ROLE OF ENTERIC GLIAL ACTIVATION IN GASTROINTESTINAL PHYSIOLOGY AND INFLAMMATION

By

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ABSTRACT

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Functional gastrointestinal (GI) and motility disorders are the most common GI disorders, affecting as many as 1 in 4 persons worldwide. GI motility dysfunction presents as a symptom in a number of diseases including Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS) and as a secondary symptom in diseases such as Parkinson's disease (PD) and diabetes mellitus. Despite this, treatments for functional GI disorders are scarce. This may be due, in part, to a lack of understanding of the underlying mechanisms that regulate GI function and dysfunction.

Normal GI functions are regulated by the enteric nervous system (ENS), an intrinsic neuronal network comprised of enteric neurons and enteric glia embedded in the gut wall. Enteric glia are a unique type of peripheral glial cell that play important roles in GI health and disease. Alterations in ENS control of GI function, underlie the pathology of many GI disorders. Indeed, enteric neuron death and subsequent disruption of the neuronal network causes motility dysfunction in a number of GI disorders. In addition, activation of enteric glia is a critical mediator of neurodegeneration during intestinal inflammation. In this dissertation, we investigate the physiological and pathophysiological roles of enteric glia in GI function regulation. The goal of this dissertation was to understand how glial activation modulates GI function and contributes to neuroinflammation.

The work in this dissertation used a combination of transgenic animal strains, immunohistochemistry (IHC), selective drugs, Ca^{2+} imaging, motility assays, and *in situ* and *in vivo* models of colitis. We found that the tachykinin neurokinin A (NKA) contributes to neuroinflammation in the ENS through a multicellular signaling cascade involving enteric neurons, nociceptors and enteric glia. Importantly, activation of neurokinin-2 receptors (NK2Rs) on nociceptive neurons drove Ca^{2+} responses on enteric glia and antagonizing NK2R signaling prevented key aspects of neuroinflammation in the ENS such as reactive gliosis and neuron death. Further, we provide the first evidence that enteric glia express two subtypes of functional muscarinic receptors and direct activation of modified muscarinic receptors on enteric glia enhances GI motility. Lastly, we studied the effects of glial fibrillary acidic protein (GFAP) upregulation in inflammation and found that GFAP upregulation may contribute to neuroinflammation in colitis.

Our data provide new evidence for an active role for enteric glial cells in GI physiology and pathophysiology. Specifically, we demonstrate that 1) communication between enteric neurons, glia and nociceptors underlies the effects of tachykinins in inflammation 2) cholinergic activation of enteric glia is a physiological mechanism that contributes to regulation of GI motility and 3) enteric glial reactivity characterized by morphological changes and GFAP upregulation drives enteric neuron death in colitis. Our findings propose new therapeutic targets for the treatment of GI motility disorders.

For my mom & dad

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KEY TO ABBREVIAITONS

5HT – serotonin

ACh – acetylcholine

ADP – adenosine diphosphate

AHP – after hyperpolarization potential

Aldh1L1 – aldehyde dehydrogenase 1 family member L1

ATP – adenosine triphosphate

BN – bombesin

BzATP – 2’(3’)-O-(4-benzoylbenzoyl)adenosine-5’-triphosphate triethylammonium salt

Ca^{2+} – Calcium

CGRP – calcitonin gene-related peptide

ChAT – choline acetyltransferase

CIPO – chronic intestinal pseudo-obstruction

CMMC – colonic migrating motor complex

CNO – clozapine-N-oxide

CNS – central nervous system

COX2 – cylclooxygenase-2

Cx43 – Connexin-43

DMEM – Dulbecco’s Modified Eagle Medium

DMSO – dimethyl sulfoxide

DNBS – 2,4-dinitrobenzene sulfonic acid

EC – enterochromaffin

ENK - encephalin

ENS – enteric nervous system

fEPSPs – fast excitatory postsynaptic potentials

FGIDs – functional gastrointestinal disorders

FLCs – fibroblast-like cells

GABA – γ-amino butyric acid

GFAP – glial fibrillary acidic protein

GI – gastrointestinal

IB4 – isolectin B4

IBD – inflammatory bowel disease

IBS – irritable bowel syndrome

ICC – interstitial cells of Cajal

IFs – intermediate filaments

IHC – immunohistochemistry

IL – interlukin

IPANs – intrinsic primary afferent neurons

LMMP – longitudinal muscle myenteric plexus

LPS – lipopolysaccharide

miRNA – microRNA

MR – muscarinic receptor

NDS – normal donkey serum

NE – norepinephrine

NGS – normal goat serum

NK1R – neurokinin 1 receptor

NK2R – neurokinin 2 receptor

NK3R – neurokinin 3 receptor

NKA – neurokinin A

NKB – neurokinin B

NO – nitric oxide

NOS – nitric oxide synthase

NPY – neuropeptide Y

PACAP – pituitary adenylyl cyclase activating peptide

Panx1 – pannexin-1

PBS – phosphate buffered saline

PD – Parkinson’s disease

PDGFR – platelet-derived growth factor receptor-α

PGE2 – prostaglandin E (2)

$PGF_{2\alpha}$ – prostaglandin F2-α

PSCs – Perisynaptic schwann cells

PSNS – parasympathetic nervous system

qPCR – quantitive real-time polymerase chain reaction

sEPSPs – slow excitatory postsynaptic potentials

SNS – sympathetic nervous system

SP – substance P

TKN – tachykinin

TRPV1 – transient receptor potential cation channel subfamily V member 1

TTX – tetrodotoxin

UTP – uridine triphosphate peptide

VIP – vasoactive intestinal peptide

WT – wildtype

CHAPTER 1

Introduction

OVERVIEW

The gastrointestinal (GI) tract is the organ system responsible for digestive function, pathogen defense, interactions with the microbiome and movement of food through the body (1). The GI system is innervated extrinsically by the central nervous system (CNS) and intrinsically by the enteric nervous system (ENS), which is embedded within the gut wall (2). Together, the CNS and ENS orchestrate GI reflexes to control digestive function. Digestive disorders or diseases can impact anywhere along the GI tract and are often debilitating. GI diseases affect an estimated 60 to 70 million Americans annually (3-6) with a financial burden of $142 billion in direct and indirect costs (3). Despite this, treatments for GI disorders are scarce and have serious adverse side effects (7). This may be due, in part, to the lack of understanding of the underlying cellular mechanisms that regulate GI function and dysfunction.

Alterations in ENS control of GI function, underlies the pathology of many GI disorders (8,9). Indeed, enteric neuron death causes motility dysfunction in a number of GI disorders including Hirschsprung's disease (10-12), chronic intestinal pseudo-obstruction (13) and Inflammatory Bowel Disease (IBD) (14,15). ENS dysfunction is also associated with secondary symptoms in other conditions including diabetes mellitus (16,17), Parkinson's disease (18-21) and Alzheimer's disease (18,19,22).

Recent evidence suggests enteric glia are important mediators in regulating gastrointestinal functions in health and disease (23,24). Enteric glia are a unique type of non-myelinating peripheral glial cells in the ENS (23). Enteric glia play active roles in enteric neuronal circuits controlling motility and secretion (25-27) and activation of enteric glia is also a critical mediator of neurodegeneration during intestinal inflammation (28).

Elucidating the mechanisms by which enteric glia regulate GI physiology and pathophysiology remain areas of intense research. This dissertation investigates the physiological and pathophysiological roles enteric glia play in regulating GI function.

INTRINSIC AND EXTRINSIC INNERVATION OF THE GI TRACT

The roles of enteric nervous system (ENS) and central nervous system (CNS) innervation vary considerably along the digestive tract (2). The upper gastrointestinal (GI) system is primarily under CNS control with the ENS playing a modulatory role. In the esophagus, neuronal innervation from the CNS through the vagus nerve regulates muscle movements (29-30). Similarly, the CNS controls contractile activity and acid secretion in the stomach through vago-vagal reflexes (2,31). In the small and large intestine, the ENS is the primary regulator over muscle activity, local blood flow and transmucosal fluid exchange (1). The CNS also has control of defecation via the defecation centers in the lumbosacral spinal cord (32). Indeed, the neuronal control of GI function is an integrated system in which local enteric reflexes and extrinsic reflexes interact (**Figure 1.1**).

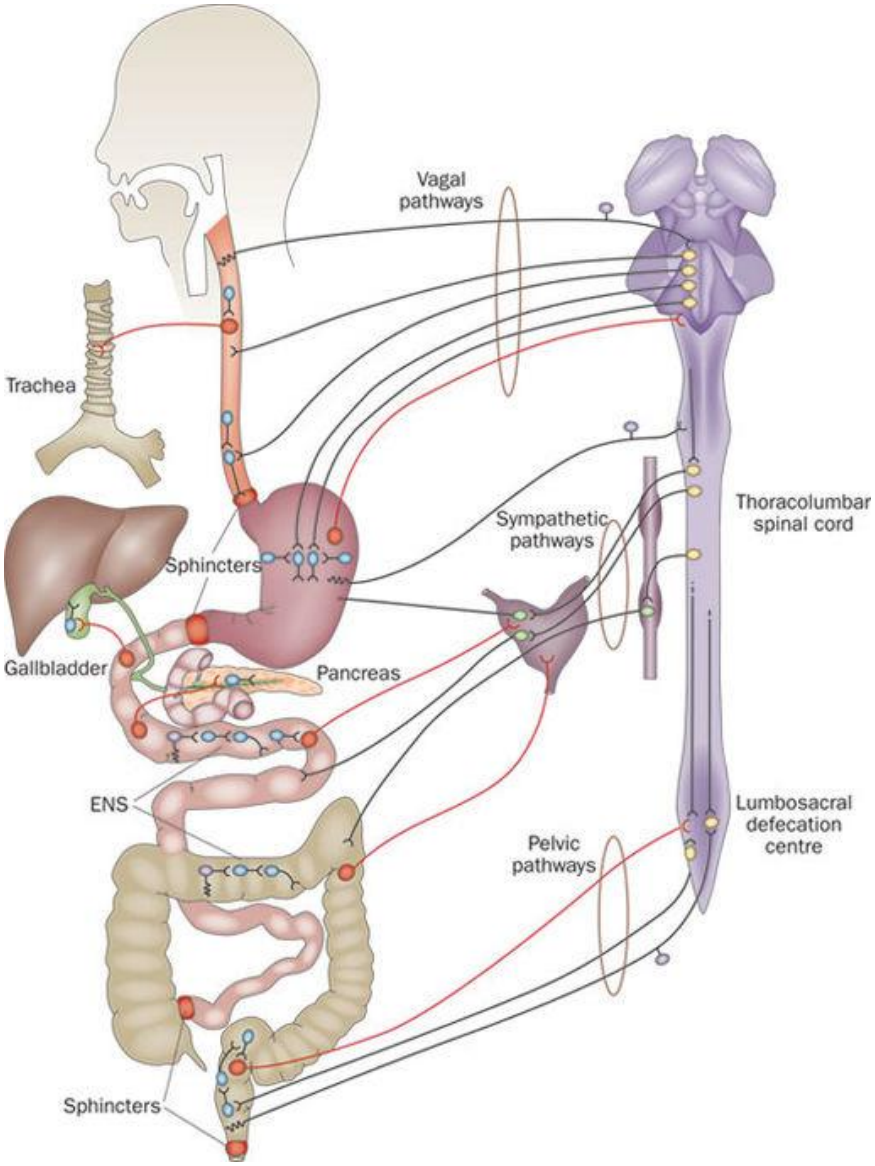

Figure 1.1: The gastrointestinal tract is innervated by extrinsic and intrinsic nerves. The GI tract has full reflex circuits of the ENS (motor neurons and interneurons in blue, sensory neurons in purple). Pathways from the gastrointestinal tract project outwards via intestinofugal neurons (red). Sensory 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 (50).

Extrinsic Innervation

The CNS provides extrinsic innervation that regulates, modulates and controls GI functions (33). Connections between the gut and the CNS can be classified as vagal, spinal thoracolumbar and spinal lumbosacral (**Figure 1.1**) and include sensory, sympathetic and parasympathetic pathways. Innervation from spinal afferent neurons with cell bodies in the dorsal root ganglia (DRG) relay sensory information to the CNS (2). Sympathetic pathways from the sympathetic nervous system (SNS) provide a tonic inhibitory influence over mucosal secretion, regulate GI blood flow, and exert a predominantly inhibitory effect upon GI muscle (34-37). In contrast, parasympathetic pathways from the parasympathetic nervous system (PSNS) exert both excitatory and inhibitory control over gastric and intestinal tone and motility (2,33).

The thoracolumbar spinal cord connects with the GI tract through spinal afferent neurons with cell bodies in the DRG and through sympathetic efferent pathways (**Figure 1.1**). A high proportion of spinal afferent neurons are immunoreactive for calcitonin gene-related peptide (CGRP), tachykinins (TKNs) and transient receptor potential cation channel subfamily V member 1 (TRPV1) (38-39). Inflammation causes long term changes in spinal afferent neurons causing neurons to become hypersensitive to GI pain sensation (40,41). In contrast, the sympathetic efferent pathways that originate from the thoracolumbar spinal cord have four primary targets: myenteric ganglia, submucosal ganglia, blood vessels and sphincter muscle (**Figure 1.1**). Sympathetic post-ganglionic (pre-enteric) neurons innervate the myenteric and submucosal ganglia and have inhibitory effects on motility and secretion, respectively, primarily through the release of

norepinephrine (NE). The sympathetic pre-ganglionic neurons contract the sphincters of the GI tract primarily through acetylcholine (ACh) neurotransmission (2).

The PSNS consists of the vagal and pelvic nerves. The vagal nerves arise from the cervical region of the spinal cord and provide sensory innervation and efferent control pathways for the upper GI tract (**Figure 1.1**). The vagal sensory innervation regulates appetite and satiety, esophageal propulsion, gastric volume, contractile activity and acid secretion in the GI system (29-30,34-35,42-45). There are three distinct types of vagal afferent endings in the GI tract: intraganglionic laminar endings that function as stretch receptors (46), intramuscular arrays that transduce specific stretch or muscle length information (45), and mucosal varicose endings responsive to low intensity stroking of the mucosa and chemical stimuli (31). Lastly, the pelvic nerves arise from the sacral region of the spinal cord and provide innervations to the distal colon and rectum. Pelvic nerves in the rectum carry afferent information from distention, including into the level of pain (47-48). Mucosal mechanoreceptors originating from the pelvic nerves in the large intestine can respond to low intensity stroking of the mucosa, but not to distension or colon contraction (49). Additionally, the efferent pathways in pelvic nerves modulate intrinsic innervations for motility control which will be discussed in a later section.

Intrinsic Innervation

The GI system is distinct from other organ systems in that it has an intrinsic nervous system that can function independent of CNS input (2). The ENS provides local neuronal control of GI functions including: determining motility patterns, modulating

gastric acid secretion, regulating fluid movement across the epithelium, changing local blood flow, and interacting with the immune and endocrine systems of the gut (1). The ENS is composed of enteric neurons and glia grouped in enteric ganglia with nerve processes innervating the muscle, epithelium, blood vessels, biliary system and pancreas. In the small and large intestine, most neurons are found in two sets of ganglionated plexuses: the myenteric plexus and the submucosal plexus (**Figure 1.2**).

The myenteric (or Auerbach's) plexus is a network of neuronal projections and ganglia that lie between the longitudinal and circular muscle layers of the gut (**Figure 1.2**). The myenteric plexus is the larger of the two ganglionated plexuses and contains two-thirds of enteric neurons (2,50). It innervates the entire GI tract and exerts control over smooth muscle contractions and relaxations and is the main regulator of GI motility (2,51). Consequently, alterations to the myenteric plexus are associated with changes in GI motility (52,53).

The submucosal plexus is located in the submucosal space between the mucosal and circular muscle layers of the gut (**Figure 1.2**) and is primarily responsible for the control of local blood flow and luminal absorptions and secretions (1). The submucosal plexus is only present in the small and large intestine, with no innervation in the esophagus or stomach. The ganglia and interconnecting fibers are smaller than those of the myenteric plexus and in humans the submucosal ganglia form two distinct but interconnected plexuses: the outer (Henle's or Schabadasch) plexus and inner (Meissner's) plexus (54-56). Ganglia in both plexuses are mainly comprised of two cell types: enteric neurons and enteric glial cells.

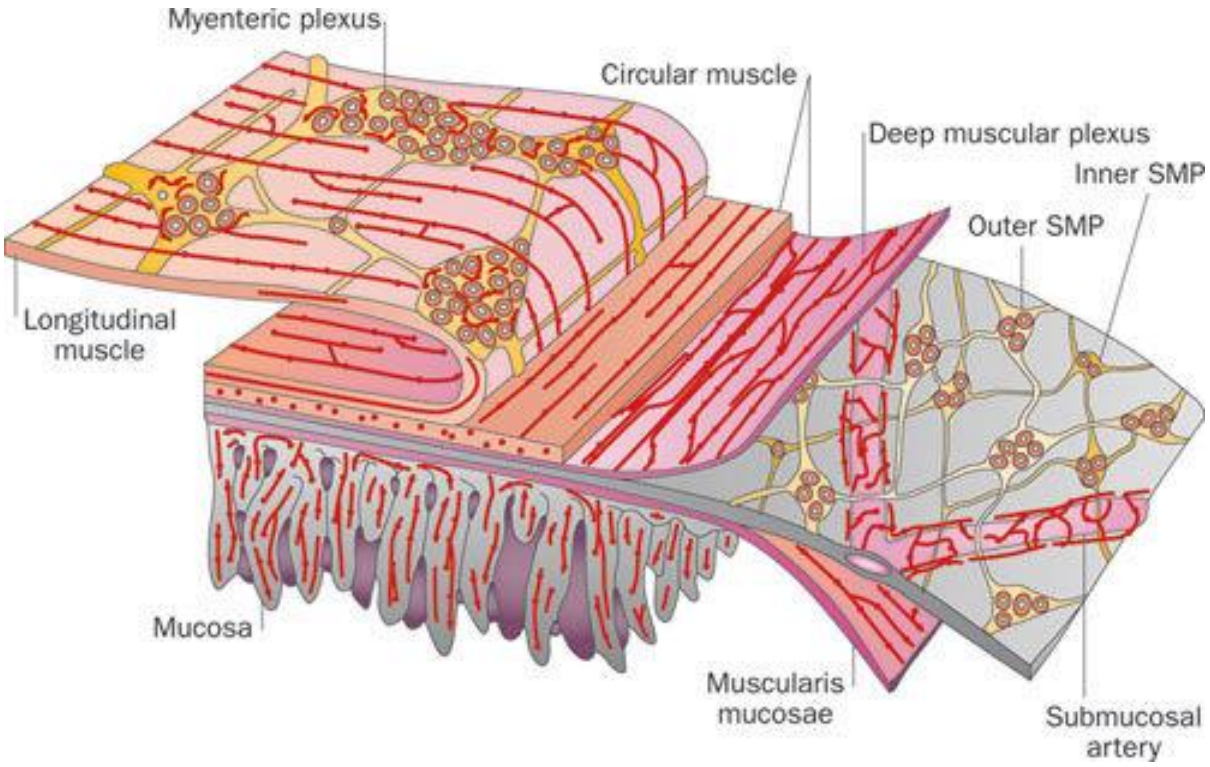

Figure 1.2: Organization of the enteric nervous system. The enteric nervous system is composed of two ganglionated plexuses, the submucosal plexus between the circular muscle and mucosal layers, and the myenteric plexus between the longitudinal and circular muscle layers. In humans, the submucosal plexus is divided into an inner and outer SMP. Figure from Furness 2012 (50).

ENTERIC NEURONS

The ENS has an estimated 200-600 million enteric neurons in humans (1). Enteric neurons are the primary component of enteric neuronal circuits that regulate GI function, and approximately 20 types of enteric neurons have been defined (57-59). Enteric neurons are classified by their morphology, electrophysiology and neurochemical coding. Enteric neurons are grouped by their functions as intrinsic primary afferent neurons (IPANs), interneurons, and motor neurons (57).

MORPHOLOGICAL CLASSIFICATIONS OF ENTERIC NEURONS

Enteric neurons were first classified according to their cellular morphology as described by Dogiel in 1899 (60-61). Dogiel described multiple neuron types, but I will focus on three types: Dogiel type I, Dogiel type II and Dogiel type III.

Dogiel Type I Neurons

Dogiel type I neurons have 4-20 short, broad, laminar dendrites (**Figure 1.3**). These dendrites are flattened in the myenteric plane and branch and end within the ganglion of origin. Their cell bodies are 13-35µm in length and 9-22µm in width. Dogiel type I neurons have a single long axon which projects to neighboring ganglia or the musculature. They include inhibitory and excitatory motor neurons and interneurons (1).

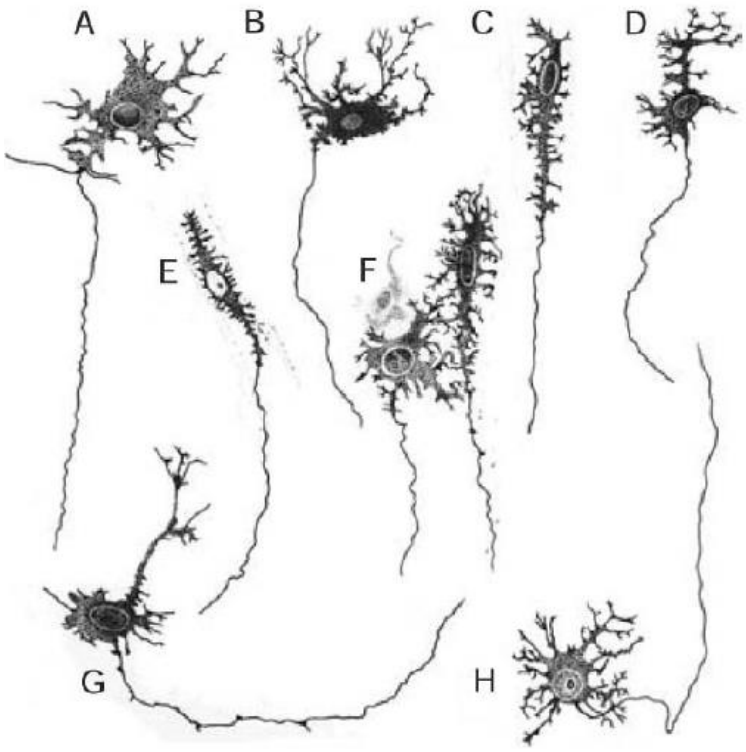

Figure 1.3: Examples of Dogiel type I neurons as defined and drawn by Dogiel (1899). (**A,C,E,F,H**) neurons from the myenteric plexus of the guinea pig intestine. (**D,G**) neurons from the myenteric plexus of human small intestine. (**H**) neuron from dog gall bladder. Figure from Furness 2006 (1).

Dogiel Type II Neurons

Dogiel type II neurons are more prominent in the myenteric and submucosal plexus of the small and large intestine, but are rare in the stomach (**Figure 1.4**). They are multi-axonal and have large round or oval cell bodies. The major diameter of type II neurons is 22-47µm and the minor diameter is between13-22µm. Dogiel type II neurons project to other neurons in the same or adjacent ganglia and to the mucosa. They are classified as sensory neurons and make up the majority of IPANs of the submucosal and myenteric plexus (1,62).

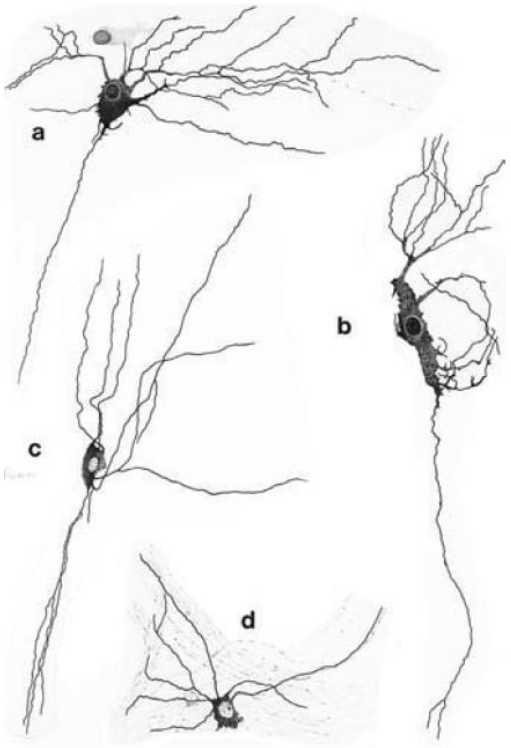

Figure 1.4: Examples of Dogiel type II neurons as defined and drawn by Dogiel (1899). (**A-B**) Neurons form the myenteric plexus of the guinea pig small intestine. (**C**) Neuron from myenteric plexus of human small intestine. (**D**) Neuron from the myenteric plexus of guinea pig large intestine. Figure from Furness 2006 (1).

Dogiel Type III Neurons

Dogiel type III neurons have 2-10 dendrites that become thinner and branched as they extend from the cell body. Th dendrites are shorter than those of type II cells and end within the ganglion of origin (1) The axon of a Dogiel type III neuron begins from a small conical protrusion of the cell body or from a dendrite. Dogiel type III neurons are primarily descending interneurons (1).

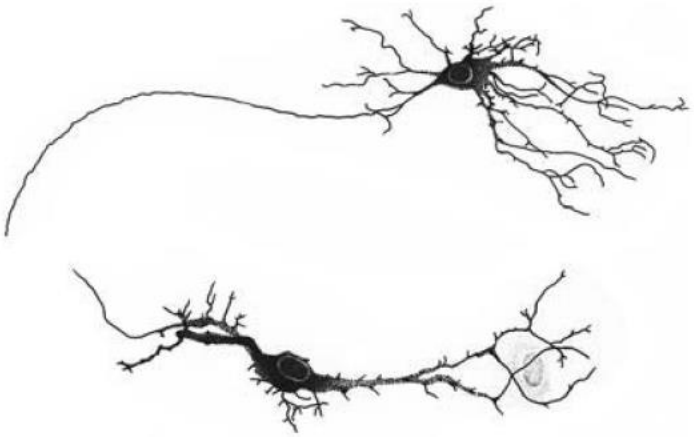

Figure 1.5: Examples of Dogiel type III neurons as defined by Dogiel (1899). Both neurons are from the myenteric plexus of the guinea pig large intestine. Figure from Furness 2006 (1).

ELECTROPHYSIOLOGICAL CLASSIFICATIONS OF ENTERIC NEURONS

Enteric neurons are classified by their electrophysiological properties into two broad classes: S-type neurons and AH-type neurons (63,64) (**Figure 1.6**). In S-type neurons, single electrical stimuli applied to interganglionic connectives elicit fast excitatory postsynaptic potentials (fEPSPs) (63). The action potentials from the soma of S-type neurons are completely blocked by the sodium channel blocker, tetrodotoxin (TTX), and most S-type neurons fire continuously when depolarized. Action potentials in S-type neurons are followed by short duration after-hyperpolarizing potentials (AHPs), lasting 20-100ms. S-type neurons have Dogiel type I morphologies and S-type electrophysiology is seen in motor neurons and interneurons. In AH-type neurons, single electrical stimuli elicit fEPSPs in some neurons, but trains of stimuli elicit slow excitatory postsynaptic potentials (sEPSPs) in all AH-type neurons (165-166). The action potentials of AH-type neurons are large, have a greater duration than those of S neurons, and have

an inflection on the falling phase. Two inward currents underlie the action potential of AH-neurons; a TTX-sensitive sodium current and a TTX-insensitive calcium current. The action potential in AH-type neurons is followed by a fast-AHP and a slow-AHP which lasts between 2-30s (65-66). The AHP is mediated by a calcium-dependent potassium channel activated by calcium entering the neuron during the action potential (1). Under resting conditions, AH-type neurons usually fire one or two action potentials when depolarized, as the AHP limits their firing rate. All AH-neurons have Dogiel type II morphology and are sensory neurons.

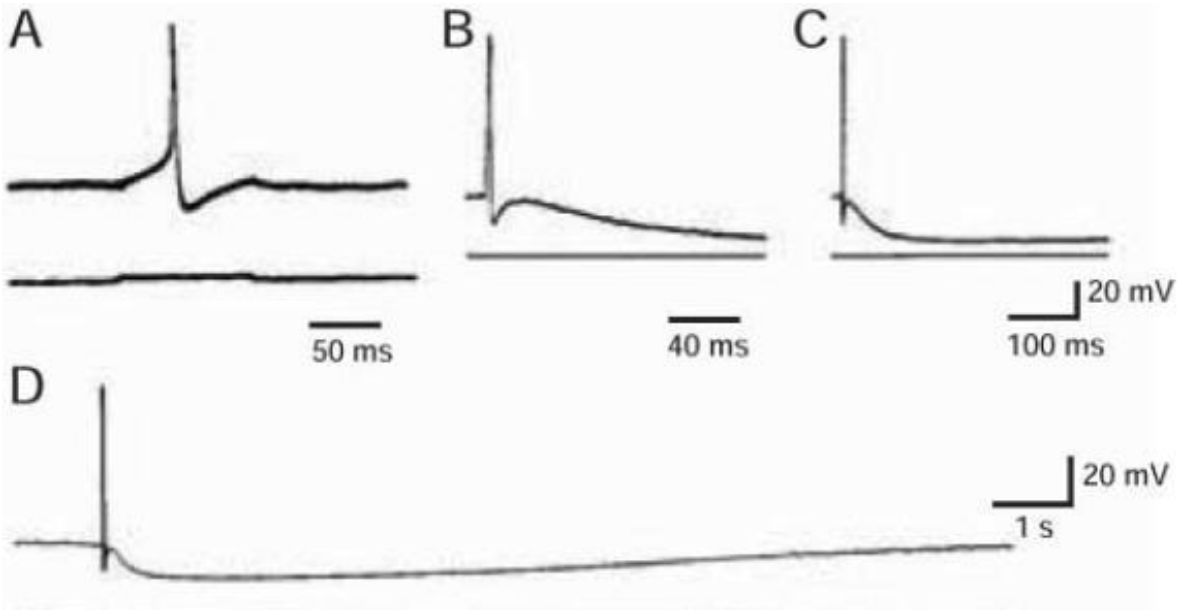

Figure 1.6: Action potentials generated by S-type neurons and AH-type neurons by intracellular current pulses. (**A**) The action potential in S-type neurons is followed by a fast after-hyperpolarization potential (AHP). (**B-D**) The action potential in AH-neurons is followed by a fast AHP and, after partial restitution of the membrane potential, by a slow AHP. Recordings are from neurons in guinea pig small intestine. Figure from Furness 2006 (1).

NEUROCHEMICAL CLASSIFICATIONS OF ENTERIC NEURONS

Three classes of enteric neurons can be described based on neurochemical coding: sensory neurons, motor neurons (excitatory or inhibitory) and interneurons (ascending or descending) (**Table 1.1**) (58,67) The primary neurotransmitters in the intrinsic sensory neurons or IPANs, are acetylcholine (ACh), tachykinins (TKNs) and calcitonin gene-related peptide (CGRP). They are immunoreactive for choline acetyltransferase (ChAT), TKNs and neurokinin 3 receptor (NK3R). Excitatory motor neurons project orally or locally to the circular or longitudinal smooth muscle to induce muscle contractions through release of ACh and substance P (SP) (68). Excitatory motor neurons projecting to the circular muscle are immunoreactive for ChAT, while excitatory motor neurons projecting to the longitudinal muscle are immunoreactive for the calcium binding protein calretinin. Inhibitory motor neurons are immunoreactive for their primary neurotransmitters: nitric oxide (NO) and vasoactive intestinal peptide (VIP). Ascending and descending interneurons, which travel along the length of the gut, primarily release ACh and are immunoreactive for ChAT. To distinguish between different types of interneurons, co-staining for other proteins and neurotransmitters such as calretinin, serotonin (5-HT) and VIP is needed (**Table 1.1**).

Functional Definition	Proportion	Neurochemical Coding	Neurotransmitters
Myenteric IPANs	26%	ChAT/TKN/orexin/IB4/NK3R	ACh,TKN
Excitatory circular muscle motor neuron	12%	ChAT/TK/ENK/GABA	ACh,TKN
Inhibitory circular muscle motor neuron	16%	NOS/VIP/PACAP/ENK/NPY/GABA	NO,ATP,VIP,PACAP
Excitatory longitudinal muscle motor neuron	25%	ChAT/calretinin/TKN	ACh,TKN
Inhibitory longitudinal muscle motor neuron	~2%	NOS/VIP/GABA	NO,ATP,VIP,PACAP
Ascending interneurons	5%	ChAT/Calretinin/TK/ENK	ACh,TKN
Descending interneurons	5%	ChAT/NOS/VIP/BN/NPY	ACh, ATP

Table 1.1: Types of neurons in the myenteric plexus. This table lists a subset of neurons found in the myenteric plexus of the guinea pig small intestine. Table modified from Furness 2006 (1). ACh (acetylcholine), ATP (adenosine triphosphate), BN (bombesin), ENK (encephalin), GABA (gamma amino butyric acid), NK3R (neurokinin 3 receptor), NOS (nitric oxide synthase), NPY (neuropeptide Y), PACAP (pituitary adenylyl cyclase activating peptide), TKN (tachykinin), VIP (vasoactive intestinal peptide).

NEURAL CONTROL OF GI MUSCLE ACTIVITY

The degree to which the ENS or the CNS regulate motility pathways vary with the region of the GI tract. Broadly, the CNS exerts control over the esophagus and stomach, and the ENS controls small intestine motility as well as large bowel motility, except for defecation which is controlled by the defecation centers in the CNS (1). The muscle layers of the GI tract direct propulsion, mixing of contents, reservoir capacity and expulsion of pathogens and noxious chemicals (1). In this section, I will focus on the intrinsic neuronal network that regulates GI motility in the small intestine and colon.

Enteric Neuronal Control Of GI Motility In The Small Intestine And Colon

The ENS directs various patterns of movement in the small and large intestine, including peristalsis, segmentation and slow orthograde propulsion (1,57). The neuronal circuits that detect and direct the activity of motor neurons are now well defined (**Figure 1.7**). The primary enteric reflex in the colon, peristalsis, is initiated when a bolus of food increases intraluminal volume and activates IPANs in the ENS (69). IPANs have cell bodies in the submucosal and myenteric plexus and projections to the mucosal layer that can respond to chemical stimuli (serotonin), mechanical deformation of the mucosa and to radial stretch and muscle tension (57). IPANs synapse onto other IPANs, motor neurons and ascending and descending interneurons (2). IPAN projections to ascending and descending interneurons activate ascending excitatory and descending inhibitory pathways, respectively. Ascending interneurons project orally to the site of distention to synapse with cholinergic and tachykinergic excitatory motor neurons

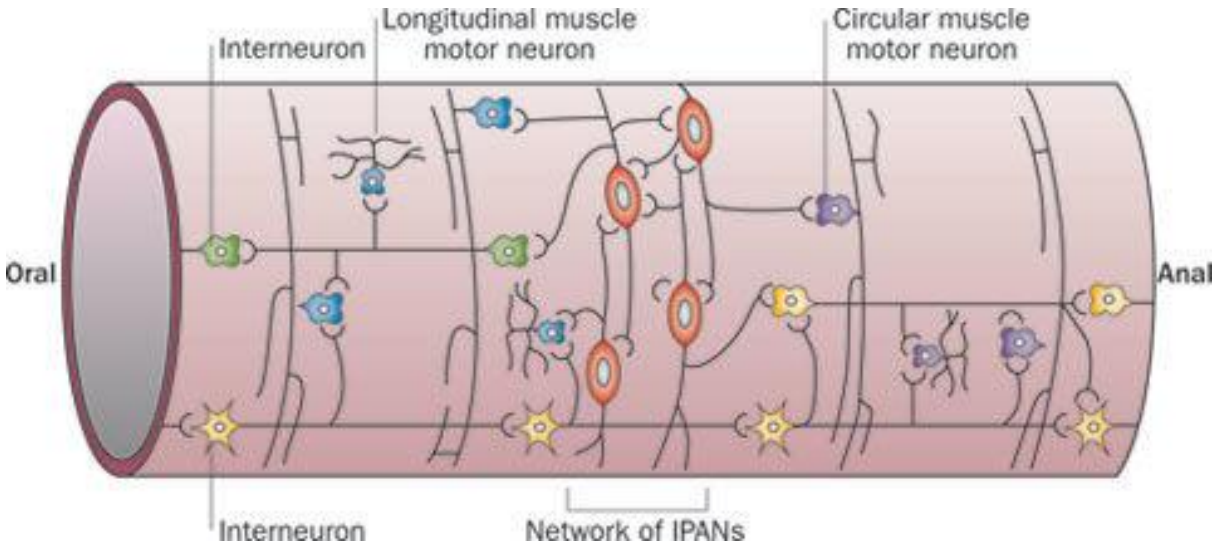

Figure 1.7: Enteric neuronal circuits for the control of motility in the intestine. Diagram is based on studies performed on guinea pig small intestine. Similar component neurons have been identified in other species and in the large intestine. Networks of IPANs (red) detect mechnaical distortion or chemical stimuli in the lumen to intiate peristalsis. IPANs synapse with ascending (green) or descending (yellow) interneurons, or directly to excitatory (blue) or inhibitory (purple) motor neurons. Excitatory motor neurons regulate smooth muscle contraction and inhibotry motor neurons regulate smooth muscle relaxation to cause propulsion. Figure from Furness 2012 (50).

projecting to the longitudinal and circular smooth muscle to cause muscle contraction oral to the food bolus. Simultaneously, the descending inhibitory interneurons project aboral to synapse with inhibitory nitrergic and purinergic motor neurons to cause smooth muscle relaxation aboral to the bolus (70-73).

