ENTERIC GLIAL CELL REGULATION OF OXIDATIVE STRESS AND IMMUNE HOMEOSTASIS DURING GASTROINTESTINAL INFLAMMATION

By

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ABSTRACT

ENTERIC GLIAL CELL REGULATION OF OXIDATIVE STRESS AND IMMUNE HOMEOSTASIS DURING GASTROINTESTINAL INFLAMMATION

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Gastrointestinal (GI) motility dysfunction is a debilitating feature that presents as a symptom in a number of conditions. These include primary GI disorders like inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) but also secondary conditions like diabetes mellitus, Parkinson's Disease and simply as a consequence of physiological aging. Although as many as 1 in 4 persons worldwide are affected by GI motility dysfunction, there is a significant lack of safe and effective drugs, due to a gap in the knowledge regarding the cellular mechanisms that contribute to GI motility dysfunction.

Normal gut function is controlled by the enteric nervous system (ENS), an intrinsic neuronal network comprised of enteric neurons and supporting glial cells, which is embedded within the walls of the GI tract. Death of enteric neurons, and the resulting disruption of this neuronal network, contributes to motility dysfunction during inflammation. In this dissertation, we investigate how increased oxidative stress and imbalanced immune homeostasis, key factors associated with GI inflammation, contribute to enteric neuropathy. We hypothesize a role for enteric glial cells, which are capable of modulating ENS oxidative stress, and also have an important immunomodulatory role in the ENS.

The work in this dissertation used a combination of transgenic animal strains, immunohistochemistry (IHC), pharmacological modulators, Ca^{2+} imaging and *in situ* and

in vivo models of colitis to investigate our hypothesis. We show a key role for enteric glial regulation of oxidative stress in mediating neuronal loss. During inflammation, purinergic activation of enteric glia drives enteric neuron death through a pathway that requires glial nitric oxide (NO) production and glial Cx43 hemichannels and demonstrates a novel pathogenic role for enteric glia. Further, glial production of the antioxidant reduced glutathione (GSH) is necessary for neuronal survival *in situ*, although whole body GSH depletion has novel protective roles against key features of murine colitis. Mice with disrupted in T cell signaling of the anti-inflammatory cytokine TGFβ had altered GI function, immune homeostasis and glial activation at the level of the myenteric plexus. Lastly, we show that key pro-inflammatory mediators differentially alter glial Cx43 channel opening in quiescent versus activated enteric glia.

Our data provide new evidence for an active role for enteric glial cells in GI (patho)physiology. Specifically, we demonstrate that enteric glia are involved in mediating the effects of oxidative stress and immune imbalance during GI inflammation and propose novel targets for the development of improved therapeutics to treat GI motility dysfunction.

