glia are major components in endocannabinoid signaling and contribute to their role in descending afferent singling (*17*, *35*, *38*, *83–85*). In this study, we demonstrate that enteric glia express components of the endocannabinoid signaling pathway, and glial activity is impacted by endocannabinoids.

We show that enteric glia readily expresses MAGL and FAAH along with CB1 and CB2 receptors under physiological and inflammatory conditions. Further, we demonstrate that the presence of these components is functionally relevant to neuro-glial signaling in the intestine. Inhibition of MAGL results in a significant decrease in Ca^{2+} signaling from enteric glia and neurons and blocking CB1 and CB2 receptors caused significant increases in Ca^{2+} signaling from glia. This suggests that increased levels of 2-AG provide inhibitory regulation of the ENS. These effects of CB1 and CB2 antagonism, however, were only observed in males where inhibition of CB1 and CB2 in females reduced Ca^{2+} responses from enteric glia. The differences observed in males and females could be associated with the sexually-dimorphic expression of endocannabinoid components on enteric glia. This is supported by the fact that we were able to identify distinct curves between males and females using KShape analysis, suggesting an overall difference in Ca^{2+} dynamics that are specific to each sex. These sex differences can again be

appreciated during colitis where we observed opposing effects between male and female mice that were mediated by endocannabinoid signaling. It is important to note that these differences observed in enteric glia and neurons can result from indirect modulation via other cell types. CB1 and CB2 receptor expression is not confined within enteric neuronal and glial cell bodies. In fact, several other cell types like immune cells and colon-projecting afferent nerves also express these receptors (*14*, *16*). Blocking cannabinoid receptors on these cells can indirectly modulate glia given that they can readily interact with each other (*32*, *33*, *86*). These components are also altered during inflammation as we can appreciate increased CB2 RNA expression during DNBS that is not confined to enteric neurons and glia. Additionally, endocannabinoids do strictly bind to CB1 and CB2 and several other non-canonical endocannabinoid receptors exist within the enteric nervous system (*14*, *16*). Anandamide and 2-AG can easily bind to TRPV1 and PPARα receptors both of which are known to directly modulate glial activity during inflammation (*32*, *39*).

Nociceptors are sensitized during inflammation and the mechanisms associated with these effects are believed to contribute to the shift from acute to chronic abdominal pain (*87*–*89*). Our Ca^{2+} imaging data from our *TRPV1*$^{Cre;GCaMP5g\text{-}tdT}$ animals show that following treatment in DNBS there is an increase in overall nociceptor Ca^{2+} responses to capsaicin. Further, we observed an increase in TRPV1+ nociceptor density within the myenteric plexus which mirrors prior work from pre-clinical models and humans (*76*–*78*, *90*–*94*). Notably, our Ca^{2+} data also showed that DNBS colitis likely induces the recruitment of silent nociceptors. Silent nociceptors are a group of sensory afferents that are not usually activated by physiologically relevant noxious stimuli (*1*, *79*, *80*). Increased levels of TRPV1+ nociceptors and the recruitment of silent afferents indicate profound

structural and functional alterations to nociceptive signaling that likely contribute to the development of chronic pain. In support of these findings, our in vivo measurements of visceral hypersensitivity indicate sustained VMR responses in females up to 3 weeks post DNBS colitis which is typically a time point where animals have recovered from inflammation. These effects were modulated by the endocannabinoid hydrolyzing enzyme MAGL, given that treatment with the MAGL inhibitor JZL184 ameliorated sensitized Ca^{2+} responses from TRPV1+ nociceptors but also reversed the recruitment of silent afferents. Although both neurons and glia express RNA for MAGL, the effects observed on nociceptors are likely primarily modulated by enteric glia. Previous work demonstrates that the contribution of glial MAGL to endocannabinoid signaling is predominant during inflammation (*38*). Our VMR recordings support this and show that genetic ablation of MAGL from glia in the *Sox10^{cre}ER^{T2}::Mgll$^{f/f}$* mice prevented the development of visceral hypersensitivity observed in females. Indicating that glial MAGL inhibition is sufficient to modulate visceral hypersensitivity during colitis.

Interestingly, *Sox10^{cre}ER^{T2}::Mgll$^{f/f}$* knock-out males showed increased VMR responses when compared with WT controls. Typically, endocannabinoids are described as being anti-inflammatory and having analgesic properties (*27*, *31*, *64*, *65*). However, AEA and 2-AG are partial agonists for TRPV1+ receptors (*95*, *96*). Under baseline conditions, endocannabinoids can activate these receptors but because they are not full agonists TRPV1+ receptors do not become desensitized as a result. It is possible that increased levels of 2-AG following genetic ablation of MAGL stimulates TRPV1+ nociceptors, increasing thresholds for nociceptive stimuli. Additionally, constant stimulation of TRPV1+ nociceptors can induce neurogenic inflammation by contributing

to increased release of proinflammatory mediators from nociceptors like tachykinins and substance P (SP) (*97*). Furthermore, other enzymes not typically associated with endocannabinoid signaling, like cyclooxygenase-2 (COX-2), can utilize 2-AG in the production of prostaglandins further contributing to the proinflammatory environment that can sensitize nociceptive signaling (*14*, *38*, *98*, *99*). Surprisingly, we did not observe a similar increase in baseline VMR responses in *Sox10^{cre}ER^{T2}::Mgll$^{f/f}$* female mice. This was unexpected since recent studies demonstrate that female mice have an increased sensitivity to prostaglandins given they highly express multiple components along the prostaglandin signaling pathway (*100*). It is possible that female mice can compensate for the loss of glial MAGL and increase in 2-AG at baseline since our data show they show an increased expression of multiple components within the endocannabinoid pathway when compared to males.

An important observation of this study is that the effects of endocannabinoids and inflammation on visceral pain are sex-dependent. DNBS induced visceral hypersensitivity in females in a manner that was dependent on glial MAGL. Sexual dimorphism in pain mechanisms is well known (*100*, *101*) and the nature of the pain-producing insult impacts males and females differentially. This could possibly be explained by an increased sensitivity to inflammatory responses in females (*102*). The sex differences observed here may be due to the differential regulation of glial signaling by endocannabinoids. Our data demonstrate profound differences in the expression of endocannabinoid signaling components and regulation of glia between male and female mice. Inhibition of CB1 receptors in male mice ablate baseline differences in Ca^{2+} signaling and result in more characteristically female responses in male glia.

Inflammation results in the sensitization of nerve afferents and increased visceral hypersensitivity which contributes to the development of chronic pain. Modulation of cannabinoid signaling is a promising approach for the treatment of visceral pain. However, new approaches to the therapeutic potential of endocannabinoids are necessary to circumvent the many side effects associated with existing drugs. Agonists for cannabinoid receptors decrease inflammation and visceral hypersensitivity. These compounds, however, readily cross the blood-brain barrier and are associated with psychoactive side effects (*21*, *23*–*25*). Our study identifies a novel glial mechanism that modulates visceral hypersensitivity through the regulation of endocannabinoids. Pharmacological inhibition MAGL reverses the sensitization of TRPV1+ nociceptors in the intestine and the recruitment of silent nociceptors. Further, genetic ablation of MAGL in glia prevents the development of visceral hypersensitivity during colitis. Previous studies show that the systemic inhibition of MAGL improves inflammation and hypersensitivity to noxious stimuli (*27*, *65*), however systemic treatment with MAGL inhibitors can produce unwanted side effects like impairments in cognitive ability (*103*) and locomotor activity in rodent models (*98*). Our studies demonstrate that the analgesic effects of MAGL are primarily mediated through glia and thus cell-specific therapies targeting glial mechanisms could avoid these side effects and represent an important step forward in limiting visceral pain at its source in the intestine.