In addition to enteric neurons, other non-neuronal cells mediate GI motility regulation. Interstitial cells of Cajal (ICC) are the pacemaker cell of the GI and are functionally interposed between the terminals of motor neurons and muscle. They have been proposed to mediate excitatory smooth muscle neurotransmission (74-76). In addition, fibroblast-like cells (FLCs) positive for platelet-derived growth factor receptor-α (PDGFR) are a distinct type of interstitial cell that have been recently proposed to transduce purinergic inhibitory neurotransmission (167). Another non-neuronal cell proposed to mediate GI regulation is enteric glia, a unique type of peripheral glial cell that will be discussed in another section in this chapter (23-25).

ENTERIC NEUROPATHIES

Disturbances in GI function range in severity from short-bouts of diarrhea to more severe symptoms, including chronic pain, nausea, vomiting and severe constipation (77). Patients with severe GI dysfunction have profound changes in GI transit and motility. These changes in motility are due to a loss in enteric neurons or functional impairment without cell death. Enteric neuropathies are diseases with histopathological evidence of enteric neuron loss or damage and are classified as congenital neuropathies, primary enteric neuropathies, secondary enteric neuropathies or predominantly inflammatory neuropathies (8,77).

Congenital Neuropathies

Hirschsprung's disease is the most well understood congenital neuropathy. It is characterized by the absence of ganglionic cells in the myenteric and submucosal plexus. Hirschsprung's affects an estimated 1 in 4000 children (78) and 20% of cases present with genetic defects associated with enteric neural crest cell proliferation, migration and differentiation (79).

Primary Enteric Neuropathies

Primary enteric neuropathies are enteric neuropathies where the ENS is the primary target of the disease. Chronic intestinal pseudo-obstruction (CIPO) is a rare, severe disease characterized by failure of the GI tract to propel its contents in the absence of an obstruction (80). CIPO is one of the most important cases of chronic intestinal failure in both pediatric and adult cases (81-85). Examination of full-thickness biopsies reveal degenerative neuropathy characterized by neuronal degeneration and loss without inflammation (86). Treatment options for CIPO are limited to supportive therapies rather than curative treatment options (87). A second example of a primary enteric neuropathy is slow-transit chronic constipation. Patients with slow-transit chronic constipation have significant impairment of propulsive colonic motor activity and significantly diminished colonic responses (88). Dysmotility in slow-transit constipation is due to a number of factors including neurodegeneration and loss of ICC cells (77).

Secondary Enteric Neuropathies

Secondary enteric neuropathies are diseases where the gut is not the primary target of disease pathology (8). The mechanisms that drive enteric neurodegeneration and GI dysfunction are not fully understood and important differences are present between different diseases. A common example of these is diabetic mellitus. Patients with diabetic mellitus present more frequently with abdominal pain, heartburn, dysphagia, nausea, diarrhea and constipation than healthy individuals (89-90). Of importance, animal models of diabetes have shown a significant loss in nitrergic myenteric neurons in the stomach, small intestine and colon (91-92). Another example of secondary enteric neuropathy is Parkinson's disease (PD). Indeed, constipation is the most common complaint reported in PD patients (93) and the underlying pathophysiology contributing to GI motility dysfunction in PD includes delayed transit and enteric neuron death (77,94).

Predominantly Inflammatory Neuropathies

A large percentage of enteric neuron death is associated with gut inflammation and the infiltration of immune cells into the GI tract. Several diseases activate immune mediated mechanisms that drive inflammation in the ENS (95). Predominantly inflammatory neuropathies include Inflammatory Bowel Diseases (IBD), Chagas's disease and paraneoplastic enteric neuropathy (96-97). The pathogenesis of inflammatory neuropathies varies among diseases; IBD pathogenesis involves multiple factors such as abnormal gut microbiota, immune response dysregulation, environmental changes, and gene variants (168); Chagas's disease is caused by infection with the

parasite *Trypanosoma cruzi* (169); and paraneoplastic enteric neuropathy is a secondary inflammatory neuropathy triggered by an autoimmune response (170). Importantly, significant neuron death is reported in animal models and biopsies for these diseases and neuron death is shown to directly contribute to dysmotility in IBD (98).

INFLAMMATION-INDUCED CHANGES IN COLONIC MOTOR CIRCUITRY

Inflammation leads to pathophysiological changes in the enteric neuronal circuit that regulates colonic motility (**Figure 1.8**) (99). In the mucosa, there is an increase in serotonin availability due to a an increased number of enterochromaffin (EC) cells and a decrease in serotonin reuptake transporters (100-102). Inflammation induces IPAN hyperexcitability in different inflammation models (103-104) characterized by a reduction in the AHP that enhances the excitability of these neurons. The increased excitability in IPANs involves a colitis-induced augmentation of the hyperpolarization-activated cation current and an increase in cyclooxygenase-2 (COX2) enzyme activation (103,171). Simultaneously, inflammation enhances ascending and descending interneuronal synaptic transmission due to an increase in the readily releasable pool of neurotransmitters (105). Although the ascending excitatory neuromuscular transmission is unchanged with inflammation, the descending inhibitory neuromuscular transmission is attenuated due to a decrease in purinergic release from inhibitory motor neurons (106). This reduction in purinergic neurotransmission is due to an oxidative stress-induced reduction in mitochondrial purine synthesis in nerve terminals of the inflamed

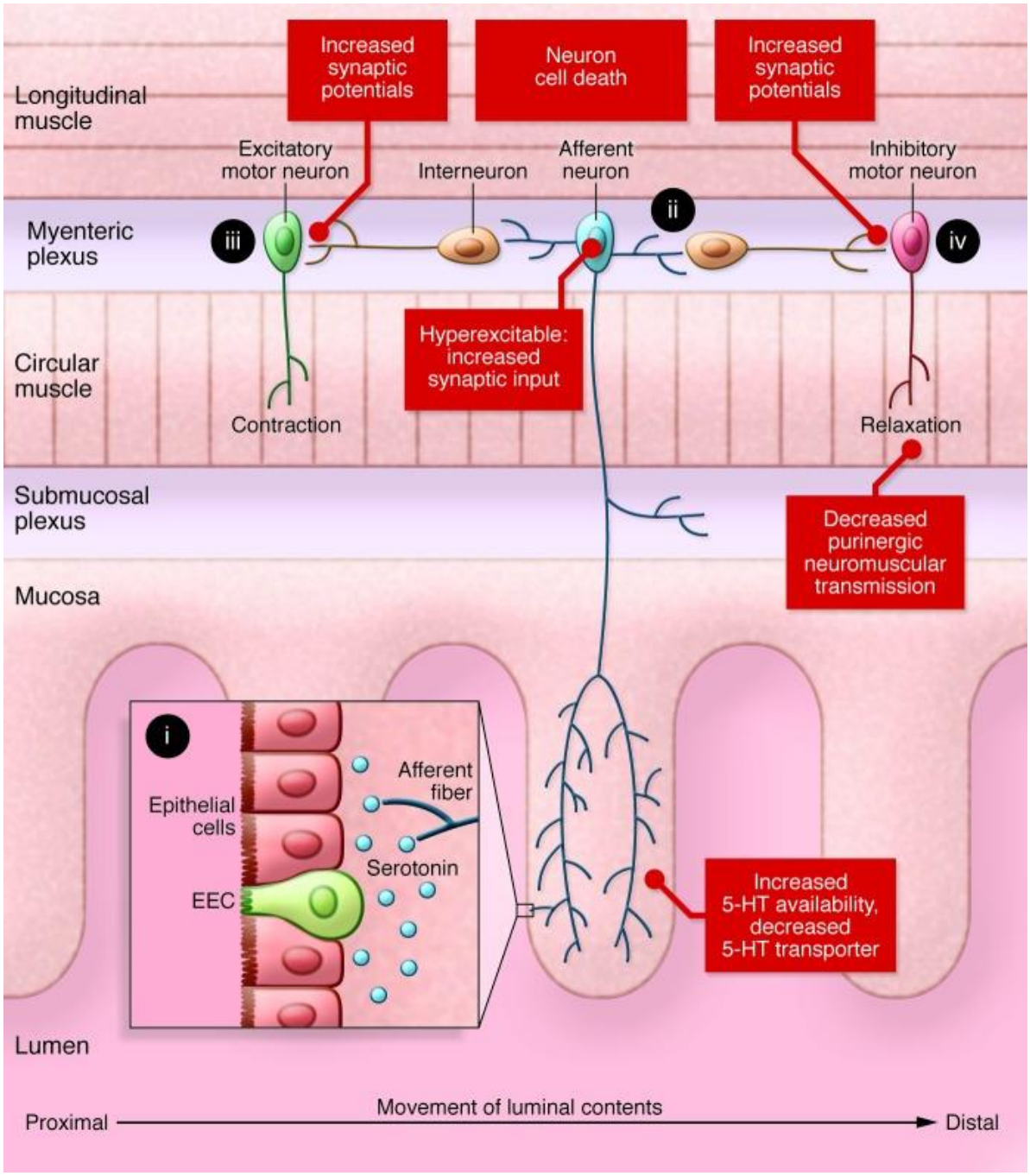

Figure 1.8: Inflammation-induced changes in intrinsic neuronal circuits that regulate peristalsis. Inflammation is characterized by increased serotonin availability om the mucosa, increased excitability of IPANs, facilitation of interneuronal synaptic activity, decreased purinergic neuromuscular transmission, and loss of myenteric neurons. Figure from Mawe 2015 (99).

colon (172). In addition, inflammation is associated with an estimated 20% loss of myenteric neurons (15,28,107). This neuron loss is an early event in the pathogenesis of inflammation (15) and involves activation of a glial signaling pathway requiring P2Y1 receptor activation and connexin 43 hemichannel activity and subsequent activation of a neuronal signaling complex composed of P2X7 receptors, pannexin-1 channels, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and caspases (28,107).

These inflammation-induced changes in neuronal physiology lead to significant alterations in peristalsis (108). In inflamed regions, IPANs are spontaneously activate and synaptic activity is enhanced, leading to overlapping excitatory and inhibitory signals throughout the inflamed region. Additionally, inhibitory neuromuscular transmission is attenuated and the decrease in neuronal populations leads to mixed signaling in the inflamed region during peristalsis (99).

ENTERIC GLIA

Enteric glia are a unique type of non-myelinating peripheral glial cell found throughout all the layers of the gut wall that surround enteric neurons and play important roles in neuronal circuits in the ENS. Enteric glia detect neuronal activity (26, 109-113) through the expression of receptors for multiple neurotransmitters (23-24) and glial activation modulates neuronal circuit activity (25-27).

Enteric glia derive from neural crest precursors that colonize the intestinal tract between embryonic (E) days 9 and 13.5 in mice (114-115). Glial precursors cells are

detected by E11.5 and express markers for terminally differentiated glial cells by E14.5 including the calcium-binding protein S100β and GFAP (116-117). Enteric glia resemble astrocytes morphologically; they display an irregular, stellar morphology dominated by extensive, highly branched processes (**Figure 1.9**). Enteric glial cells have small cell bodies with large nuclei (2-3μm in diameter in guinea pig myenteric glia) and very complex processes which, similar to astrocytes, receive synaptic contacts from neurons, indicating these are the primary points of neuron-glia communication (24). Importantly, not all astrocytic properties can be generalized to enteric glia and vice versa. Enteric glia are derived from the neural crest while astrocytes are derived from precursor cells in the neural tube (118). Furthermore, enteric glia lack expression of key astrocytic properties such as aldehyde dehydrogenase 1 family member L1 (Aldh1L1) (119) and express non-astrocytic molecules like SOX10 (120). Therefore, although enteric glia share similarities with astrocytes, they are a distinct glial cell.

Enteric glia to neuron ratio varies depending on the gut region and species (121). Generally, the number of myenteric glia is equal or greater than the number of myenteric neurons while the reverse is seen in the submucosal plexus (122). In mice, the model used in this dissertation, enteric glia and neurons have a 1:1 ratio in the myenteric plexus. In guinea pigs, a widely used model in gastroenterology, the enteric glia to neuron ratio is closer to 2:1 in the myenteric plexus. This ratio of glia to neurons increases with species size. Humans have up to seven times more myenteric glia than neurons. This difference may highlight increasingly complex roles of enteric glia in higher order mammals and is worth considering when translating from animal models to human disease.

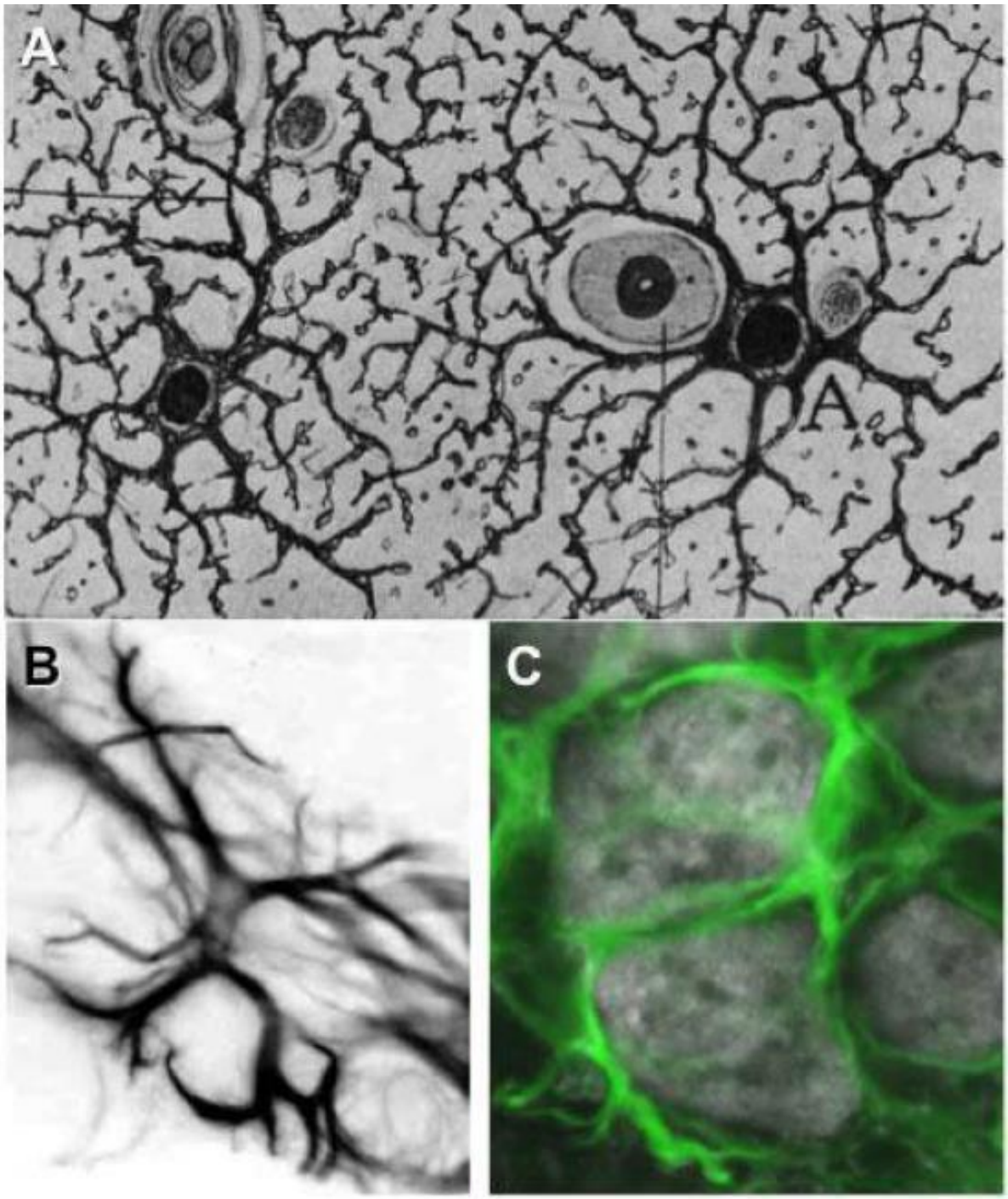

Figure 1.9: Enteric glial morphology resembles astrocyte morphology. (**A**) Drawing of astrocytes by Santiago Ramon y Cajal. (**B-C**) Representative images of enteric glial cells in the myenteric plexus of the mouse colon labeled with GFAP (black in **B** and green in **C**). Figure from Gulbransen 2014 (24).

Enteric Glial Receptors

Enteric glia express numerous receptors allowing them to “listen to” neuron to neuron communication, respond to changes in neurotransmitter concentration, and may mediate glia to glia communication (**Table 1.2**) (123). To date, enteric glia are known to express receptors for adenosine diphosphate (ADP; P2Y1 receptors) (28,123), adenosine triphosphate (ATP) and uridine triphosphate (UTP) (both through P2Y4 receptors) (109), adenosine (A2B receptors) (124), norepinephrine (α2a adrenergic receptors) (125), glutamate (mGluR5, AMPA, NMDA) (126-127), serotonin ($5\text{-}HT_2$) (113), bioactive lipids (SP1R, LPA1) (128-129), endothelin (likely ETB receptors) (46), protease-activate receptors (PAR1, PAR2) (130) and bradykinin (B2 receptors) (131).

Enteric Glial Channels

Similarly, enteric glia express a number of cell surface ion channels. Enteric glia express both voltage-gated sodium and potassium channels (132-134), and aquaporin-4 channels (**Table 1.2**) (135). However, their functional significance is not well understood. Similar to astrocytes, enteric glia also express connexin-43 (Cx43) hemichannels and we have previously shown that glial Cx43 hemichannel activity is important for glia to glia signaling and in mediating enteric neuron death in inflammation (28,123).

Channels		**References**
Voltage-gated sodium	Type unknown	132-133
Delayed rectifier potassium	Kv1.1 and Kv1.2	132-134
Inward rectifier potassium	Type unknown	133
Aquaporins	Aquaporin-4	135
Connexins	Connexin 43 hemichannels	123
Neurotransmitter receptors		
ATP	P2Y4	109,111
ADP	P2Y1	107,110,123
UTP	P2Y4	109,111
Adenosine	A2B	124
Glutamate	mGluR5	126
Norepinephrine	A2a	125
Bioactive lipids	SP1R, LPA1	128-129
Interaction with vasculature		
Endothelin	ETB	130
Bradykinin	B2	131
Protease-activated receptors	PAR1, PAR2	142
Neurotransmitter uptake/degradation		
Small peptides	PEPT2	147
GABA	GAT2	148
Ectonucleotidases	eNTPDase2	145-146

Table 1.2: Summary of known receptors, channels, transporters and releasable factors expressed by enteric glial cells.

Table 1.2 (cont'd)

Trophic Factors		
Receptors	TrkB	173
Growth Factors	Pro-epidermal growth factor, nerve growth factor	127
Immune interactions		
Toll-like receptors	TLR4	174
Major histocompatibility complex	HLA-DR class II complex	175
Releasable factors		
Purines	ATP	28,136
Nitric oxide	iNOS	28,137,174
Antioxidants	15-deoxy-$\Delta^{12,14}$-prostaglandin J2, S-nitrosoglutathione (GSNO)	139-140
Prostaglandins	Prostaglandin E(2)	131
Cytokines	Interlukin-1β, TGF-β, Interlukin-6	161,176-177

Table modified from Gulbransen 2014 (24). A2A (α-2 adrenergic receptor), A2B (adenosine 2B receptor), ADP (adenosine diphosphate), ATP (adenosine triphosphate), B2 (bradykinin receptor B2), eNTPDase2 (ecto-nucleoside triphosphate diphosphohydrolase 2), ETB (endothelin receptor type B), GABA (γ-aminobutyric acid), GAT2 (GABA transporter 2), HLA-DR (Human Leukocyte Antigen- antigen D related), iNOS (inducible nitric oxide), LPA1 (lysophosphatidic acid receptor), mGluR5 (metabotropic glutamate receptor 5), PAR1 (protease activated receptor 1), PAR2 (protease activated receptor 2), PEPT2 (peptide transporter 2), SP1R (sphingosine-1-phosphate receptor), TGFβ (transforming growth factor beta), TLR4 (toll-like receptor 4), TRK (tyrosine receptor kinase), UTP (uridine triphosphate peptide)

Enteric Glial Releasable Factors

Similar to astrocytes, enteric glia were classically viewed as passive supporting cells but recent evidence has suggested enteric glia can release compounds to modify surrounding cells (**Table 1.2**). The most well characterized substance released from enteric glia is ATP (136). Enteric glia release ATP through Cx43 hemichannel activity and ATP release triggers intracellular Ca^{2+} waves from neighboring glial cells (24,123). In addition, selectively attenuating glial ATP release by deleting glial Cx43 hemichannels alters GI motility (123). Importantly, ATP release through glial Cx43 hemichannels drives neuron death in the myenteric plexus during inflammation (24). These findings suggest that glial ATP release plays important roles in modulating physiological and pathological pathways in the ENS.

Enteric glia are also capable of producing nitric oxide (NO); the main inhibitory neurotransmitter in the ENS. Physiologically, glial derived NO modulates epithelial ion transport in mice (137). Importantly, inflammation drives glial NO production which potentiates ATP release through Cx43 hemichannels and contributes to neuroinflammation (24). Similarly, enteric glia also produce prostaglandins such as prostaglandin E (2) (PGE2) (138) which may act to modulate neurotransmission or contribute to disease development, but the role of PGE2 in the ENS is currently unknown.

Lastly, enteric glia also secrete neuroprotective compounds such as reduced glutathione and the prostaglandin derivative 15-deoxy-$\Delta^{12,14}$-prostaglandin J2, S-nitrosoglutathione (**Table 1.2**) (139-140). Importantly, enteric glia produce these compounds under physiological conditions. This suggests that enteric glial mediators can

promote neuron health and modify function under physiological conditions but pathological stimuli may drive release of toxic compounds from enteric glia.

FUNCTIONAL ROLES OF ENTERIC GLIA

Regulation of Neurotransmission

Enteric glial activity is recruited by multiple neurotransmitters and modulators in the ENS. ATP and related purines are the most ubiquitous signaling molecules involved in neuron-to-glia transmission (24,26,107,109-111,123,141), but enteric glia have the potential to detect and respond to multiple substances including norepinephrine (NE) (125), glutamate (126), thrombin (142), lipid-signaling molecules (128-129), serotonin (111,113), bradykinin (111), histamine (111) and endothelin (130). How glial signaling mechanisms affect neurotransmission is still not fully understood but recent evidence demonstrates that activation of glial Ca^{2+} responses modulates neuronal circuits that underlie colonic peristalsis (25).

The close association between enteric glial processes and enteric neurons in the ganglia suggests that enteric glia can function to modulate enteric neurotransmission by either regulating the availability of neurotransmitters or by releasing neuroactive substances upon stimulation in a process called gliotransmission. Indeed, Ca^{2+} responses in enteric glia drives ATP release in vitro (136) and propagation of glial Ca^{2+} responses depends on ATP release through Cx43 hemichannels (123,136). Importantly, the conditional ablation of glial Cx43 hemichannels delays GI transit in vivo and selective elimination of enteric glia alters intestinal motility in female mice (123,178). In addition,

direct activation of enteric glia modulates excitatory enteric circuits, enhances colonic motility in vivo and contributes to the regulation of electrogenic ion transport in the intestine (25,27). These findings provide strong evidence that enteric glia have the potential to release gliotransmitters that can influence gut function through actions on enteric neurons.

Support Of Enteric Neurons

Enteric glia are actively involved in homeostatic regulation to maintain and support enteric neurotransmission (143). Enteric glia provide essential precursors for the synthesis of neurotransmitters including NO (144), glutamate and γ-amino butyric acid (GABA) (117). In health, enteric glia supply enteric neurons with antioxidants such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 and S-nitrosoglutathione (140) and growth factors such as pro-epidermal growth factor and nerve growth factor (127). Additionally enteric glia can regulate the bioavailability of neurotransmitters in the extracellular environment. Enteric glia express cell surface enzymes such as ectonucletidases (145-146) and transporters for peptides (147) and GABA (148) which are essential for removing neuroactive compounds from the environment. Similarly, glial potassium channels can regulate and buffer extracellular potassium and prevent excitotoxic neuron death (133-134). Thus, enteric glia are essential for homeostatic regulation in enteric ganglia and disruption of enteric glial cells could alter GI physiology.

ENTERIC GLIA IN PATHOPHYSIOLOGY

Enteric glia are emerging as key mediators of GI pathophysiology (149). 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.

Morphological Changes

Changes in expression of glial markers such as GFAP, S100β and SOX10 are traditionally measured to indicate glial alterations in disease. In the gut, changes in glial markers are linked with IBD (Crohn's and ulcerative colitis) (150-151), CIPO (152), slow-transit constipation (153), diverticular disease (154), necrotizing enterocolitis (53), Chagas disease (155) diabetes (156) and Parkinson's disease (157). Although the most common abnormality reported is decreased glial cell density, changes in enteric glia vary widely. For example, GFAP levels are increased in inflamed regions in both Crohn's and ulcerative colitis, but are decreased in uninflamed regions in Crohn's (150-151). Likewise, GFAP levels are increased in Chagas disease while S100β levels are decreased (155). Clearly enteric glia can respond to intestinal pathology, but how changes in glial morphology contribute to disease progression are unknown.

Functional Changes

Although changes in morphology do not necessarily correlate to functional changes, there is evidence that enteric glia are involved in disease pathophysiology. Inflammation alters expression of glial receptors (126) and enzymes (158) and increases glial-proliferation (159). Importantly, inflammation drives glial alterations that contribute to neuron death (28) primarily through ATP release. Additionally, loss of glial neuroprotective functions can result in a decrease in enteric neuronal populations (160). Enteric glia can also contribute to inflammation by secreting proinflammatory cytokines and immunomodulatory signals (161). Lipopolysaccharide (LPS) activation drives glial secretion of multiple cytokines and chemokines including interferon-γ, chemokine ligand 20, and prostaglandin D_2 which could play important roles in the activation and recruitment of immune cells to modulate inflammation (162-164). Together, these data suggest that enteric glia are active participants in gut pathophysiology in inflammation.

SUMMARY AND AIMS OF DISSERTATION

The enteric nervous system (ENS) is the principal regulator of gastrointestinal (GI) function (1). Traditionally, the functional outputs of the ENS have been attributed to enteric neuronal signaling, but recent evidence demonstrates that enteric glial activation modulates the activity of neural circuits (25-27). Disruptions of this neuronal network, due to enteric neuron death , contributes to motility dysfunction in various GI diseases (8,77,99,149). While some mechanisms that contribute to neuron loss and GI dysfunction

are known, there is still a lack in knowledge in other key mechanisms that could be involved in neuroinflammation.

Enteric glia are unique peripheral glial cells that regulate GI function and have important roles in GI pathophysiology (23-24,149). In health, glial activation can influence enteric circuits involved in the regulation of motility and secretion and activation of enteric glia in inflammation drives enteric neuron death through purinergic pathways (25,27-28). 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 the intestine in health and disease. Chapter 2 investigates how communication between enteric glia, neurons and nociceptors underlies the effects of tachykinins on neuroinflammation. Chapter 3 studies the contribution of cholinergic activation on enteric glia in gastrointestinal physiology and pathophysiology. The final data chapter, Chapter 4, studies the role of GFAP in enteric glial responses to inflammation. Together, these chapters aim to improve the understanding of glial signaling mechanisms that contribute to GI physiology and pathophysiology. Further, this dissertation serves to improve broader understanding of neuron-glia interactions in health and disease.

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CHAPTER 2

Communication Between Enteric Neurons, Glia, and Nociceptors Underlies the Effects of Tachykinins on Neuroinflammation

ABSTRACT

Background & Aims

Tachykinins are involved in physiological and pathophysiological mechanisms in the gastrointestinal (GI) tract. The major sources of tachykinins in the gut are intrinsic enteric neurons in the enteric nervous system (ENS) and extrinsic nerve fibers from the dorsal root and vagal ganglia. Although tachykinins are important mediators in the ENS, how they contribute to neuroinflammation through effects on neurons and glia is not fully understood. Here, we tested the hypothesis that tachykinins contribute to enteric neuroinflammation through mechanisms that involve intercellular neuron-glia signaling.

Methods

We used immunohistochemistry and quantitative real-time polymerase chain reaction (qPCR) to study tachykinin and tachykinin receptor expression, and selectively studied cellular activity using genetically encoded calcium indicators in *TRPV1*$^{tm1(cre)Bbm}$ /J::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ and *Sox10*CreER^{T2}::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice or Fluo-4. We probed glial signaling pathways using *Sox10*::CreER$^{T2+/-}$ / Cx43$^{f/f}$ mice and selective drugs. We used the 2,4-di-nitrobenzene sulfonic acid (DNBS) model of colitis to study neuroinflammation, glial reactivity, and neurogenic contractility. We used *Sox10*::CreER$^{T2+/-}$ / *Rpl22*$^{tm1.1Psam}$/J mice to selectively study glial transcriptional changes.

Results

Tachykinins are predominantly expressed by intrinsic neuronal varicosities while NK2Rs are predominantly expressed by enteric neurons and nociceptive neurons. Stimulation of

NK2Rs drives responses in neuronal varicosities that are propagated to enteric glia and neurons. The NK2R antagonist GR 159897 enhanced recovery from DNBS colitis and prevented the development of reactive gliosis, neuroinflammation and enhanced neuronal contractions. Inflammation drove changes in enteric glia gene expression and function and antagonizing NK2R signaling mitigated these changes in larger-scale cellular function. NKA-induced neurodegeneration requires glial Cx-43 hemichannel activity.

Conclusions

Our results show that tachykinins drive enteric neuroinflammation through a multicellular cascade involving enteric neurons, nociceptors, and enteric glia. Therapies targeting components of this pathway could broadly benefit the treatment of dysmotility and pain following acute inflammation in the intestine.

INTRODUCTION

Functional gastrointestinal disorders (FGIDs) are highly prevalent disorders characterized by dysmotility and visceral hypersensitivity (1). Irritable bowel syndrome (IBS) is the most common FGID affecting approximately 11% of the population (2). Broad abnormalities in the brain-gut axis contribute to the pain component of FGIDs (3), but it is clear that changes in gut motility are primarily driven by alterations to the enteric nervous system (ENS). The enteric nervous system (ENS) is a complex network of enteric neurons and glia embedded within the gut wall that provides local control over reflexive gut functions such as fluid exchange across the mucosa, local blood flow and patterns of motility (4). Inflammation can disrupt the control of these gastrointestinal (GI) reflexes by altering both the function and survival of enteric neurons that then contributes to the development of FGIDs (5). Despite having a clear involvement in the pathogenesis of inflammation, the mechanisms that drive and sustain ENS dysfunction remain unresolved.

Inflammation is a particularly potent driving force for the induction of neuroplasticity in the periphery and contributes to both altered motility and the sensitization of peripheral nociceptors in functional GI disorders (5,6). Histamine, proteases, poly-unsaturated fatty acid metabolites and tachykinins are now recognized as some of the key inflammatory mediators that modify peripheral neurons in functional GI disorders (7-9). Tachykinins are particularly important because of the prominent roles that they play in both pain transmission and intestinal motility (10,11).

Tachykinins, including substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), are important neuropeptide mediators that contribute to GI functions such as motility and secretion, and pathophysiological processes that contribute to gut

inflammation and visceral pain (10,11). Enteric neurons are the major source of tachykinins in the gut followed by innervating nerve fibers from the dorsal root and vagal ganglia and to a lesser extent, immune and enterochromaffin cells (12). There are at least eight types of tachykinin-immunoreactive neurons in the ENS, with the majority of tachykinin containing neurons of intrinsic origin, primarily intrinsic primary afferent neurons (IPANs), oral and aboral projecting interneurons, and motor neurons projecting to the GI smooth muscle (12). The effects of SP, NKA and NKB are preferentially mediated through activation of neurokinin-1 (NK1R), neurokinin-2 (NK2R) and neurokinin-3 (NK3R) receptors, respectively (13). Physiologically, tachykinins function as co-neurotransmitters of excitatory neurons innervating the muscle (14); serve as transmitters of the ascending contractile component of peristaltic reflexes (15); are involved in neuro-neuronal transmission between IPANs and interneurons (11); and have been proposed to be involved in the transmission of sensory information from the gut to the central nervous system (12). During inflammation, tachykinins induce secretion of proinflammatory cytokines in the mucosa (16); contribute to secretion associated with inflammation (17) and increased NKA has been shown to produce visceral hypersensitivity in rats (19).

Given their important roles in gut physiology and disease, there is increasing interest in targeting tachykinin receptors for therapeutic benefit. Importantly, antagonists for the neurokinin-2 receptor (NK2R) have been proven to reduce visceral pain associated with inflammation in rats (19). Recent success in clinical trials with the drug ibodutant, a neurokinin-2 receptor antagonist, show that tachykinins acting through NK2Rs play an important role in the sustained pathophysiology of diarrhea-predominant IBS (20).

Although these findings are exciting, the mechanisms underlying the beneficial actions of NK2R antagonists in FGIDs are still poorly understood.

Here, we tested the hypothesis that the effects of tachykinins on neuroinflammation are mediated, in part, by signaling between enteric neurons, glia and nociceptors in the ENS. We tested our hypothesis by studying the expression and function of tachykinin peptides and receptors with immunohistochemistry, molecular biology, and calcium (Ca^{2+}) imaging. We found that SP and NKA are predominantly expressed by neuronal varicosities in close proximity to enteric glia, and NK2Rs are predominantly expressed by extrinsic and intrinsic neuronal varicosities surrounding enteric glia. Stimulation of NK2Rs with NKA produces robust activity from transient receptor potential vanilloid-1 (TRPV1) positive varicosities, enteric glia and enteric neurons throughout the myenteric plexus and glial response to NKA is critically dependent upon the activation of both NK2Rs and connexin-43 (Cx43) hemichannels. We further tested our hypothesis by assessing the effects of NK2R antagonism in an *in vivo* model of colitis and found that NK2R antagonism protected against glial reactivity and enteric neurodegeneration associated with colitis. Collectively, our results show that activation of NK2Rs is a critical mechanisms that drives neuron-to-glia signaling and reducing enteric neuroinflammation and glial reactivity likely contributes to the beneficial effects of NK2R antagonist drugs during inflammation.

MATERIALS AND METHODS

Declaration of Animal Use Approval

All work involving animals was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University (MSU).

Animals

Male and female C57BL/6 mice between 8-10 weeks of age were used for experiments unless stated otherwise (Charles River Laboratories, Hollister, CA). All mice were maintained on a 12-hour light/dark cycle in a temperature-controlled environment with access to food and water *ad libitum*. Transgenic mice expressing the genetically-encoded Ca^{2+} indicator GCaMP5g in enteric glia (*Sox10*CreER^{T2}::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$) were bred in house and were generated as previously described (21) by crossing *Sox10*::CreER$^{T2+/-}$ mice (gift from Dr. Vassilis Pachnis, The Francis Crick Institute, London, England) with *Polr2a*$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice [PC::G5-tdT(Jackson Laboratory, Bar Harbor, ME; stock number: 024477; RRID: IMSR_JAX:024477)]. Transgenic mice expressing the genetically-encoded Ca^{2+} indicator GCaMP5g in nociceptors (T*RPV1*$^{tm1(cre)Bbm}$ /J::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$) were bred in house and were generated by crossing B6.129-*Trpv1*$^{tm1(cre)Bbm}$/J mice [TRPV1^{Cre}(Jackson Laboratory; stock number: 017769; RRID: IMSR_JAX:017769)] with *Polr2a*$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice. Mice with a tamoxifen-sensitive deletion of connexin-43 in enteric glia (*Sox10*::CreER$^{T2+/-}$ / Cx43$^{f/f}$) were also bred in house and

generated as previously described (22) by crossing *Sox10*::CreER^{T2} mice with *Gja1*^{tm1Dlg} mice [Cx43$^{f/f}$ (Jackson Laboratory; stock number: 008039; RRID: IMSR_JAX:008039)]. Mice with a tamoxifen-sensitive targeted mutation of the ribosomal protein L22 (*Rpl22*) in enteric glia (*Sox10*::CreER$^{T2+/-}$ / *Rpl22*$^{tm1.1Psam}$/J mice) were also bred in house and generated by crossing *Sox10*::CreER^{T2} mice with *Rpl22*$^{tm1.1Psam}$/J mice [RiboTag (Jackson Laboratory; stock number: 011029; RRID: IMSR_JAX:011029)]. All double transgenic mice were maintained as hemizygous for Cre (*Sox10*::CreER$^{T2+/-}$) and homozygous for the floxed allele (*Polr2a*$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$, TRPV1^{Cre}, Cx43$^{f/f}$ or *Rpl22*$^{tm1.1Psam}$/J). CreER^{T2} activity was induced in *Sox10*CreER^{T2}::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice by 4 intraperitoneal (i.p.) injections of 4-hydroxitamoxifen (0.1mg/g) every 12 hours 7 days prior to conducting experiments. CreER^{T2} activity was induced in *Sox10*::CreER$^{T2+/-}$ / Cx43$^{f/f}$ and *Sox10*::CreER$^{T2+/-}$ / *Rpl22*$^{tm1.1Psam}$/J mice by feeding the animals with chow containing tamoxifen citrate (400 mg/kg) for 2 weeks followed by 1 week of normal chow before use. Genotyping was performed by the Research Technology Support Facility (RTSF) Genomics Core at MSU and Transnetyx (Cordova, TN).