For my mom

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KEY TO ABBREVIATIONS

γ-GC – gamma glutamyl cysteine

15d-PGJ2 – 15-deoxy-Δ12,14-prostaglandin J2

AAV – adeno-associated virus

ACh – acetylcholine

ADP – adenosine diphosphate

AFU – arbitrary fluorescence units

AH – afterhyperpolarization

APC – antigen-presenting cell

ATP – adenosine triphosphate

BSO – L-buthionine sulfoximine

BN – bombesin

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

Ca^{2+} - Calcium

ChAT – choline acetyltransferase

CIPO – chronic idiopathic pseudo-obstruction

CNS – Central Nervous System

Cx43 – Connexin 43

DHE - dihydroethidium

DMEM – Dulbecco's Modified Eagle Medium

DNBS – 2,4-dinitrobenzene sulfonic acid

ENK – encephalin

ENS – enteric nervous system

EtBr – Ethidium Bromide

FLEX – flip excision

GABA – gamma amino butyric acid

GCL – glutamate-cytsteine ligase

GDNF – glial-derived neurotrophic factor

GFAP – glial fibrillary acidic protein

GI – gastrointestinal

GSH – reduced glutathione

GPX – glutathione peroxidase

GS – glutathione synthethase

GSNO – S-nitrosoglutathione

IBD – inflammatory bowel disease

ICC – interstitial cells of Cajal

IFN-γ – interferon gamma

IHC – immunohistochemistry

IL – interleukin

iNOS – inducible nitric oxide synthase

IPANs – intrinsic primary afferent neurons

LMMP – longitudinal muscle myenteric plexus

MHC – major histocompatibility complex

miRNA – microRNA

MPO – myeloperoxidase

NAC – N-acetyl cysteine

NDS – normal donkey serum

NEM – N-ethyl maleimide

NGS – normal goat serum

NK3 – neurokinin 3

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

NPY – neuropeptide Y

Nrf2 – nuclear factor-erythroid 2- relatated factor 2

nTYR – nitrotyrosine

panx1 – pannexin-1

PACAP – pituitary adenylyl cyclase activating peptide

PAPA NONOate – Propylamine Propylamine NONOate

PAR1 – protease –activated receptor 1

SMP – submucosal plexus

SP – substance P

TK - tachykinin

TLR4 – Toll-like receptor 4

TNFα – tumor necrosis factor alpha

UC – ulcerative colitis

UTP – uridine triphosphate

VIP – vasoactive intestinal peptide

CHAPTER 1

Introduction

Overview

The gastrointestinal (GI) tract is the organ system responsible for controlling digestion and the movement of food through the body. Disorders and diseases of the GI tract may impact anywhere along the alimentary canal and are often debilitating. GI diseases are estimated to affect as many as 1 in 4 persons worldwide[1-3], with prevalence increasing annually. Despite this, current drug treatments for GI diseases are few, are often ineffective in large patient populations, and pose serious side effects[4]. This is in part due to the lack of understanding of the underlying cellular mechanisms that contribute to GI motility dysfunction.

The pathology of many GI diseases involves alterations within the enteric nervous system (ENS)[5]; the heterogeneous neuronal network embedded within the gut wall that controls intrinsic GI function [6-8]. Disruption of this neuronal network, by irreversible neuron death, is an underlying cause of motility dysfunction in a number of GI disorders including Inflammatory Bowel Disease (IBD)[9,10], infectious diseases[11,12], irritable bowel syndrome (IBS)[13], the congenital condition Hirschsprung's Disease[14] and chronic idiopathic pseudo-obstruction (CIPO)[15]. GI dysfunction and enteric neuropathy are also secondary symptoms in other conditions such as diabetes mellitus[16,17], central nervous system diseases[18] including Parkinson's Disease[19,20] and Alzheimer's Disease[21], and simply as a consequence of physiological aging[22,23].

Research has begun to identify some mechanisms that contribute to enteric neuropathy[24]. However, a number of other factors are associated with neuron death and GI dysfunction, and their mechanisms of action are less clearly understood. For example, increased oxidative stress in the ENS is a key factor associated with enteric

neuron death in IBD[25-27], aging[28,29] and diabetes mellitus[17]. In addition, inflammatory mediators and disruption of the immune balance in the gut are also associated with enteric neuropathies[30-32]. Enteric glial cells, specialized cells that surround and support enteric neurons and maintain the neuronal extracellular environment[33-35], can regulate ENS oxidative stress by regulating antioxidant[36,37] and oxidant production[38-40] and also interact with immune signaling molecules and components[41-44]. This dissertation investigates how ENS oxidative stress and immune dysfunction contribute to enteric neuropathy during inflammation, and the role of enteric glial cells in mediating those effects.

The Enteric Nervous System (ENS)

The enteric nervous system (ENS) or “brain of the gut” is one of three components of the autonomic nervous system[45] and is responsible for local and moment-to-moment control of gastrointestinal (GI) functions such as motility, blood flow and luminal absorptions and secretions. Early work by Auerbach (1862)[46] identified the existence of nerve cells within the wall of the GI tract. Bayliss and Sterling[47] showed that these intrinsic nerves are capable of controlling the reflexive behaviors of the intestine, without innervation from the CNS, writing: “Local stimulation of the gut produces excitation above and inhibition below the excited spot. These effects are dependent on the activity of the local nervous mechanism”. Ultimately, it was British physiologist John Newport Langley who coined the term ‘enteric nervous system’ to describe this intrinsic nerve network[45].

The progenitor cells of the ENS are derived from the vagal and sacral neural crest[48] and colonize the gut in a rostral to caudal manner, moving from the foregut to the hindgut[49] in a process completed by embryonic day 15[50]. Following this initial colonization, progenitor cells actively proliferate and differentiate into the neural components of the ENS. Improper colonization results in a lack of enteric neural cells (aganglionosis) in the hindgut, as seen in Hirschsprung's disease patients[51].