REFERENCES

1. L. Grundy, A. Erickson, S. M. Brierley, Visceral Pain. *Annu Rev Physiol*. 81, 1–24 (2018).

2. M. Ledergerber, B. M. Lang, H. Heinrich, L. Biedermann, S. Begré, J. Zeitz, N. Krupka, A. Rickenbacher, M. Turina, T. Greuter, P. Schreiner, R. Roth, A. Siebenhüner, S. R. Vavricka, G. Rogler, N. Beerenwinkel, B. Misselwitz, C. Anderegg, P. Bauerfeind, C. Beglinger, S. Begré, D. Belli, J. M. Bengoa, L. Biedermann, B. Bigler, J. Binek, M. Blattmann, S. Boehm, J. Borovicka, C. P. Braegger, N. Brunner, P. Bühr, B. Burnand, E. Burri, S. Buyse, M. Cremer, D. H. Criblez, P. de Saussure, L. Degen, J. Delarive, C. Doerig, B. Dora, G. Dorta, M. Egger, T. Ehmann, A. El-Wafa, M. Engelmann, J. Ezri, C. Felley, M. Fliegner, N. Fournier, M. Fraga, P. Frei, R. Frei, M. Fried, F. Froehlich, C. Funk, R. I. Furlano, S. Gallot-Lavallée, M. Geyer, M. Girardin, D. Golay, T. Grandinetti, B. Gysi, H. Haack, J. Haarer, B. Helbling, P. Hengstler, D. Herzog, C. Hess, K. Heyland, T. Hinterleitner, P. Hiroz, C. Hirschi, P. Hruz, R. Iwata, R. Jost, P. Juillerat, V. K. Brondolo, C. Knellwolf, C. Knoblauch, H. Köhler, R. Koller, C. Krieger-Grübel, G. Kullak-Ublick, P. Künzler, M. Landolt, R. Lange, F. S. Lehmann, A. Macpherson, P. Maerten, M. H. Maillard, C. Manser, M. Manz, U. Marbet, G. Marx, C. Matter, V. McLin, R. Meier, M. Mendanova, C. Meyenberger, P. Michetti, B. Misselwitz, D. Moradpour, B. Morell, P. Mosler, C. Mottet, C. Müller, P. Müller, B. Müllhaupt, C. Münger-Beyeler, L. Musso, A. Nagy, M. Neagu, C. Nichita, J. Niess, N. Noël, A. Nydegger, N. Obialo, C. Oneta, C. Oropesa, U. Peter, D. Peternac, L. M. Petit, F. Piccoli-Gfeller, J. B. Pilz, V. Pittet, N. Raschle, R. Rentsch, S. Restellini, J.-P. Richterich, S. Rihs, M. A. Ritz, J. Roduit, D. Rogler, G. Rogler, J.-B. Rossel, M. Sagmeister, G. Saner, B. Sauter, M. Sawatzki, M. Schäppi, M. Scharl, M. Schelling, S. Schibli, H. Schlauri, S. S. Uebelhart, J.-F. Schnegg, A. Schoepfer, F. Seibold, M. Seirafi, G.-M. Semadeni, D. Semela, A. Senning, M. Sidler, C. Sokollik, J. Spalinger, H. Spangenberger, P. Stadler, M. Steuerwald, A. Straumann, B. Straumann-Funk, M. Sulz, J. Thorens, S. Tiedemann, R. Tutuian, S. Vavricka, F. Viani, J. Vögtlin, R. V. Känel, A. Vonlaufen, D. Vouillamoz, R. Vulliamy, J. Wermuth, H. Werner, P. Wiesel, R. Wiest, T. Wylie, J. Zeitz, D. Zimmermann, Abdominal pain in patients with inflammatory bowel disease: association with single-nucleotide polymorphisms prevalent in irritable bowel syndrome and clinical management. *Bmc Gastroenterol*. 21, 53 (2021).

3. R. Tolba, J. Shroll, A. Kanu, M. K. Rizk, Chronic Abdominal Pain. *springer*, 13–24 (2015).

4. A. Deiteren, A. de Wit, L. van der Linden, D. J. Man, P. Pelckmans, D. B. Winter, Irritable bowel syndrome and visceral hypersensitivity : risk factors and pathophysiological mechanisms. *Acta gastro-enterologica Belgica*. 79, 29–38 (2016).

5. C. Ibeakanma, M. Miranda-Morales, M. Richards, F. Bautista-Cruz, N. Martin, D. Hurlbut, S. Vanner, Citrobacter rodentium colitis evokes post-infectious hyperexcitability of mouse nociceptive colonic dorsal root ganglion neurons. *The Journal of Physiology*. 587, 3505–3521 (2009).

6. M. M. Wouters, D. Balemans, S. Wanrooy, J. Dooley, V. Cibert-Goton, Y. A. Alpizar, E. E. Valdez-Morales, Y. Nasser, P. P. Veldhoven, W. Vanbrabant, S. der Merwe, R. Mols, B. Ghesquière, C. Cirillo, I. Kortekaas, P. Carmeliet, W. E. Peetermans, S. Vermeire, P. Rutgeerts, P. Augustijns, P. W. Hellings, A. Belmans, S. Vanner, D. C. Bulmer, K. Talavera, P. Berghe, A. Liston, G. E. Boeckxstaens, Histamine Receptor H1–Mediated Sensitization of TRPV1 Mediates Visceral Hypersensitivity and Symptoms in Patients With Irritable Bowel Syndrome. *Gastroenterology*. 150, 875-887.e9 (2016).

7. S. M. Brierley, D. R. Linden, Neuroplasticity and dysfunction after gastrointestinal inflammation. *Nat Rev Gastroentero*. 11, 611–627 (2014).

8. P. Hughes, Brierley, L. Blackshaw, Post-inflammatory modification of colonic afferent mechanosensitivity. *Clinical and Experimental Pharmacology and Physiology*. 36, 1034–1040 (2009).

9. G. Grabauskas, X. Wu, J. Gao, J.-Y. Li, D. K. Turgeon, C. Owyang, Prostaglandin E2, Produced by Mast Cells in Colon Tissues from Patients with Irritable Bowel Syndrome, Contributes to Visceral Hypersensitivity in Mice. *Gastroenterology* (2020), doi:10.1053/j.gastro.2020.02.022.

10. R.-R. Ji, A. Chamessian, Y.-Q. Zhang, Pain regulation by non-neuronal cells and inflammation. *Science*. 354, 572–577 (2016).

11. J. BouSaba, W. Sannaa, M. Camilleri, Pain in irritable bowel syndrome: Does anything really help? *Neurogastroenterol Motil*. 34, e14305 (2022).

12. M. Camilleri, A. Lembo, D. A. Katzka, Opioids in Gastroenterology: Treating Adverse Effects and Creating Therapeutic Benefits. *Clinical Gastroenterology and Hepatology*. 15, 1338–1349 (2017).

13. J.-F. Colombel, N. Narula, L. Peyrin-Biroulet, Management Strategies to Improve Outcomes of Patients With Inflammatory Bowel Diseases. *Gastroenterology*. 152, 351-361.e5 (2017).

14. L. López-Gómez, A. Szymaszkiewicz, M. Zielińska, R. Abalo, The Enteric Glia and Its Modulation by the Endocannabinoid System, a New Target for Cannabinoid-Based Nutraceuticals? *Molecules*. 27, 6773 (2022).

15. K. A. Sharkey, J. W. Wiley, The Role of the Endocannabinoid System in the Brain–Gut Axis. *Gastroenterology*. 151, 252–266 (2016).

16. H. Cuddihey, W. K. MacNaughton, K. A. Sharkey, Role of the Endocannabinoid System in the Regulation of Intestinal Homeostasis. *Cell Mol Gastroenterology Hepatology*. 14, 947–963 (2022).

17. I. Svíženská, P. Dubový, A. Šulcová, Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures — A short review. *Pharmacol Biochem Be*. 90, 501–511 (2008).

18. U. Binzen, W. Greffrath, S. Hennessy, M. Bausen, S. Saaler-Reinhardt, R.-D. Treede, Co-expression of the voltage-gated potassium channel Kv1.4 with transient receptor potential channels (TRPV1 and TRPV2) and the cannabinoid receptor CB1 in rat dorsal root ganglion neurons. *Neuroscience*. 142, 527–539 (2006).

19. J. Ahluwalia, L. Urban, S. Bevan, M. Capogna, I. Nagy, Cannabinoid 1 receptors are expressed by nerve growth factor- and glial cell-derived neurotrophic factor-responsive primary sensory neurones. *Neuroscience*. 110, 747–753 (2002).