Whole-Mount Immunohistochemistry

Longitudinal muscle–myenteric plexus (LMMP) whole-mount preparations were microdissected from mouse colonic tissue preserved in Zamboni's fixative. Processing of LMMPs via immunohistochemistry was conducted as described previously (23) with the primary and secondary antibodies listed in Tables 1 and 2, respectively. Briefly, LMMP preparations underwent three 10-minute washes in 0.1% Triton X-100 in phosphate-

buffered saline (PBS) followed by a 45-minute incubation in blocking solution containing 4% normal goat or donkey serum, 0.4% Triton X-100 and 1% bovine serum in PBS. Preparations were incubated with primary antibodies for 48 hours at 4°C and secondary antibodies for 2 hours at room temperature before mounting. Antibody specificity was confirmed by preadsorption with the corresponding control peptides. Fluorescent labeling was visualized by confocal imaging through the Plan-Apochromat 60x oil immersion objective (1.42 numerical aperture) of an inverted Olympus Fluoview FV1000 microscope (Olympus, Center Valley, PA).

Myenteric Whole Mount Tissue Culture

LMMP whole-mount preparations were microdissected from mouse colons and incubated in DMEM in 95% air, 5% CO_2 at 37ºC for 3 days. DMEM was changed daily. After 3 days, samples were fixed overnight in Zamboni's fixative and processed for immunohistochemistry.

Ca^{2+} Imaging

LMMP whole-mount preparations were microdissected from mouse colons and incubated for 15 minutes at room temperature in an enzyme mixture consisting of 150 U/mL Collagenase type II and 1 U/mL Dispase (Life Techonologies). Samples from C57BL/6 and *SOX10*::CreER$^{T2+/-}$/Cx43$^{f/f}$ mice were loaded in the dark with 4 μM Fluo-4 AM, 0.02% Pluronic F-127 and 200 μM Probenecid (Life Technologies) in DMEM at 37°C (13). Images were acquired every 1- 2 seconds through the 40X water immersion objective [LUMPlan N, 0.8 numerical aperture (n.a.)] of an upright Olympus BX51WI fixed stage

microscope (Olympus, Center Valley, PA) using IQ3 software, MetaMorph or NIS Elements software and a Neo sCMOS camera or a Andor Zyla sCMOS camera (Andor, South Windsor, CT). We continually perfused whole-mounts at 2-3 mL min^{-1} with buffer using a gravity flow perfusion system. Agonists were dissolved in buffer and bath applied for 30 seconds. Antagonists were dissolved in buffer and applied for either 20 minutes or 3 minutes before imaging.

Mouse Model of Colitis

Acute colitis was induced in male and female mice under isoflurane anesthesia by an enema of 0.1 mL of a solution containing 5 mg di-nitrobenzene sulfonic acid (DNBS) dissolved in 50% ethanol (24). Control animals were given enemas of saline. Subsets of mice received daily injections (i.p.) of either 0.5 mg/kg GR159897 (Tocris, Minneapolis, MN) in 0.1% dimethyl sulfoxide (DMSO) or 0.1% DMSO over 1, 3, 7 or 21 days beginning 24 hours prior to the induction of colitis. Mice were monitored closely and weight loss was recorded daily for the first week and once every other day for the following 2 weeks. Mice were sacrificed at 24 hours, 48 hours, 7 days or 3 weeks post induction of colitis. Macroscopic damage was recorded with an established scoring system (25).

Haemotoxylin and Eosing (H&E) staining

Whole colonic tissue was collected 24 hours after induction of colitis. Tissue was fixed in 10% neutral buffered formalin for 72 hours. 4 mm thick sections were cut and rinsed in 50% ethanol for 24 hours. Tissue was processed and stained by the Investigative Histopathology Laboratory at MSU. For each section, the following parameters were

scored: loss of mucosal architecture (0, 1, 2 or 3 from mild to severe); cell infiltration (0 = none, 1 = in muscularis mucosae, 2 = in lamina propria/villi, 3 = in serosa); muscle thickening (0 = < ½ of mucosal thickness, 1 = ½ - ¼ of mucosal thickness, 2 = mucosal thickness, 3 = full thickness); goblet cell depletion (0 = absent; 1 = present) and crypt abscess formation (0 = absent, 1 = present).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Colonic tissue was flash frozen and total messenger RNA (mRNA) was isolated from 1 mg colon samples using the RNeasy Micro Kit (Qiagen, Valencia, CA) and reverse transcribed (High-Capacity cDNA Reverse Transcript Kit; Thermofisher) per the manufacturer's protocol. qPCR was performed using a Taqman gene expression assay for mouse GFAP, TACR-1, TACR-2, and TAC-1 genes. Amplification was performed by the MSU RTSF Genomics Core. Fold changes were calculated using the 2-ΔΔCT method (55) and normalized to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Contractility Studies

Mouse colons were collected 7 days following the induction of colitis. Longitudinally oriented muscle strips were mounted in organ baths, and one end was attached to a force transducer (Grass Instruments, Quincy, MA). Electrical field stimulation (EFS) was supplied by two platinum electrodes and a GRASS stimulator (S88; GRASS telefactor, West Warwick, RI). Data were charted with LabChart 8 software (ADInstruments, Colorado Springs, CO) as described previously (26). Tissue segments were equilibrated for 20 minutes under 0.5 g initial tension. Neurogenic relaxations were studied in tissues

precontracted with 5 µM prostaglandin F2-α ($PGF_{2\alpha}$). Relaxations were induced when the contractile response to $PGF_{2\alpha}$ was stable for at least 5 minutes.

Glial Morphology Analysis

We assessed changes in glial morphology using the FIJI-Image J Simple Neurite Tracer (SNT) plugin as described by Tavares et al. (2016) (27). After immunostaining with GFAP, Z-stacks of confocal images were obtained using a 60x objective and 1 µm z-step interval. We analyzed a total of 16 enteric glia per treatment group. Total process length was determined by the sum of the length of individual paths reconstructed, process thickness was estimated from GFAP thickness provided by "Fill Out" Analysis and morphology complexity was estimated from the number of intersections at each radial distance from the starting point provided by the Sholl analysis.

In Situ Model of Neuroinflammation

Enteric neuron death was driven as previously described (28). LMMP preparations were incubated with the P2X7R agonist 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (BzATP) (300µM) or the NK2R agonist neurokinin-A (1µM) for 2 hours in 95% air, 5% CO_2 at 37ºC. LMMP preparations were then rinsed with fresh buffer, incubated for an additional 2 hours in buffer only, and fixed overnight in Zamboni's fixative.

RiboTag Procedure

For immunoprecipitation, 200 µL of protein A/G magnetic beads (Thermofisher Scientific, Waltham,WA) were washed 3 times with 500 µL homogenization buffer before coupled

directly to 5 µL mouse monoclonal anti-HA antibody for 2-hours before they were added to homogenates. Homogenates were prepared as follows: Colonic tissue from *Sox10*::CreER$^{T2+/-}$ / *Rpl22*$^{tm1.1Psam}$/J mice was removed and placed in a bath containing DMEM/F-12 maintained at 4ºC. Luminal contents were flushed, and a plastic rod (3.2 mm-diameter) was inserted into the lumen. The LMMP was isolated from the underlying circular muscle by gently removing the mesenteric border and gently teasing away the LMMP using a cotton swab wetted with cold DMEM/F-12. LMMP preps were then flash frozen before homogenization. Samples were homogenized in 1 mL ice cold supplemented homogenization buffer adjusted for enteric neuron use and centrifuged at 10,000g for 10 minutes at 4ºC (63-64). Supernatants (800 µL) were then added directly to the antibody-coupled protein A/G magnetic beads and rotated overnight at 4ºC. The following day, samples were placed in a magnet on ice and the pellet was washed three times with 800 µL high salt buffer. After washing, 350 µL of Qiagen RLT buffer was added to the pellet, vortexed for 30 s and supernatant was recovered and flash frozen for RNA extraction. Total RNA was prepared according to manufacturer's instructions using an RNeasy Mini kit (Qiagen). RNA quality and sequencing were performed by LC Sciences (Houston,TX). Briefly, RNA quality was assessed by 18S and 28S rRNA peaks generated by the 2100 Bioanalyzer and the RNA 6000 Nano LabChip Kit (Agilent, Santa Clara, CA). For sequencing, 1ng mRNA was used to generate cDNA with the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech Laboratories, Mountain View, CA). Paired-end libraries were constructed from 1ng cDNA utilizing Nextera DNA Library Preparation Kit (Ilumina, San Diego, CA) and sequenced on an Illumina Hiseq X Ten following

manufacturer's specification. HISAT2 (65) was used to map reads to mouse genome assembled using StringTie (66).

Solutions

Live tissue was maintained in DMEM/F-12 nutrient mixture (Life Technologies) during collection and microdissection. Ca^{2+} imaging experiments were performed in modified Krebs buffer containing (in mM): 121 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 10 HEPES, 21.2 $NaHCO_3$, 1 pyruvic acid, and 8 glucose (pH adjusted to 7.4 with NaOH). Ribotag experiments were performed with homogenization buffer (50mM Tris, pH 7.4, 100mM KCl, 12mM $MgCl_2$ and 1% Nonidet P-40); supplemented homogenization buffer (50mM Tris, pH 7.4, 100mM KCl, 12mM $MgCl_2$, 1% Nonidet P-40, 100µg/mL cycloheximide, protease inhibitor mix, 3mg/mL heparin, SUPERase RNase inhibitor and 100mM DTT) and high salt buffer (50mM Tris, pH 7.4, 100mM KCl, 12mM $MgCl_2$, 1% Nonidet P-40, 100µg/mL cycloheximide and 0.5mM 1,4-Dithiothreitol).

Chemicals and Reagents

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). GR 159897 (5-Fluoro-3-[2-[4-methoxy-4-[[(*R*)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1*H*-indole), MRS 2500 tetraammonium salt ((1*R**,2*S**)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxyl)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt) and CP 96345 ((2S,3S)-*N*-(2-Methoxyphenyl)methyl-2-diphenylmethyl-1-azabicyclo[2.2.2]octan-2-amine) were purchased from Tocris (Minneapolis, MN). Neurokinin-A was purchased from Abcam (Cambridge, MA). The connexin-43 mimetic peptide, 43Gap 26, was purchased from

Anaspec (Fremont, CA). Di-nitro benzenesulfonic acid (2,4-Dinitrobenzenesulfonic acid dihydrate) was purchased from MP Biomedicals (Solon, OH).

Statistics

For Ca^{2+} imaging, traces represent the average change in fluorescence ($\Delta F/F$) over time for individual cell responses. Integrated density was quantified using the measure function in ImageJ Software, version 1.48 (NIH). Neuron packing density was determined by counting the number of HuC/D-immunoreactive neurons per ganglionic area in 10 ganglia per LMMP preparation using the cell counter plug-in tool on ImageJ. For RNA sequencing, low quality bases and adapter-contaminated reads were removed using Cutadapt (67) and LC sciences in-house perl scripts. Sequence quality was confirmed using the web-based platform FastQC (https://www.bioinformatics.babraham.ac.uk/). Transcript expression levels as FPKMs were estimated using StringTie (66) and differential expression was determined in the R package Ballgown (68) with $P< 0.005$ considered statistically significant. All results are presented as mean ± standard error of the mean (SEM) and statistically significant differences were determined using an analysis of variance (ANOVA) or t-test, as appropriate with $P < 0.05$ considered statistically significant (GraphPad Prism; GraphPad Software, San Diego, CA).

RESULTS

Nerve processes in the myenteric plexus contain tachykinins

The ENS is the major source of intestinal tachykinins (11,12) and SP and NKA are the predominant tachykinins that exert control over motility, secretion, inflammation, and pain (10,12). Although the cellular localization of SP is well characterized in the mouse ENS (29), the localization of NKA is less well defined. Dual-label immunohistochemistry on myenteric whole-mount tissue preparations shows that SP and NKA are localized to nerve varicosities in the myenteric plexus of the mouse colon (**Figure 2.1**). Varicosities containing SP (**Figure 2.1A-A'''**) and NKA (**Figure 2.1B-B'''**) are closely apposed to enteric glia in the myenteric plexus and dual labeling with SP and NKA shows broad overlap between SP and NKA immunoreactivity (**Figure 2.1C-C'''**), implying that these neurokinins are released by the same class of neurons. Varicosities expressing SP in the myenteric plexus of the mouse colon belong to enteric neurons and are of intrinsic in origin (29, 30). In agreement, our results show that varicosities expressing SP are distinct from tdTomato-tagged TRPVI varicosities belonging to extrinsic spinal afferent neurons from T*RPV1*$^{tm1(cre)Bbm}$ /J::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ transgenic mice (**Figure 2.1D-D''**). To confirm varicosities expressing SP and NKA are of intrinsic origin, we cultured myenteric whole-mount tissue preparations for three days to remove extrinsic innervation. Under these conditions, we found that SP and NKA are still present in the myenteric plexus and observed robust expression in neuronal cell bodies (**Figure 2.1E-E'''**). Together, these results show that NKA is localized to varicosities of myenteric neurons that also contain SP in the mouse colon and both NKA and SP are of intrinsic origin.

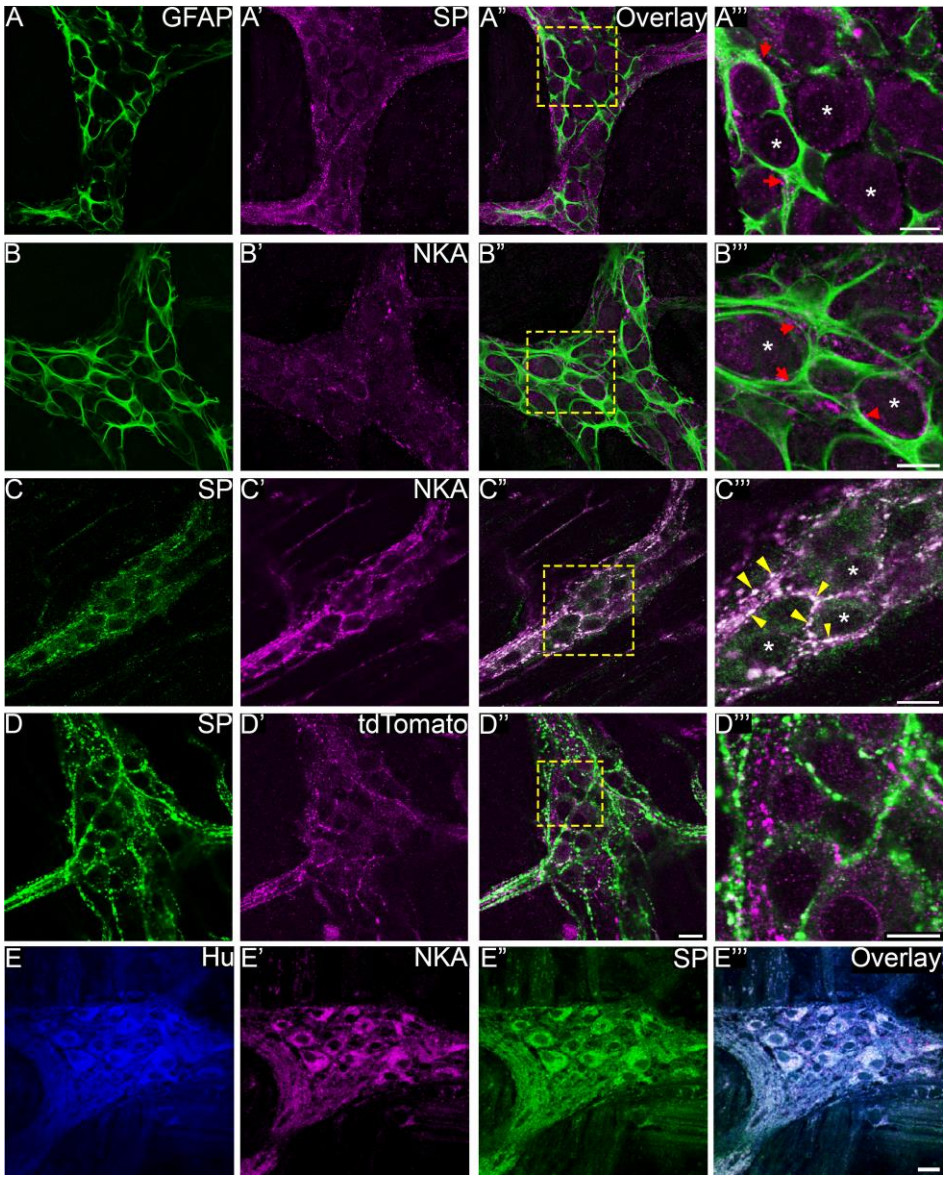

Figure 2.1. Intrinsic varicosities surrounding enteric glia in the mouse myenteric plexus contain substance P (SP) and neurokinin A (NKA). Images are single optical sections (1 μm) through myenteric ganglia from the distal colons of mice. (**A-B**) Varicosities labeled with antibodies against SP (magenta, **A'-A'''**) or NKA (magenta, **B'-B'''**) surround GFAP-immunoreactive enteric glia (green, **A-B**). (**C**) Immunoreactivities for SP (green, **C-C'''**) and NKA (magenta, **C-C'''**) colocalize in the same population of varicosities. (**D**) Varicosities labeled with antibodies against SP (green, **D-D'''**) do not colocalize with tdTomato-tagged transient receptor potential cation channel subfamily V member 1 (TRPV1)-positive varicosities (magenta, **D-D'''**).

Figure 2.1 (cont'd)

Overlays of each combination are shown in **A''- D''**. The scale bar in **D''** represent 20μm and applies to (**A-D''**). Images are representative of labeling performed on tissue from a minimum of three mice. Areas demarcated by the dashed boxes in **A''- D''** are enlarged in panels **A'''- D'''**. Asterisks in **A'''-C'''** are placed within the nuclei of representative neurons. Red arrowheads in **A''' – B'''** highlight varicosities closely associated with enteric glia. Yellow arrowheads in **C'''** highlight areas of colocalization. All scale bars in the enlarged images represent 10μm. (**E - E'''**) LMMP cell culture shows intrinsic expression of tachykinins in the myenteric plexus. Immunoreactivities for NKA (magenta, **E'**) and SP (green, **E"**) colocalize to neuronal cell bodies labeled with antibodies against HuC/D (blue, **E**). The scale bar in **E'''** represents 20μm and applies to (**E-E'''**).

NKA drives neuron and glial Ca^{2+} responses

Neuronal SP release contributes to both central and peripheral neurogenic inflammation (11, 21), but the contribution of NKA to peripheral neurogenic inflammation is not understood. To understand how NKA release from enteric varicosities affects the activity of neurons and glia within the ENS, we used Ca^{2+} imaging in whole mount preparations of myenteric plexus loaded with the Ca^{2+} indicator dye Fluo-4AM. Challenging these samples with 1 µM NKA, the preferred ligand for NK2Rs (13), drove Ca^{2+} responses in both enteric neurons and enteric glia (**Figure 2.2A**). However, the most striking effect was the stimulation of robust activity throughout the glial network that persisted for several minutes (**Figure 2.2A**). Analyzing the time course of neuron and glial responses revealed that some enteric neurons responded prior to the surrounding glia (**Figure 2.2B**), while others responded directly after (**Figure 2.2C**). Individual neurons mounted numerous Ca^{2+} responses (31 peaks on average, average magnitude of 0.48 ±0.17 ∆F/F) (**Figure 2.2D-E**) while individual glial cells mounted numerous individual Ca^{2+} transients (47 peaks on average, average magnitude of 0.49 ±0.11 ∆F/F) (**Figure 2.2D-E**) that were seemingly stochastic. The seemingly random pattern of activity evoked by NKA is in stark contrast to glial responses to other neurotransmitters, like purines, that drive uniform wave-like events through the glial network (**Figure 2.2F-G**) (21,32). This difference may suggest that complex intercellular signaling mechanisms underlie glial response to NKA.

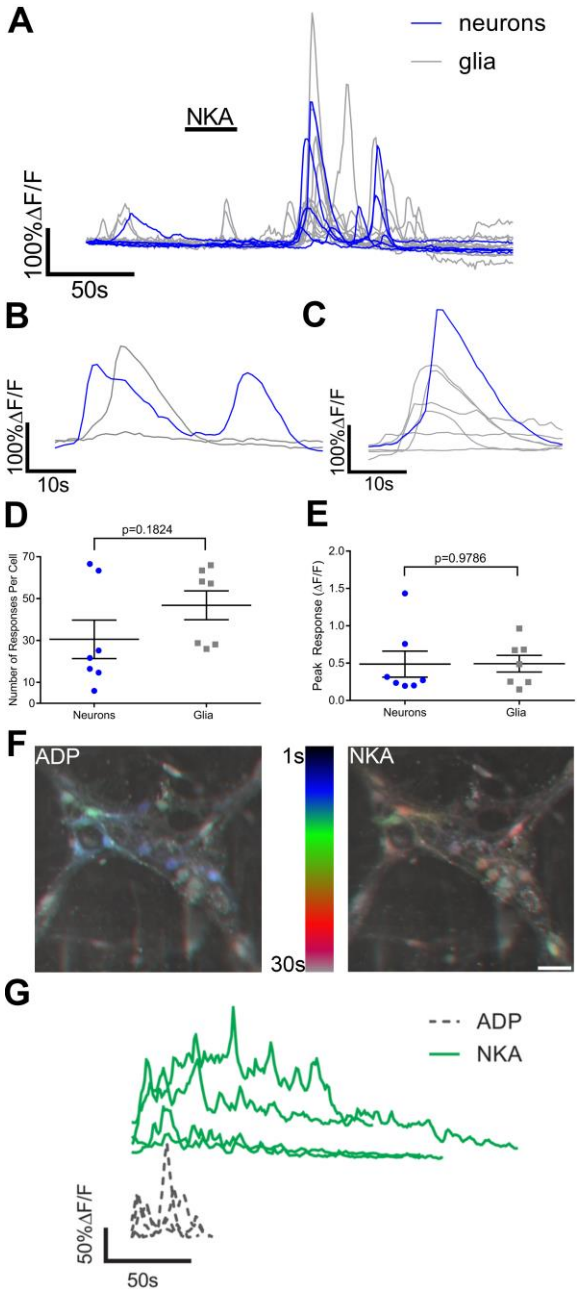

Figure 2.2: NKA drives Ca^{2+} responses in both neurons and enteric glia. (**A**) Representative Ca^{2+} responses evoked by NKA in enteric neurons and glia. Gray traces show the responses of individual glial cells and blue traces show the responses of individual neuronal cells. (**B-C**) Representative Ca^{2+} responses evoked by NKA in a single enteric neuron (blue traces) and the surrounding enteric glia (gray traces) showing examples of a neuron response that proceeds glial responses (**B**) and a neuronal response that follows glial responses (**C**).

Figure 2.2 (cont'd)

(**D**) Number of responses and (**E**) peak response per neuron or glia when challenged with NKA (n=7 cells from 2 mice; Student's t-test). (**F**) Temporal-code images of enteric glial responses to ADP or NKA. Images are Z-projections from peak responses of enteric glia to ADP or NKA where cells that respond first are colored in blue (1s after drug application) and cells that respond later are colored in red (30s after drug application). Note that ADP responding cells respond faster, and at the same time compared to NKA responding cells. Scale bar represents 50 μM. (**G**) Representative glial Ca^{2+} responses evoked by either ADP (dashed gray traces, bottom) or NKA (solid green traces, top).

Given that NKA evokes responses in enteric glia and neurons, we tested if both enteric glia and neurons express the receptors required to detect NKA. To this end, we used triple label immunohistochemistry with antibodies against GFAP and Hu to identify glia and neurons, respectively, in combination with antibodies against NK1Rs or NK2Rs (**Figure 2.3**). In agreement with previous studies, our results show that NK1Rs are predominantly localized to enteric neurons (**Figure 2.3A-A'''**) (29). In addition, we found that NK2Rs are localized to neuronal varicosities that surround enteric glia **(Figure 2.3C-C'''**). Surprisingly, NK2R immunoreactivity also colocalized with TRPV1, a marker of extrinsic primary afferent neurons (**Fig 2.3E-E'''**).

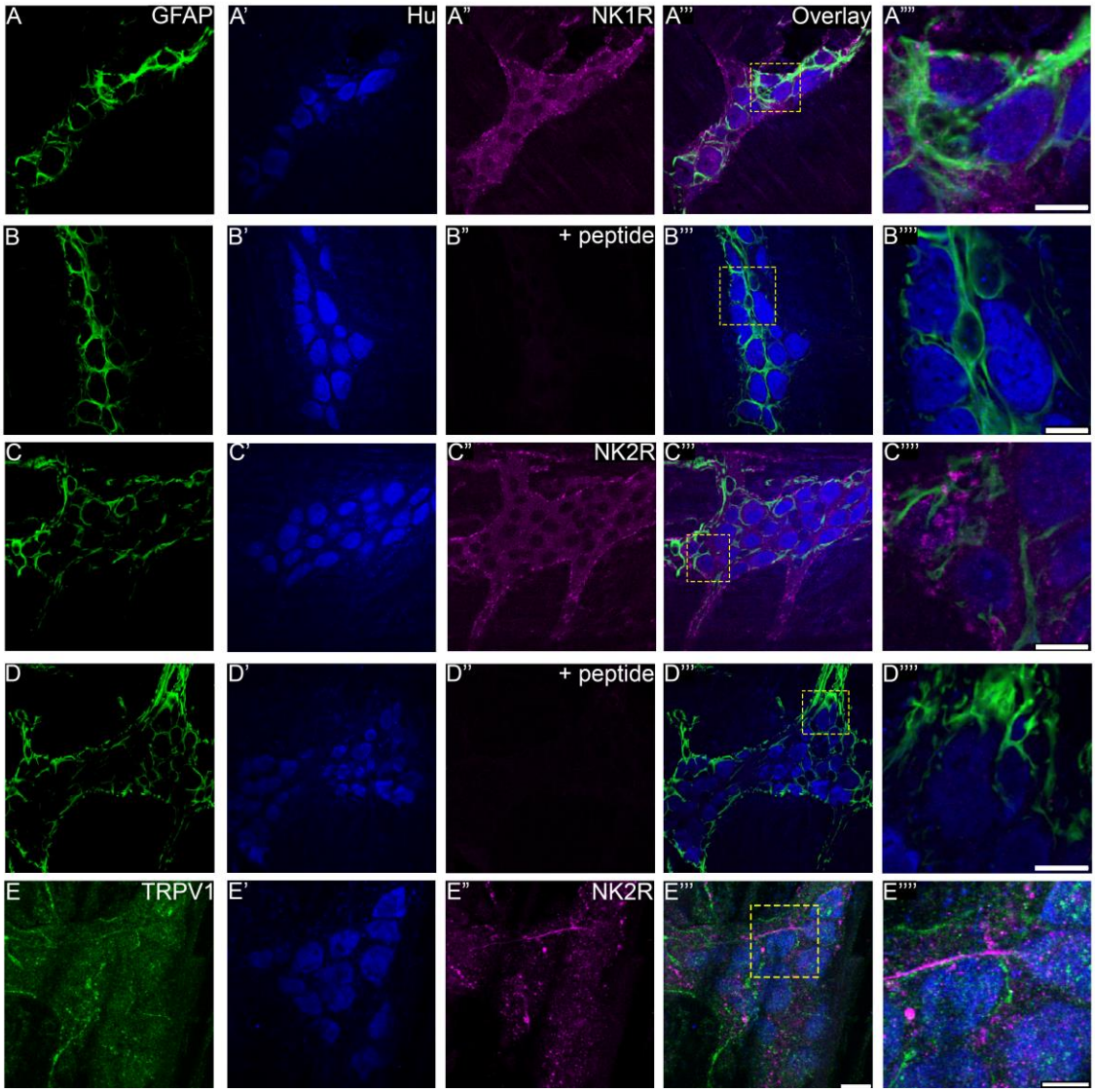

Figure 2.3. NK1R expression is localized to enteric neuron cell bodies and varicosities while NK2R is primarily expressed by neuronal varicosities surrounding enteric glia. Images (confocal single optical planes, 1 μm) show triple-label immunohistochemistry for GFAP (green, **A-D**), Hu (blue, **A'-D'**) and either NK1R (magenta, **A''-B''**) or NK2R (magenta, **C''-D''**) in the distal colon myenteric plexus of mice. Preadsorption control for NK1R is shown in **B-B'''' ** and for NK2R in **D-D''''**. Images show triple-label immunohistochemistry for TRPV1 (green, **E**), Hu (blue, **E'**) and NK2R (magenta, **E''**) in the distal colon myenteric plexus of mice. Overlays of each combination are shown in **A'''- E'''**. Areas demarked by the dashed boxes in A'''-E''' are enlarged in panels **A''''-E''''**.

Figure 2.3 (cont'd)

The scale bar in **E'''** represents 20 μm and applies to **A-E'''**. All scale bars in the enlarged images represent 10 μm. Labeling is representative of experiments performed on a minimum of three mice.

TRPV1-positive neuronal varicosities express functional NK2Rs

Our immunohistochemical data above suggest that NK2Rs are expressed by extrinsic nociceptors that innervate the myenteric plexus. To test this hypothesis more directly, we generated mice that conditionally express the genetically-encoded Ca^{2+} indicator GCaMP5g in TRPV1 positive cells (*TRPV1*$^{tm1(cre)Bbm}$ /J::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice) to specifically study the activity of this population of nerve fibers in the myenteric plexus (**Figure 2.4A**). Tachykinin receptor activation primarily induces phospholipase C mediated increases in intracellular Ca^{2+} (11,13) that are measured by changes in GCaMP fluorescence. Nerve fibers expressing tdTomato in this model traverse through the myenteric plexus and are closely apposed to enteric glia (**Figure 2.4B**). These nerve fibers exhibited robust responses to capsaicin (1 µM), confirming that they do indeed express TRPV1 receptors (**Figure 2.4C**). Importantly, all TRPV1+ nerve fibers (n=3-5 nerve fibers from 3 mice) responded to NKA (1 µM) (**Fig.2.4C'**). Responses to NKA were on average 82% as large as responses to capsaicin in the same nerve fiber. Taken together with our above immunohistochemical and Ca^{2+} imaging data, these results show that NKA released by enteric neurons activates TRPV1+ nociceptors that, in turn, drive glial and neuronal activity in the myenteric plexus.

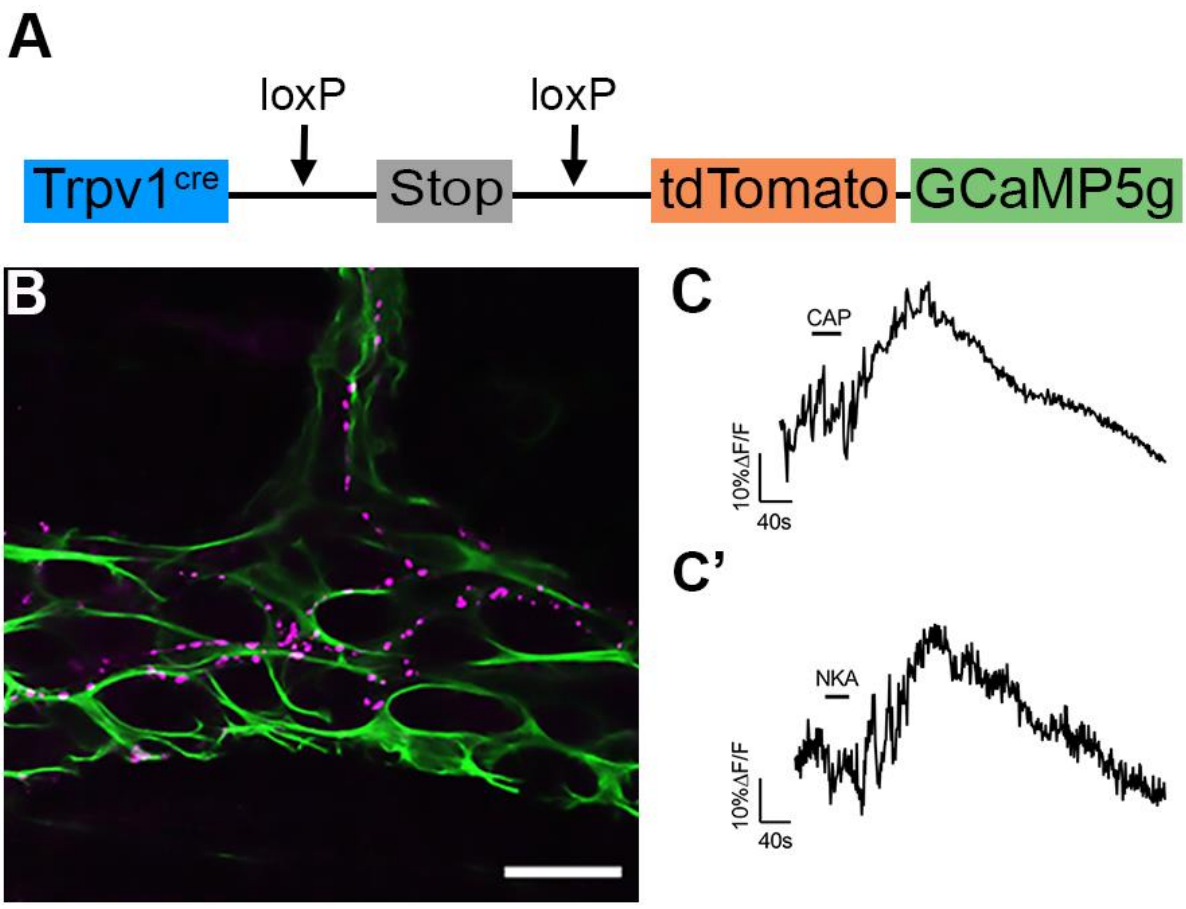

Figure 2.4: NKA drives Ca^{2+} responses in TRPV1-positive neuronal varicosities. (**A**) Schematic of transgene containing the tdTomato-tagged genetically-encoded Ca^{2+} indicator GCaMP5g driven by the transient receptor potential cation channel subfamily V member 1 (TRPV1) promoter. (**B**) Confocal image of dual-label immunohistochemistry (single optical plane, 1µm) for the tdTomato-tagged GCaMP5g (magenta) and enteric glia (GFAP, green) in the myenteric plexus of the *TRPV1*$^{tm1(cre)Bbm}$ /J::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mouse distal colon. Scale bar represents 20µm. (**C-C'**) Representative traces of average Ca^{2+} responses from TRPV1-positive neuronal fiber varicosities in response to 1µM capsaicin (CAP, **C**) or 1µM neurokinin-A (NKA, **C'**). Recordings were obtained from 3-5 ganglia from 3 mice.