The Ganglionated Plexuses of the ENS

The neural cells of the ENS are organized into enteric ganglia, or collections of neural bodies, that are connected by interganglionic fiber tracts to form ganglionated networks or plexuses. The ENS is subdivided into two plexuses: the submucosal plexus (SMP) and the myenteric plexus (***Figure 1.1***). The SMP, as its name suggests, is located in the submucosal space between the mucosal and circular muscle layers of the gut (***Figure 1.1***) and is primarily responsible for the control of local blood flow and luminal absorptions and secretions. SMP innervation is only present within the small and large intestines and does not extend to the esophagus or stomach. In higher order animals with a larger ENS[52], the SMP is organized into two distinct layers: an inner (Meissner's) and an outer (Schabadasch or Henle's) SMP [53-56].

The myenteric (or Auerbach's) plexus is the larger of the two ganglionated plexuses and contains two-thirds of the neurons in the ENS. Unlike the SMP, the myenteric plexus innervates the entire length of the GI tract from esophagus to anus [6,57]. It is located between the circular and longitudinal smooth muscle layers within gut (***Figure 1.1***) and controls smooth muscle contractions and relaxations, and thus exerts

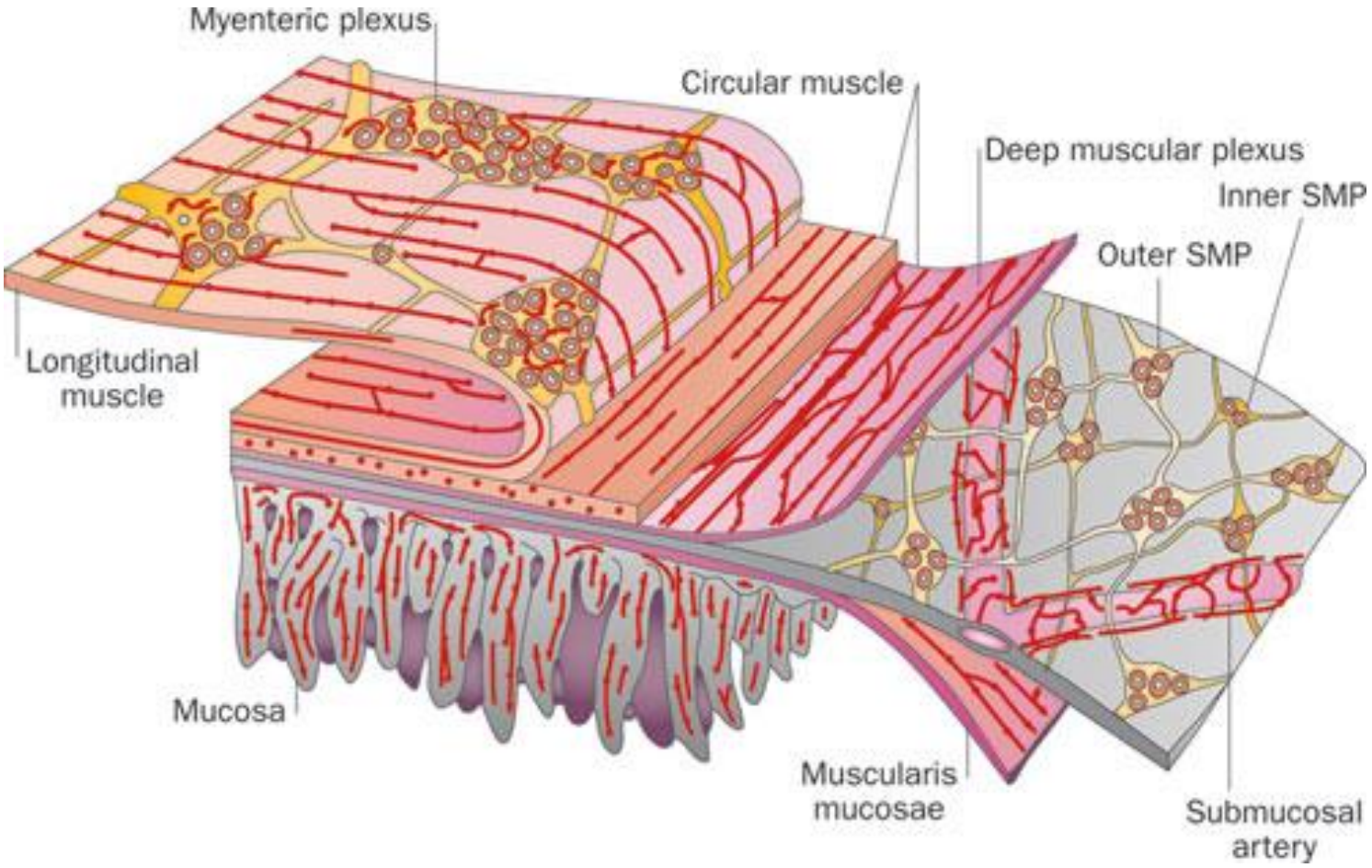

Figure 1.1: Organization of the enteric nervous system (ENS) in humans and higher order mammals. The ENS is comprised of two ganglionated plexuses the SMP, which regulates blood flow and luminal absorptions, and the myenteric plexus, which controls motility. In larger mammals, the SMP is divided into an inner and outer SMP. Figure from Furness (2012)[6].