20. J. Ahluwalia, L. Urban, M. Capogna, S. Bevan, I. Nagy, Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons. *Neuroscience*. 100, 685–688 (2000).

21. A. Kikuchi, K. Ohashi, Y. Sugie, H. Sugimoto, H. Omura, Pharmacological Evaluation of a Novel Cannabinoid 2 (CB2) Ligand, PF-03550096, In Vitro and In Vivo by Using a Rat Model of Visceral Hypersensitivity. *J Pharmacol Sci*. 106, 219–224 (2008).

22. M. Sanson, L. Bueno, J. Fioramonti, Involvement of cannabinoid receptors in inflammatory hypersensitivity to colonic distension in rats. *Neurogastroenterol Motil*. 18, 949–956 (2006).

23. M. Naguib, P. Diaz, J. J. Xu, F. Astruc-Diaz, S. Craig, P. Vivas-Mejia, D. L. Brown, MDA7: a novel selective agonist for CB2 receptors that prevents allodynia in rat neuropathic pain models. *Brit J Pharmacol*. 155, 1104–1116 (2008).

24. Y. Iwata, K. Ando, K. Taniguchi, N. Koba, A. Sugiura, M. Sudo, Identification of a highly potent and selective CB2 agonist, RQ-00202730, for the treatment of irritable bowel syndrome. *Bioorg Med Chem Lett*. 25, 236–240 (2015).

25. S. Han, J. Thatte, D. J. Buzard, R. M. Jones, Therapeutic Utility of Cannabinoid Receptor Type 2 (CB2) Selective Agonists. *J Med Chem*. 56, 8224–8256 (2013).

26. C. Cremon, V. Stanghellini, M. R. Barbaro, R. F. Cogliandro, L. Bellacosa, J. Santos, M. Vicario, M. Pigrau, C. A. Cotoner, B. Lobo, F. Azpiroz, S. B. des Varannes, M. Neunlist, D. DeFilippis, T. Iuvone, S. Petrosino, V. D. Marzo, G. Barbara, Randomised clinical trial: the analgesic properties of dietary supplementation with palmitoylethanolamide and polydatin in irritable bowel syndrome. *Aliment Pharm Therap*. 45, 909–922 (2017).

27. M. S. Crowe, E. Leishman, M. L. Banks, R. Gujjar, A. Mahadevan, H. B. Bradshaw, S. G. Kinsey, Combined inhibition of monoacylglycerol lipase and cyclooxygenases synergistically reduces neuropathic pain in mice. *Brit J Pharmacol*. 172, 1700–1712 (2015).

28. Y. S. Sakin, A. Dogrul, F. Ilkaya, M. Seyrek, U. H. Ulas, M. Gulsen, S. Bagci, The effect of FAAH, MAGL, and Dual FAAH/MAGL inhibition on inflammatory and colorectal distension-induced visceral pain models in Rodents. *Neurogastroenterol Motil*. 27, 936–944 (2015).

29. J. Desroches, J. Guindon, C. Lambert, P. Beaulieu, Modulation of the anti-nociceptive effects of 2-arachidonoyl glycerol by peripherally administered FAAH and MGL inhibitors in a neuropathic pain model. *Brit J Pharmacol*. 155, 913–924 (2008).

30. A. Gil-Ordóñez, M. Martín-Fontecha, S. Ortega-Gutiérrez, M. L. López-Rodríguez, Monoacylglycerol lipase (MAGL) as a promising therapeutic target. *Biochem Pharmacol*. 157, 18–32 (2018).

31. J. Guindon, A. Guijarro, D. Piomelli, A. G. Hohmann, Peripheral antinociceptive effects of inhibitors of monoacylglycerol lipase in a rat model of inflammatory pain. *Brit J Pharmacol*. 163, 1464–1478 (2011).

32. N. M. Delvalle, C. Dharshika, W. Morales-Soto, D. E. Fried, L. Gaudette, B. D. Gulbransen, Communication between enteric neurons, glia, and nociceptors underlies the effects of tachykinins on neuroinflammation. *Cellular and Molecular Gastroenterology and Hepatology*, 321–344 (2018).

33. V. Grubišić, J. L. McClain, D. E. Fried, I. Grants, P. Rajasekhar, E. Csizmadia, O. A. Ajijola, R. E. Watson, D. P. Poole, S. C. Robson, F. L. Christofi, B. D. Gulbransen, Enteric Glia Modulate Macrophage Phenotype and Visceral Sensitivity following Inflammation. *Cell Reports*. 32, 108100 (2020).

34. E. Lucarini, L. Seguella, M. Vincenzi, C. Parisio, L. Micheli, A. Toti, C. Corpetti, A. D. Re, S. Squillace, D. Maftei, R. Lattanzi, C. Ghelardini, L. D. C. Mannelli, G. Esposito, Role of Enteric Glia as Bridging Element between Gut Inflammation and Visceral Pain Consolidation during Acute Colitis in Rats. *Biomed*. 9, 1671 (2021).

35. A. Covelo, A. Eraso-Pichot, I. Fernández-Moncada, R. Serrat, G. Marsicano, CB1R-dependent regulation of astrocyte physiology and astrocyte-neuron interactions. *Neuropharmacology*. 195, 108678 (2021).

36. N. Stella, Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia*. 58, 1017–1030 (2010).

37. A.-C. Schmöle, R. Lundt, B. Gennequin, H. Schrage, E. Beins, A. Krämer, T. Zimmer, A. Limmer, A. Zimmer, D.-M. Otte, Expression Analysis of CB2-GFP BAC Transgenic Mice. *Plos One*. 10, e0138986 (2015).

38. A. Viader, J. L. Blankman, P. Zhong, X. Liu, J. E. Schlosburg, C. M. Joslyn, Q.-S. Liu, A. J. Tomarchio, A. H. Lichtman, D. E. Selley, L. J. Sim-Selley, B. F. Cravatt, Metabolic

Interplay between Astrocytes and Neurons Regulates Endocannabinoid Action. *Cell Reports*. 12, 798–808 (2015).

39. G. Esposito, E. Capoccia, F. Turco, I. Palumbo, J. Lu, A. Steardo, R. Cuomo, G. Sarnelli, L. Steardo, Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR-α activation. *Gut*. 63, 1300–1312 (2014).

40. A. Stanzani, G. Galiazzo, F. Giancola, C. Tagliavia, M. D. Silva, M. Pietra, F. Fracassi, R. Chiocchetti, Localization of cannabinoid and cannabinoid related receptors in the cat gastrointestinal tract. *Histochem Cell Biol*. 153, 339–356 (2020).

41. F. Progatzky, M. Shapiro, S. H. Chng, B. Garcia-Cassani, C. H. Classon, S. Sevgi, A. Laddach, A. C. Bon-Frauches, R. Lasrado, M. Rahim, E.-M. Amaniti, S. Boeing, K. Shah, L. J. Entwistle, A. Suárez-Bonnet, M. S. Wilson, B. Stockinger, V. Pachnis, Regulation of intestinal immunity and tissue repair by enteric glia. *Nature*. 599, 125–130 (2021).

42. Y. Chen, X. Liu, C. R. Vickstrom, M. J. Liu, L. Zhao, A. Viader, B. F. Cravatt, Q. Liu, *Eneuro*, in press, doi:10.1523/eneuro.0048-16.2016.

43. A. Wangzhou, C. Paige, S. V. Neerukonda, D. K. Naik, M. Kume, E. T. David, G. Dussor, P. R. Ray, T. J. Price, A ligand-receptor interactome platform for discovery of pain mechanisms and therapeutic targets. *Sci Signal*. 14 (2021), doi:10.1126/scisignal.abe1648.

44. M. M. Ahmadzai, L. Seguella, B. D. Gulbransen, Circuit-specific enteric glia regulate intestinal motor neurocircuits. *P Natl Acad Sci Usa*. 118, e2025938118 (2021).

45. J. L. McClain, D. E. Fried, B. D. Gulbransen, Agonist-Evoked Ca2+ Signaling in Enteric Glia Drives Neural Programs That Regulate Intestinal Motility in Mice. *Cell Mol Gastroenterology Hepatology*. 1, 631–645 (2015).