Intercellular communication between nociceptors and enteric glia driven by NKA involves neuronal depolarization, purines, and glial connexin-43 activity

The expression of NK2Rs by TRPV1+ nociceptors and the complex glial responses evoked by NKA suggests intercellular signaling between the two cell populations. We interrogated these mechanisms by using a number of selective drugs and mutant mice with targeted deletions in glia. In these experiments, we used *Sox10*CreER^{T2}::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice to selectively study glial Ca^{2+} responses (21,33) and avoid the confounding effects of other nearby cells. First, we used 1 µM tetrodotoxin (TTX) to test whether glial responses to NKA require reciprocal interactions with neurons that require neuronal depolarization. Although TTX did not abolish NKA-induced Ca^{2+} response (**Figure 2.5A**), it did significantly reduce the number of responses per glia by 69% (p=0.0038; **Figure 2.5G**) and the duration of the response by 58% (p=0.0054; **Figure 2.5H**). Next, we tested the contribution of NK1Rs and NK2Rs by blocking their activity with the specific antagonists CP 96345 or GR 159897, respectively. NKA still reliably evoked glial Ca^{2+} response in the presence of CP 96345 (10 µM; **Figure 2.5B**) that were similar in duration as NKA alone (164s vs 278s) (**Figure 2.5H**). Interestingly, CP 96345 increased the number of responses per enteric glia as compared to NKA alone by 51% (p=0.0001; **Figure 2.5G**). GR 159897 (1 µM) alone reduced glial responses by 61% (p=0.0001; **Figure 2.5G**), but was not sufficient to completely abolish glial responses to NKA (**Figure 2.5C**). However, glial responses in the presence of GR 159897 were 55% shorter in duration (p=0.0086; **Figure 2.5H**). In prior work, we showed that intercellular neuron-to-glia communication involves the stimulation of glial P2Y1 receptors by purines released through neuronal channels (24,28). To

determine if purines contribute to glial Ca^{2+} responses evoked by NKA, we used MRS 2500 (10μM) to antagonize P2Y1Rs (**Figure 2.5D**). Although MRS 2500 did not abolish NKA-induced Ca^{2+} responses (**Figure 2.5D**), it did significantly reduce the number of responses per glia by 50% (p=0.0008; **Figure 2.5G**) and the duration of glial responses by 57% (p=0.0035; **Figure 2.5H**). Enteric glia utilize hemichannels composed of connexin-43 (Cx43) for intercellular communication (32). To determine if intercellular signaling through Cx43 contributes to glial Ca^{2+} response evoked by NKA, we used a genetic model to selectively ablate glial Cx43 [*SOX10*::CreER$^{T2+/-}$/Cx43$^{f/f}$ (22)] and monitored glial activity evoked by NKA with Fluo-4AM. We still observed glial responses to NKA in samples from *SOX10*::CreER$^{T2+/-}$/Cx43$^{f/f}$ mice (**Figure 2.5E**). However, similar to the effects above with GR 159897, the number of responses per glia and duration of response were reduced by 60% (0.0001) and 63% (p=0.0027), respectively, in samples from *SOX10*::CreER$^{T2+/-}$/Cx43$^{f/f}$ mice (**Figure 2.5G-H**). Applying GR 159897 to samples from *SOX10*::CreER$^{T2+/-}$/Cx43$^{f/f}$ mice completely abolished glial responses to NKA (**Figure 2.5F,G–H**). Together, these data show that the release of NKA from enteric neurons drives a multicellular signaling cascade through mechanisms that include the activation of NK2R+ nociceptors and the subsequent recruitment of glial activity through the generation of extracellular purines and intercellular glial signaling involving Cx43 hemichannels.

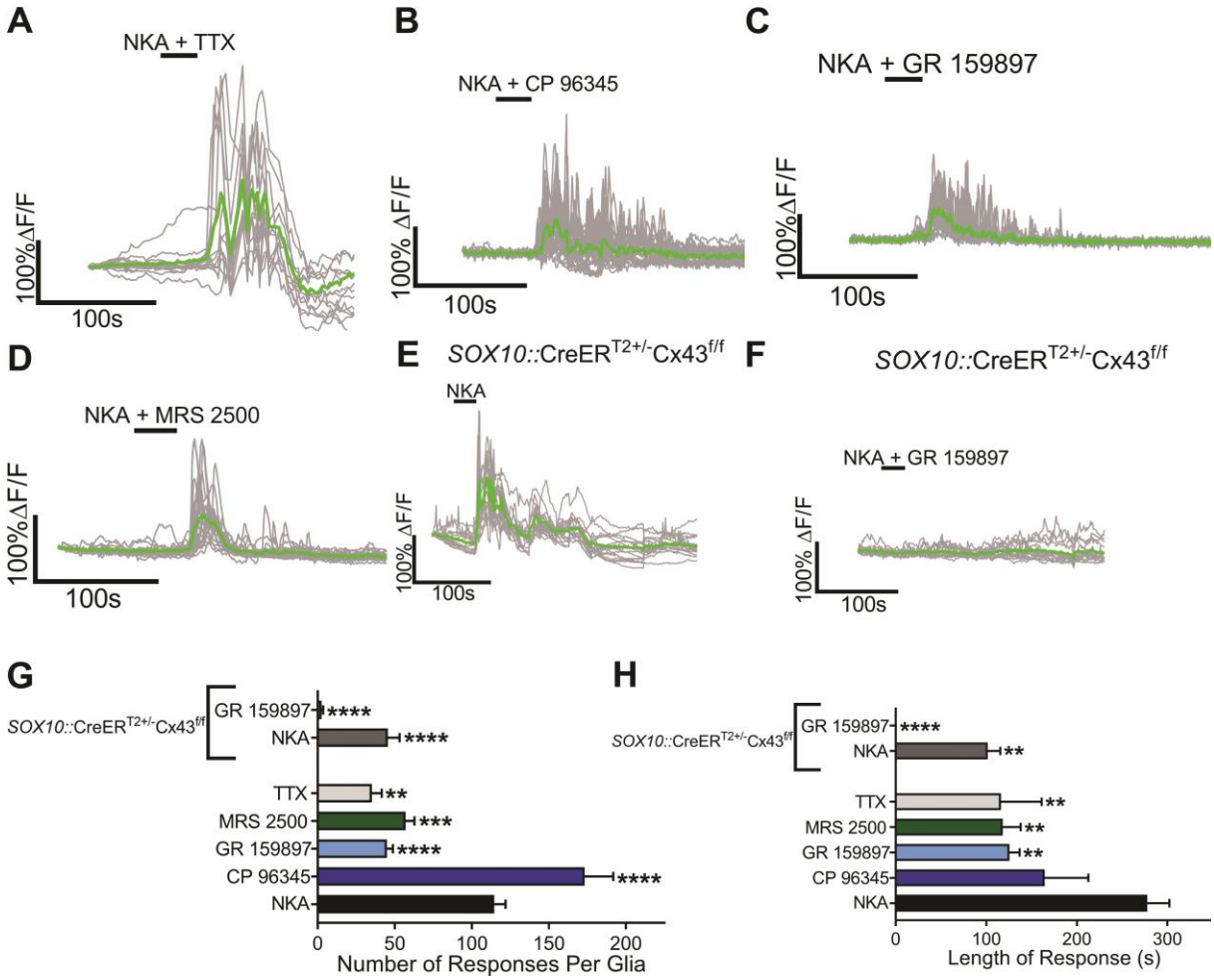

Figure 2.5: Glial Ca^{2+} responses driven by NKA require the activation of NK2Rs and purinergic intercellular signaling mediated by Cx43. (**A-D**) Representative glial Ca^{2+} responses evoked by NKA in the presence of tetrodotoxin (**A**); the NK1R antagonist CP 96345 (**B**); the NK2R antagonist GR 159897 (**C**) or the P2Y1R antagonist MRS 2500 (**D**). Gray traces show the responses of individual glial cells and the averaged response of all glia within the ganglion is shown in the green trace. (**E-F**) Representative glial Ca^{2+} responses evoked by NKA in tissues from *SOX10*::$CreER^{T2+/-}Cx43^{f/f}$ mice in the absence (**E**) or presence of GR 159897 (**F**). (**G-H**) Quantification of the effects of CP 96345, GR 159897, tetrodotoxin, MRS 2500 or ablation of glial Cx43 in *SOX10*::$CreER^{T2+/-}Cx43^{f/f}$ mice on the number (**G**) and length of response (**H**) of glial Ca^{2+} responses induced by NKA (n=15-33 glia from 3-5 mice; one-way ANOVA; *p < 0.05; **p < 0.01; ****p < 0.0001).

Contribution of NK2Rs to inflammation during colitis

Tachykinins are key mediators of neurogenic inflammation (11,31) and our data show that NKA drives the activation of glial mechanisms that contributes to neuroinflammation in the ENS (28). Therefore, we speculated that intercellular communication mediated by NKA could contribute to inflammatory processes in the intestine. We tested this hypothesis by assessing the effects of the NK2R antagonist GR 159897 (0.5mg/kg i.p.) (34,35) in the DNBS model of mouse colitis (**Figure 2.6A**). This is a well-characterized model that is widely used to study neuroplasticity and neuroinflammation in the ENS (36). Active inflammation in murine DNBS colitis peaks between days two and three and is completely resolved by three weeks (24). We found that blocking NK2Rs did not prevent body weight loss in mice at the peak of inflammation (**Figure 2.6B**), but mice treated with GR 159897 recovered from weight loss associated with inflammation within 9 days while vehicle treated mice did not recover until day 12, suggesting that GR 159897 accelerates the recovery from inflammation. Treatment with GR 159897 had no effect on the overall pattern of macroscopic damage to the colon in healthy or inflamed mice at peak inflammation or following recovery (**Figure 2.6C**) and no effect on histological damage at the initiation of inflammation (**Figure 2.6D**). Interestingly, we observed that TAC-1 mRNA levels are significantly elevated by 43% in animals with active inflammation ($p=0.0368$; **Figure 2.6D**). Given that TAC-1 encodes NKA, the primary agonist of NK2Rs, this outcome strongly suggests that higher levels of NK2R agonists are present during active inflammation in the colon. Importantly, treatment with GR 159897 during active inflammation was sufficient to prevent the increase in TAC-1 levels during colitis (**Figure 2.6D**). Mice treated with GR 159897 tended to have higher levels of TAC-1 at 3 weeks

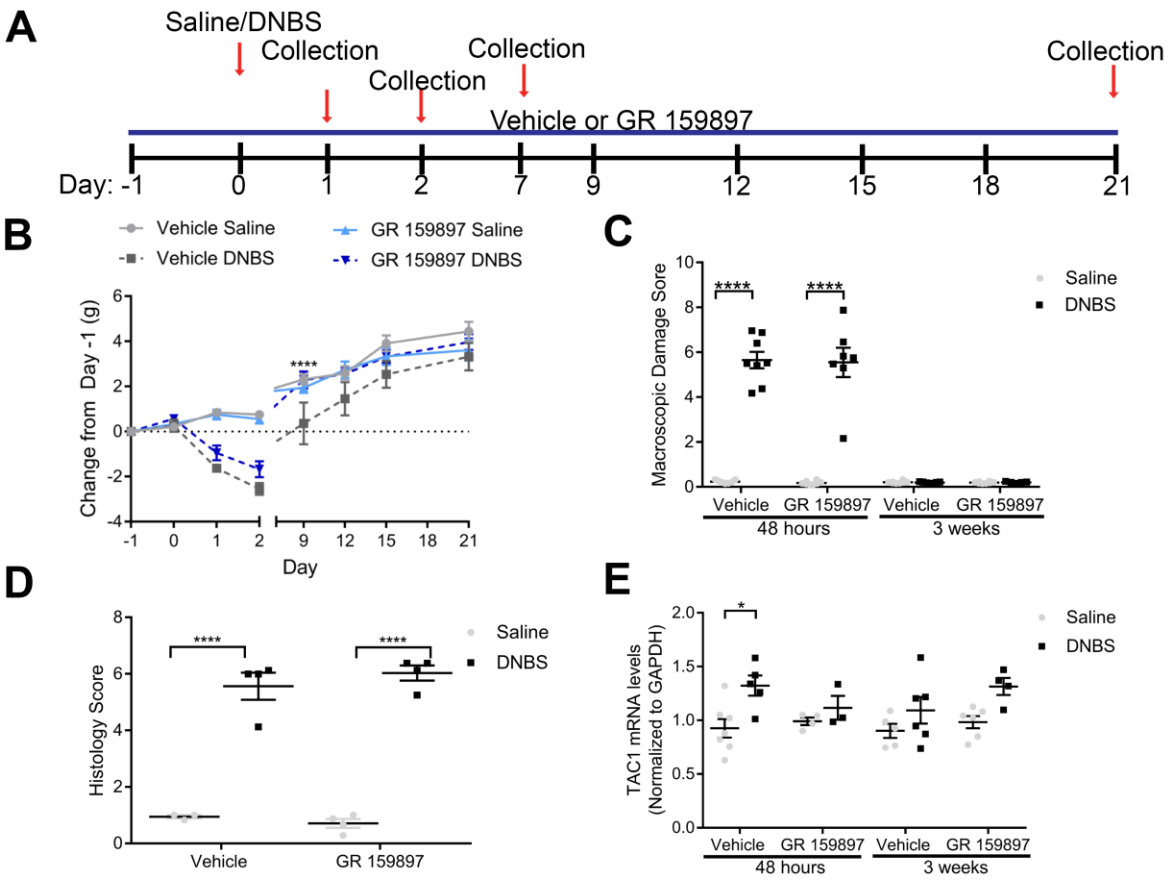

Figure 2.6: Effects of the NK2R antagonist GR 159897 on colonic inflammation and TAC-1 mRNA levels during acute colitis and recovery in mice. **(A)** Schematic representation of the timeline of treatments during colitis. (**B**) Weight loss pattern at 3 weeks after induction of DNBS colitis in mice (n=5-16 mice; two-way ANOVA with multiple comparisons; ***p = 0.001 for vehicle treated mice; **** p<0.0001). Note that inflamed mice treated with GR 159897 recover normal body weight sooner than inflamed mice treated with vehicle. (**C**) GR 159897 did not modify macroscopic damage to the colon at 48 hours or 3 weeks after induction of DNBS colitis in mice (n = 5-16 mice; two-way ANOVA with multiple comparisons; ****p < 0.0001). (**D**) GR 159897 did not modify histology scoring to the colon at 24 hours after induction of DNBS colitis in mice (n=3-4 mice; two-way ANOVA with multiple comparisons; ****p<0.0001). (**E**) TAC-1 mRNA levels (normalized to GAPDH) in the distal colon myenteric plexus of healthy (vehicle) and inflamed (DNBS) mice treated with vehicle or GR 159897 at 48 hours or 3 weeks after induction of DNBS colitis. (n = 4-7 mice; two-way ANOVA with multiple comparisons; *p =0.0368).

post-induction of colitis, but this did not reach significance (**Figure 2.6D**). Together, these results indicate that higher levels of NK2R agonists are present during active inflammation and that signaling through NK2Rs contributes to the production of NK2R agonists.

Tachykinin Peptide and Receptor Expression are not Significantly Altered During Inflammation

To test if SP or NKA levels are altered during active inflammation or recovery from inflammation, we performed triple-label immunohistochemistry (**Figure 2.7A**) and measured NKA and SP immunoreactivity (**Figure 2.7B-C**). Although we found increased TAC-1 levels during acute inflammation (**Figure 2.1E**), tachykinin immunoreactivity was not significantly altered. Indeed, we found that during acute inflammation, NKA and SP immunoreactivity trended to decrease by 25% and 12% respectively. To determine if receptor expression is altered by inflammation, we measured changes in tachykinin receptor mRNA levels (**Figure 2.7C-D**) in DNBS mice treated with either vehicle or GR 159897 and at 48 hours and 3 weeks post induction of colitis. TACR1 mRNA levels were not significantly altered during inflammation and treatment with GR 159897 trended to increase TACR1 mRNA levels by 58% at 48 hours (**Figure 2.7C**). Additionally, TACR2 mRNA levels trended to decrease during acute inflammation by 36% and were significantly reduced during inflammation in mice treated with GR 159897 ($p=0.0432$; **Figure 2.7D**). Together, these data suggest that neuroinflammation in the gut is associated with changes in tachykinin peptide expression, but is not associated with significant changes in tachykinin receptor expression.

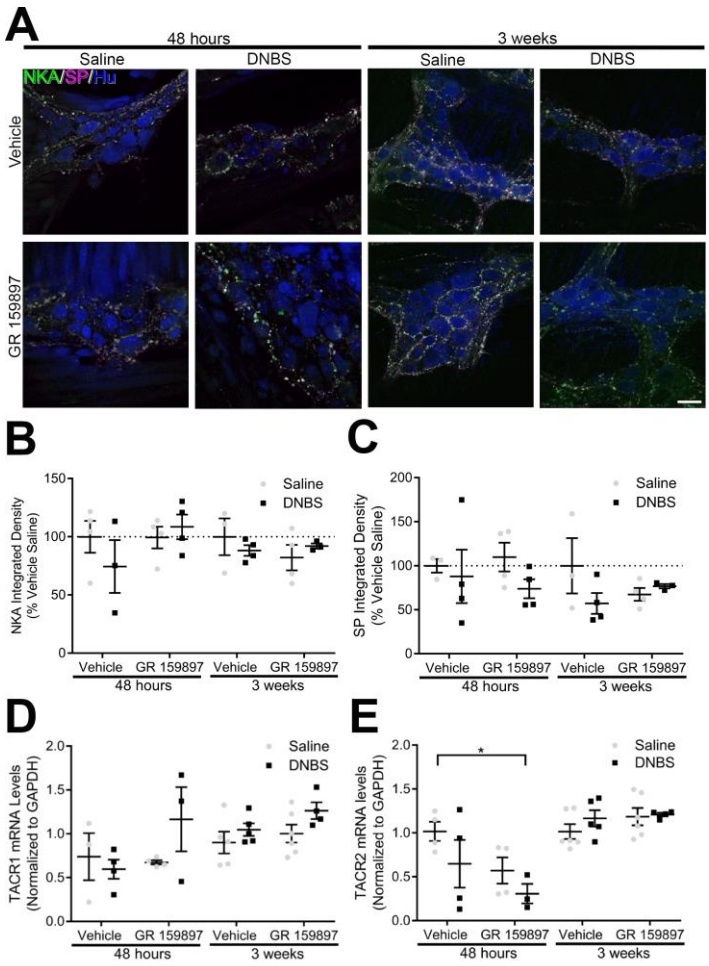

Figure 2.7: The expression of tachykinins and their receptors is not altered during inflammation. (**A**) Representative single optical section (1µm) images of triple-label immunohistochemistry for the pan-neuronal marker Hu (blue); NKA (green) and SP (magenta) in saline and DNBS mice treated with vehicle or GR 159897 at 48 hours or 3 weeks post-induction of colitis. Scale bar represents 30 µm and applies to all images. Labeling is representative of experiments performed on a minimum of three mice. Quantification of NKA (**B**) and SP (**C**) integrated density in saline and DNBS mice treated with vehicle or GR 159897 at 48 hours or 3 weeks post-induction of colitis (n= 3-4 mice; two-way ANOVA with multiple comparisons, normalized to group vehicle saline). Quantification of TACR1 (**D**) and TACR2 (**E**) protein expression from the distal colons of healthy or inflamed mice treated with vehicle or GR 159897 at 48 hours or 3 weeks post-induction of colitis (n= 3-6 mice; two-way ANOVA with multiple comparisons; normalized to GAPDH; *p= 0.0344).

NK2R antagonism prevents neurodegeneration and reactive gliosis in the ENS

Our data show that intercellular communication driven by NKA activates glial mechanisms that are involved in neuroinflammation in the ENS (28). Therefore, we hypothesized that blocking NK2Rs during inflammation would protect against neuroinflammation. In agreement, we found that mice treated with GR 159897 were protected against neurodegeneration during and after recovery from colitis (**Figure 2.8A** and **2.8B**). Likewise, inflamed mice treated with GR 159897 did not develop reactive gliosis (**Figure 2.8A, 2.8D-H**). This is important because enteric glia are responsible for driving neuron death during colitis (28). Reactive gliosis is a complex response that involves changes in glial fibrillary acidic protein (GFAP) expression (37,38) and glial morphology (27,39). In agreement, we observed a significant 63% ($p=0.0114$) increase in GFAP mRNA levels in mice during active inflammation and a significant 46% ($p<0.0001$) and 81% ($p=0.0003$) increase in glial process length and process thickness, respectively (**Figure 2.8D-F**). Importantly, all of these indicators of reactive gliosis were prevented by treatment with GR 159897 during active inflammation. Similarly, after recovery from inflammation, GFAP mRNA levels and glial process length and thickness are not significantly different from healthy controls (**Figure 2.8D-F**). Surprisingly, we did not observe significant changes in total GFAP immunoreactivity levels in healthy or inflamed animals or those treated with GR 159897 at either time point (**Figure 2.8C**). This may indicate that glial GFAP expression is mainly affected at the transcriptional level during the active phase of inflammation. Glial process branching trended to increase during inflammation by 23% at 10 µm from the nucleus, but did not reach significance (**Figure 2.8G**). Although it did not reach significance, glia from inflamed mice exposed to GR 159897 exhibited 6% less

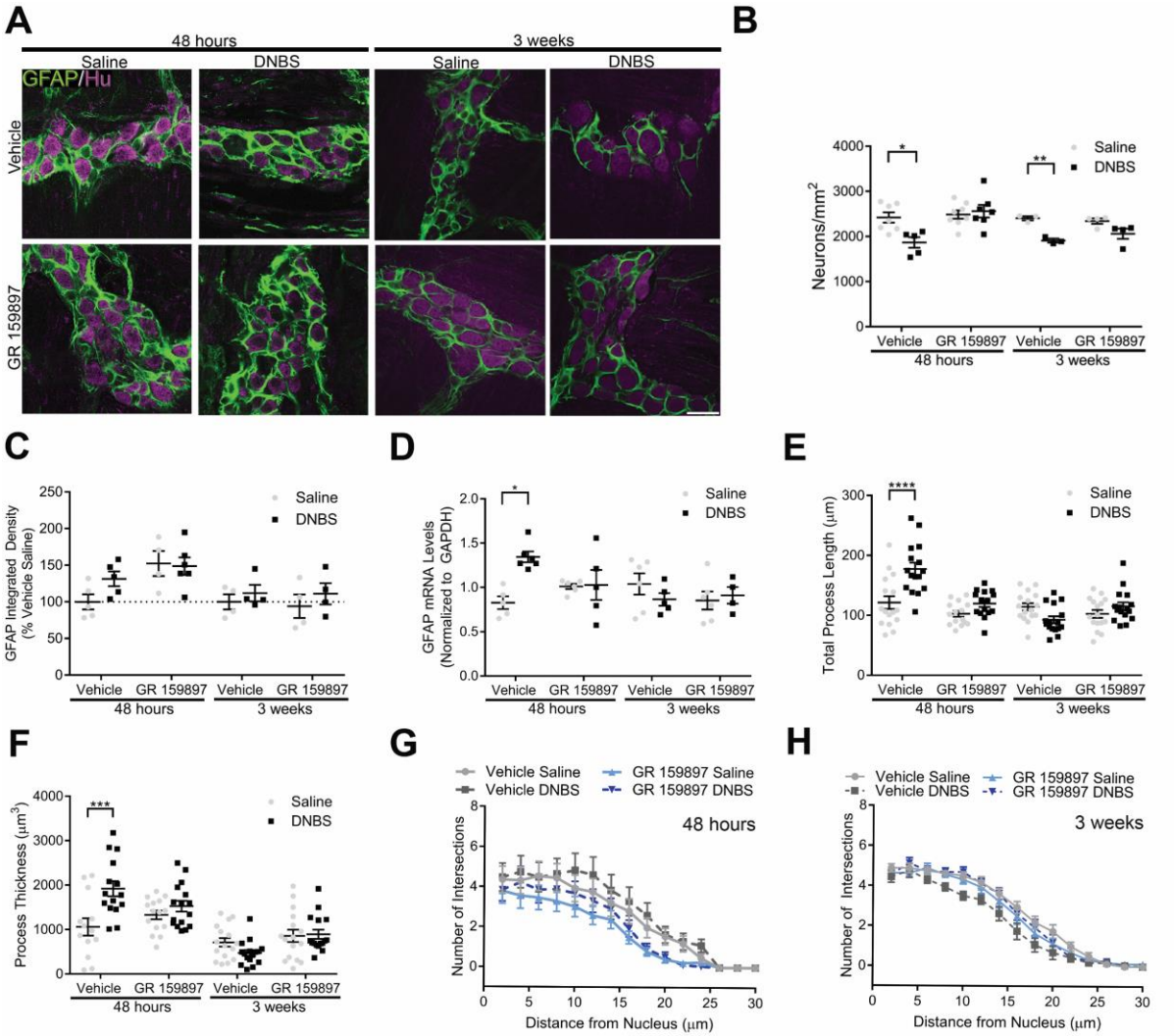

Figure 2.8: The NK2R antagonist GR 159897 limits neuropathy and reactive gliosis in the myenteric plexus during inflammation. (**A**) Representative confocal microscopy images of dual-label immunohistochemistry for GFAP (green) and the pan-neuronal marker Hu (magenta) in healthy (saline) and inflamed (DNBS) mice treated with vehicle or GR 159897 at 48 hours or 3 weeks post-induction of colitis. Scale bar represents 30 μm and applies to all images. Labeling is representative of experiments performed on a minimum of four mice. (**B**) Quantification of myenteric neuron packing density in healthy and inflamed mice treated with vehicle or GR 159897 at 48 hours or 3 weeks post-induction of colitis (n= 3-8 mice; two-way ANOVA with multiple comparisons; *p= 0.02239; **p= 0.0097).

Figure 2.8 (cont'd)

(**C-D**) Quantification of GFAP protein expression (**C**; integrated density, n=4-6 mice; two-way ANOVA with multiple comparisons, normalized to group vehicle saline) and mRNA levels (**D**; n=4-7 mice; two-way ANOVA with multiple comparisons, normalized to GAPDH *p= 0.0114) in samples of myenteric plexus from the distal colons of healthy and inflamed mice treated with vehicle or GR 159897 at 48 hours or 3 weeks post-induction of colitis. (**E-H**) Quantification of glial morphology including total process length (**E**), process thickness (**F**), and Scholl analysis for process branching at 48 hours (**G**) or 3 weeks (**H**) post-induction of colitis. (n=16 glia; two-way ANOVA with multiple comparisons; ***p = 0.0003; ****p<0.0001).

process branching than healthy controls and 24% less process branching than glia from inflamed mice treated with vehicle at 10 µm from the nucleus (**Figure 2.8G**). After recovery from inflammation, glial process branching trended to decrease by 6% at 10 µm from the nucleus, but did not reach significance (**Figure 2.8H**). Collectively, these results show that the antagonism of NK2Rs with GR 159897 prevents key changes associated with neuroinflammation in the ENS such as neurodegeneration and reactive gliosis. Additionally, we demonstrate that enteric glia become reactive during active inflammation and these changes are reversed after recovery from colitis.

NKA drives enteric neurodegeneration through mechanisms that require glial Cx43 hemichannels

The protective effects of GR 159897 on reactive gliosis and neurodegeneration during colitis strongly suggest that NK2Rs contribute to neuroinflammation in the ENS through glial mechanisms. Especially when taken into consideration with our mechanistic studies showing that intercellular communication driven by NKA involves glial mechanisms that drive neuron death. To test this notion, we used an *in situ* model of neuroinflammation in whole mount preparations of myenteric plexus to study the underlying intracellular mechanisms (**Figure 2.9**). Similar to our published data with purine-driven neurodegeneration in this model, we found that 1µM NKA was sufficient to drive myenteric neuron death to an equal extent as the P2X7R agonist BzATP (300µM) (29% reduction with NKA vs 31% with BzATP; **Figure 2.9**). P2X7R-driven enteric neuron death required the activation of enteric glia and subsequent mechanisms that involve purine release through glial Cx43 hemichannels (28). Similarly, blocking glial Cx43 hemichannel

activity with the mimetic peptide 43Gap-26 (100μM) completely protected against NKA-driven neuron death (**Figure 2.9**). Antagonizing NK2Rs with GR 159897 (10μM) partially protected against neuron death driven by NKA (32%; p=0.2524) and the combination of GR 159897 and 43Gap26 completely protected against neuron loss. Enteric neurodegeneration during inflammation requires the activation of neuronal P2X7 receptors and neuronal ATP release through Panx1 channels (24,28). Blocking these mechanisms with the P2X7 receptor antagonist A74003 (10μM) or the Panx1 inhibitor probenecid (2mM) protected against neuron death driven by NKA (by 77% and 82%, respectively). Together, these data show that NKA drives neurodegeneration through similar mechanisms as purine driven neurodegeneration. These mechanisms include the activation of neuronal P2X7 receptors, neuronal Panx1 channels, and glial Cx43 hemichannels.

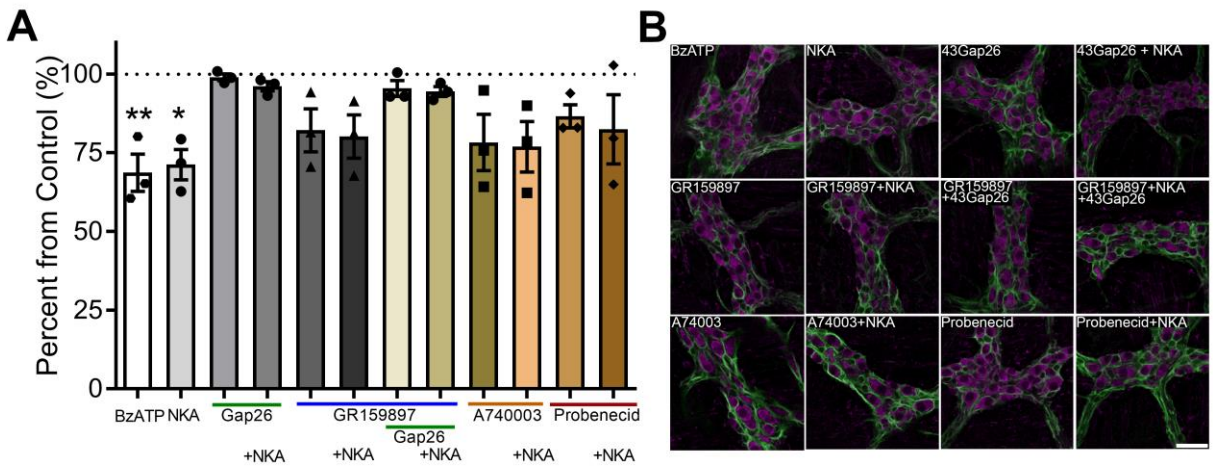

Figure 2.9: NKA driven enteric neurodegeneration requires the activation of NK2Rs and Cx43 hemichannels, but not P2X7Rs or pannexin-1. Quantification (**A**) and representative images (**B**) of mean packing density of HuC/D-immunoreactive neurons in myenteric ganglia after *in situ* activation of P2X7Rs with BzATP or activation of NK2Rs with NKA in the presence or absence of the Cx43 inhibitor 43Gap26; the NK2R antagonist GR 159897; the P2X7 antagonist A74003; or the pannexin-1 inhibitor probenecid (n= 3 mice; one-way ANOVA with multiple comparisons; *p= 0.0157; **p= 0.0073). Enteric neurons are labeled with HuC/D (magenta) and enteric glia are labeled with glial fibrillary acidic protein (GFAP, green) in all panels. Scale bar represents 20 µm and applies to all images.

Glial Transcriptome Alterations Driven by Acute Inflammation

To understand the glial response to inflammation in more depth, we specifically isolated and sequenced glial ribosomal-associated mRNA (translating mRNA) from healthy and inflamed animals (**Figure 2.10**). We accomplished this by crossing our glial *Sox10*::CreER$^{T2+/-}$ driver line with *Rpl22*$^{tm1.1Psam}$/J mice (*Sox10*::CreER$^{T2+/-}$ / *Rpl22*$^{tm1.1Psam}$/J mice) to generate mice with hemagglutinin-tagged ribosomes in Sox10 positive enteric glia (**Figure 2.10A**). Dual-label immunohistochemistry with antibodies against GFAP and hemagglutinin (HA) on LMMP preparations from the distal colon of *Sox10*::CreER$^{T2+/-}$ / *Rpl22*$^{tm1.1Psam}$/J mice confirmed HA-tagged ribosomes were predominantly localized to enteric glia (**Figure 2.10A'**). Although, SOX10 protein is essential in the development of both enteric neurons and enteric glia (69), we found that *Sox10*::CreER$^{T2+/-}$ / *Rpl22*$^{tm1.1Psam}$/J mice had low levels of relative mapped neuronal genes including *Syp*, *Syt1* and *Syn1* and had high levels of relative mapped glial genes including *GFAP*, *SOX10* and *S100β* (**Figure 2.10B**). Together with our immunohistochemistry data, this confirms HA-tagged ribosomes are predominantly from enteric glia. We next wanted to determine if inflammation drives differential expression of enteric glial genes. Our data shows that DNBS-colitis drives an upregulation of 184 and a downregulation of 233 enteric glial genes in vehicle DNBS mice compared to vehicle saline mice (**Figure 2.10C**). Importantly, mice treated with the NK2R antagonist GR 159897 in DNBS-colitis had 516 upregulated and 62 downregulated enteric glial genes compared to vehicle DNBS mice (**Figure 2.10D**). To determine what glial gene functions are affected in inflammation alone, we explored fold changes in gene ontology (GO) terms associated with biological processes (**Figure 2.10E**). We found that DNBS-colitis drives

significant dysregulation in genes related to neuron apoptotic processes, sensory perception of pain, positive regulation of cell differentiation, cell adhesion, cytokine activity, antigen processing and presentation, immune response and astrocyte differentiation compared to saline controls ($p<0.005$; **Figure 2.10E**). Next we wanted to understand if antagonizing NK2R signaling had effects on larger-scale cellular function in inflammation. We found that inflammation drives changes in genes associated with development, localization, cell death and immune processes and antagonizing NK2R signaling with GR 159897 mitigated these changes in enteric glial genes ($p<0.005$; **Figure 2.10F**). Together, our data suggest inflammation drives significant changes in enteric glial gene expression and gene function, and antagonizing NK2R signaling mitigates the effects of inflammation on important cell processes.

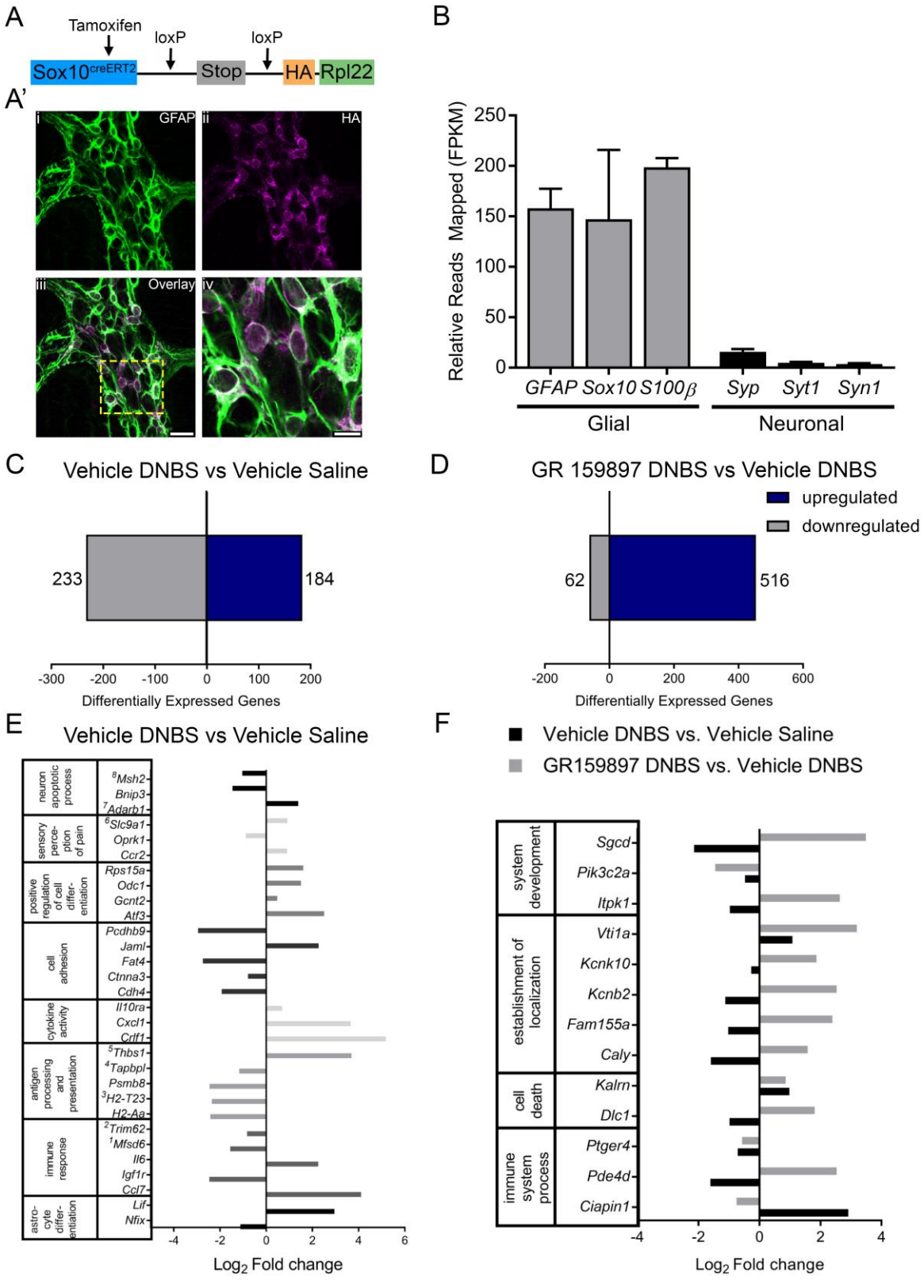

Figure 2.10: Glial transcriptome alteration driven by acute inflammation

Figure 2.10 (cont'd)

(**A**) Schematic of transgene containing the hemagglutinin (HA)-tagged ribosomal protein L22 (Rpl22) driven by the tamoxifen inducible glial promoter (SOX10^{creERT2}). (**A'**) Confocal image of dual label immunohistochemistry (single optical plane, 1µm) for the HA-tagged Rpl22 (magenta) and enteric glia (GFAP, green) in the myenteric plexus of the *Sox10*::CreER$^{T2+/-}$ / *Rpl22*$^{tm1.1Psam}$/J mouse distal colon. The area demarked in **iii** is enlarged in panel **iv**. Scale bar in **iii** represents 20µm and applies to **i-iii**. Scale bar in **iv** represents 10µm. (**B**) Mean Fragments per Kilobase of transcript per Million mapped reads (FPKM) values of sentinel glia and neuronal genes from vehicle saline samples depicting increased relative expression of glial to neuronal transcripts (n=3 mice). (**C**) Significant differentially expressed genes in the colon of vehicle DNBS mice compared to vehicle saline mice (n=3 mice; $p<0.005$). (**D**) Significant differentially expressed genes in the colon of GR 159897 DNBS mice compared to vehicle DNBS mice (n=3 mice; $p<0.005$). Upregulated genes are shown in blue and downregulated genes are shown in grey for both **C-D**. (**E**) Significant differentially regulated genes classified by gene ontology (GO) terms in biological processes from the colon of vehicle DNBS mice compared to vehicle saline mice (n=3 mice; $p<0.005$). Superscripts denote actual categorization in similar, more specific GO terms, which were grouped together under a broader category GO term: [1]adaptive immune response, [2]innate immune response in mucosa, [3]antigen processing and presentation of peptide antigen via MHC (major histocompatibility complex) class I, [4]negative regulation of antigen processing and presentation of peptide antigen via MHC class I, [5]negative regulation of peptide or polysaccharide antigen via MHC class II. (**F**) Significant differentially regulated genes classified by GO categories in biological processes from the colon of vehicle DNBS mice compared to vehicle saline mice and from the colon of GR 159897 mice compared to vehicle DNBS mice (n=3 mice; $p<0.005$).