control over GI motility[6,57,58]. Accordingly, changes or alterations to the myenteric plexus are associated with changes in GI motility[59,60]. Ganglia in both plexuses are comprised of two primary cell types: enteric neurons and enteric glial cells.

Enteric Neurons

The ENS is estimated to contain up to 100 million enteric neurons – as many as found in the spinal cord[52]. Enteric neurons are the primary building block of the neural circuits of the ENS and are a heterogeneous cellular population with an estimation of up 20 different types of neurons in the ENS[61-63]. Enteric neurons have been traditionally classified according to their morphology and/or electrophysical properties, and in more recent years, by neurochemical coding.

Morphological Classification of Enteric Neurons

Before the methodological advance of immunohistochemistry, enteric neurons were primarily classified according to cellular morphology as described by Dogiel in 1899[64,65] (***Figure 1.2***). Dogiel Type I neurons have multiple short dendrites and a single long axon which projects to neighboring ganglia or to the musculature. Type I neurons can be further classified by the direction of their axonal projection as either orally projection or aborally projecting. This feature was important in identifying at least some Type I neurons as motor neurons, responsible for innervating and controlling smooth muscle contraction and relaxation. Dogiel Type II neurons are identified by multiple short and long processes, which project circumferentially from the entire cell body. Dogiel type II neurons feature a large cell body and at least one dendrite with ends residing in the submucosa or mucosa, lending to their classification as sensory neurons capable of