46. L. Seguella, J. L. McClain, G. Esposito, B. D. Gulbransen, *J Neurosci*, in press, doi:10.1523/jneurosci.2379-20.2022.

47. B. D. Gulbransen, M. Bashashati, S. A. Hirota, X. Gui, J. A. Roberts, J. A. MacDonald, D. A. Muruve, D. M. McKay, P. L. Beck, G. M. Mawe, Activation of neuronal P2X7 receptor–pannexin-1 mediates death of enteric neurons during colitis. *Nature medicine*. 18 (2012).

48. B. D. Gulbransen, M. Bashashati, S. A. Hirota, X. Gui, J. A. Roberts, J. A. MacDonald, D. A. Muruve, D. M. McKay, P. L. Beck, G. M. Mawe, R. J. Thompson, K. A. Sharkey, *Nature Medicine*, in press, doi:10.1038/nm.2679.

49. S. Wirtz, C. Neufert, B. Weigmann, M. F. Neurath, Chemically induced mouse models of intestinal inflammation. *Nat Protoc*. 2, 541–546 (2007).

50. M. M. Ahmadzai, J. L. McClain, C. Dharshika, L. Seguella, F. Giancola, R. D. Giorgio, B. D. Gulbransen, LPAR1 regulates enteric nervous system function through glial signaling and contributes to chronic intestinal pseudo-obstruction. *J Clin Investigation*. 132, e149464 (2022).

51. X. Zhang, S. A. Thayer, Monoacylglycerol lipase inhibitor JZL184 prevents HIV-1 gp120-induced synapse loss by altering endocannabinoid signaling. *Neuropharmacology*. 128, 269–281 (2018).

52. L. Scalvini, D. Piomelli, M. Mor, Monoglyceride lipase: Structure and inhibitors. *Chem Phys Lipids*. 197, 13–24 (2016).

53. S. Hernandez, W. Morales-Soto, V. Grubišić, D. Fried, B. D. Gulbransen, Pyridostigmine bromide exposure creates chronic, underlying neuroimmune disruption in the gastrointestinal tract and brain that alters responses to palmitoylethanolamide in a mouse model of Gulf War Illness. *Neuropharmacology*. 179, 108264 (2020).

54. F. Molina-Holgado, E. Molina-Holgado, C. Guaza, N. J. Rothwell, Role of CB1 and CB2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures. *J. Neurosci. Res.* 67, 829–836 (2002).

55. M. Larauche, G. Gourcerol, L. Wang, K. Pambukchian, S. Brunnhuber, D. W. Adelson, J. Rivier, M. Million, Y. Taché, Cortagine, a CRF1 agonist, induces stresslike alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. *Am J Physiology Gastrointest Liver Physiology*. 297, G215-27 (2009).

56. N. J. Spencer, E. I. Magnúsdóttir, J. E. Jakobsson, G. Kestell, B. N. Chen, D. Morris, S. J. Brookes, M. C. Lagerström, CGRPα within the Trpv1-Cre population contributes to visceral nociception. *American journal of physiology. Gastrointestinal and liver physiology*. 314, G188–G200 (2018).

57. K. Kolar, D. Dondorp, J. C. Zwiggelaar, J. Høyer, M. Chatzigeorgiou, Mesmerize is a dynamically adaptable user-friendly analysis platform for 2D and 3D calcium imaging data. *Nat Commun*. 12, 6569 (2021).

58. J. J. Galligan, Cannabinoid signalling in the enteric nervous system. *Neurogastroenterol Motil*. 21, 899–902 (2009).

59. N. Acharya, S. Penukonda, T. Shcheglova, A. T. Hagymasi, S. Basu, P. K. Srivastava, Endocannabinoid system acts as a regulator of immune homeostasis in the gut. *Proc National Acad Sci*. 114, 5005–5010 (2017).

60. A. A. May-Zhang, E. Tycksen, A. N. Southard-Smith, K. K. Deal, J. T. Benthal, D. P. Buehler, M. Adam, A. J. Simmons, J. R. Monaghan, B. K. Matlock, D. K. Flaherty, S. S. Potter, K. S. Lau, E. M. Southard-Smith, Combinatorial Transcriptional Profiling of Mouse

and Human Enteric Neurons Identifies Shared and Disparate Subtypes In Situ. *Gastroenterology*. 160, 755-770.e26 (2020).

61. M. Duncan, A. Mouihate, K. Mackie, C. M. Keenan, N. E. Buckley, J. S. Davison, K. D. Patel, Q. J. Pittman, K. A. Sharkey, Cannabinoid CB2 receptors in the enteric nervous system modulate gastrointestinal contractility in lipopolysaccharide-treated rats. *Am J Physiol-gastr L*. 295, G78–G87 (2008).

62. M. Storr, A. Sibaev, G. Marsicano, B. Lutz, V. Schusdziarra, J.-P. Timmermans, H. D. Allescher, Cannabinoid receptor type 1 modulates excitatory and inhibitory neurotransmission in mouse colon. *Am J Physiol-gastr L*. 286, G110–G117 (2004).

63. A. A. Coutts, A. J. Irving, K. Mackie, R. G. Pertwee, S. Anavi-Goffer, Localisation of cannabinoid CB1 receptor immunoreactivity in the guinea pig and rat myenteric plexus. *J Comp Neurol*. 448, 410–422 (2002).

64. K. L. Leinwand, M. E. Gerich, E. J. Hoffenberg, C. B. Collins, Manipulation of the Endocannabinoid System in Colitis. *Inflamm Bowel Dis*. 23, 192–199 (2017).

65. M. Alhouayek, D. M. Lambert, N. M. Delzenne, P. D. Cani, G. G. Muccioli, Increasing endogenous 2-arachidonoylglycerol levels counteracts colitis and related systemic inflammation. *Faseb J*. 25, 2711–2721 (2011).

66. A. C. Johnson, A. D. Farmer, T. J. Ness, B. G.-V. Meerveld, Critical evaluation of animal models of visceral pain for therapeutics development: A focus on irritable bowel syndrome. *Neurogastroenterol Motil Official J European Gastrointest Motil Soc*. 32, e13776 (2019).

67. J. Castro, S. Garcia-Caraballo, J. Maddern, G. Schober, A. Lumsden, A. Harrington, S. Schmiel, B. Lindstrom, J. Adams, S. M. Brierley, Olorinab (APD371), a peripherally acting, highly selective, full agonist of the cannabinoid receptor 2, reduces colitis-induced acute and chronic visceral hypersensitivity in rodents. *Pain*. Publish Ahead of Print (2021), doi:10.1097/j.pain.0000000000002314.

68. L. Marquéz, J. Suárez, M. Iglesias, F. J. Bermudez-Silva, F. R. de Fonseca, M. Andreu, Ulcerative Colitis Induces Changes on the Expression of the Endocannabinoid System in the Human Colonic Tissue. *Plos One*. 4, e6893 (2009).

69. M. Grill, C. Hasenoehrl, M. Kienzl, J. Kargl, R. Schicho, Cellular localization and regulation of receptors and enzymes of the endocannabinoid system in intestinal and systemic inflammation. *Histochem Cell Biol*. 151, 5–20 (2019).

70. M. Bashashati, Y. Nasser, C. M. Keenan, W. Ho, F. Piscitelli, M. Nalli, K. Mackie, M. A. Storr, V. D. Marzo, K. A. Sharkey, Inhibiting endocannabinoid biosynthesis: a novel approach to the treatment of constipation. *Brit J Pharmacol*. 172, 3099–3111 (2015).

71. M. Alhouayek, G. G. Muccioli, The endocannabinoid system in inflammatory bowel diseases: from pathophysiology to therapeutic opportunity. *Trends Mol Med*. 18, 615–625 (2012).

72. N. Roy, M. Barnett, B. Knoch, Y. Dommels, W. McNabb, Nutrigenomics applied to an animal model of Inflammatory Bowel Diseases: Transcriptomic analysis of the effects of eicosapentaenoic acid- and arachidonic acid-enriched diets. *Mutat Res Fundam Mol Mech Mutagen*. 622, 103–116 (2007).