NK2R antagonism protects against changes in muscle contractility during colitis

Acute inflammation drives persistent dysmotility in large part by changing the function (6,40) and survival (24,28) of enteric neurons. Given the beneficial actions of NK2R antagonism on motility in functional GI disorders (8,20) and our current data showing neuroprotective effects during inflammation, we hypothesized that antagonizing NK2Rs with GR 159897 would protect against increased neurogenic contractions during colitis (24). To test our hypothesis, we performed contractility studies from mice treated with vehicle or GR 159897 at 7 days post-induction of colitis. We found that inflammation significantly enhanced neurogenic contractions by 217% ($p=0.0261$) and treatment with GR 159897 prevented this increase (**Figure 2.11A-B**). Inflammation increased neurogenic relaxations by 70%, but did not reach significance and treatment with GR 159897 did not significantly alter neurogenic relaxations (**Figure 2.11C-D**). These results confirm the hypothesis that NK2R antagonism protects against functional changes associated with inflammation in the gut.

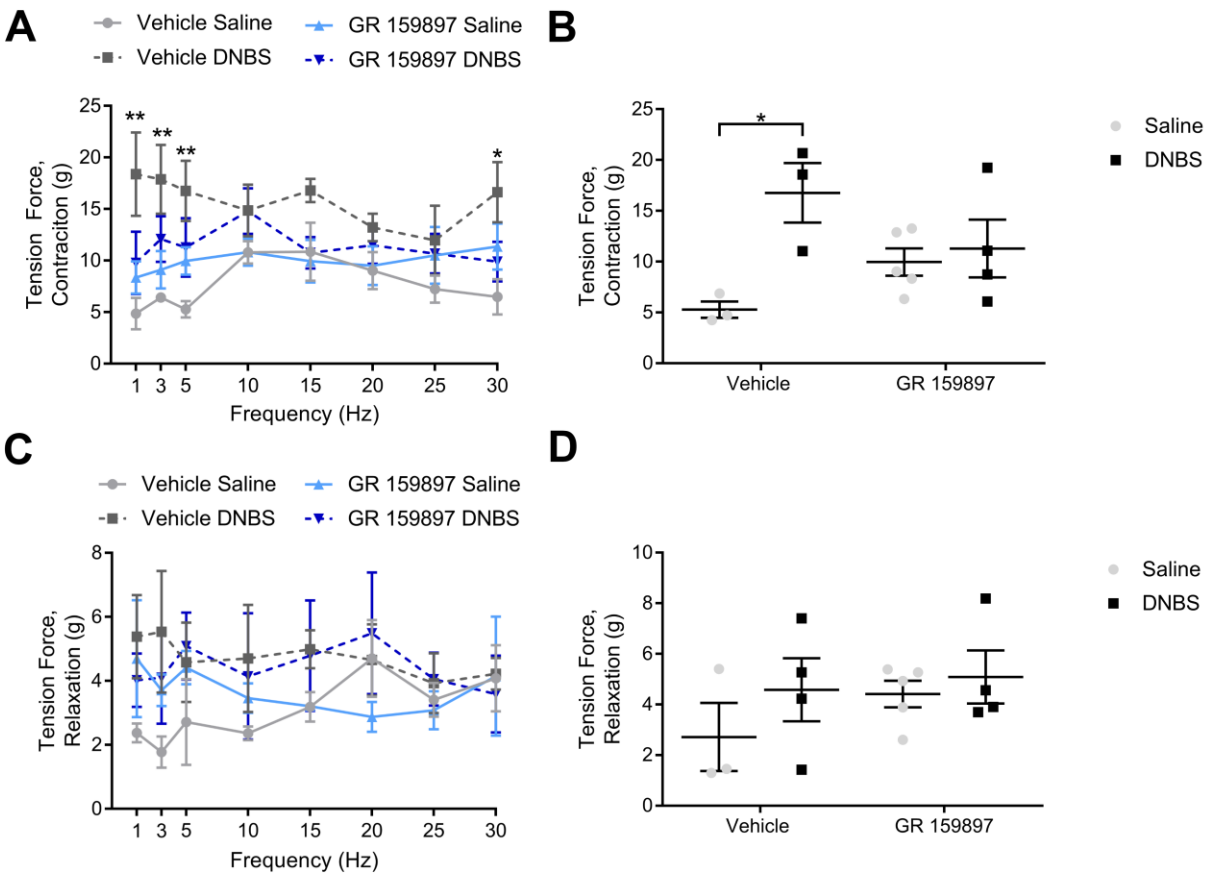

Figure 2.11: NK2R antagonism prevents changes in muscle contraction following colitis. Neurogenic contractions (**A-B**) and relaxations (**C-D**) driven by EFS (20 V, 0.3ms, 5 Hz) in colons from mice treated with vehicle or GR 159897 at 7 days post-induction of colitis. DNBS enhanced neurogenic contractions (**A-B**), but did not significantly alter neurogenic relaxations (**C-D**). Treatment with GR 159897 diminished the effects of DNBS colitis on neurogenic contractions (**A-B**) (n=3-4; two-way ANOVA with multiple comparisons; *p<0.05; **p<0.01).

DISCUSSION

Tachykinins are a family of endogenous peptides that include SP and NKA. They are widely expressed in the GI tract and are involved in both physiological and pathological conditions (10-13). Physiologically, they are involved in the modulation of motor activity (41-43), secretion (10,11,44) and immune responses (10,45). In pathological conditions, tachykinins are implicated in modulating inflammation (45-47) and inflammation-induced changes in motility and secretion (44-46,48-49). The ENS and primary afferent neurons are the major sources of tachykinins in the gut and noxious stimulus can drive tachykinin release and neurogenic inflammation in the ENS (11-12). However, how tachykinin signaling interacts with nociceptive neurons, enteric neurons, and glia is not fully understood. Here, we tested the hypothesis that tachykinins are involved in signaling between nociceptive neurons and enteric neurons and glia. Our results show that the tachykinin NKA contributes to neuroinflammation in the ENS through a multicellular signaling cascade involving enteric neurons, nociceptors, and enteric glia (**Figure 2.12**). Our data confirm earlier reports showing that intrinsic neuronal varicosities are the main source of tachykinins in the ENS and expand upon these findings to show that NK2R are localized to nociceptors and that their stimulation drives activity in enteric neurons and glia. Importantly, antagonizing NK2R signaling with GR 159897 prevents key aspects of neuroinflammation in the ENS such as reactive gliosis and neurodegeneration. Interestingly, the beneficial effects of antagonizing NK2R signaling are primarily mediated by their suppressive action on neuro-glia communication because NKA-driven neurodegeneration was Cx43 hemichannel dependent. Together, our data

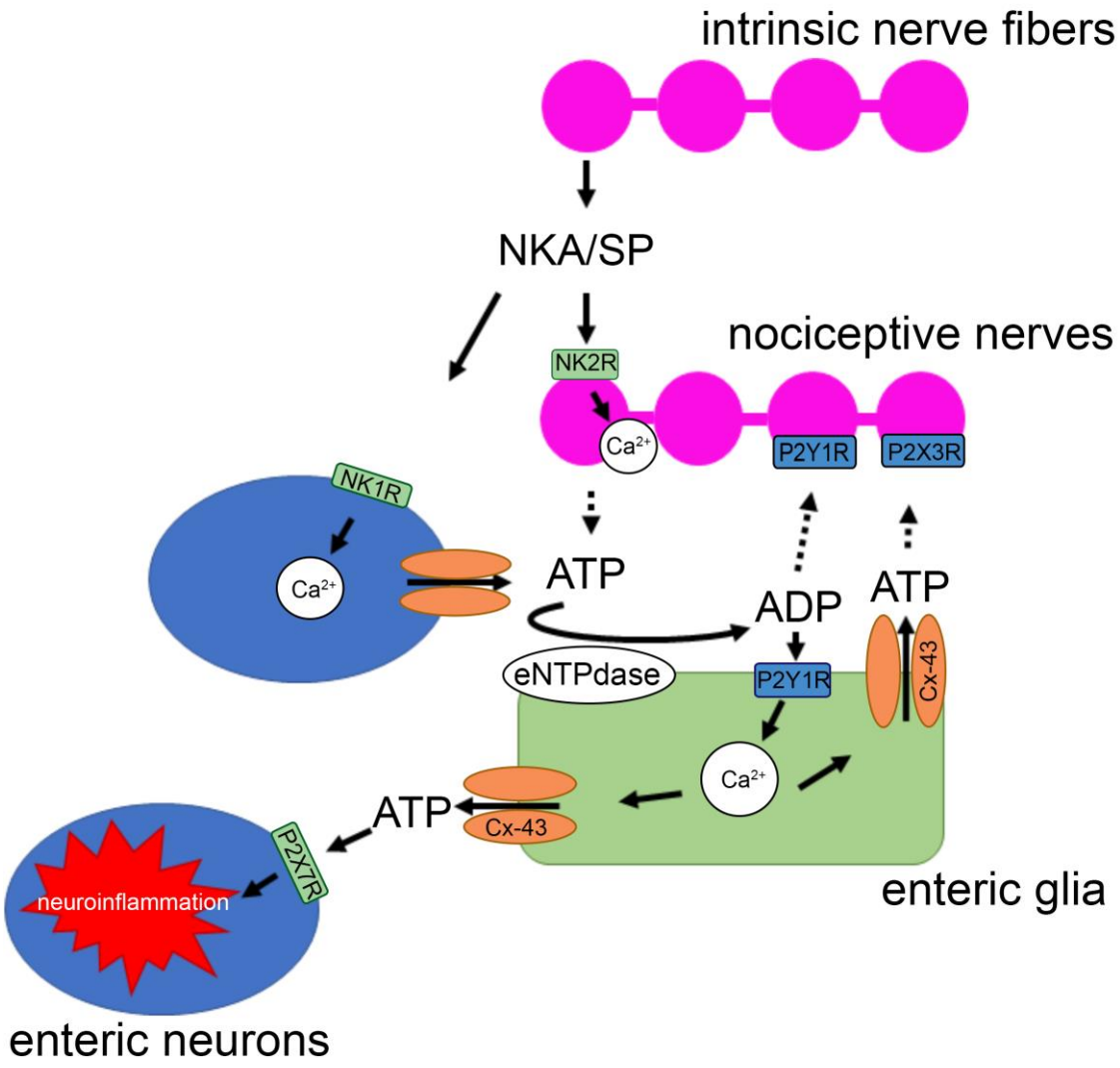

Figure 2.12: Schematic model of the intercellular signaling mechanisms that underlie the effects of NKA on neuroinflammation in the enteric nervous system. NKA and SP released from intrinsic neuronal varicosities drive NK1R activation on enteric neurons and NK2R activation on nociceptive neurons. Activation of NKRs drives adenosine triphosphate (ATP) release from enteric neurons that recruit activity in surrounding enteric glia by activating P2Y1Rs. Intracellular signaling pathways downstream of P2Y1R activation drive glial Ca^{2+} responses that contribute to connexin-43 (Cx-43) hemichannel opening and ATP release that further enhances glial responses, drives P2X7R-mediated neuroinflammation, and P2Y1R activation on nociceptive neurons.

show that tachykinins are novel mediators of enteric neuron-glia communication that could play an important role in the pathogenesis of gastrointestinal dysfunction.

Our findings suggest that tachykinins are key mediators between nociceptive sensory neurons and enteric neurons and glia. We found that enteric neurons predominantly express NK1R while NK2Rs are predominantly expressed by nociceptive sensory neurons, although we cannot completely rule out the possibility that some enteric neurons also express NK2Rs. This is consistent with previous data showing that in the mouse distal colon NK1Rs are predominantly expressed by myenteric intrinsic primary afferent neurons, descending interneurons, and inhibitory motor neurons (11). To this point, the localization of NK2Rs in the mouse distal colon has remained unclear. Studies in guinea pig ileum show that NK2Rs are primarily expressed by smooth muscle cells, nerve varicosities, and epithelial cells (11,13), but species differences preclude extrapolations to mice. Our data show that the activation of NK2Rs with NKA induces Ca^{2+} responses in nociceptive neurons that subsequently activate enteric neurons and glia. Interestingly, enteric glia could respond before or after enteric neuronal response, suggesting that nociceptors directly interact with enteric glia. This is consistent with our immunohistochemical data showing that NK2R-immunoreactive neuronal varicosities are in close proximity to enteric glia.

The pattern of glial activity evoked by NKA is entirely different from what we (28,32) and others (38-39,50) have observed with purinergic agonists. Purinergic agonists typically evoke a relatively uniform Ca^{2+} transient among enteric glia that peaks and concludes in a matter of seconds. In contrast, NKA evokes stochastic glial Ca^{2+} transients and drives activity that persists for several minutes. We studied the mechanisms

underlying these unique glial responses and found that they require the activation of nociceptive neuronal NK2Rs, glial P2Y1Rs, and Cx43. The participation of glial Cx43 is not entirely surprising given the key role of this protein in glial intercellular communication and specifically in the propagation of responses among enteric glia (28,32). However, we were surprised that glial responses to NKA were not totally abolished in the presence of GR 159897 and were increased in the presence of the NK1R antagonist CP 96345. One explanation for this observation is that although NKA preferentially interacts with NK2Rs, it can potentially interact with both NK1 and NK2Rs which might affect agonist availability or feedback signaling from enteric neurons. In support, the inhibition of P2Y1Rs with MRS 2500 significantly attenuated the duration and number of glial responses to NKA. Together, these results indicate that glial responses to nociceptive neuronal NK2R activation are mediated through mechanisms that involve neuron-to-glia and glia-to-glia communication.

Tachykinins are an attractive class of candidate mediators of acute inflammatory responses in the ENS because they are released from hyperactive intrinsic primary afferent neurons (IPANs) (40) and their expression is altered during intestinal inflammation in animal models and humans (11,13,47,51). In support, our results show an upregulation of TAC-1 mRNA during acute colitis and a trending decrease in both NKA and SP immunoreactivity. Prior studies have indicated that NK2Rs contribute to gut inflammation in animal models because NK2R antagonism with SR 48698 prevented weight loss and reduced macroscopic damage during TNBS colitis in rats and guinea pigs (46). In this study, we did not observe a significant effect of GR 159897 on the initial inflammatory insult, but GR 159897 did enhanced the recovery from inflammation as

reflected by animal weight. However, we did not observe significant improvement in the colonic macroscopic damage score or in histological scoring. The additional beneficial effects observed by treatment with SR 48698 could be driven by several factors including species differences in receptor expression or structural difference between the antagonists. Importantly, SR 48698 also acts on μ-opioid receptors (52), calcium channels (53) and other neurokinin-receptors (54). Thus, the participation of these other systems on the anti-inflammatory benefits observed cannot be excluded. To our knowledge, GR 159897 has not been shown to interact with other systems.

Interestingly, treatment with GR 159897 was also sufficient to prevent the upregulation of TAC-1 mRNA during colitis. Surprisingly, we found that both SP and NKA immunoreactivity trended to decrease during inflammation and after recovery from inflammation, but did not reach significance. Although changes in mRNA levels do not necessarily correspond to changes in protein expression (56), these data suggest that changes in tachykinin levels could contribute to neuroinflammation. This is important because it suggests that the regulation of tachykinins depends on positive feedback loops driven by tachykinins acting on NK2Rs and this could be an important mechanism in disease progression. We found that treatment with the NK2R antagonist GR 159897 trended to increase NK1R mRNA levels and significantly decreased NK2R mRNA levels. This suggests that antagonizing NK2Rs stimulates a downregulation of NK2R receptor expression that in turn induces an upregulation of NK1R expression.

Inflammation alters neuromuscular transmission and disrupts GI motility (57,58). For example, DNBS colitis creates persistent changes such as enhanced EFS-elicited neurogenic contractions and attenuated EFS-elicited neurogenic relaxations that persist

after resolution of colitis (24). In support, we found that DNBS colitis enhanced EFS-elicited neurogenic contractions, but we did not observe significant effects of DNBS colitis on neurogenic relaxations. This may be due to the fact that contractility studies were performed during the active phase of inflammation (7 days post-DNBS) while the previous study performed contractility studies during the resolution of inflammation (21 days post-DNBS). Importantly, we found that NK2R antagonism was able to protect against the enhanced neurogenic contractions associated with DNBS. This is consistent with previous data showing that NK2R agonists mediate circular muscle contractions in the human colon and ileum (11,59). Together, these data demonstrate that antagonizing NK2Rs could provide functional benefits in colitis-induced dysmotility.

It is now clear that enteric glia play an important role in immune responses during inflammation (22). Enteric glia both respond to and secrete inflammatory mediators such as interleukin (IL)-1, IL-6 (60), and purines (28). However, the mechanisms that trigger glial responses to gut inflammation are still unclear. Although our results show that nociceptive neurons are the primary site of activation for NK2Rs, we found that tachykinins could drive neuro-to-glia signaling and this communication plays a major role in the generation of gliosis during inflammation. GR 159897 had a major attenuating effect on reactive gliosis and prevented key changes in GFAP expression and morphological modifications that are indicative of reactive glia. Although inflammation significantly increased GFAP mRNA levels at the peak of inflammation, we did not observe corresponding changes in GFAP immunoreactivity. This difference is not surprising given that changes in mRNA levels do not necessarily directly correspond to changes in protein expression (56). However, we performed additional studies where we reconstructed

individual glial cells to study glial morphology and confirmed that reactive gliosis was occurring. Our findings show that glial process length and thickness are significantly increased during inflammation and this observation is consistent with changes in reactive astrocytic processes that occur during injury or inflammation (27). In agreement with our GFAP expression analysis, we found that GR 159897 prevented the extension of glial processes during inflammation. In addition, we found that inflammation drove significant changes in enteric glial gene expression and function, and antagonizing NK2R signaling mitigated some changes in larger-scale cellular function. Importantly, this was an exploratory study and further smaller-scale studies are required to confirm and further elucidate the effects of antagonizing NK2R signaling on enteric glial gene expression and function.

We recently showed that glial activation during colitis drives enteric neurodegeneration through mechanisms that involve glial Ca^{2+} responses and the potentiation of Cx43 hemichannel opening by nitric oxide (28). Similarly, our present data demonstrate that the NK2R agonist NKA drives enteric neuron loss in situ that is dependent on glial Cx43 hemichannel activity. This is important, because it suggests that the activation of nociceptive NK2Rs drives neuron-to-glia communication that can trigger ATP release from glial Cx43 hemichannels, leading to P2X7-mediated enteric neurodegeneration (24). In support, we found that animals treated with GR 159897 were protected against neurodegeneration during colitis. Given that enteric glia are a major source of purines in the ENS (28), purines released from enteric glia could activate $P2X_3$ and $P2Y_1$ receptors on nociceptive neurons and contributing to visceral hypersensitivity in many gastrointestinal disorders (61,62) (**Figure 2.11**).

In conclusion, our results show that nociceptive neurons predominantly express NK2Rs in the ENS and that the stimulation of these receptors by NKA released from enteric neurons drives signaling to enteric glia and neurons. Antagonizing NK2R-signaling from nociceptive neurons prevented reactive gliosis, neurodegeneration, and protected against enhanced neurogenic contractions during colitis. NK2R antagonism prevented enteric neuroinflammation and glial reactivity by interfering with neuron-to-glia and glia-to-neuron signaling. Our findings show that neuron-to-glia communication is induced by tachykinin receptor activation on nociceptive nerve fibers and implies that interfering with neuron-to-glia activation could be an important therapeutic approach in the treatment of functional GI disorders.

APPENDIX

Antibody	Source	Dilution	Catalog No.	Resource ID No.
Chicken anti-GFAP	Abcam, Cambridge, MA	1:1000	AB4674	AB_304558
Rabbit anti-NK1R	Alomone Labs, Jerusalem, Israel	1:500	ATR-001	AB_11219139
Rabbit anti-NK2R	Alomone Labs	1:500	ATR-002	AB_2341078
Biotinylated mouse anti-human HuC/D	Invitrogen, Carlsbad, CA	1:200	A21272	AB_2535822
Rabbit anti-HA-Tag (C29F4)	Cell Signaling Technology, Danvers, MA	1:500	3724	AB_1549585
DsRed Polyclonal	Clontech Laboratories, Mountain View, CA	1:1000	632496	AB_10015246
Rabbit anti-TRPV1	Alomone Labs	1:200	ACC-030	AB_2313819

Table 2.1: Primary antibodies used in Chapter 2. GFAP, glial fibrillary acidic protein; NK1R, Neurokinin-1 Receptor; NK2R, Neurokinin-2 receptor.

Antibody	Source	Dilution	Catalog No.	Resource ID No.
Alexa Fluor 594 donkey anti-guinea pig	Jackson ImmunoResearch, West Grove, PA	1:400	706-585-148	AB_2340474
Alexa Fluor 568 goat anti-rabbit	Invitrogen	1:400	A-11036	AB_2534094
Alexa Fluor 594-conjugated streptavidin	Jackson ImmunoResearch	1:400	016-580-084	AB_2337250
Alexa Fluor 488 goat anti-chicken	Invitrogen	1:400	A-11039	AB_2534096
Dylight 405-conjugated streptavidin	Jackson ImmunoResearch	1:400	016-470-084	AB_2337248
Alexa Fluor 488 donkey anti-guinea pig	Jackson ImmunoResearch	1:400	706-545-148	AB_2340472

Table 2.2: Secondary antibodies used in Chapter 2.

Table 2.2 (cont'd)

Alexa Fluor 488 donkey anti-rabbit	Jackson ImmunoResearch	1:400	711-545-152	AB_2313584
Alexa Fluor 594 donkey anti-rabbit	Jackson ImmunoResearch	1:400	711-585-152	AB_2340621

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CHAPTER 3

The Cholinergic Activation Of Enteric Glia Is A Physiological Mechanism That Contributes To The Regulation Of Gastrointestinal Motility

ABSTRACT

The reflexive activities of the gastrointestinal (GI) tract are regulated, in part, by precise interactions between neurons and glia in the enteric nervous system (ENS). Enteric glia are a unique type of peripheral glia that surround enteric neurons and regulate neuronal function, activity, and survival. Enteric glia express numerous neurotransmitter receptors that allow them to sense neuronal activity, but it is not clear if enteric glia monitor acetylcholine (ACh), the primary excitatory neurotransmitter in the ENS. Here, we tested the hypothesis that enteric glia detect ACh and that glial activation by ACh contributes to the physiological regulation of gut functions. Our results show that enteric glia express both M3 and M5 receptors, and that muscarine drives intracellular Ca^{2+} signaling predominantly through M3R activation. To elucidate the functional effects of activation of glial M3Rs, we used *GFAP::hM3Dq* mice that express a modified human M3R (hM3Dq) exclusively on glia to directly activate glial hM3Dqs using clozapine-N-oxide. Using spatiotemporal mapping analysis, we found that the activation of glial hM3Dq receptors enhances motility reflexes ex vivo. Continuous stimulation of hM3Dq receptors in vivo, drove changes in GI motility without affecting neuronal survival in the ENS and glial muscarinic receptor activation did not alter neuron survival in vitro. Our results provide the first evidence that enteric glia express functional muscarinic receptors and suggest that the activation of glial muscarinic receptors contributes to the physiological regulation of GI functions.

INTRODUCTION

The enteric nervous system (ENS) is the principal regulator of gastrointestinal (GI) function. The ENS has multiple roles including regulating fluid exchange across the mucosa, changing local blood flow, and regulating patterns of motility (1). The importance of the ENS is emphasized by a wide range of enteric neuropathies that are caused by failure in ENS function. The major inhibitory transmitters within the ENS are nitric oxide (NO), adenosine triphosphate (ATP) and vasoactive intestinal peptide (VIP). The major excitatory pathways within the ENS are cholinergic and tachykinergic, with acetylcholine (ACh) and tachykinins (TKNs) producing excitatory potentials in post-synaptic effector cells (2). Importantly, the majority of functional outputs of the ENS are mediated by the integration of signaling between enteric neurons and enteric glia (3,7).

Enteric glia are a distinct type of peripheral glia cell that surround enteric neurons and interact with neural circuits in the ENS (3). Indeed, enteric glia detect neuronal activity (4-9) through the expression of receptors for multiple neurotransmitters (2,9) and glial activation subsequently modulates the activity of neural circuits (7-11). Glial activation has a major influence over enteric circuits involved in the regulation of motility (11) and secretion (42) and the direct activation of enteric glia is sufficient to drive intestinal reflexes (7,11,42).Therefore, understanding the circumstances under which glial activation is triggered and the downstream consequences of various glial signal transduction pathways is important to understand the functional regulation of the intestine.

Enteric glial activity is recruited by multiple neurotransmitters and modulators in the ENS. To date, enteric glia are known to express receptors for adenosine diphosphate (ADP; P2Y1 receptors) (12-13), adenosine triphosphate (ATP) and uridine triphosphate

(UTP) (both through P2Y4 receptors) (4), adenosine (A2B receptors) (14), norepinephrine (α2a adrenergic receptors) (15), glutamate (mGluR5, AMPA, NMDA) (16,43), serotonin (5-HT_2) (9), bioactive lipids (SP1R, LPA1) (44,45), endothelin (likely ETB receptors) (46), protease-activate receptors (PAR1, PAR2) (47) and bradykinin (B2 receptors) (48). Thus, enteric glia can respond to multiple neurotransmitters and modulators within the ENS, but it is still unknown whether they respond to the principal excitatory neurotransmitter in the ENS: ACh. Importantly, cholinergic receptor expression is well established in other types of neuroglia such as perisynaptic schwann cells (PSCs) in the neuromuscular junction (17), cortical astrocytes (18-20), and cochlear astrocytes (21). The similarities between enteric glia and these related populations of neuroglia suggest that enteric glia may also have the potential to sense cholinergic transmission.

Here, we addressed this issue by testing the hypothesis that enteric glia detect ACh and that glial activation by ACh contributes to the physiological regulation of gut functions. Our results show that enteric glia express the M3 and M5 subtypes of muscarinic receptors (MRs) and that muscarine drives glial calcium (Ca^{2+}) responses that are primarily driven by M3Rs and are independent of neuronal activation. We assessed the functional consequences of glial M3R activation using *GFAP*::hM3Dq+/- mice that express a modified human M3R (hM3Dq) exclusively on glia and found that the selective activation of glial M3Rs with clozapine-N-oxide (CNO) enhances distension-induced motility reflexes. Chronic stimulation of glial M3Rs in vivo altered intestinal motility, but did not cause overt damage or affect neuron survival in the ENS. Overall, our results provide the first evidence that enteric glia express functional muscarinic receptors and

suggest that the activation of glial muscarinic receptors contributes to the functional regulation of intestinal reflexes.

MATERIALS AND METHODS

Declaration of Animal Use Approval

All work involving animals was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University (MSU).

Animals

Male and female C57BL/6 mice between 8-10 weeks of age were used for immunohistochemistry and in situ model of neuroinflammation (Charles River Laboratories, Hollister, CA). All mice were maintained on a 12-hour light/dark cycle in a temperature-controlled environment with access to food and water *ad libitum*. Transgenic mice expressing the genetically-encoded Ca^{2+} indicator GCaMP5g in enteric glia (*Sox10*CreER^{T2}::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice) were bred in house and were generated as previously described (22) by crossing *Sox10*::CreER^{T2} mice (gift from Dr. Vassilis Pachnis, The Francis Crick Institute, London, England) with *Polr2a*$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice [PC::G5-tdT (Jackson Laboratory, Bar Harbor, ME; stock number: 024477; RRID: IMSR_JAX:024477)]. Transgenic mice expressing the designer receptor hM3Dq under the transcriptional control of the glial fibrillary acidic protein (GFAP) promotor [*GFAP*::hM3Dq+/- (RRID: MMRRC_042286-UNC)] were a gift from Dr. Ken

McCarthy (University of North Carolina Chapel Hill) and were bred in house as heterozygous as previously described (11). All double transgenic mice were maintained as hemizygous for Cre (*Sox10*::CreER$^{T2+/-}$) and homozygous for the floxed allele (*Polr2a*$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$). CreER^{T2} activity was induced in *Sox10*CreER^{T2}::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice by feeding animals with chow containing tamoxifen citrate (400 mg/kg) for 2 weeks followed by 1 week of normal chow before use. Genotyping was performed by the Research Technology Support Facility (RTSF) Genomics Core at Michigan State University and by Transnetyx (Cordova, TN).

Whole-Mount Immunohistochemistry

Longitudinal muscle myenteric plexus (LMMP) whole-mount preparations were microdissected from mouse colonic tissue preserved in Zamboni's fixative. Processing of LMMPs via immunohistochemistry was conducted as previously described (23) with the primary and secondary antibodies listed in Tables **3.1** and **3.2**, respectively. Briefly, LMMP preparations were washed three times for 10 minutes in 0.1% Triton X-100 in phosphate-buffered saline (PBS) followed by a 45-minute incubation in blocking solution containing 4% normal goat serum, 0.4% Triton X-100 and 1% bovine serum in PBS. Preparations were incubated with primary antibodies overnight at room temperature and with secondary antibodies for 2-hours at room temperature before mounting. Antibody specificity was confirmed by preadsorption with the corresponding control peptides. Fluorescent labeling was visualized by confocal imaging through the Plan-Apochromat 60x oil immersion objective (1.42 numerical aperture) of an inverted Olympus Fluoview FV1000 microscope (Olympus, Center Valley, PA).

Ca^{2+} Imaging

LMMP whole-mount preparations from *Sox10*CreER^{T2}::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice were microdissected from mouse colons and incubated for 15 minutes at room temperature in an enzyme mixture consisting of 150 U/mL Collagenase type II and 1 U/mL Dispase (Life Technologies). Images were acquired at 1 Hz through the 40X water immersion objective [LUMPlan N, 0.8 numerical aperture (n.a.)] of an upright Olympus BX51WI fixed stage microscope (Olympus, Center Valley, PA) using MetaMorph software (Molecular Devices, Sunnyvale, CA) and a Neo sCMOS camera (Andor, South Windsor, CT). We continually perfused whole-mounts at 2-3 mL min^{-1} with buffer using a gravity flow perfusion system. Agonists were dissolved in buffer and bath applied for 30 seconds. Antagonists were dissolved in buffer and applied for either 20 minutes or 3 minutes before imaging.

In situ Model of Neuroinflammation

Enteric neuron death was driven as previously described (13). LMMP preparations were incubated with the P2X7R agonist 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (BzATP) (300μM) or (+)-(2S,4R,5S)-Tetrahydro-4-hydroxy-N,N,N,5-tetramethyl-2-furanmethanammonium chloride [(+)-Muscarine] (20μM) in the presence or absence of the nonselective muscarinic acetylcholine antagonist (-)-Scopolamine hydrochloride (10μM) for 2 hours in 95% air, 5% CO_2 at 37ºC. LMMP preparations were then rinsed with fresh buffer, incubated for an additional 2 hours in buffer only, and fixed overnight in Zamboni's fixative.

Motility Reflex Recordings Using Spatiotemporal Maps

Mouse colons from *GFAP*::hM3Dq+/- and WT mice were removed and placed in a bath containing DMEM/F-12 maintained at 37ºC. Luminal contents were flushed, the colon was cannulated at both ends (oral and anal), and secured using silk suture. Intraluminal pressure was hydrostatically maintained using DMEM/F-12 at room temperature. After a 30-minute acclimation period, tissue movements were recorded for 30-45 minutes using a 2 megapixel digital camera (Olympus DP21, Olympus Corporation, Tokyo, Japan) at a rate of 15 frames/s, sampled at a pixel density of 1600x1200) and saved in the AVI format. The recordings were subsequently processed offline to generate and analyze spatiotemporal maps.

Clozapine-N-Oxide (CNO) Administration in Drinking Water

GFAP::hM3Dq+/- mice and WT littermates were administered 0.13mg/mL of CNO dissolved in 0.1% dimethyl sulfoxide (DMSO) and 3.5% sucrose in drinking water. Mice were monitored closely and weight was recorded daily. Mice were sacrificed after 4 days of treatment with CNO. Macroscopic damage was recorded with an established scoring system (24).

CMMC Recordings

Mouse colons were removed and placed in a bath containing DMEM/F-12 maintained at 37ºC. Luminal contents were flushed and a stainless-steel rod was inserted into the lumen. Tissue was secured at both ends with surgical silk and force transducers (Grass Instruments) were placed 2 cm apart and attached by metal hooks. The initial tension was

adjusted to 0.5 g, and the development of spontaneous CMMCs was monitored over an acclimatization period of 20-minutes using LabChart8. The last 6-minute interval of this acclimation period was used as baseline. Drugs were added after the acclimation period, and CMMCs were recorded for an additional 10-minute interval.

Endogenous Pellet Production

Mice were individually housed, and fecal pellet output was measured on three consecutive days after two days of acclimation. Pellets were collected for 1 hour beginning at 9:00 AM (Zeitgeber +3). The wet weight of fecal matter was measured immediately, and the dry weight was obtained the next day after dehydration. Data from three days was averaged.

Colon Bead Assay

Mice were lightly anesthetized with isoflurane and a 2 mm plastic bead was inserted 3 cm into the colon. Distal colonic transit time was assessed by recording the time to expulsion of the bead. Data from two days was averaged.

Solutions

Live tissue was maintained in DMEM/F-12 nutrient mixture (Life Technologies) during collection and microdissection. Ca^{2+} imaging experiments were performed in modified Krebs buffer containing (in mM): 121 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 10 HEPES, 21.2 $NaHCO_3$, 1 pyruvic acid, and 8 glucose (pH adjusted to 7.4 with NaOH).

Chemicals and Reagents

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich(St.Louis,MO). 8-chloro-11-(4-methyl-4-oxido-1-piperazinyl)-5H-dibenzo[b,e][1,4]diazepine [clozapine N-oxide (CNO)] was obtained from the National Institute on Drug Abuse Supply Program (NDSP). 1-(4-Methoxybenzyl)-5-trifluotomethoxyisatin (VU 0238429) and (α*R*)-α-Cyclopentyl-α-hydroxy-*N*-[1-(4-methyl-3-pentenyl)-4-piperidinyl]benzeneacetamide fumarate (J104129-fumarate) were purchased from Tocris (Minneapolis, MN). 5-[(3-Acetylphenoxy)methyl]-N-methyl-N-[(1S)-1-pyridin-2-ylethyl]-1,2-oxazole-3-carboxamide [ML381 (VU0488130) Fumarate] was purchased from Aobious (Glousester, MA).

Statistical Analysis

For Ca^{2+} imaging, traces represent the average change in fluorescence ($\Delta F/F$) over time for all glial cells within a single ganglion. Neuron packing density was determined by counting the number of HuC/D-immunoreactive neurons per ganglionic area in 10 ganglia per LMMP preparation using the cell counter plug-in tool on ImageJ Software, version 1.48 (NIH). All results are presented as mean ± standard error of the mean (SEM) and statistically significant differences were determined using an analysis of variance (ANOVA) or t-test, as appropriate with $P < 0.05$ considered statistically significant (GraphPad Prism; GraphPad Software, San Diego, CA).