73. M. A. Storr, C. M. Keenan, H. Zhang, K. D. Patel, A. Makriyannis, K. A. Sharkey, Activation of the cannabinoid 2 receptor (CB2) protects against experimental colitis. *Inflamm Bowel Dis*. 15, 1678–1685 (2009).

74. R. Pihlaja, J. Takkinen, O. Eskola, J. Vasara, F. R. López-Picón, M. Haaparanta-Solin, J. O. Rinne, Monoacylglycerol lipase inhibitor JZL184 reduces neuroinflammatory response in APdE9 mice and in adult mouse glial cells. *J Neuroinflamm*. 12, 81 (2015).

75. M. Kyloh, S. Nicholas, V. P. Zagorodnyuk, S. J. Brookes, N. J. Spencer, Identification of the Visceral Pain Pathway Activated by Noxious Colorectal Distension in Mice. *Frontiers in Neuroscience*. 5, 16 (2011).

76. Y. Luo, C. Feng, J. Wu, Y. Wu, D. Liu, J. Wu, F. Dai, J. Zhang, P2Y1, P2Y2, and TRPV1 Receptors Are Increased in Diarrhea-Predominant Irritable Bowel Syndrome and P2Y2 Correlates with Abdominal Pain. *Digest Dis Sci*. 61, 2878–2886 (2016).

77. K. Matsumoto, F. Sugimoto, T. Mizuno, T. Hayashi, R. Okamura, T. Nishioka, H. Yasuda, S. Horie, M. A. Kido, S. Kato, Immunohistochemical characterization of transient receptor potential vanilloid types 2 and 1 in a trinitrobenzene sulfonic acid-induced rat colitis model with visceral hypersensitivity. *Cell Tissue Res.*, 1–17 (2022).

78. A. Akbar, Y. Yiangou, P. Facer, W. G. Brydon, J. R. F. Walters, P. Anand, S. Ghosh, Expression of the TRPV1 receptor differs in quiescent inflammatory bowel disease with or without abdominal pain. *Gut*. 59, 767 (2010).

79. K. M. Smith-Edwards, J. J. DeBerry, J. L. Saloman, B. M. Davis, C. J. Woodbury, Profound alteration in cutaneous primary afferent activity produced by inflammatory mediators. *Elife*. 5, e20527 (2016).

80. V. Prato, F. J. Taberner, J. Hockley, G. Callejo, A. Arcourt, B. Tazir, L. Hammer, P. Schad, P. A. Heppenstall, E. S. Smith, S. G. Lechner, Functional and Molecular Characterization of Mechanoinsensitive “Silent” Nociceptors. *Cell Reports*. 21, 3102–3115 (2017).

81. M. Larauche, J. Erchegyi, C. Miller, M. S. Sim, J. Rivier, D. Behan, Y. Taché, Peripheral CRF-R1/CRF-R2 antagonist, astressin C, induces a long-lasting blockade of acute stress-related visceral pain in male and female rats. *Peptides*. 157, 170881 (2022).

82. S. M. Brierley, B. G.-V. Meerveld, G. Sarnelli, K. A. Sharkey, M. Storr, J. Tack, Targeting the endocannabinoid system for the treatment of abdominal pain in irritable bowel syndrome. *Nat Rev Gastroentero*. 20, 5–25 (2023).

83. E. Rojo-Bustamante, I. Íñigo-Marco, M. A. Abellanas, R. Vinueza-Gavilanes, A. Baltanás, E. Luquin, M. Arrasate, M. S. Aymerich, CB2 Receptors and Neuron–Glia Interactions Modulate Neurotoxicity Generated by MAGL Inhibition. *Biomol*. 10, 1198 (2020).

84. A. Eraso-Pichot, S. Pouvreau, A. Olivera-Pinto, P. Gomez-Sotres, U. Skupio, G. Marsicano, Endocannabinoid signaling in astrocytes. *Glia*. 71, 44–59 (2023).

85. S. Haj-Dahmane, R.-Y. Shen, M. W. Elmes, K. Studholme, M. P. Kanjiya, D. Bogdan, P. K. Thanos, J. T. Miyauchi, S. E. Tsirka, D. G. Deutsch, M. Kaczocha, Fatty-acid–binding protein 5 controls retrograde endocannabinoid signaling at central glutamate synapses. *Proc National Acad Sci*. 115, 201721339 (2018).

86. A. K. Chow, V. Grubišić, B. D. Gulbransen, Enteric Glia Regulate Lymphocyte Activation via Autophagy-Mediated MHC-II Expression. *Cell Mol Gastroenterology Hepatology*. 12, 1215–1237 (2021).

87. L. Bueno, J. Fioramonti, Effects of Inflammatory Mediators on Gut Sensititvity. *Canadian Journal of Gastroenterology and Hepatology*. 13, 42A-46A (1999).

88. M. Defaye, N. S. Abdullah, M. Iftinca, A. Hassan, F. Agosti, Z. Zhang, M. Cumenal, G. W. Zamponi, C. Altier, Gut-innervating TRPV1+ Neurons Drive Chronic Visceral Pain via Microglial P2Y12 Receptor. *Cell Mol Gastroenterology Hepatology*. 13, 977–999 (2022).

89. F. Azpiroz, M. Bouin, M. Camilleri, E. Mayer, P. Poitras, J. Serra, R. Spiller, Mechanisms of hypersensitivity in IBS and functional disorders. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*. 19, 62–88 (2007).

90. C. Chan, P. Facer, J. Davis, G. Smith, J. Egerton, C. Bountra, N. Williams, P. Anand, Sensory fibres expressing capsaicin receptor TRPV1 in patients with rectal hypersensitivity and faecal urgency. *The Lancet*. 361, 385–391 (2003).

91. S. Mathivanan, I. Devesa, J.-P. Changeux, A. Ferrer-Montiel, Bradykinin Induces TRPV1 Exocytotic Recruitment in Peptidergic Nociceptors. *Frontiers in Pharmacology*. 7, 178 (2016).

92. K. M. Smith-Edwards, S. A. Najjar, B. S. Edwards, M. J. Howard, K. M. Albers, B. M. Davis, Extrinsic Primary Afferent Neurons Link Visceral Pain to Colon Motility Through a Spinal Reflex in Mice. *Gastroenterology*. 157, 522-536.e2 (2019).

93. D. Balemans, J. Aguilera-Lizarraga, M. V. Florens, P. Jain, A. Denadai-Souza, M. F. Viola, Y. A. Alpizar, S. V. D. Merwe, P. V. Berghe, K. Talavera, S. Vanner, M. M. Wouters, G. E. Boeckxstaens, Histamine-mediated potentiation of transient receptor potential (TRP) ankyrin 1 and TRP vanilloid 4 signaling in submucosal neurons in patients with irritable bowel syndrome. *Am J Physiol-gastr L*. 316, G338–G349 (2019).

94. W. Ma, L. Li, S. Xing, PGE2/EP4 receptor and TRPV1 channel are involved in repeated restraint stress-induced prolongation of sensitization pain evoked by subsequent PGE2 challenge. *Brain Res*. 1721, 146335 (2019).

95. C. Potenzieri, T. S. Brink, D. A. Simone, Excitation of cutaneous C nociceptors by intraplantar administration of anandamide. *Brain Res*. 1268, 38–47 (2009).

96. P. M. Zygmunt, A. Ermund, P. Movahed, D. A. Andersson, C. Simonsen, B. A. G. Jönsson, A. Blomgren, B. Birnir, S. Bevan, A. Eschalier, C. Mallet, A. Gomis, E. D. Högestätt, Monoacylglycerols Activate TRPV1 – A Link between Phospholipase C and TRPV1. *Plos One*. 8, e81618 (2013).

97. A. B. Petrenko, M. Yamazaki, K. Sakimura, M. Kano, H. Baba, Augmented tonic pain-related behavior in knockout mice lacking monoacylglycerol lipase, a major degrading enzyme for the endocannabinoid 2-arachidonoylglycerol. *Behav Brain Res*. 271, 51–58 (2014).

98. S. Martínez-Torres, L. Cutando, A. Pastor, A. Kato, K. Sakimura, R. de la Torre, E. Valjent, R. Maldonado, M. Kano, A. Ozaita, Monoacylglycerol lipase blockade impairs fine motor coordination and triggers cerebellar neuroinflammation through cyclooxygenase-2. *Brain Behav Immun*. 81, 399–409 (2019).

99. X. Nie, S. Kitaoka, M. Shinohara, A. Kakizuka, S. Narumiya, T. Furuyashiki, Roles of Toll-like receptor 2/4, monoacylglycerol lipase, and cyclooxygenase in social defeat stress-induced prostaglandin E2 synthesis in the brain and their behavioral relevance. *Sci Rep-uk*. 9, 17548 (2019).

100. D. Tavares-Ferreira, P. R. Ray, I. Sankaranarayanan, G. L. Mejia, A. Wangzhou, S. Shiers, R. Uttarkar, S. Megat, P. Barragan-Iglesias, G. Dussor, A. N. Akopian, T. J. Price, Sex Differences in Nociceptor Translatomes Contribute to Divergent Prostaglandin Signaling in Male and Female Mice. *Biol Psychiat*. 91, 129–140 (2022).

101. D. K. Prusator, B. G.-V. Meerveld, Sex-related differences in pain behaviors following three early life stress paradigms. *Biol Sex Differ*. 7, 29 (2016).

102. Y. Jang, M. Kim, S. W. Hwang, Molecular mechanisms underlying the actions of arachidonic acid-derived prostaglandins on peripheral nociception. *J Neuroinflamm*. 17, 30 (2020).

103. G. Griebel, P. Pichat, S. Beeské, T. Leroy, N. Redon, A. Jacquet, D. Françon, L. Bert, L. Even, M. Lopez-Grancha, T. Tolstykh, F. Sun, Q. Yu, S. Brittain, H. Arlt, T. He, B. Zhang, D. Wiederschain, T. Bertrand, J. Houtmann, A. Rak, F. Vallée, N. Michot, F. Augé, V. Menet, O. E. Bergis, P. George, P. Avenet, V. Mikol, M. Didier, J. Escoubet, Selective blockade of the hydrolysis of the endocannabinoid 2-arachidonoylglycerol impairs learning and memory performance while producing antinociceptive activity in rodents. *Sci Rep-uk*. 5, 7642 (2015).

Chapter 5:

SUMMARY AND CONCLUSIONS

KEY FINDINGS

Enteric glia modulate visceral hypersensitivity by directly communicating with nociceptors

In Chapter 3 of this dissertation, we made a significant contribution to the field by establishing a specific mechanism whereby enteric glia sensitize nociceptors under pro-inflammatory conditions. We demonstrated an in-depth cytokine-immune profile for the dinitro-benzene sulfonic acid (DNBS) model of colitis and demonstrated how these mediators impact enteric glia permeability and behavior. We found that in the presence of interleukin 1-β (IL1-β), enteric glia sensitize TRPV1+ nociceptor responses to capsaicin through the Cx-43 dependent release of prostaglandin-E2 (PGE2). Further, we determined that these mechanisms were relevant in vivo by showing that IL1-β induced visceral hypersensitivity requires glial Cx43 hemichannel signaling.

Endocannabinoids modulate visceral hypersensitivity through glia-dependent mechanisms

In Chapter 4 we demonstrate that visceral hypersensitivity induced by inflammation is regulated through enteric glia-dependent modulation endocannabinoids and that these effects were sexually dimorphic. This work provides the first evidence of the expression of monoacylglycerol lipase (MAGL) in enteric glia. Furthermore, we demonstrate that pharmacological inhibition of MAGL and cannabinoid receptors has a direct impact on glial signaling in health and disease. Our studies characterize enteric glia into subgroups via their Ca^{2+} signaling properties and show that these are dependent on endocannabinoids, inflammation, and sex. Acute inflammation with DNBS results in the sensitization of nociceptive nerve fibers and visceral hypersensitivity in mice. The effects

of DNBS on nociceptive signaling were ameliorated by the pharmacological inhibition and genetic ablation of glial MAGL.

SIGNIFICANCE

Abdominal (visceral) pain is the most common GI issue (*1*) and the dominant symptom of irritable bowel syndrome (IBS) (*2*, *3*) and inflammatory bowel disease (IBD) in remission (*4*, *5*). IBS is a pain disorder under Rome IV and affects between 7-15% of Americans with a higher prevalence in women than men (*3*, *6*). Abdominal pain is also common in active and inactive IBD and reduces the quality of life (*7*–*10*). Pain severity predicts health status and healthcare resource usage in both IBS and IBD (*11*) and is responsible for driving increased care-seeking behavior in patients with IBS more so than altered bowel habits (*12*). Pain management remains a significant challenge in IBS and IBD and yearly healthcare expenditures exceed $10 billion (*13*, *14*).

Despite its high prevalence, analgesic management options for visceral pain are few, suboptimal, and limited by adverse side effects. Frontline drugs such as antidepressants (neuromodulators), antispasmodics, and opioids (*14*) have significant adverse effects on the central nervous system (CNS) and on gut functions. Opioids, which are often used to control chronic visceral pain, have serious GI complications and chronic use produces a condition called narcotic bowel syndrome that paradoxically worsens pain symptoms (*15*). Controlling inflammation with aminosalicylates, corticosteroids, immunomodulators, and biological agents clearly benefits the treatment of active inflammation, but these therapies do not address abdominal pain (*16*). The lack of effective therapies and the high prevalence of visceral pain create a serious and growing

issue that highlights a clear need for a more sophisticated understanding of the underlying causal mechanisms.

Visceral hypersensitivity associated with the sensitization of afferent nociceptors that project from the spinal cord to the intestine is regarded as the key mechanism associated with the development of abdominal pain (*17–20*). However, the underlying mechanisms describing visceral hypersensitivity are incompletely understood. The work defined in this thesis identifies two possible mechanisms whereby enteric glia modulate visceral hypersensitivity in the intestine. The visceral nociceptors that project from the dorsal root ganglia to the intestine densely innervate the myenteric plexus (*21–23*). These nociceptors gate the transmission of noxious stimuli from the intestine, but very little is known about how these nerve endings become sensitized. In Chapter 3 we show that enteric glia have the ability to directly sensitize nociceptive nerve endings during acute inflammation. We used novel transgenic animal models that incorporated chemogenetics on enteric glia and the genetically encoded Ca^{2+} indicator *GCaMP5g* on nociceptors to assess the effect of glial activation on nociceptor Ca^{2+} responses. Our studies show that acute inflammation modulated enteric glia permeability through the actions of IL1-β on Cx43 hemichannels. This surrounding proinflammatory environment promotes the release of PGE2 from enteric glia in a Cx43-dependent manner which directly acts on nociceptor EP4 receptors. We were able to validate these mechanisms in vivo by measuring visceromotor responses to colorectal distensions in a glial-specific Cx43 knockout model. This effect was limited to female mice.

In Chapter 4, we provide the first evidence for MAGL and cannabinoid receptors on enteric glia using RNAscope labeling. MAGL is a major component of

endocannabinoid signaling and is responsible for the hydrolysis of 2-arachidonoylglycerol (2-AG) (*24*). Endocannabinoid signaling regulates gut function (*25*) and visceral hypersensitivity primarily through CB1 and CB2-dependent mechanisms (*26*–*29*). We discovered that endocannabinoids modulate enteric neurons and glia signaling in the myenteric plexus in a sex-dependent manner. Our studies demonstrate that these effects are altered during colitis. Inflammation is a key component in the development of visceral hypersensitivity. We find that following chemically induced colitis mice induces sensitization of nociceptors and the recruitment of silent nociceptors in the myenteric plexus and these effects can be reversed through the inhibition of MAGL. We also demonstrated that inflammatory colitis sensitizes visceromotor responses to colorectal distension in female mice. Further, this effect is ameliorated in glial-specific MAGL knockout animals. Interestingly, our studies show that baseline visceromotor responses in male MAGL knock out are significantly increased when compared to wild-type animals. These findings further highlight the dramatic sex differences observed in endocannabinoid signaling.