RESULTS

Enteric glia express M3 and M5 receptors

Acetylcholine is the predominant neurotransmitter in the ENS and muscarinic acetylcholine receptors (MRs) are involved in multiple intestinal reflexes (25-27). To test whether enteric glia have the potential to respond to acetylcholine, we co-labeled with antibodies against glial fibrillary acidic protein (GFAP) and the different MR subtypes (**Figure 3.1**). Our results show that enteric glia primarily express muscarinic receptor subtype 3 (M3R) (**Figure 3.1C-C'''**) and muscarinic receptor subtype 5 (M5R) (**Figure 3.1E-E'''**). Preadsorption controls for the different MR subtypes (**Figure 3.2**) confirmed labeling. These results demonstrate that enteric glia express muscarinic receptors and have the potential to respond to acetylcholine.

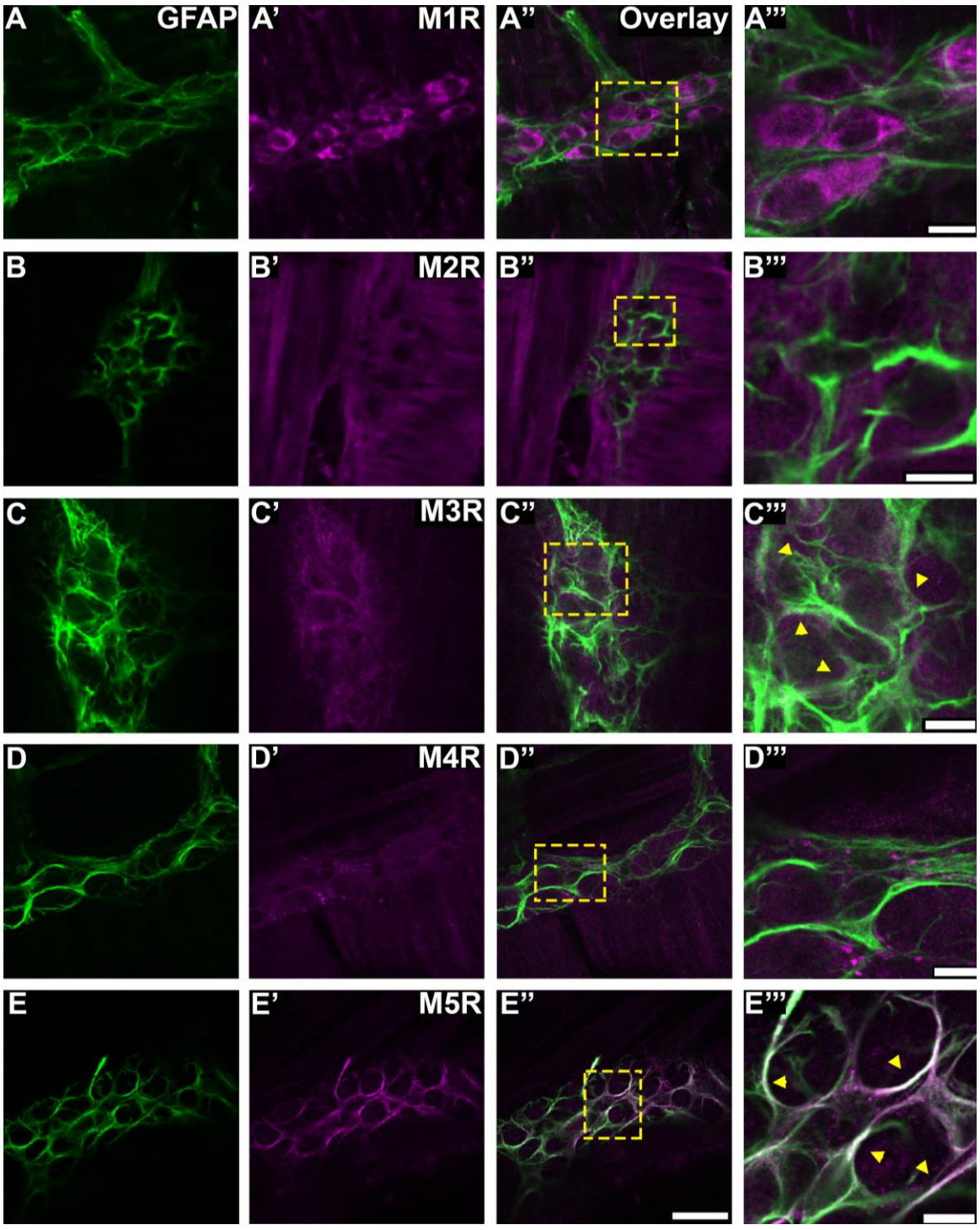

Figure 3.1: Enteric glia in the mouse myenteric plexus express M3 and M5 receptors. Images (confocal single optical planes, 1µm) show dual-label immunohistochemistry for GFAP (green, **A-E**) and either M1R (magenta, **A'**), M2R (magenta, **B'**), M3R (magenta, **C'**), M4R (magenta, **D'**) or M5R

Figure 3.1 (cont'd)

(magenta, **E'**) in the distal myenteric plexus of mice. Overlays of each combination are shown in **A" – E"**. Areas demarcated by the dashed boxes in **A" – E"** are enlarged in panels **A"' – E"'**. Asterisks in **A"' – E"'** highlight areas of colocalization. The scale bar in **E''** represents 20µm and applies to **A' – E''**. All scale bars in the enlarged images represent 10µm. Images are representative of labeling performed on tissue from a minimum of three mice.

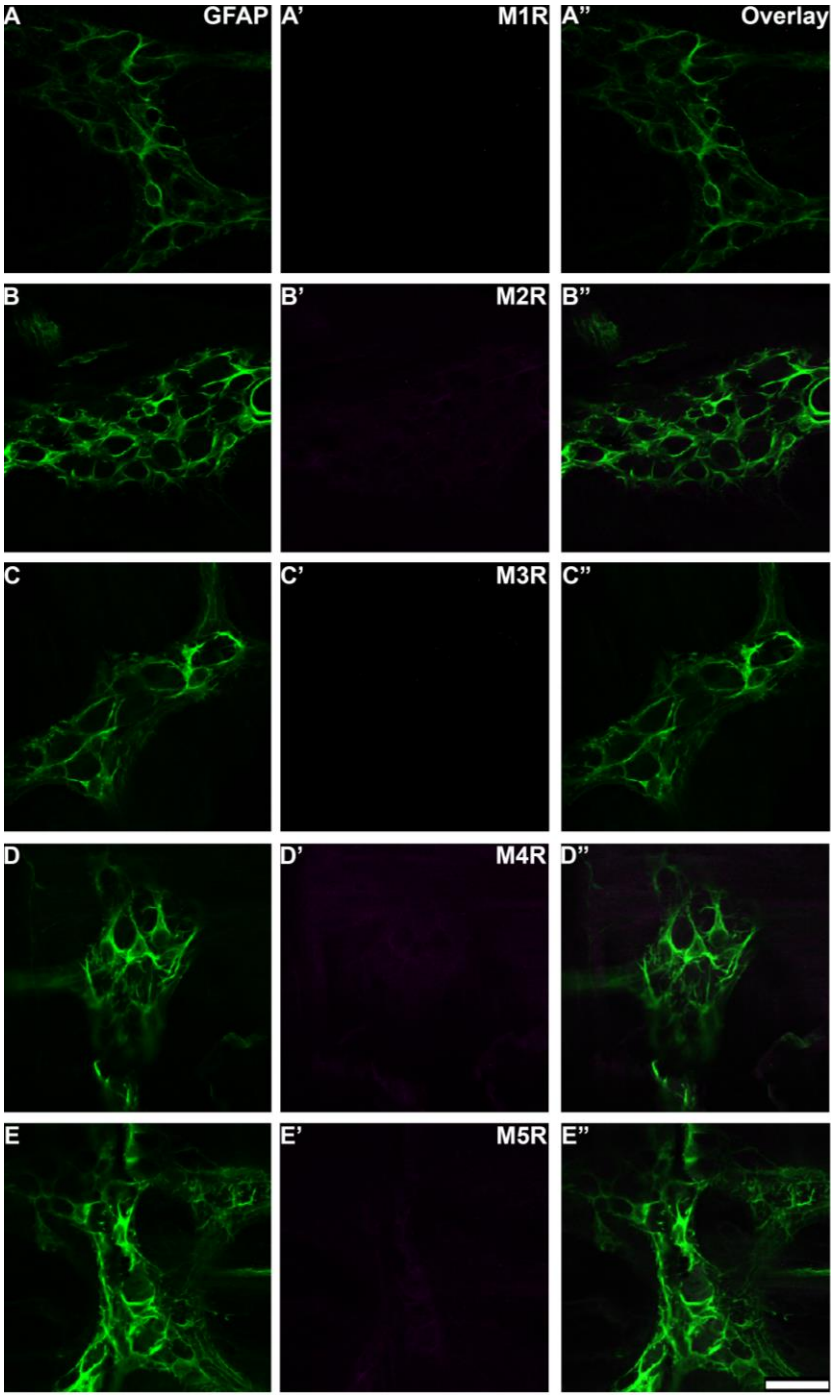

Figure 3.2: Preabsorption controls for muscarinic receptor antibodies confirm specificity. Images are single optical sections (1µm) through myenteric ganglia from the distal colons of mice. Dual-label immunohistochemistry for GFAP (green, A – E) and preabsoprtion controls for M1R (magenta, A'), M2R (magenta, B'), M3R (magenta, C'), M4R (magenta, D') or M5R (magenta, E').

Figure 3.2 (cont'd)

Overlays of each combination are shown in A" – E". Scale bar in E" represents 20µm and applies to all images. Images are representative of labeling performed on tissue from a minimum of three mice.

Muscarine drives robust glial Ca^{2+} responses independent of neuronal depolarization

M3 and M5Rs couple to Gq signal transduction pathways that lead to increases in intracellular Ca^{2+} (28). Therefore, we used Ca^{2+} imaging to determine whether glial muscarinic receptors are functional. In these experiments, we used *Sox10*CreER^{T2}::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice to selectively study glial Ca^{2+} responses (22) and avoid the confounding effects of other nearby cells. We first stimulated myenteric plexus preparations with the prototypical muscarinic agonist muscarine (20 µM; **Figure 3.3A**). A 30s application of muscarine evoked numerous individual glial Ca^{2+} responses (75 on average) with an average magnitude of 0.62 ±0.06 ΔF/F (**Figure 3.3C-D**). We next used tetrodotoxin (1 µM; TTX) to test whether glial responses to muscarine require reciprocal interactions with neurons (**Figure 3.3B**). Although TTX did not abolish glial-Ca^{2+} responses to muscarine, it did significantly reduce the number of responses per glia by 46% (p=0.0026; **Figure 3.3C**) and significantly increased the glial peak response by 43% (p=0.0059; **Figure 3.3D**). Together with our immunohistochemistry data above, these data show that glia are capable of directly responding to muscarinic agonists. However, the bath application of muscarine also evokes intercellular neuron-glia communication that contributes to cholinergic transmission in the ENS.

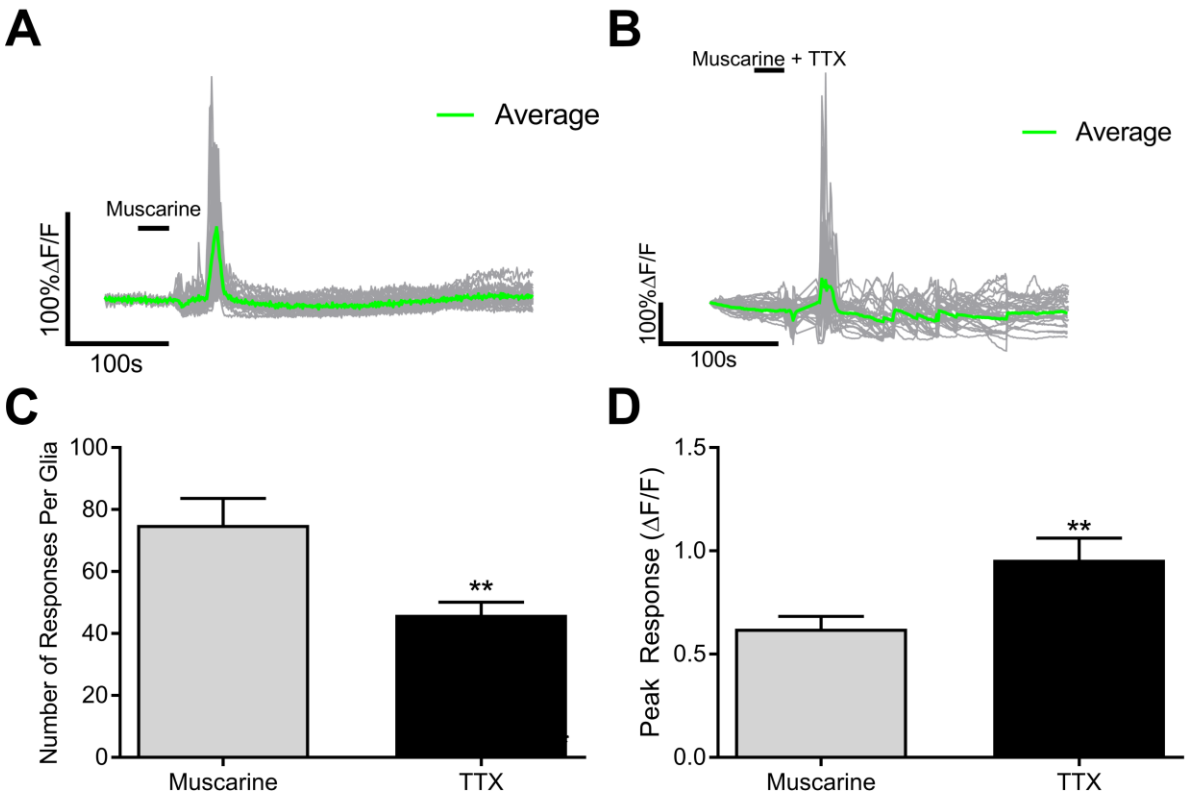

Figure 3.3: Muscarine drives Ca^{2+} responses from enteric glia independent of neuronal activation. Representative Ca^{2+} responses evoked by muscarine in enteric glia in the absence (**A**) or presence of tetrodotoxin (TTX) (**B**). Gray traces show the responses of individual glial cells and the averaged response of all glia within the ganglion is shown in the green trace. (**C – D**) Quantification of the effects of TTX in the number (**C**) and peak response (**D**) of glial Ca^{2+} responses induced by muscarine (n=26-34 glia from 3-4 mice; student's t-test; **p<0.01).

Glial Ca^{2+} responses evoked by muscarine are primarily mediated through M3 receptors.

Next, we conducted experiments with M3 and M5 selective drugs to test the relative contribution of each receptor subtype to muscarine driven glial Ca^{2+} responses. In these experiments, we again used *Sox10*CreER^{T2}::Polr2a$^{tm1(CAG-GCaMP5g,-tdTomato)Tvrd}$ mice to selectively study glial Ca^{2+} responses. We began by testing the contribution of M5Rs to glial Ca^{2+} responses by using the selective M5R antagonist ML381, and the selective positive allosteric modulator of M5Rs, VU0238429 (**Figure 3.4**). Muscarine still reliably evoked glial Ca^{2+} responses in the presence of 10µM ML381 (**Figure 3.4A**), but the number of responses per enteric glia was significantly reduced by 65% (p=0.0001; **Figure3.4D**) and glial peak responses were significantly reduced by 64% compared to muscarine alone (p=0.0002; **Figure 3.4E**). Interestingly, 10µM VU0238429 (**Figure 3.4B**) also significantly reduced the number of glial responses by 75% (p=0.0001; **Figure 3.4D**) and glial peak responses were significantly reduced by 88% compared to muscarine alone (p=0.0001; **Figure 3.4E**). To determine the contribution of M3Rs to muscarine-induced glial Ca^{2+} responses, we used the potent and selective M3R antagonist J104129 (100 nM). Treatment with J104129 alone was sufficient to abolish glial Ca^{2+} responses (**Figure 3.4C**), significantly reducing the number of individual glial responses (p=0.0001; **Figure 3.4D**) and the peak response (p=0.0001; **Figure 3.4C**). Together, these data suggest that glial Ca^{2+} responses to muscarine are primarily mediated through M3Rs, but M5Rs also contribute to the response.

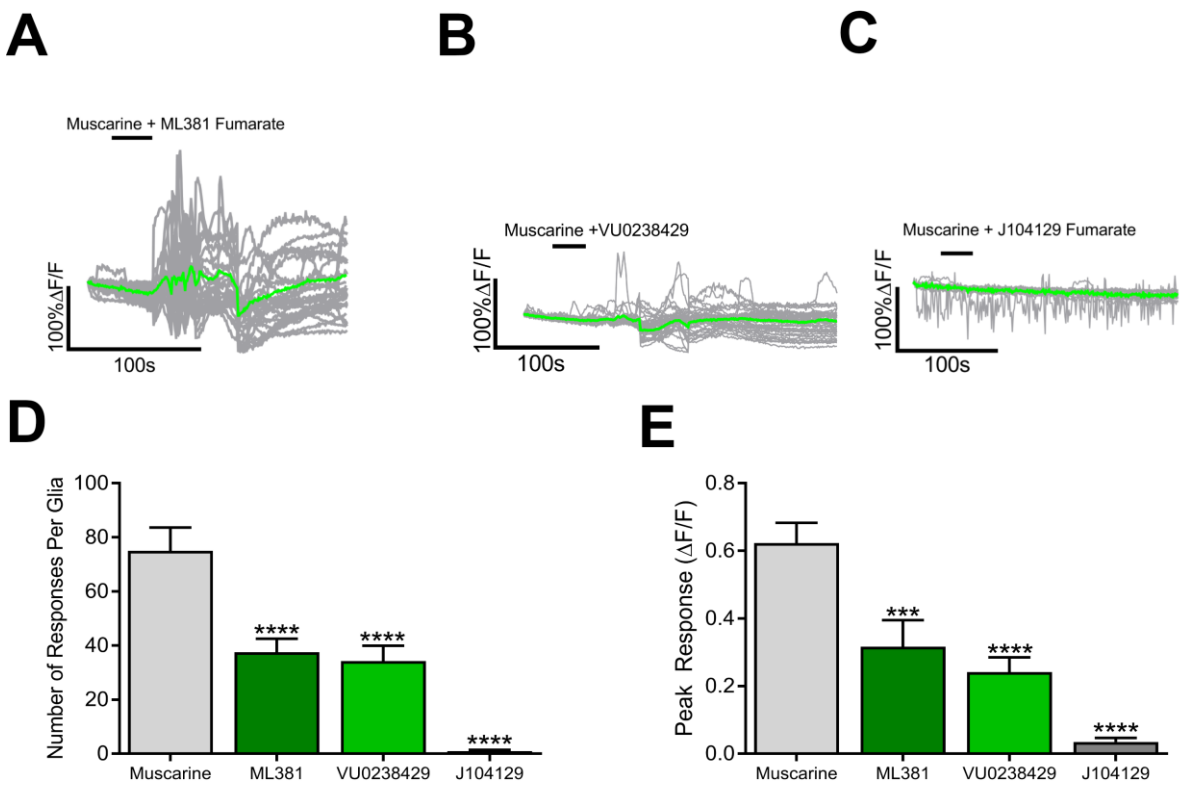

Figure 3.4: Glial Ca^{2+} responses driven by muscarine are primarily mediated through M3 receptors. (**A-C**) Representative glial Ca^{2+} responses evoked by muscarine in the presence of the M5R selective antagonist ML381 Fumarate (**A**); the M5R selective positive modulator VU0238429 (**B**) or the M3R selective antagonist J104129 Fumarate (**C**). Gray traces show the responses of individual glial cells and the averaged response of all glia within the ganglion is shown in the green trace. (**D – E**) Quantification of the effects of ML381 Fumarate, VU0238429 or J104129 Fumarate on the number (**D**) and peak response (**E**) of glial Ca^{2+} responses induced by muscarine. (n=21-39 glia from 4-5 mice; one-way ANOVA with multiple comparisons; ***p =0.0002; ****p <0.0001).

The selective activation of glial M3Rs in GFAP::hM3Dq mice modulates distension evoked motility reflexes

The observation that M3R activation is the major component of glial calcium responses suggest that M3R activation on enteric glia plays a role in the functional regulation of ENS reflexes. To test this notion, we used a chemogenetic model that expresses a modified human M3 (hM3Dq) receptor under the transcriptional control of the GFAP promoter (*GFAP::*hM3Dq) (11). This is an ideal model to specifically study the effects of glial M3 receptor activation because the hM3Dq is a M3R that can be selectively activated by the agonist clozapine-n-oxide (CNO). This allows a clear assessment of the downstream effects subsequent to the stimulation of glial M3R signaling. We observed clear colonic patterns after filling the entirety of the colon segment. Although there were no significant changes in colon segment diameter between baseline recordings and treatment with 10µM CNO ($p=0.4110$; n=3 mice; **Figure 3.5A**), we did find trending changes in propagation velocity between baseline and CNO treated colonic preparations ($p=0.0571$; n=3 mice; **Figure 3.5B**). Overall, this suggests that activation of modified M3Rs on enteric glia modulates distention evoked motility reflexes in the mouse colon.

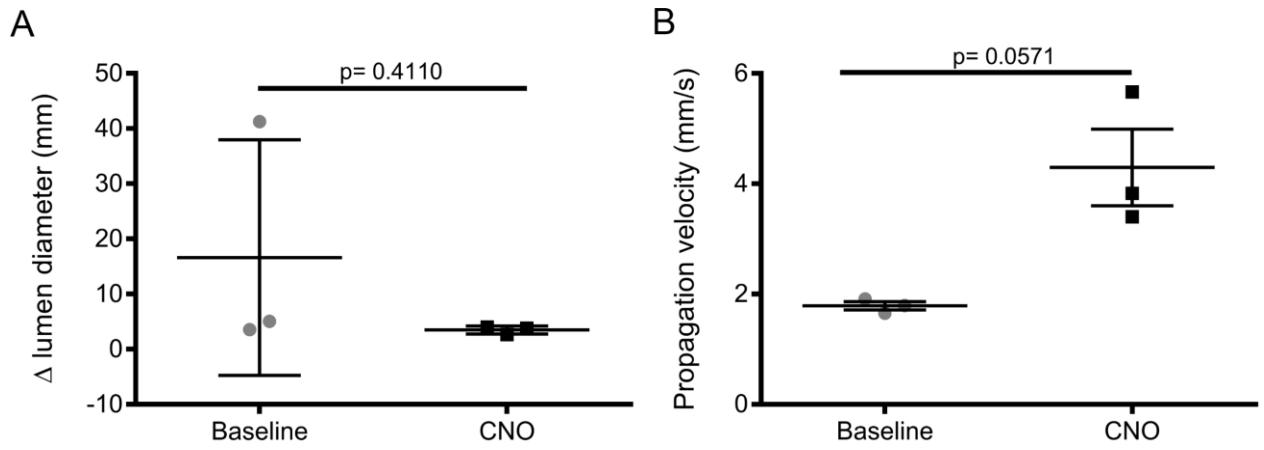

Figure 3.5: Effects of CNO on colonic distention reflexes of *GFAP*::hM3Dq mice using spatiotemporal mapping. (**A**) Changes in colon segment diameter at baseline (4.25±0.42mm) or after treatment with 10µM CNO (3.5±0.42mm) (paired t-test; p=0.4110; n=3 mice). (**B**) Propagation velocity at baseline (1.79±0.07mm/s) or after treatment with 10µM CNO (4.30±0.69mm/s) (paired t-test; p=0.0571; n=3 mice).

Cholinergic activation of enteric glia does not modify purine–driven neurodegeneration

Purinergic signaling has a pivotal role in the modulation of inflammation in the colon (29). We and others have shown that adenosine triphosphate (ATP) levels are significantly elevated during colitis (13,30) and that this increase in ATP levels drives enteric neuron death through neuronal P2X7 receptors (P2X7Rs) (31) and decreases cholinergic signaling in the myenteric plexus (32). In addition, cholinergic transmission affects immune cell proliferation, cytokine production, T helper differentiation and antigen presentation providing anti-inflammatory benefits (49-51). This suggests that cholinergic anti-inflammatory pathways could compete and protect against purinergic pro-inflammatory pathways. To determine if ACh signaling through MRs modulates P2X7R-mediated neuron death in inflammation, we used an in situ model of neuroinflammation in whole mount preparations of myenteric plexus to study potential interactions between the purinergic and cholinergic activation of glia (**Figure 3.6**). Similar to our previous data, 300μM BzATP, a P2X7R agonist, was sufficient to drive a significant 36% reduction in myenteric neuron packing density ($p=0.0082$) (13). 20μM muscarine alone was not sufficient to drive neuron loss in situ and neurodegeneration in the presence of 20μM muscarine and 300μM BzATP was comparable (40% reduction, $p=0.0054$) to neurodegeneration in the presence of BzATP alone. This suggests that glial MR activation does not potentiate or protect against P2X7R-mediated neurodegeneration.

Increases in ATP during inflammation contribute to decreases in ACh levels (32). Therefore, we tested if decreasing ACh signaling through MRs potentiates P2X7R-mediated neuron death. To this end, we used 10μM scopolamine, a non-specific

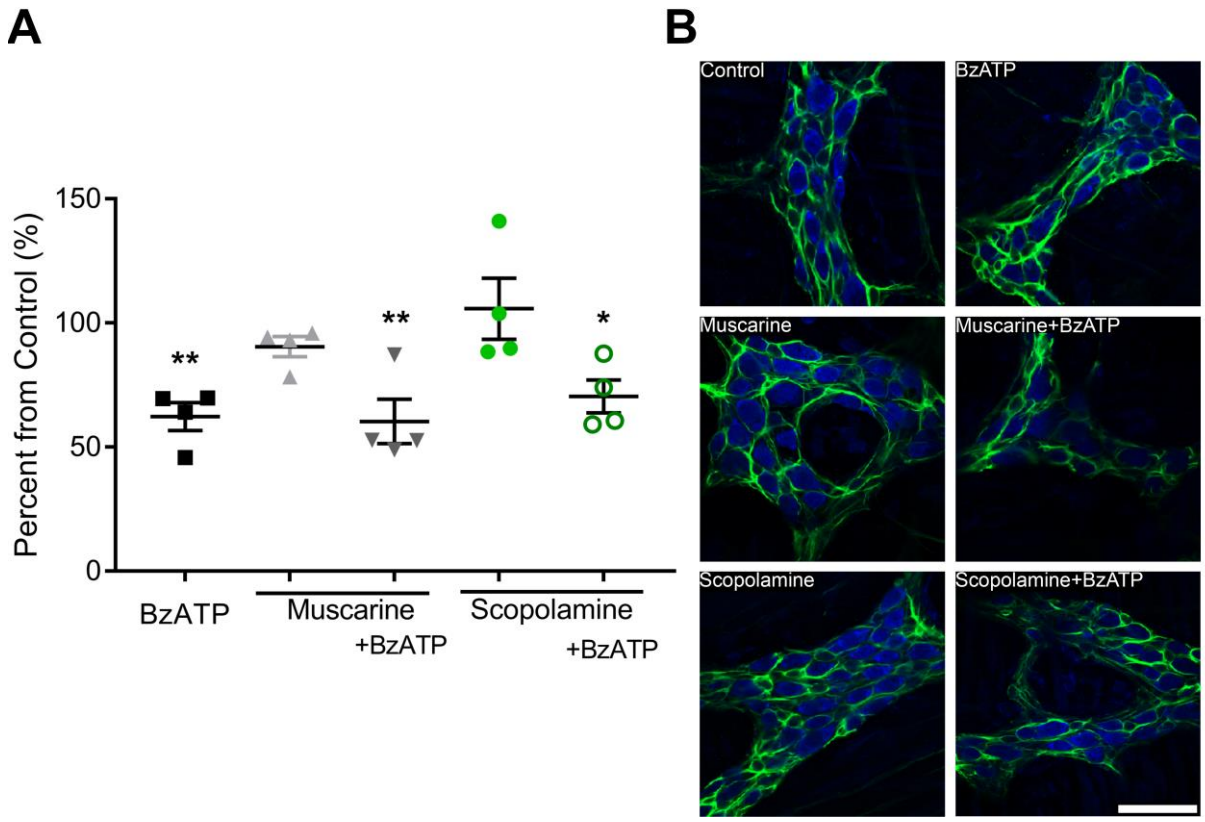

Figure 3.6: Muscarine does not drive neurodegeneration in situ. Quantification (**A**) and representative images (**B**) of mean packing density of HuC/D-immunoreactive neurons in the myenteric ganglia after in situ activation of P2X7Rs with BzATP; activation of muscarinic receptors with muscarine in the presence or absence of BzATP; or the nonselective muscarinic receptor antagonist scopolamine in the presence or absence of BzATP. (n=4 mice; one-way ANOVA with multiple comparisons; *p <0.05; **p <0.01). Enteric neurons are labeled with HuC/D (magenta) and enteric glia are labeled with GFAP (green) in all panels. Scale bar represents 20µm and applies to all images.

muscarinic receptor antagonist, to prevent muscarine receptor activation. 10μM scopolamine alone did not drive neurodegeneration and treatment of 10μM scopolamine in conjunction with 300μM BzATP did not potentiate or prevent neuronal loss (30% neuron loss, p=0.042). Together, these data suggest muscarinic receptors are not involved in pathophysiological signaling pathways in the ENS.

The chronic stimulation of glial M3Rs in vivo drives weight loss, but does not induce overt inflammation or affect neuron survival

To assess potential pathophysiological contributions of glial muscarinic receptor activation pathways, we chronically activated glial M3R pathways over 4 days by administering CNO (0.13mg/mL) to the drinking water of *GFAP*::hM3Dq+/- mice and WT littermates (**Figure 3.7A**). In agreement with earlier reports (34), CNO treatment alone drove significant body weight loss in *GFAP*::hM3Dq+/- mice compared to WT littermates ($p<0.0001$; **Figure 3.7B**). This effect is likely caused by increased heart rate in this model (33,34). However, the chronic stimulation of glial M3 receptors did not increase macroscopic damage to the distal colon ($p=0.7231$; **Figure 3.7C**) or drive neurodegeneration ($p=0.1823$; **Figure 3.7D**). These in vivo data are in agreement with our in vitro data and show that cholinergic signaling in enteric glia mediates functional, but not pathophysiological pathways.

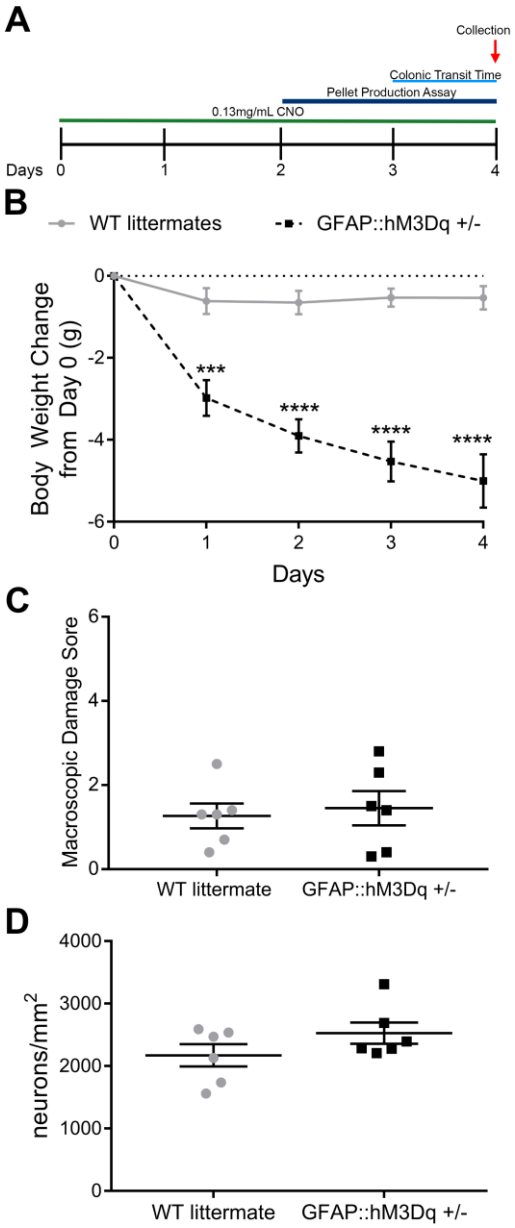

Figure 3.7: Continuous stimulation of glial hM3Dq receptors with clozapine-n-oxide (CNO) in vivo decreases mouse body weight without decreasing neuronal survival. (**A**) Schematic representation of the timeline of assays performed on WT littermate and GFAP::hM3Dq mice. (**B**) Weight loss pattern at 4 days after start of treatment with 0.13mg/mL CNO in water (n=6 mice; two-way ANOVA with multiple comparisons; ***p =0.002; ****p <0.0001). In vivo stimulation of GFAP::hM3Dq with CNO does not increase macroscopic damage (**C**) or decrease neuronal survival (**D**) (n=6 mice; student's t-test).

The chronic activation of glial cholinergic pathways alters GI motility

We next assessed how the chronic stimulation of glial M3Rs affects gastrointestinal motility in vivo (**Figure 3.8**). The chronic treatment with CNO did not significantly alter colonic transit time in *GFAP*::hM3Dq+/- mice (**Figure 3.8A**), but fecal pellet production was significantly reduced by 120% compared to WT littermates (p=0.0098; **Figure 3.8B**). Pellet dry weight was not significantly different between groups (**Figure 3.8C**), but chronic glial M3R stimulation did significantly reduce pellet fluid content in *GFAP::*hM3Dq+/- mice by 87% compared to WT littermates (p=0.04; **Figure 3.8D**).

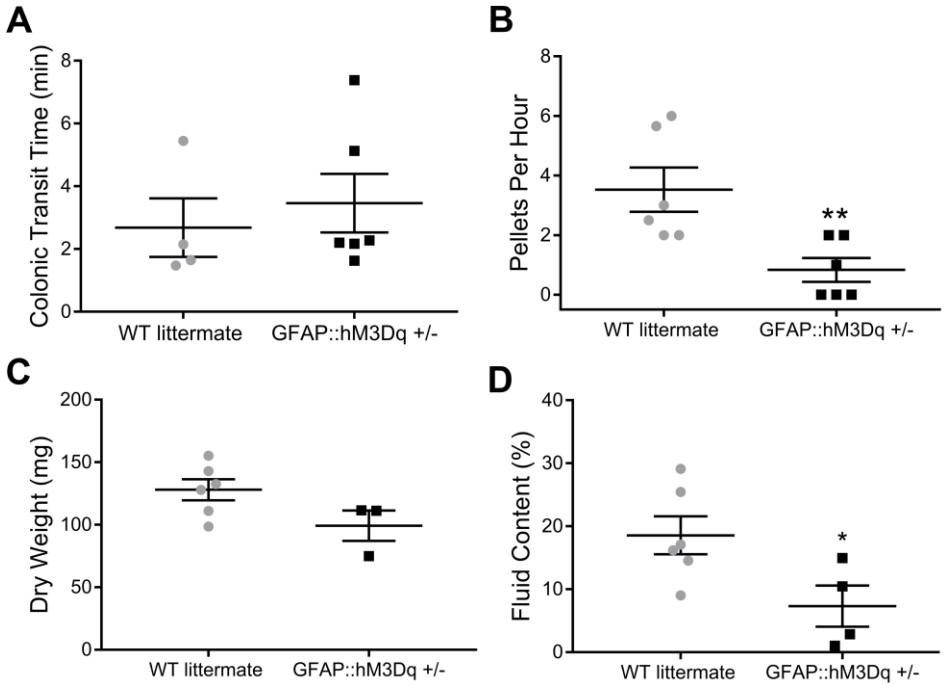

Figure 3.8: Continuous activation of glial hM3Dq receptors in vivo decreases pellet production and pellet fluid content without increasing colonic transit time. Effects of 0.13mg/mL CNO in drinking water on colonic transit time (**A**); endogenous pellet production (**B**); pellet dry weight (**C**) and pellet water content (**D**) from GFAP::hM3Dq mice and WT littermates. (n=3-6 mice; student's t-test; *p <0.05; **p <0.02).

We also tested how chronic glial M3R activation affects the ongoing pattern of CMMCs in the isolated colon. Similar to our published data, we observed spontaneous, regular CMMCs in both *GFAP*::hM3Dq+/- mice and WT littermates (11). However, oral CMMC contraction amplitude was reduced by 93% in colons from mice with chronic glial M3R stimulation as compared to WT littermate controls ($p=0.0402$; **Figure 3.9A**). Although chronic CNO treatment trended to decrease other contractile aspects of CMMCs, we did not observe any other significant changes (**Figure 3.9**). This is in agreement with other studies demonstrating that muscarinic receptors are involved in the excitatory, but not inhibitory, component of CMMCs (27). Overall, our data suggest that the cholinergic activation of enteric glial cells is primarily a physiological receptor pathway.