Our study identifies enteric glial signaling as a novel mechanism that promotes visceral hypersensitivity at the initial site of sensory transduction. Inflammation-induced changes in enteric glia increase their potential to modulate surrounding cells such as intrinsic and extrinsic neurons through effects on glial transmitter synthesis and release mechanisms. Inflammation is a major component associated with the disease pathology of IBS and IBD. Thus, the mechanisms described in this thesis are considerable drug targets for the treatment of both conditions. Our data show that perturbing these glial mechanisms limits visceral sensitization. Nonsteroidal anti-inflammatory drugs (NSAIDs)

are the most frequently used analgesics that suppress inflammatory pain by inhibiting COX-1 and/or COX-2 and reducing the production of prostanoids including PGE2 (*30*). However, products of COX-2 activity, such as PGE2, are involved in diverse physiological processes and current drugs have serious side effects on gastrointestinal, renal, and cardiovascular systems (*31*). Similarly, agonists for cannabinoid receptors and systemic inhibition of MAGL show promise in the modulation of inflammation and pain. However, many of these compounds come with unwanted psychotropic reactions (*32*–*35*) and impairments to memory (*36*) and locomotor activity (*37*). Therefore, cell-specific therapies targeting glial mechanisms could avoid these side effects and represent an important step forward in limiting visceral pain at its source in the intestine.

LIMITATIONS OF THE STUDY

DNBS model of colitis

We primarily used the DNBS model to induce murine colitis in our studies. This chemical model is driven by a TH1-mediated immune response and is characterized by infiltration of the lamina propria with CD4+ T cells, neutrophils, and macrophages as well as the development of diarrhea and weight loss (23). Thus, it is important to interpret findings within the context of features of this model and consider they may not be applicable to all inflammatory enteric neuropathies. Thus, the validity of our findings would benefit from the repetition of our colitis studies in other models of murine colitis (24-27).

Use of Pharmacological Modulators

Studies in this dissertation used a number of drugs to modulate and antagonize the behavior of channels, receptors, and signaling molecules of interest. Specifically, studies in Chapter 3 relied heavily on the pharmacological properties of the EP4

receptor antagonist L-161,982 and 43Gap26. Studies in Chapter 4 relied on the actions of the CB1 receptor antagonist SR141716A, the CB2 receptor antagonist SR144528, and the MAGL inhibitor JZL184. Furthermore, many of the experiments that formed part of these studies relied on the pharmacological properties of clozapine-n-oxide (selective hM3Dq receptor agonist). Hence, the conclusions of these chapters rely on the assumption that these pharmacological modulators perform as intended and have little to no off-target effects.

Inducible knock-out models

Our studies in Chapter 3 and Chapter 4 utilize glial specific knock out models, the *Sox10^{creERT2};Cx43$^{f/f}$* and *Sox10^{creERT2};Mgll$^{f/f}$*, to assess the contribution of enteric glia to visceral hypersensitivity signaling mechanisms. Both models presented in these studies rely on the Sox10^{cre} promoter to induce the glial-specific ablation of Cx43 and MAGL. However, Sox10 is not specific to enteric glia and is also expressed by other glial cell types like Shwan cells (*38*), satellite glia (*39*), and oligodendrocytes (*40*). Hence, it is possible that these cell types contribute to the effects observed in vivo.

FUTURE DIRECTIONS

This dissertation presented a number of key findings that warrant further investigation in future studies. Our work highlights two glial-dependent mechanisms that contribute to visceral hypersensitivity. In Chapter 3 we show that enteric glia directly sensitize nociceptors through the Cx43-dependent release of PGE2 under proinflammatory conditions. These mechanisms were described utilizing acute models of inflammation with IL1-β and DNBS. A key follow-up study would be to investigate whether these glia-dependent mechanisms are relevant in chronic inflammatory models. Our in

vivo studies in Chapter 3 showed that visceral hypersensitivity is induced in the presence of proinflammatory mediators and that these effects can be inhibited by ablation of glial-Cx43. To support that these mechanisms are facilitated by the glial release of PGE2, it would be interesting to incorporate experiments in the glial cyclooxygenase-2 (COX2) knock-out model.

Our studies described in Chapter 4 show that enteric glia are an important component of endocannabinoid signaling in the enteric nervous system and that these pathways had sex-specific effects. Our Ca^{2+} imaging data from these studies demonstrated that modulating endocannabinoid signaling components via the pharmacological inhibition of MAGL, CB1, and CB2. Additionally, we demonstrate that inhibition of MAGL is able to reverse the sensitizing effects of inflammation on TRPV1+ nociceptors and prevents the recruitment of silent nociceptors. In addition to enteric glia and neurons, multiple other cell types express endocannabinoid signaling components (*24*). For this reason, these studies would benefit from cell-specific inhibitors in order to identify the specific contributions of glia and neurons to these sex-specific pathways.

The data presented in this dissertation begins to address the role of enteric glia in the modulation of nociceptive signaling in the intestine and their contribution to visceral hypersensitivity. In combination with proposed future work, these findings will aid in the identification of therapeutic targets to treat GI motility disorders.

REFERENCES

1. A. F. Peery, S. D. Crockett, A. S. Barritt, E. S. Dellon, S. Eluri, L. M. Gangarosa, E. T. Jensen, J. L. Lund, S. Pasricha, T. Runge, M. Schmidt, N. J. Shaheen, R. S. Sandler, Burden of Gastrointestinal, Liver, and Pancreatic Diseases in the United States. *Gastroenterology*. 149, 1731-1741.e3 (2015).

2. L. Chang, C. D. Lorenzo, G. Farrugia, F. A. Hamilton, G. M. Mawe, P. J. Pasricha, J. W. Wiley, "Functional Bowel Disorders: A Roadmap to Guide the Next Generation of Research." in (2018), vol. 154 of *Gastroenterology*, pp. 723–735.

3. F. Mearin, B. E. Lacy, L. Chang, W. D. Chey, A. J. Lembo, M. Simren, R. Spiller, Bowel Disorders. *Gastroenterology*. 150, 1393-1407.e5 (2016).

4. M. Regueiro, J. B. Greer, E. Szigethy, Etiology and Treatment of Pain and Psychosocial Issues in Patients With Inflammatory Bowel Diseases. *Gastroenterology*. 152, 430-439.e4 (2017).

5. A. D. Lee, B. M. Spiegel, R. D. Hays, G. Y. Melmed, R. Bolus, D. Khanna, P. P. Khanna, L. Chang, Gastrointestinal symptom severity in irritable bowel syndrome, inflammatory bowel disease and the general population. *Neurogastroenterology & Motility*. 29, e13003 (2017).

6. M. Camilleri, Diagnosis and Treatment of Irritable Bowel Syndrome. *Jama*. 325, 865–877 (2021).

7. W. R. Best, J. M. Becktel, J. W. Singleton, F. Kern, Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology*. 70, 439–444 (1976).

8. J. Keohane, C. O'Mahony, L. O'Mahony, S. O'Mahony, E. M. Quigley, F. Shanahan, Irritable bowel syndrome-type symptoms in patients with inflammatory bowel disease: a real association or reflection of occult inflammation? *The American journal of gastroenterology*. 105, 1788-1789-94-quiz 1795 (2010).

9. I. M. Minderhoud, B. Oldenburg, J. A. Wismeijer, G. P. van B. Henegouwen, A. J. P. M. Smout, IBS-like symptoms in patients with inflammatory bowel disease in remission; relationships with quality of life and coping behavior. *Digestive diseases and sciences*. 49, 469–474 (2004).

10. S. J. Halpin, A. C. Ford, Prevalence of symptoms meeting criteria for irritable bowel syndrome in inflammatory bowel disease: systematic review and meta-analysis. *The American journal of gastroenterology*. 107, 1474–1482 (2012).

11. C. Y. FRANCIS, J. MORRIS, P. J. WHORWELL, The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. *Aliment Pharm Therap*. 11, 395–402 (1997).

12. V. Yu, S. Ballou, R. Hassan, P. Singh, E. Shah, V. Rangan, J. Iturrino, J. Nee, A. Lembo, Abdominal Pain and Depression, Not Bowel Habits, Predict Health Care Utilization in Patients With Functional Bowel Disorders. *Am J Gastroenterology*. 116, 1720–1726 (2021).