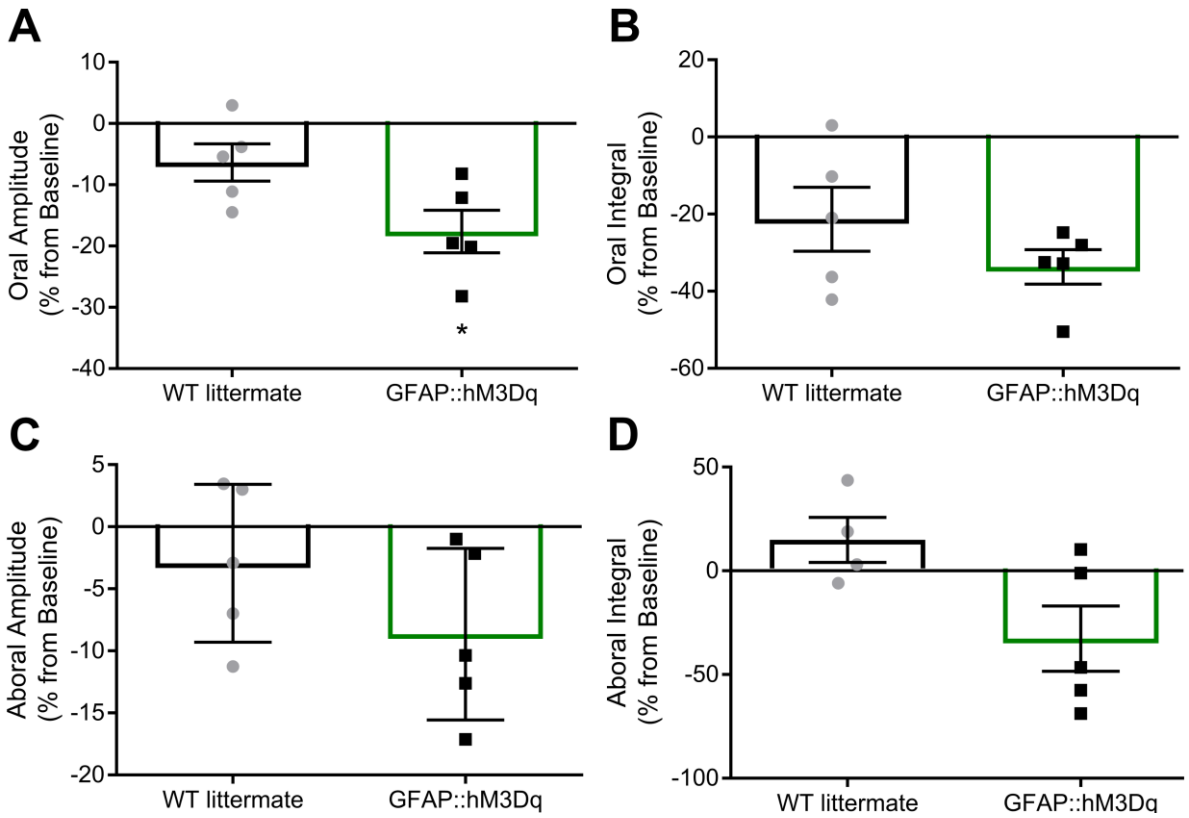

Figure 3.9: Continuous activation of glial hM3Dq receptors in vivo decreases oral contraction amplitude of colonic migrating motor complexes (CMMCs). Effects of 0.13mg/mL CNO in drinking water on CMMC characteristics including oral contraction amplitude (**A**) and integral (**B**); and aboral contraction amplitude (**C**) and integral (**D**). All data are expressed as percentage change from baseline after acclimation period. (n=4-6 mice; student's t-test; *p <0.05).

DISCUSSION

The ENS regulates GI reflexes by precise interactions between enteric neurons and glia (1). Enteric glia are a distinct type of peripheral glia that surround enteric neurons and play important roles in regulating neuronal function, activity and survival (3). Enteric glial activity is recruited by multiple neurotransmitters and modulators. However, it is still not clear if enteric glia can sense and respond to ACh, the principal excitatory neurotransmitter in the ENS. Here, we addressed this issue by testing the hypothesis that enteric glia detect ACh and that glial activation by ACh contributes to physiological regulation of gut functions. Our results show that enteric glia express two subtypes of muscarinic receptors and the stimulation of these receptors induces Ca^{2+} responses independent of neuronal depolarization, primarily through M3Rs. Importantly, the direct activation of glial hM3Dq receptors enhanced distension-evoked motility reflexes and the continuous stimulation of glial hM3Dq receptors in vivo drove changes in GI motility without affecting neuronal survival in the ENS. Together, our data provide the first evidence for functional muscarinic receptors on enteric glia and suggest that activation of glial muscarinic receptors contributes to cholinergic pathways in the ENS.

Muscarinic receptor signaling is well established in other glial cells including perisynaptic schwann cells (PSCs), astrocytes, oligodendrocytes, and Müller cells (17,18,21,25,37,36). PSCs express MRs that when activated can protect against GFAP upregulation associated with glial reactivity (17). Cortical and choclear astrocytes express multiple MR subtypes and muscarine stimulation drives intracellular Ca^{2+} responses (18,21,35). Similarly, Müller cells, a type of retinal glial cell, express functional M1Rs in culture (36). The myelinating glial cells of the central nervous system, oligodendrocytes,

also express functional MRs that upon activation enhance oligodendrocyte proliferation (37).

The similarities between enteric glia and these other population of neuroglia provided strong rationale for our original hypothesis that enteric glia express functional muscarinic receptors. In support, we found that enteric glia predominantly express M3Rs and M5Rs. Both M3Rs and M5Rs signal through Gq transduction cascades that involve phosphoinositol and the release of Ca^{2+} from intracellular stores. Therefore, we used Ca^{2+} imaging to understand the mechanisms of MR activation on enteric glia. We found that muscarine drives Ca^{2+} responses in enteric glia. Interestingly, TTX treatment significantly reduced the number of responses per glia to muscarine, suggesting there is a neuronal component to muscarine induced glial Ca^{2+} signaling. Our data using MR subtype selective drugs demonstrate that muscarine-mediated glial Ca^{2+} responses are primarily driven through M3Rs because antagonizing M3Rs completely abolished glial responses. However, antagonizing M5Rs also significantly reduced glial Ca^{2+} responses. These data suggest that glial M5Rs may also play a role in cholinergic signaling, but to a lesser extent.

ACh is the principal excitatory neurotransmitter in the ENS and the stimulation of MRs is involved in neurotransmission mediating intestinal muscle reflexes and ion secretion within the gut (38-39). Importantly, enteric glia modulate enteric neuronal networks involved in GI motility and the stimulation of glial Ca^{2+} responses has strong, excitatory effects on motor circuits (11). Additionally, enteric glial activity contributes to the regulation of electrogenic ion transport in the intestine through effects on enteric neuronal circuits (42). Our current data suggest that the cholinergic activation of glia contributes to the regulation of these GI reflexes. Enteric glia could function similar to

astrocytes in tripartite synapses to detect ACh and respond with release of gliotransmitters to modulate synaptic strength and neuronal excitability (52-53). In support, stimulation of muscarinic receptors on cortical astrocytes elicits gliotransmitter release to enhance synaptic plasticity in the somatosensory cortex (54). This suggests that glial M3R activation may function to enhance synaptic strength and potentiate excitatory reflexes through gliotransmitter release. However, more work is needed to decipher the exact mechanisms and gliotransmitters involved.

Enteric glia are actively involved in pathophysiological pathways during inflammation in the ENS. Purines are an important mediator that drives pathophysiological glial mechanisms including reactive gliosis (13) and neurodegeneration (31). Increased purine levels during colitis (13,32) also contribute to decreases in ACh levels (32). This suggests that changes in the cholinergic activation of glia could contribute to pathophysiological pathways in the ENS. Our data show that MRs on enteric glia primarily mediate physiological and not pathophysiological pathways in the ENS. The activation of muscarinic receptors in an in situ model of neuroinflammation did not drive neuron death and did not potentiate P2X7R-mediated neuron loss. Similarly, antagonizing muscarinic receptors had no effect on P2X7R-mediate neuron death. This is important because MRs are important mediators of enteric neurotransmission (27,38-39). In addition, both MRs and the purinergic P2Y1 receptor (P2Y1R) signal through Gq transduction cascades that involve phosphoinositol and the release of Ca^{2+} from intracellular stores (28,56). Activation of glial P2Y1 drives enteric neuron death (13) in contrast, MR activation on enteric glia did not drive enteric neuron death in this study suggesting enteric glia have intracellular signaling specificity. However, more work is

needed to elucidate the mechanisms that drive this intracellular signaling specificity. Similarly, chronic stimulation of glial M3Rs did not drive inflammation or neuron death. Together, these results strongly suggest that enteric glial MRs are primarily involved in physiological signaling in the ENS.

MRs, particularly M3Rs, are functionally significant in mediating ACh-induced smooth muscle contraction and motility reflexes (40). However, the majority of studies on M3Rs in the intestine have focused on isolated smooth muscle cell preparations. Here we show that M3Rs are also expressed by enteric glia. This observation suggests that some of the functional effects of M3Rs on GI motility could be mediated through MRs on enteric glia. In this study, we used a chemogenetic model that expresses a modified human M3 (hM3Dq) receptor under the transcriptional control of a GFAP promotor (*GFAP*::hM3Dq+/-) (11) to specifically assess the role of glial M3Rs and avoid the potential confounding effects of MRs expressed by other cell types. These modified M3 receptors are designer receptors that are activated by the hM3Dq agonist clozapine-n-oxide (CNO) and allowed us to selectively stimulate M3R pathways in glial cells. Importantly, hM3Dq receptors stimulate the same phosphoinositol cascade as endogenous M3Rs and provide an effective model to study downstream mechanisms (33). Using this model, we demonstrate that direct activation of M3R pathways on enteric glia enhances distension-evoked motility reflexes. These data support the conclusion that glial M3R signaling contributes to the regulation of GI motility.

Enteric glia play an active role in the regulation of enteric circuits that exert control over motility and manipulating enteric glia affects GI functions (7,11-12,22,41). The chronic stimulation of glia M3Rs in vivo did not drive neurodegeneration, overt intestinal

inflammation, or other major changes to gut health. This is important, because it shows that the effects of glial activation on neural circuits are receptor dependent and not merely an effect of general glial activation. For example, purinergic pathways may have a more prominent role driving glial responses involved in neuroinflammation while cholinergic pathways may be more prominent in modulating physiological reflexes. However, the chronic stimulation of glial M3Rs did drive some alterations to motility and to body weight. Changes in body weight are likely caused by the activation of other populations of peripheral and central glia in this in vivo model that modulate metabolism, cardiovascular function, locomotor activity, salivation, and body temperature (33-34). These effects also likely explain the decrease in fecal pellet fluid content and endogenous pellet production observed in the chronic stimulation model. However, the decrease in CMMC oral contractile amplitude suggests that local changes to enteric glia, such as the desensitization of enteric glial M3Rs, could contribute to the effects on motility.

Together, our results provide the first evidence of functional muscarinic receptors on enteric glia. Importantly, our results show that activation of glial hM3Dq receptors has effects on gut physiology through modulation of excitatory reflexes involved in GI motility. Thus, enteric glia could detect ACh release and enhance enteric neuron excitability possibly through the release of glial mediators. This is particularly important when considering gastrointestinal disorders such as slow transit constipation where glial network activity is disrupted (55). In these disorders, modifying M3 receptor activity on enteric glia could provide a novel therapeutic target to restore GI motility.

APPENDIX

Antibody	Source	Dilution	Catalog No.	Resource ID No.
Chicken anti-GFAP	Abcam, Cambridge, MA	1:1000	AB4674	AB_304558
Biotinylated mouse anti-human HuC/D	Invitrogen, Carlsbad, CA	1:200	A21272	AB_2535822
Rabbit anti-M1 muscarinic receptor	Alomone labs, Jerusalem, Israel	1:200	AMR-010	AB_2340994
Rabbit anti-M2 muscarinic receptor	Alomone labs	1:200	AMR-002	AB_2039995
Rabbit anti-M3 muscarinic receptor	Alomone labs	1:200	AMR-006	AB_2039997
Rabbit anti-M4 muscarinic receptor	Alomone labs	1:200	AMR-004	AB_11219338
Rabbit anti-M5 muscarinic receptor	Alomone labs	1:200	AMR-005	AB_10658757

Table 3.1: Primary antibodies used in Chapter 3.

Antibody	Source	Dilution	Catalog No.	Resource ID No.
Alexa Fluor 488 goat anti-chicken	Invitrogen	1:400	A-11039	AB_2534096
Alexa Fluor 594 donkey anti-rabbit	Jackson ImmunoResearch, West Grove, PA	1:400	711-585-152	AB_2340621
Dylight 405-conjugated streptavidin	Jackson ImmunoResearch	1:400	016-470-084	AB_2337248
Alexa Fluor 594-conjugated streptavidin	Jackson ImmunoResearch	1:400	016-580-084	AB_2337250

Table 3.2: Secondary antibodies used in Chapter 3.

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CHAPTER 4:

Role Of GFAP In Enteric Glial Response To Inflammation

ABSTRACT

Gastrointestinal (GI) reflexes are regulated, in part, by interactions between enteric neurons and glia in the enteric nervous system (ENS). Enteric glia are a unique type of peripheral glia that play active roles in maintaining ENS function and regulating enteric neuron death. Inflammation disrupts the control of GI reflexes and drives glial transformations that include hypertrophy and increases in glial fibrillary acidic protein (GFAP). Although the inflammation-induced increase in GFAP expression is well described, the pathophysiological consequences of altered GFAP expression remains unknown. Here, we tested the hypothesis that glial GFAP upregulation contributes to neuroinflammation. We tested our hypothesis using in vivo and in vitro models of neuroinflammation, *GFAP* null mice (*GFAP*$^{-/-}$), and *GFAP::hM3Dq+/-* mice that have an impaired ability to upregulate GFAP. Our results show that *GFAP::hM3Dq+/-* mice fail to increase GFAP expression during inflammation, potentially due to high transgene copy number. *GFAP::hM3Dq+/-* mice failed to exhibit neurodegeneration during DNBS-colitis in vivo and in response to the activation of neuroinflammatory pathways in vitro. *GFAP*$^{-/-}$ mice exhibited comparable neuron death to a similar extent as wild type animals during DNBS-colitis and in vitro. Our data suggest that the glial upregulation of GFAP contributes to neuroinflammation during colitis. However, neuroprotective effects were only observed when glial GFAP expression was impaired and not completely ablated. This may suggest compensatory mechanisms in the constitutive GFAP null model.

INTRODUCTION

The enteric nervous system (ENS) is a complex network of enteric neurons and glial cells embedded within the gut wall that provide local control of gastrointestinal (GI) reflexes. The ENS modulates gastric acid secretion, regulates movement of fluid across the epithelium, and determines patterns of motility (1). Inflammation disrupts the control of GI reflexes by altering the function and/or survival of enteric neurons and these changes contribute to common functional GI motility disorders (FGIDs) (2-5). However, the mechanisms that drive ENS dysfunction remain unresolved.

Enteric glia are the most abundant cells in the ENS and play important roles in the regulation of GI functions (6-8). Indeed, enteric glial cells detect neuronal activity (9-14) through multiple neurotransmitter pathways (6,14) and, subsequently, modulate enteric reflexes involved in motility (15) and secretion (34). Glia react to inflammation in a process called reactive gliosis (16) that is characterized by glial hypertrophy (Chapter 2) and increased expression levels of the intermediate filament (IF) protein glial fibrillary acidic protein (GFAP) (16). Reactive enteric glia also contribute to neuroinflammation by driving enteric neuron death during inflammation (17-18). Although the increase in glial GFAP expression during inflammation is well described, the pathophysiological consequences of altered GFAP expression remain unknown.

Similar to astrocytes, enteric glial cytoskeleton is primarily formed by the IF protein GFAP (7,35). In astrocytes, GFAP help maintain the mechanical strength and shape of the cell and are implicated in playing a role in cell motility and migration (36-37), astrocyte proliferation (38-39) and vesicle trafficking (40). In response to injury astrocytes undergo a process known as reactive gliosis that is characterized by an increase in GFAP

regulation (41), altered glial function (42), and glial regulation of neuronal signaling (43-44). Given the similarities between enteric glia and astrocytes, increased GFAP in enteric glia may contribute to the inflammatory processes by altering glial function.

Here, we tested the hypothesis that the glial upregulation of GFAP contributes to neuroinflammation, in part, through mechanisms that contribute to neuron death in inflammation. We tested our hypothesis using in vivo and in vitro models of enteric neuroinflammation and transgenic mice that lack GFAP (*GFAP*$^{-/-}$) or that have an impaired ability to upregulate GFAP (*GFAP::hM3Dq+/-*). Our results show that *GFAP*::*hM3Dq+/-* mice fail to increase GFAP expression during inflammation, potentially due to high transgene copy number. *GFAP*::*hM3Dq+/-* mice did not exhibit neurodegeneration during DNBS-colitis or in response to neuroinflammatory pathways in vitro. *GFAP*$^{-/-}$ mice exhibited comparable neuron death to a similar extent as wild type animals during DNBS-colitis and in vitro. Our data suggest that the glial upregulation of GFAP contributes to neuroinflammation during colitis. However, neuroprotective effects were only observed when glial GFAP expression was impaired and not completely ablated.

MATERIAL AND METHODS

Declaration of Animal Use Approval

All work involving animals was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University (MSU).

Animals

Male and female mice between 8-10 weeks of age were used for experiments unless otherwise stated. All mice were maintained on a 12-hour light/dark cycle in a temperature-controlled environment with access to food and water *ad libitum*. Transgenic mice expressing the designer receptor hM3Dq under the transcriptional control of the glial fibrillary acidic protein (GFAP) promotor [*GFAP::hM3Dq+/-* (RRID: MMRRC_042286-UNC)] were a gift from Dr. Ken McCarthy (University of Carolina Chapel Hill) and were bred in house as heterozygous as previously described (15). Transgenic mice with a targeted ablation of *GFAP* (*GFAP*$^{-/-}$) [B6;129S-Gfap^{tm1Mes} (RRID:IMSR_JAX:002642)] were purchased from Jackson Laboratory (Bar Harbor, ME) and were bred in house as homozygous (*GFAP*$^{-/-}$) or heterozygous (*GFAP*$^{+/-}$). Genotyping was performed by the Research Technology Support Facility (RTSF) Genomics Core at Michigan State University or by Transnetyx (Cordova, TN). Genotyping of GFAP transgene copy number was performed by Transnetyx using real-time PCR.

Mouse Model of Colitis

Acute colitis was induced in mice under isoflurane anesthesia by an enema of 0.1 mL of a solution containing 5 mg di-nitrobenzene sulfonic acid (DNBS) dissolved in 50% ethanol (2). Control animals were given enemas of saline. Mice were monitored closely and weight loss was recorded daily. Mice were sacrificed at 48 hours post-induction of DNBS colitis. Macroscopic damage was recorded with an established scoring system (19).

Whole-Mount Immunohistochemistry

Longitudinal muscle myenteric plexus (LMMP) whole-mount preparations were microdissected from mouse colonic tissue preserved in Zamboni's fixative. Processing of LMMPs via immunohistochemistry was conducted as previously described (20) with the primary and secondary antibodies listed in Tables 1 and 2, respectively. Briefly, LMMP preparations were washed three times for 10 minutes in 0.1% Triton X-100 in phosphate-buffered saline (PBS) followed by a 45-minute incubation in blocking solution containing 4% normal goat serum, 0.4% Triton X-100 and 1% bovine serum in PBS. Preparations were incubated with primary antibodies overnight at room temperature and with secondary antibodies for 2-hours at room temperature before mounting. Antibody specificity was confirmed by preadsorption with the corresponding control peptides. Fluorescent labeling was visualized by confocal imaging through the Plan-Apochromat 60x oil immersion objective (1.42 numerical aperture) of an inverted Olympus Fluoview FV1000 microscope (Olympus, Center Valley, PA).

In situ Model of Neuroinflammation

Enteric neuron death was driven as previously described (17). LMMP preparations were incubated with the P2X7R agonist 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (BzATP) (300μM) or adenosine 5'-diphosphate (ADP) (100μM) for 2 hours in 95% air, 5% CO_2 at 37ºC., The connexin-43 hemichannel mimetic peptide 43Gap26 (100μM) was added in a subset of experiments to block neurotoxic glial pathways. LMMP preparations were then rinsed with fresh buffer, incubated for an additional 2 hours in buffer, and fixed overnight in Zamboni's fixative.

Solutions

Live tissue was maintained in DMEM/F-12 nutrient mixture (Life Technologies) during collection and microdissection. In vitro neuroinflammation experiments were performed in modified Krebs buffer containing (in mM): 121 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 10 HEPES, 21.2 $NaHCO_3$, 1 pyruvic acid, and 8 glucose (pH adjusted to 7.4 with NaOH).

Chemicals and Reagents

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). 43Gap 26, Connexin mimetic was purchased from Anaspec (Fremont, CA). 8-chloro-11-(4-methyl-4-oxido-1-piperazinyl)-5H-dibenzo[b,e][1,4]diazepine [clozapine N-oxide (CNO)] was obtained from the National Institute on Drug Abuse Supply Program (NDSP). Di-nitro benzenesulfonic acid (2,4-Dinitrobenzenesulfonic acid dihydrate) was purchased from MP Biomedicals (Solon, OH).

Statistical Analysis

Neuron packing density was determined by counting the number of HuC/D-immunoreactive neurons per ganglionic area in 10 ganglia per LMMP preparation using the cell counter plug-in tool on ImageJ Software, version 1.48 (NIH). All results are presented as mean ± standard error of the mean (SEM) and statistically significant differences were determined using an analysis of variance (ANOVA) or t-test, as appropriate with $P < 0.05$ considered statistically significant (GraphPad Prism; GraphPad Software, San Diego, CA).

RESULTS

GFAP transgene copy number is significantly increased in GFAP::hM3Dq+/- mice

In the central nervous system, inflammation drives glial hypertrophy, proliferation, and the upregulation of cytoskeletal GFAP (21,35,41). Similarly, inflammation in the gut drives glial hypertrophy (Chapter 2) and increases in GFAP expression levels (16, 22-23). However, the consequences of increased GFAP in inflammation are unclear. To begin testing the role of glial GFAP upregulation during inflammation, we took advantage of a transgenic mouse model (*GFAP::hM3Dq+/-* mice) where the ability of glia to upregulate GFAP is impaired due to low promoter availability. *GFAP::hM3Dq+/-* mice are a chemogenetic model that express a modified designer receptor (hM3Dq) on enteric glia that can only be activated by the selective hM3Dq agonist clozapine-N-oxide (CNO). Activation of this receptor drives strong enteric glial Ca^{2+} responses, and regulates intestinal motility and secretion (15,34). In addition, this model has also been used to study activation of other populations of peripheral and central glia that modulate metabolism, cardiovascular function, locomotor activity, salivation, and body temperature (45-46). We assessed transgene copy number as part of our initial characterization of this line and found that transgene copy number in *GFAP::hM3Dq+/-* mice is significantly higher than other common transgenic lines that utilize the GFAP promoter such as *GFAP::Cre^{ERT2}* mice and *GFAP::Cre* mice (**Figure 4.1**). Specifically, transgene copy number in *GFAP::hM3Dq+/-* is 118% greater than in *GFAP::Cre^{ERT2}* mice (p=0.0099) and 170% greater than in *GFAP::Cre* mice (p=0.0026). High numbers of transgene copies utilize a greater proportion of the GFAP promoters and the dilution effect limits the ability to increase GFAP protein expression. In support, *GFAP::hM3Dq+/-* mice failed to exhibit

an increase in GFAP expression during colitis (**Figure 4.2**). Thus, while *GFAP::hM3Dq+/-* mice express normal GFAP levels in health, they have a limited ability to upregulate GFAP expression during inflammation.

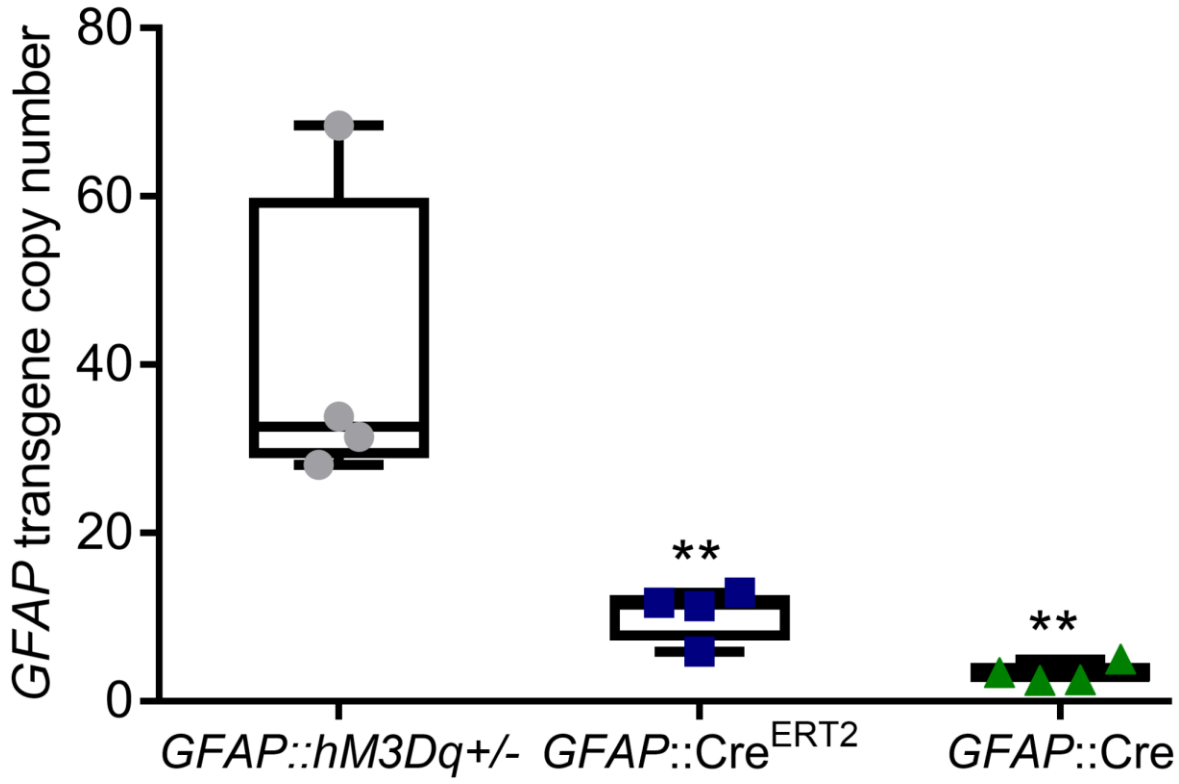

Figure 4.1: *GFAP* transgene copy number is significantly increased in *GFAP::hM3Dq+/-* mice. Quantification of *GFAP* transgene copy number from *GFAP::hM3Dq+/-*; *GFAP*::Cre^{ERT2}; and *GFAP*::Cre mice (n=4 mice; one-way ANOVA with multiple comparisons; **p<0.005).

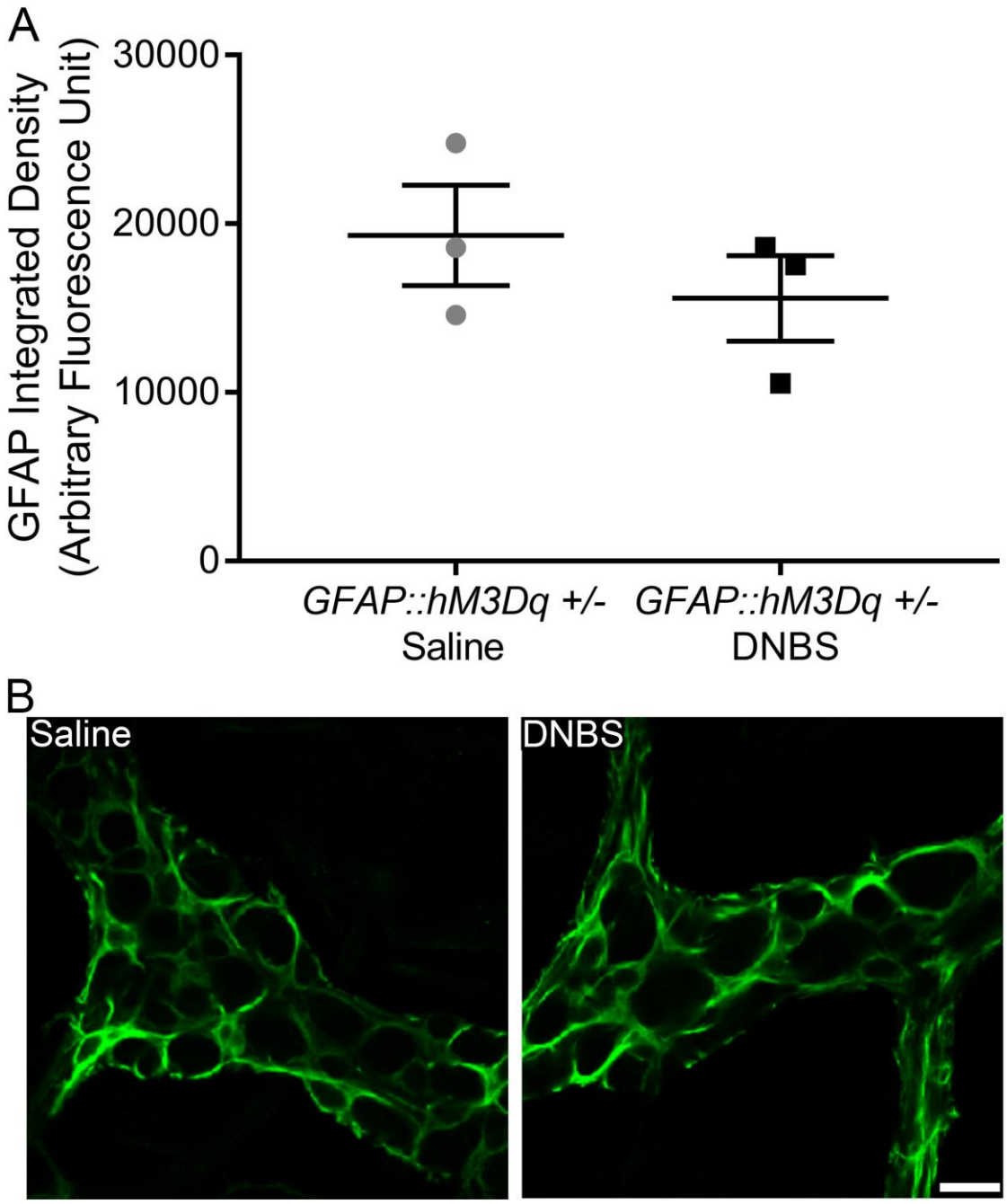

Figure 4.2: DNBS-colitis fails to drive GFAP upregulation in *GFAP::hM3Dq+/-* mice. Quantification (A) and representative images (B) of glial fibrillary acidic protein (GFAP) integrated density in myenteric ganglia after induction of DNBS-colitis on *GFAP::hM3Dq+/-* mice (n=3 mice; Student's t-test). Enteric glia are labeled with antibodies against GFAP (green). Scale bar represents 20µm and applies to all images.

An impaired ability to upregulate GFAP is neuroprotective

We took advantage of the *GFAP::hM3Dq+/-* model to test how glial GFAP upregulation contributes to neuroinflammation in the DNBS mouse model of colitis. DNBS colitis is a well-characterized model that is widely used to study neuroplasticity and neuroinflammation in the ENS (24). Importantly, the inflammatory response driven by DNBS colitis induces increases in GFAP expression levels and glial reactivity (Chapter 2). Active inflammation in murine DNBS colitis peaks between days two and three and is completely resolved by three weeks (2). *GFAP::hM3Dq +/-* and wildtype (WT) littermates exhibit a similar pattern of weight loss after induction of DNBS colitis ($p<0.0001$; **Figure 4.3A**) and a similar gross inflammatory response as measured by macroscopic damage to the colon ($p<0.0001$; **Figure 4.3A**). Surprisingly, *GFAP::hM3Dq+/-* mice treated with saline alone lost a significant 6.3% in body weight from Day 0 ($p=0.0113$; **Figure 4.3A**). In agreement with our prior findings, DNBS-colitis drove a 35% neuron loss in WT littermates ($p=0.0141$; **Figure 4.4A-B**) (17). Importantly, *GFAP::hM3Dq+/-* mice were protected against neuron death during colitis (**Figure 4.4A-B**). Overall, our data suggests that glial GFAP upregulation does not make a major contribution to gross inflammation, but does contribute to neuroinflammation.

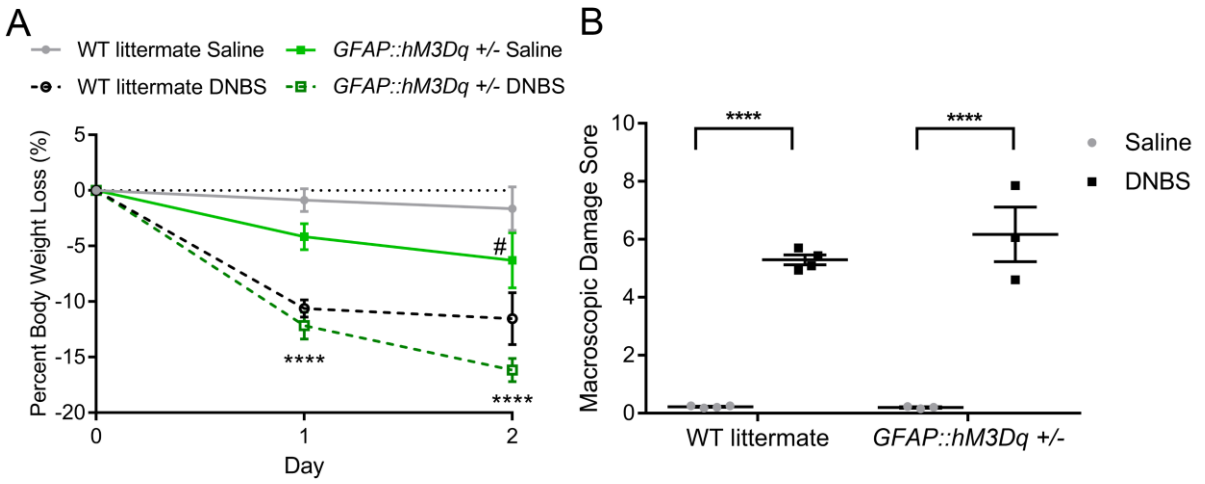

Figure 4.3: *GFAP::hM3Dq+/-* and WT littermate mice exhibit comparable weight loss and gross inflammation during DNBS colitis. (**A**) Weight loss pattern at 2 days after induction of DNBS-colitis on WT littermates and GFAP::hM3Dq +/- mice (n=3-4 mice; two-way ANOVA with multiple comparisons; #p=0.0113 applies to GFAP::hM3Dq +/- saline mice; ****p<0.0001 applies to DNBS treated mice). (**B**) Macroscopic damage scoring at 2 days post induction of DNBS-colitis (n=3-4 mice; two-way ANOVA with multiple comparisons; ***p<0.0001).

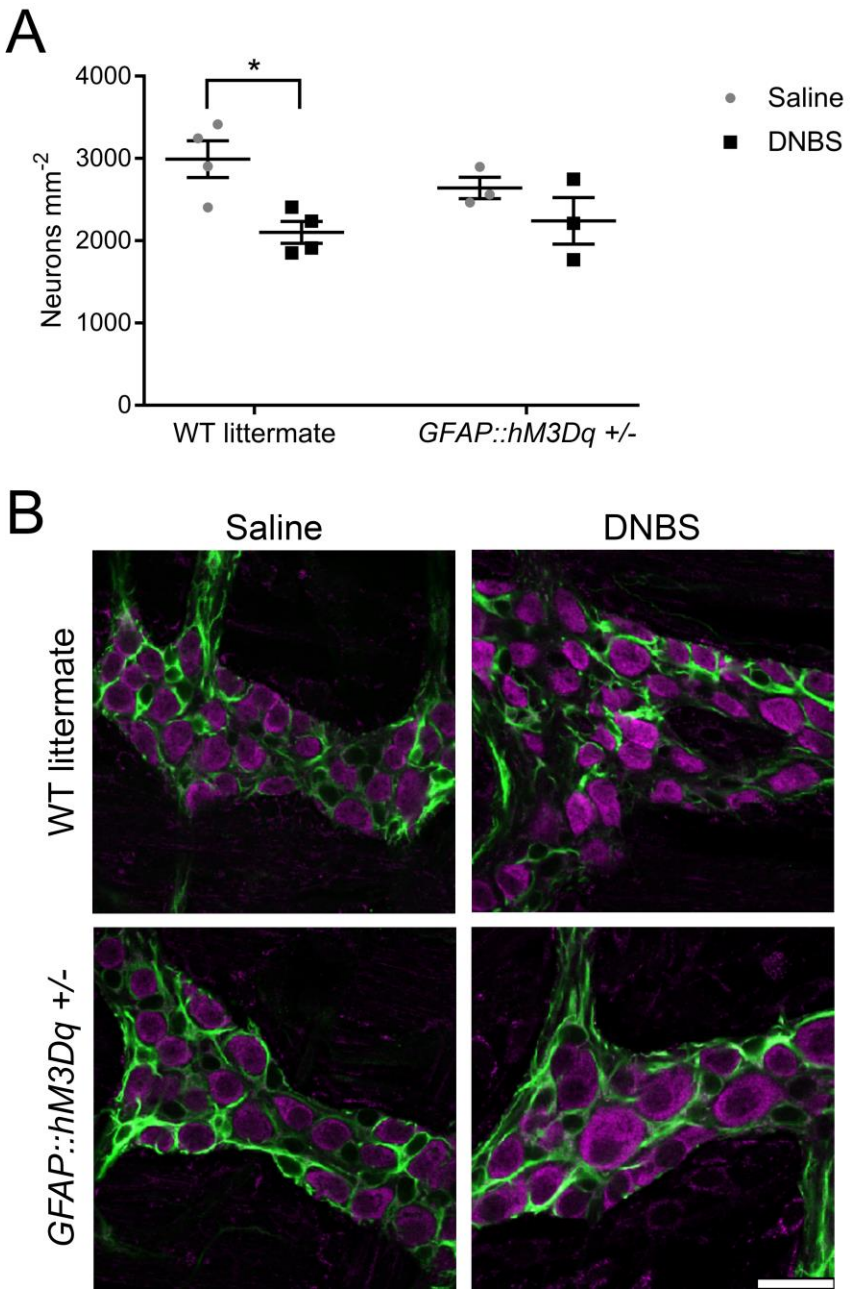

Figure 4.4: DNBS-colitis drives neurodegeneration in WT littermates, but not in *GFAP::hM3Dq+/-* mice. Quantification (**A**) and representative images (**B**) of mean packing density of HuC/D-immunoreactive neurons in myenteric ganglia after induction of DNBS-colitis on WT littermates and *GFAP::hM3Dq*+/- mice (n=3-4 mice; two-way ANOVA with multiple comparisons; *p= 0.0141). Enteric neurons are labeled with HuC/D (magenta) and enteric glia are labeled with glial fibrillary acidic protein (GFAP, green) in all panels. Scale bar represents 20µm and applies to all images.

GFAP::hM3Dq+/- mice are protected against neuron death in situ

Enteric glia mediate enteric neuron death through purinergic pathways that involve the activation of glial P2Y1 receptors (P2Y1Rs) and neuronal P2X7 receptors (P2X7Rs) (2,17). The activation of these pathways in vitro models mechanisms that drive neuron death in vivo (17). We used this in vitro model of neuroinflammation to determine how altered GFAP regulation in *GFAP::hM3Dq+/-* mice influences glial-mediated neuron death (**Figure 4.5**). In whole mount preparations of myenteric plexus from wild type animals, the activation of neuronal P2X7Rs with 300μM BzATP or the activation of glial P2Y1Rs with 100μM ADP drive enteric neurodegeneration (Chapter 2). However, neither mechanism was sufficient to drive neuron death in myenteric plexus whole mount preparations from *GFAP::hM3Dq+/-* mice (**Figure 4.5**). In addition, the direct stimulation of glial G-protein coupled receptors (GPCRs) with the hM3Dq agonist clozapine-N-oxide (CNO, 10 μM) (25) was not sufficient to drive neurodegeneration in samples from *GFAP::hM3Dq+/-* mice (**Figure 4.5**). These data suggest that changes in the *GFAP::hM3Dq+/-* model that include an impaired ability to upregulate GFAP are neuroprotective during inflammation.

Genetic ablation of GFAP does not alter neuroinflammation

The observation that *GFAP::hM3Dq+/-* mice are protected against neuroinflammation in vivo and in situ, suggests that the dysregulation of GFAP levels affects glial signaling pathways that mediate neuron death. To more directly test the role of GFAP, we studied DNBS colitis in GFAP null mice(26). Mice with a partial ablation of

GFAP (*GFAP*$^{+/-}$) still express enteric glial GFAP immunoreactivity but mice with a full ablation of GFAP (*GFAP*$^{-/-}$) show decreased expression of GFAP immunoreactivity

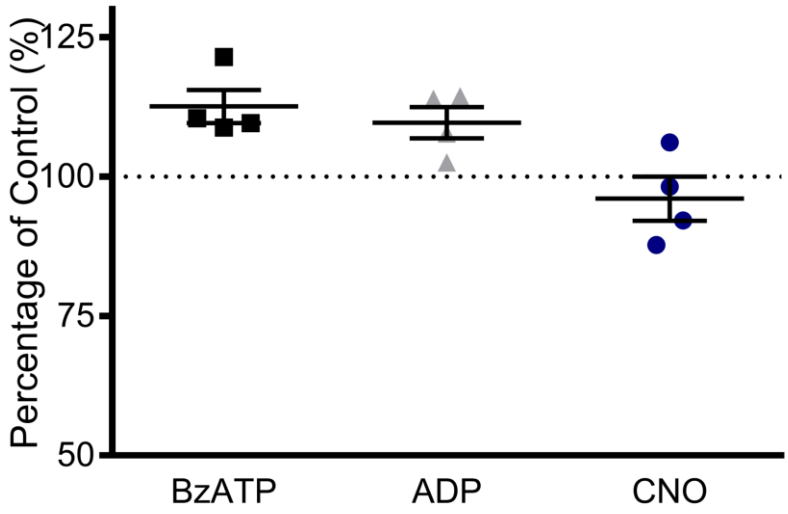

Figure 4.5: *GFAP::hM3Dq+/-* mice are protected against neurodegeneration in situ. Quantification of mean packing density of HuC/D-immunoreactive neurons in the myenteric ganglia after in situ activation of P2X7Rs with 300µM BzATP; of P2Y1Rs with 100µM ADP; or hM3Dq receptors with 10µM CNO. (n=4 mice; one-way ANOVA with multiple comparisons).

(**Figure 4.6**). Hemizygous (*GFAP*$^{+/-}$ mice) and homozygous (*GFAP*$^{-/-}$ mice) GFAP null mice exhibited similar patterns of weight loss and macroscopic damage as WT mice (**Figure 4.7A-B**). Similarly, inflammation significantly decreased neuronal survival in WT mice ($p=0.0012$); *GFAP*$^{+/-}$ mice ($p=0.0056$) and *GFAP*$^{-/-}$ mice ($p<0.0001$) (**Figure 4.8**). Together, these data show that the ablation of GFAP does not protect against neurodegeneration during colitis.

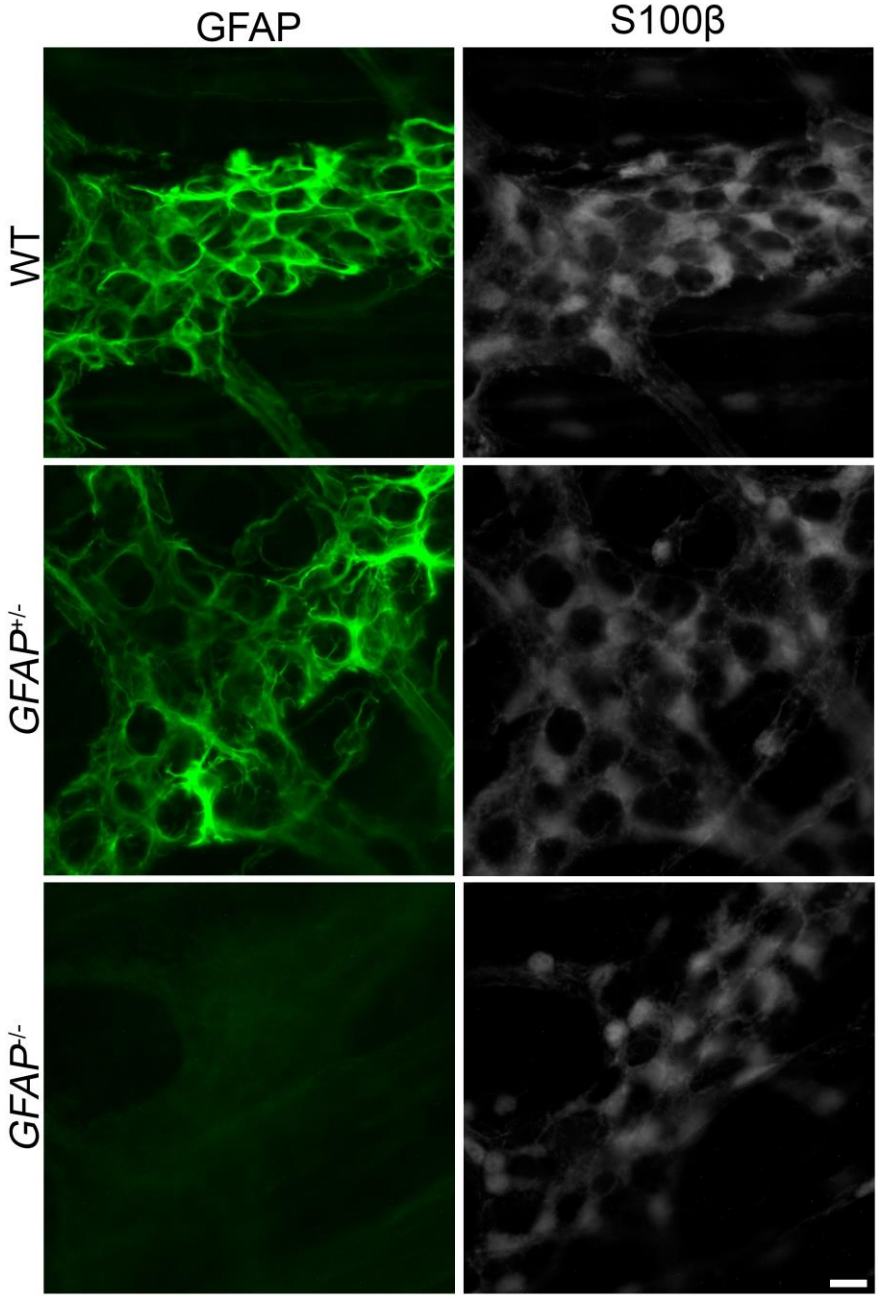

Figure 4.6: *GFAP*$^{-/-}$ mice exhibit low levels of GFAP immunoreactivity. Representative images of GFAP immunoreactivity on WT, *GFAP*$^{+/-}$ and *GFAP*$^{-/-}$ mice. Enteric glia were labeled with antibodies against GFAP (green) and the enteric glial cell marker S100β (grey). Labeling is representative of experiments performed on a minimum of 3 mice. Scale bar represents 20µm and applies to all images.

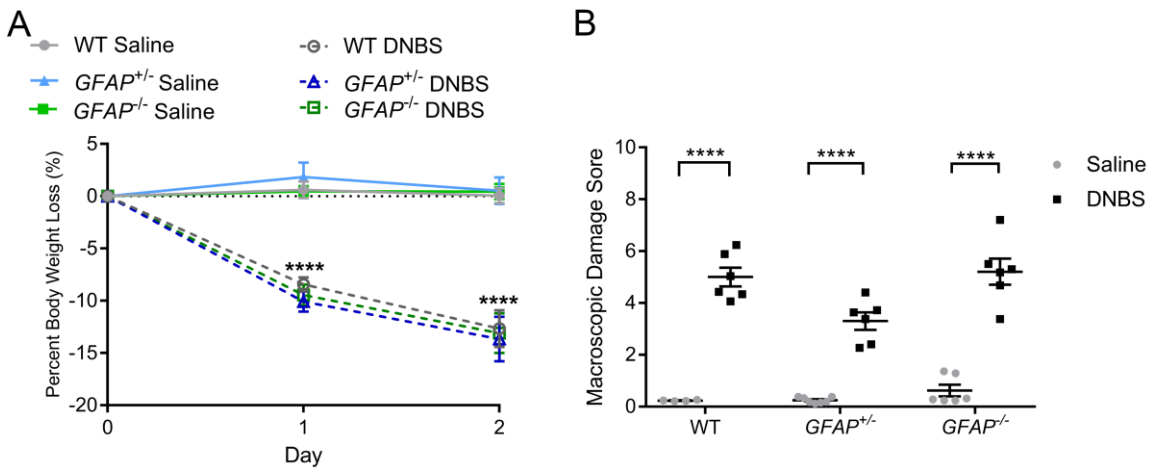

Figure 4.7: *GFAP*$^{+/-}$, *GFAP*$^{-/-}$ and WT mice exhibit similar patterns of weight loss and macroscopic inflammation during DNBS-colitis. (**A**) Weight loss pattern at 2 days after induction of DNBS-colitis on *GFAP*$^{+/-}$, *GFAP*$^{-/-}$ and WT mice (n=4-8 mice; two-way ANOVA with multiple comparisons; ****p<0.0001 applies to all DNBS groups). (**B**) Macroscopic damage scoring at 2 days post induction of DNBS-colitis (n=4-8 mice; two-way ANOVA with multiple comparisons; ***p<0.0001).

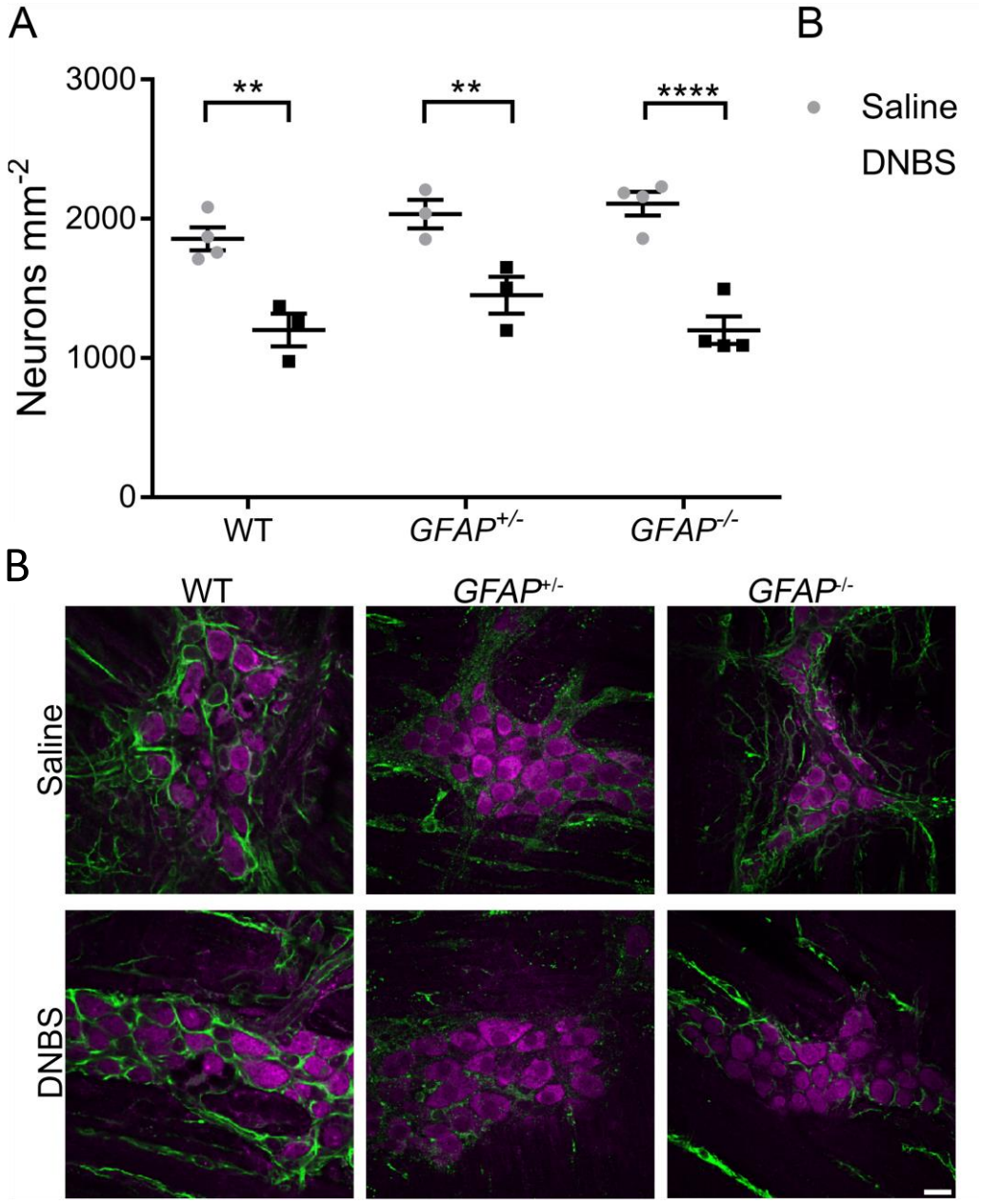

Figure 4.8: DNBS colitis significantly decreases neuronal survival in GFAP$^{+/-}$, GFAP$^{-/-}$ and WT mice. Quantification (**A**) and representative images (**B**) of mean packing density of HuC/D-immunoreactive neurons in myenteric ganglia after induction of DNBS-colitis on GFAP$^{+/-}$, GFAP$^{-/-}$ and WT mice (n=3-4 mice; two-way ANOVA with multiple comparisons; **p<0.01; ****p<0.0001). Enteric neurons are labeled with HuC/D (magenta) and enteric glia are labeled with vimentin (green) in all panels. Scale bar represents 20µm and applies to all images.

Effects of decreased GFAP expression in an in situ model of neuroinflammation

To more specifically assess how the ablation of GFAP affects the ability of glia to drive neuron death, we, again, used an in situ model of neuroinflammation in whole mount preparations of myenteric plexus to study the underlying mechanisms that drive neuron death (**Figure 4.9**). In WT littermates, the stimulation of neuronal P2X7Rs with BzATP (300μM) and of glial P2Y1Rs with ADP (100μM) significantly decreased neuronal survival by 23% (p=0.0071) and 20% (p=0.0202), respectively (**Figure 4.9A,D**). P2X7R-mediated enteric neuron death requires the activation of enteric glia and subsequent mechanisms that involve purine release through glial connexin-43 (Cx43) hemichannels (17). In WT littermates, blocking glial Cx43 hemichannel activity with the mimetic peptide 43Gap26 (100μM) protected against ADP-driven neuron loss, but not BzATP-driven neuron loss (p=0.0149; **Figure 4.9A,D**). Similarly, both BzATP (p=0.0132) and ADP (p=0.0062) drove enteric neuron death in *GFAP*$^{+/-}$ mice (**Figure 4.9B,E**). Additionally, 43Gap26 protected against neuroinflammation with either BzATP or ADP in *GFAP*$^{+/-}$ mice (**Figure 4.9B,E**). In mice with a full knockout of GFAP (*GFAP*$^{-/-}$ mice), only ADP drove a significant reduction in neuronal survival by 22% (p=0.225) and blocking glial Cx43 hemichannel activity protected against ADP-induced neurodegeneration (**Figure 4.9C,F**). Together, our data suggest that changes in the *GFAP::hM3Dq*+/- model that include an impaired ability to upregulate GFAP are neuroprotective during inflammation but a partial or complete ablation of GFAP expression does not provide protection against neuron death during inflammation.

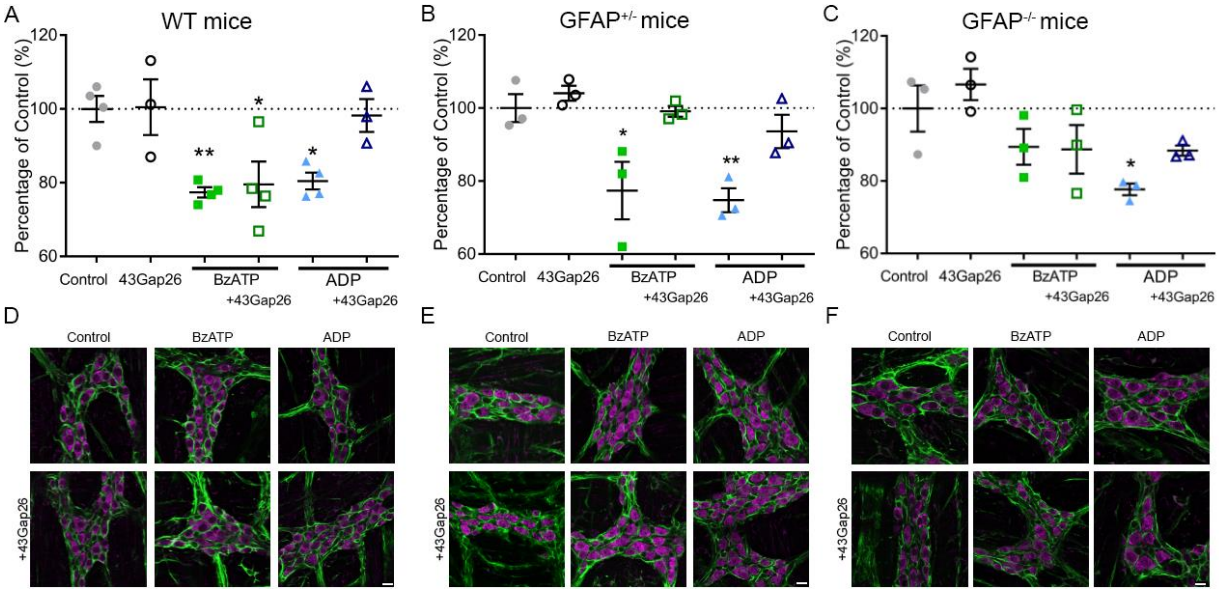

Figure 4.9: BzATP and ADP drive enteric neurodegeneration on WT and *GFAP*$^{+/-}$ mice but only ADP drives enteric neurodegeneration on *GFAP*$^{-/-}$ mice. Quantification (**A-C**) and representative images (**D-E**) of mean packing density of HuC/D-immunoreactive neurons in myenteric ganglia from WT (**A,D**); *GFAP*$^{+/-}$ (**B,E**) and *GFAP*$^{-/-}$ mice (**C,F**) after in situ activation of P2X7Rs with 300µM BzATP or activation of P2Y1Rs with 100µM ADP in the presence or absence of the Cx43 inhibitor 43Gap26 (100µM) (n=3-4 mice; two-way ANOVA with multiple comparisons; *p<0.05; **p<0.01). Enteric neurons are labeled with HuC/D (magenta) and enteric glia are labeled with vimentin (green) in all panels. Scale bars represent 20µm and apply to all images within the group.

DISCUSSION

Enteric glia are important mediators of GI function (2,6-8). They respond to neuronal activation, express multiple receptors for neurotransmitters, and are active regulators of GI motility (9-15). Importantly, enteric glia are linked to pathophysiological pathways in the ENS during inflammation (16,17,22,23). Inflammation drives reactive gliosis in the ENS (16), characterized by glial hypertrophy (Chapter 2) and increased expression levels of the intermediate filament protein, GFAP (16). Lipopolysaccharide (LPS) induction of systemic inflammation drives upregulation of GFAP expression from enteric glia in rats (16,22) and in cultured human enteric glial cells (23). Additionally, enteric glial cells contribute to neuroinflammation by driving enteric neuron death during inflammation (6,17). In this study, we tested the role of GFAP upregulation in neuroinflammation. We found that in a mouse model with impaired regulation of GFAP, inflammation did not drive neurodegeneration in vivo or in situ. However, mice with a partial or full ablation of GFAP were not protected against neuroinflammation.

We first identified a mouse model with dysregulation of GFAP expression levels by quantifying *GFAP* transgene copy numbers in different transgenic models of GFAP. In yeast, increased transgene copy number expression has a negative impact on cell growth and decreases cell viability (27). We identified *GFAP::hM3Dq+/-* mice as having significantly increased *GFAP* transgene copies. High numbers of transgene copies utilize a greater proportion of the GFAP promoters and the dilution effect limits the ability to increase GFAP protein expression. In support, *GFAP::hM3Dq+/-* mice were unable to upregulate GFAP protein expression in colitis.

Intermediate filaments (IFs) are actively involved in transducing biomechanical and molecular signals in astrocytes (21,28) and astrocytes in mice with a GFAP ablation lack IF networks in the hippocampus, corpus callosum and spinal cord (47-48). In culture, GFAP ablation reduces astrocyte motility (49) and increases proliferation (39). In addition, mice with an overexpression of GFAP have hyperactive astrocytes with intracytoplasmic aggregates of IFs (50). Inflammation drives increases in the IF GFAP (29) and GFAP upregulation is associated with increased cytokine production, glial reactivity and increased neuroinflammation (30-32). This highlights the importance of GFAP regulation in glial cells and the potential role GFAP dysregulation has in the ENS during inflammation. The observation that *GFAP::hM3Dq+/-* mice might have decreased GFAP regulation, suggested these mice might be protected from the effects of GFAP upregulation in colitis. Interestingly, *GFAP::hM3Dq+/-* mice were not protected against macroscopic damage and body weight changes associated with inflammation, suggesting that glial GFAP upregulation does not make a major contribution to gross inflammation. Importantly, *GFAP::hM3Dq+/-* mice were protected against neurodegeneration in colitis. This is consistent with our previous studies were colitis drives glial hypertrophy and subsequent enteric neuron death (Chapter 2). This suggests that glial upregulation of GFAP contributes to enteric neuron death in inflammation.

Enteric glia mediate enteric neuron death through purinergic pathways that involve the activation of glial P2Y1 receptors (P2Y1Rs) and neuronal P2X7 receptors (P2X7Rs) (2,17). We found that *GFAP::hM3Dq+/-* mice were protected against purinergic mediated enteric neuron death. In addition, direct activation of hM3Dq receptors failed to drive

enteric neuron death. This suggests that the decreased regulation of GFAP expression in these mice alters glial signaling pathways downstream of receptor activation.

The observation that *GFAP::hM3Dq+/-* mice were protected against neuroinflammation, suggests that the dysregulation of GFAP levels affects glial signaling pathways that mediate neuron death. To more directly test the role of GFAP, we studied DNBS colitis in GFAP null mice.(26). DNBS-colitis induced significant macroscopic damage, body weight loss and neuron death in mice with a partial or full ablation of GFAP. Similarly, activation of purinergic pathways with ADP in an in situ model of neuroinflammation drove significant neuron loss in $GFAP^{+/-}$ and $GFAP^{-/-}$ mice. Interestingly, $GFAP^{-/-}$ mice were partially protected against BzATP induced neuroinflammation *in situ*. This suggests that although the inability to upregulate GFAP protein in *GFAP::hM3Dq+/-* mice is neuroprotective in inflammation, the ablation of GFAP is only partially neuroprotective. However, GFAP null mice have a developmental knockout of GFAP, thus, there may be a genetic compensation in response to GFAP knockout (33). Although upregulation of related genes has not been observed in astrocytes in this model in physiological conditions (26), upregulation of genes may be occurring in enteric glia during inflammation. Importantly, models with complete genetic ablations tend to have no obvious phenotype while models with a knockdown phenotype show more significant changes in function (33). Alternatively, overexpression of hM3Dq receptors on *GFAP::hM3Dq+/-* mice may alter intracellular signaling pathways in enteric glia inhibiting glial-driven neuron death (61). However, enteric glia from *GFAP::hM3Dq+/-* mice and WT littermates exhibit comparable Ca^{2+} responses to ADP suggesting intracellular signaling pathways are not altered in these mice (15). This implies that

decreased regulation of GFAP protein in *GFAP::hM3Dq+/-* mice may show a more clear effect because other mechanisms may be compensating for GFAP loss in GFAP null mice. However, more work is needed to decipher the exact mechanisms involved in the observed neuroprotection in *GFAP::hM3Dq +/-* mice.

Overall, our data suggest that glial upregulation of GFAP contributes to neuroinflammation during colitis. However, neuroprotective effects were only observed when glial GFAP expression was impaired and not completely ablated. This may suggest compensatory mechanisms in the constitutive GFAP null model.

APPENDIX

Antibody	Source	Dilution	Catalog No.	Resource ID No.
Chicken anti-GFAP	Abcam, Cambridge, MA	1:1000	AB4674	AB_304558
Biotinylated mouse anti-human HuC/D	Invitrogen, Carlsbad, CA	1:200	A21272	AB_2535822
Chicken anti-vimentin	Millipore, Burlington, MA	1:1000	AB5733	AB_11212377
Rabbit anti-S100 beta	Abcam	1:200	AB52641	AB_882734
Rabbit anti-connexin 43	Sigma-Aldrich St. Louis, MO	1:500	C6219	AB_476857

Table 4.1. Primary antibodies used in Chapter 4

Antibody	Source	Dilution	Catalog No.	Resource ID No.
Alexa Fluor 488 goat anti-chicken	Invitrogen	1:400	A-11039	AB_2534096
Dylight 405-conjugated streptavidin	Jackson ImmunoResearch, West Grove, PA	1:400	016-470-084	AB_2337248
Alexa Fluor 568 goat anti-rabbit	Invitrogen	1:400	A-11036	AB_10563566

Table 4.2. Secondary antibodies used in Chapter 4

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CHAPTER 5

Key Findings, Study Limitations And Future Directions

KEY FINDINGS

Tachykinins are novel mediators of enteric neuro-glia communication

In Chapter 2 of my dissertation we advanced the field by demonstrating that the tachykinin neurokinin A (NKA) contributes to neuroinflammation in the enteric nervous system (ENS) through a multicellular signaling cascade involving enteric neurons, nociceptors and enteric glia. We found that antagonizing neurokinin-2 receptor (NK2R) signaling prevents key aspects of neuroinflammation in the ENS such as reactive gliosis and neurodegeneration. In addition, we demonstrated that the beneficial effects of antagonizing NK2R signaling are primarily mediated by their suppressive action on neuro-glia communication because NKA-driven neurodegeneration was connexin-43 hemichannel dependent.

Muscarinic receptors on enteric glia contribute to motility regulation

In Chapter 3 of my dissertation we provide the first evidence that enteric glia express two subtypes of functional muscarinic receptors and stimulation of these receptors induces Ca^{2+} responses independent of neuronal depolarization primarily through the muscarinic 3 receptor (M3R). We found that direct activation of modified muscarinic receptors on enteric glia enhances distention evoked motility reflexes and continuous stimulation of these receptors in vivo drives changes in gastrointestinal (GI) motility.

Glia upregulation of glial fibrillary acidic protein (GFAP) may contribute to neuroinflammation in colitis

Lastly, in Chapter 4 of my dissertation we identified the *GFAP*::hM3Dq+/- mouse as a model with impaired regulation of GFAP. We found that impaired GFAP regulation protects against neurodegeneration in inflammation. However, partial or full ablation of GFAP does not protect against neurodegeneration in inflammation. This may suggest compensatory mechanisms in the constitutive GFAP null model.

SIGNIFICANCE

Our study shows that tachykinins are key mediators between nociceptive sensory neurons and enteric neurons and glia. Tachykinins are a family of endogenous peptides that are widely expressed in the GI tract and are involved in both physiological and pathological conditions (1-4). Tachykinins are implicated in modulating inflammation (5-7) and inflammation-induced changes in motility and secretion (5-6,8-10). The ENS and primary afferent neurons are the major sources of tachykinins in the gut and noxious stimulus can drive tachykinin release and neurogenic inflammation in the ENS (2-3). We found that nociceptive neurons predominantly express NK2Rs in the ENS and that the stimulation of these receptors by NKA released from enteric neurons drives signaling to enteric glia and neurons. We demonstrate that antagonizing NK2R-signaling from nociceptive neurons prevents reactive gliosis and neurodegeneration by interfering with neuron-to-glia and glia-to-neuron signaling. These findings show that neuron-to-glia communication is induced by tachykinin receptor activation on nociceptive nerve fibers

and implies that interfering with neuron-to-glia activation could be an important therapeutic approach in the treatment of functional gastrointestinal disorders.

In Chapter 3, we provide the first evidence for functional muscarinic receptors on enteric glia. Acetylcholine (ACh) is the principal excitatory neurotransmitter in the ENS and the stimulation of muscarinic receptors is involved in neurotransmission mediating intestinal muscle reflexes and ion secretion within the gut (11-12). Importantly, enteric glia modulate enteric neuronal networks involved in GI motility and the stimulation of glial Ca^{2+} responses has strong, excitatory effects on motor circuits (13). Our results show that activation of modified muscarinic receptors on enteric glia has effects on gut physiology through modulation of excitatory reflexes involved in GI motility. This suggests enteric glia can detect ACh release and enhance enteric neuron excitability possibly through the release of glial mediators. This is particularly important when considering gastrointestinal disorders such as slow transit constipation where glial network activity is disrupted (14). In these disorders, modifying M3 receptor activity on enteric glia could provide a novel therapeutic target to restore GI motility.

Lastly, our study suggests that enteric glial upregulation of GFAP contributes to neuroinflammation during colitis. Enteric glia are important mediators of GI function (15-18) and are involved in pathophysiological pathways in the ENS during inflammation (19-22). Inflammation drives reactive gliosis in the ENS (19), characterized by glial hypertrophy and increased expression levels of the intermediate filament protein, GFAP (19). We found that a mouse model with dysregulation of GFAP expression failed to exhibit neurodegeneration in inflammation. However, the neuroprotective effects were not

observed when glial expression was partially or fully abolished. This may suggest compensatory mechanisms in the constitutive GFAP null model.

STUDY LIMITATIONS

DNBS Model of Colitis

We primarily used the 2,4-dinitrobenze (DNBS) model to induce murine colitis in our studies. This chemical model is driven by a T_H1-mediated immune response and is characterized by infiltration of the lamina propria with $CD4^+$ T cells, neutrophils, and macrophages as well as 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. The validity of our findings would benefit from repetition of our colitis studies in other model 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 2 relied heavily on the pharmacological properties of the neurokinin-2 receptor antagonist GR159897 and studies in Chapters 3 and 4 relied on the pharmacological properties of clozapine-n-oxide (selective hM3Dq receptor agonist). Additionally, studies in Chapter 3 relied on the pharmacological properties and specificity

of muscarinic receptor antagonists for muscarine receptor subtypes 3 and 5 and a positive allosteric modulator for the muscarine receptor subtype 5. Thus, the conclusions of these chapters rely on the assumption that these pharmacological modulators perform as intended and have little to no off target effects. Thus, the validity of our findings would benefit from repetition of our colitis studies in other model of murine colitis

Constitutive Knockout Mouse Model

For studies in Chapter 4 we used $GFAP^{-/-}$ mice which have a developmental knockout of GFAP. Thus, there may be a genetic compensation in response to GFAP knockout (28). Importantly, models with complete genetic ablations tend to have no obvious phenotype while models with a knockdown phenotype show more significant changes in function (28). The validity of our findings in Chapter 4 would benefit from repetition of our studies in a GFAP knockdown model to reduce genetic compensatory effects.

FUTURE DIRECTIONS

This dissertation presented a number of key findings that warrant further investigation in future studies:

Signaling pathways between nociceptive neurons and enteric glia in inflammation

Experiments in Chapter 2 demonstrate that nociceptive neurons express NK2Rs and activation of NK2Rs drives enteric glial Ca^{2+} responses. However, how nociceptive

neurons communicate to enteric glia remains unknown. A key follow-up study would be to investigate potential mediators of nociceptive neuron-to-glia communication. Nociceptive neurons that innervate the ENS are immunoreactive for calcitonin gene-related peptide (CGRP) (29) and astrocytes express functional receptors for CGRP (30-33). A key experiment would question whether enteric glia express functional receptors for CGRP. This will add substantial knowledge to our understanding of nociceptive neuron-to-glia signaling in the ENS.

Gliotransmitter release after M3R activation on enteric glia

Our studies in Chapter 3 demonstrate that activation of modified M3 receptors on enteric glia enhances colonic motility through excitatory pathways. However, how enteric glia modulate these reflexes remains unanswered. Astrocytes in tripartite synapses detect ACh and respond with release of gliotransmitters to modulate synaptic strength and neuronal excitability (34-35). In addition, purinergic activation of enteric glia drives ATP release through Cx43 hemichannels (20). Thus, enteric glia have the potential to release mediators upon stimulation. A key experiment would be to determine if muscarinic receptor activation on enteric glia drives gliotransmitter release. These studies would help determine how enteric glia modulate enteric neuron reflex pathways.

Role of GFAP upregulation on neuroinflammation

Glia react to inflammation in a process called reactive gliosis that is characterized by glial hypertrophy and increased expression levels of the intermediate filament (IF) protein glial fibrillary acidic protein (GFAP) (19). In Chapter 4 we demonstrate that mice

with an inability to upregulate GFAP (GFAP::hM3Dq+/- mice) are protected against neurodegeneration. A key follow-up experiment would be to determine if GFAP::hM3Dq+/- mice are also protected against glial hypertrophy in inflammation. Additionally, mice with a full or partial ablation of GFAP expression were not protected against neurodegeneration. Thus, the validity of our findings in Chapter 4 would benefit from repetition of our studies in a GFAP knockdown model to reduce genetic compensatory effects.

The data presented in this dissertation begins to address the role of enteric glial cells and enteric glia-to-neuron signaling in gastrointestinal physiology and pathophysiology. In combination with proposed future work, these findings will aid in the identification of therapeutic targets to treat GI motility disorders.

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