13. A. F. Peery, E. S. Dellon, J. Lund, S. D. Crockett, C. E. McGowan, W. J. Bulsiewicz, L. M. Gangarosa, M. T. Thiny, K. Stizenberg, D. R. Morgan, Y. Ringel, H. P. Kim, M. D. Dibonaventura, C. F. Carroll, J. K. Allen, S. F. Cook, R. S. Sandler, M. D. Kappelman, N. J. Shaheen, Burden of gastrointestinal disease in the United States: 2012 update. *Gastroenterology*. 143, 1179-87.e1–3 (2012).

14. J. BouSaba, W. Sannaa, M. Camilleri, Pain in irritable bowel syndrome: Does anything really help? *Neurogastroenterol Motil*. 34, e14305 (2022).

15. M. Camilleri, A. Lembo, D. A. Katzka, Opioids in Gastroenterology: Treating Adverse Effects and Creating Therapeutic Benefits. *Clinical Gastroenterology and Hepatology*. 15, 1338–1349 (2017).

16. J.-F. Colombel, N. Narula, L. Peyrin-Biroulet, Management Strategies to Improve Outcomes of Patients With Inflammatory Bowel Diseases. *Gastroenterology*. 152, 351-361.e5 (2017).

17. M. D. Coates, M. Lahoti, D. G. Binion, E. M. Szigethy, M. D. Regueiro, K. Bielefeldt, Abdominal Pain in Ulcerative Colitis. *Falk Symp*. 19, 2207 (2013).

18. M. Defaye, N. S. Abdullah, M. Iftinca, A. Hassan, F. Agosti, Z. Zhang, M. Cumenal, G. W. Zamponi, C. Altier, Gut-innervating TRPV1+ Neurons Drive Chronic Visceral Pain via Microglial P2Y12 Receptor. *Cell Mol Gastroenterology Hepatology*. 13, 977–999 (2022).

19. E. A. Mayer, K. Tillisch, The Brain-Gut Axis in Abdominal Pain Syndromes. *Annual Review of Medicine*. 62, 381–396 (2011).

20. L. Grundy, A. Erickson, S. M. Brierley, Visceral Pain. *Annu Rev Physiol*. 81, 1–24 (2018).

21. N. M. Delvalle, C. Dharshika, W. Morales-Soto, D. E. Fried, L. Gaudette, B. D. Gulbransen, Communication between enteric neurons, glia, and nociceptors underlies the effects of tachykinins on neuroinflammation. *Cellular and Molecular Gastroenterology and Hepatology*, 321–344 (2018).

22. K. Matsumoto, T. Hosoya, K. Tashima, T. Namiki, T. Murayama, S. Horie, Distribution of transient receptor potential vanilloid 1 channel-expressing nerve fibers in mouse rectal and colonic enteric nervous system: relationship to peptidergic and nitrergic neurons. *Neuroscience*, 518–34 (2011).

23. N. J. Spencer, M. Kyloh, M. Duffield, Identification of Different Types of Spinal Afferent Nerve Endings That Encode Noxious and Innocuous Stimuli in the Large Intestine Using a Novel Anterograde Tracing Technique. *Plos One*. 9, e112466 (2014).

24. H. Cuddihey, W. K. MacNaughton, K. A. Sharkey, Role of the Endocannabinoid System in the Regulation of Intestinal Homeostasis. *Cell Mol Gastroenterology Hepatology*. 14, 947–963 (2022).

25. J. J. Galligan, Cannabinoid signalling in the enteric nervous system. *Neurogastroenterol Motil*. 21, 899–902 (2009).

26. K. A. Sharkey, J. W. Wiley, The Role of the Endocannabinoid System in the Brain–Gut Axis. *Gastroenterology*. 151, 252–266 (2016).

27. J. Castro, S. Garcia-Caraballo, J. Maddern, G. Schober, A. Lumsden, A. Harrington, S. Schmiel, B. Lindstrom, J. Adams, S. M. Brierley, Olorinab (APD371), a peripherally acting, highly selective, full agonist of the cannabinoid receptor 2, reduces colitis-induced acute and chronic visceral hypersensitivity in rodents. *Pain*. Publish Ahead of Print (2021), doi:10.1097/j.pain.0000000000002314.

28. S. M. Brierley, B. G.-V. Meerveld, G. Sarnelli, K. A. Sharkey, M. Storr, J. Tack, Targeting the endocannabinoid system for the treatment of abdominal pain in irritable bowel syndrome. *Nat Rev Gastroentero*. 20, 5–25 (2023).

29. Y. S. Sakin, A. Dogrul, F. Ilkaya, M. Seyrek, U. H. Ulas, M. Gulsen, S. Bagci, The effect of FAAH, MAGL, and Dual FAAH/MAGL inhibition on inflammatory and colorectal distension-induced visceral pain models in Rodents. *Neurogastroenterol Motil*. 27, 936–944 (2015).

30. A. Kawabata, Prostaglandin E2 and Pain—An Update. *Biol Pharm Bull*. 34, 1170–1173 (2011).

31. C. Patrono, Cardiovascular effects of cyclooxygenase-2 inhibitors: a mechanistic and clinical perspective. *Brit J Clin Pharmaco*. 82, 957–964 (2016).

32. M. Naguib, P. Diaz, J. J. Xu, F. Astruc-Diaz, S. Craig, P. Vivas-Mejia, D. L. Brown, MDA7: a novel selective agonist for CB2 receptors that prevents allodynia in rat neuropathic pain models. *Brit J Pharmacol*. 155, 1104–1116 (2008).

33. A. Kikuchi, K. Ohashi, Y. Sugie, H. Sugimoto, H. Omura, Pharmacological Evaluation of a Novel Cannabinoid 2 (CB2) Ligand, PF-03550096, In Vitro and In Vivo by Using a Rat Model of Visceral Hypersensitivity. *J Pharmacol Sci*. 106, 219–224 (2008).

34. Y. Iwata, K. Ando, K. Taniguchi, N. Koba, A. Sugiura, M. Sudo, Identification of a highly potent and selective CB2 agonist, RQ-00202730, for the treatment of irritable bowel syndrome. *Bioorg Med Chem Lett*. 25, 236–240 (2015).

35. S. Han, J. Thatte, D. J. Buzard, R. M. Jones, Therapeutic Utility of Cannabinoid Receptor Type 2 (CB2) Selective Agonists. *J Med Chem*. 56, 8224–8256 (2013).

36. G. Griebel, P. Pichat, S. Beeské, T. Leroy, N. Redon, A. Jacquet, D. Françon, L. Bert, L. Even, M. Lopez-Grancha, T. Tolstykh, F. Sun, Q. Yu, S. Brittain, H. Arlt, T. He, B. Zhang, D. Wiederschain, T. Bertrand, J. Houtmann, A. Rak, F. Vallée, N. Michot, F. Augé, V. Menet, O. E. Bergis, P. George, P. Avenet, V. Mikol, M. Didier, J. Escoubet, Selective blockade of the hydrolysis of the endocannabinoid 2-arachidonoylglycerol impairs learning and memory performance while producing antinociceptive activity in rodents. *Sci Rep-uk*. 5, 7642 (2015).

37. S. Martínez-Torres, L. Cutando, A. Pastor, A. Kato, K. Sakimura, R. de la Torre, E. Valjent, R. Maldonado, M. Kano, A. Ozaita, Monoacylglycerol lipase blockade impairs fine motor coordination and triggers cerebellar neuroinflammation through cyclooxygenase-2. *Brain Behav Immun*. 81, 399–409 (2019).

38. E. A. Fogarty, J. O. Kitzman, A. Antonellis, SOX10-regulated promoter use defines isoform-specific gene expression in Schwann cells. *Bmc Genomics*. 21, 549 (2020).

39. D. Wang, J. Lu, X. Xu, Y. Yuan, Y. Zhang, J. Xu, H. Chen, J. Liu, Y. Shen, H. Zhang, Satellite Glial Cells Give Rise to Nociceptive Sensory Neurons. *Stem Cell Rev Rep*. 17, 999–1013 (2021).

40. E. Sock, M. Wegner, Using the lineage determinants Olig2 and Sox10 to explore transcriptional regulation of oligodendrocyte development. *Dev Neurobiol*. 81, 892–901 (2021).