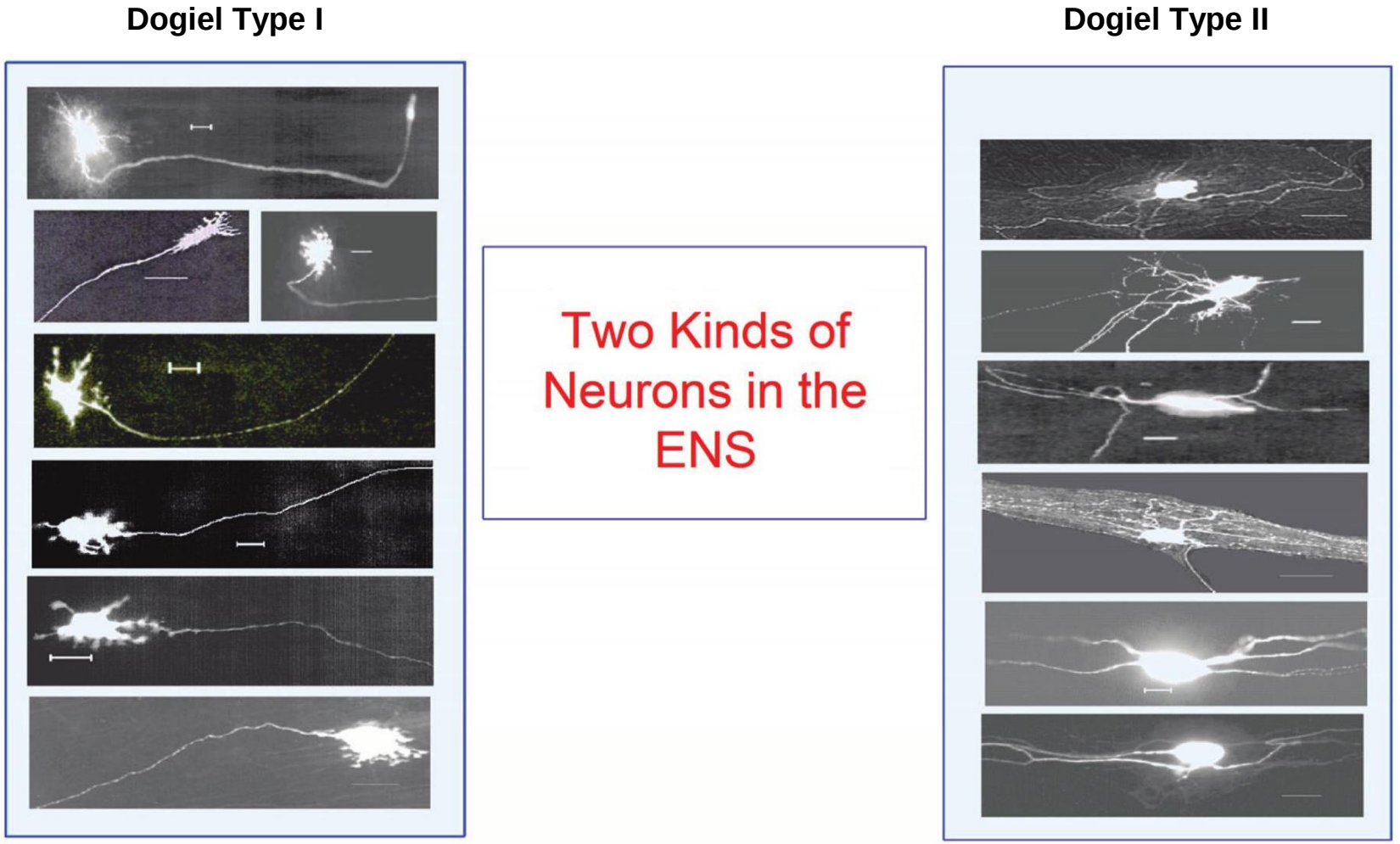

Figure 1.2: Morphological Classification of Neurons in the ENS. Dogiel Type I neurons are primarily identified by their uniaxonal morphology while Dogiel Type II neurons have a large cell body and multipolar morphology with circumferential projection of processes. Figure modified from Enteric Nervous System (Wood, 2011)[66].

detecting mechanical or chemical stimuli in the mucosa[67].

Electrophysical Classification of Enteric Neurons

Enteric neurons can also be classified according to electrophysical properties into two broad classes: AH-type neurons and S-type neurons[68,69]. After the firing of an action potential, AH neurons produce an extended afterhyperpolarisation lasting on the magnitude of seconds[70]. This characteristic electrophysiological feature is only evident in neurons with Dogiel Type II morphology[69,71,72]. The extended hyperpolarization limits the firing rate of these AH-type neurons and is further evidence for the designation of Type II/AH neurons as sensory neurons[73]. S-type neurons are distinguished from AH neurons by the lack of the prominent afterhyperpolarisation. Instead, they are characterized by a fast excitatory synaptic input, which results in an excitatory post synaptic potential[68]. S-type neurons have Dogiel Type I morphology and S-type electrophysiology is seen in multiple classes of enteric neurons including motor neurons and interneurons (described further below).

Immunohistochemical Classification of Enteric Neurons

Currently, enteric neurons are primarily classified by immunohistochemistry/ neurochemical coding [74,75] (***Table 1.1***). Three broad classes of neurons involved in the control of GI motility can be described based on neurochemical coding: motor neurons (excitatory and inhibitory), interneurons and sensory neurons. Excitatory motor neurons project to orally or locally to circular and longitudinal smooth muscle where they cause muscle contraction via release of excitatory neurotransmitters acetylcholine (ACh) and substance P (SP)[76]. These neurons are immunoreactive for choline acetyltransferase (ChAT) and the calcium binding protein calretinin. The primary neurotransmitters in

	Proportion	Neurochemical Coding	Shape
Excitatory circular muscle motor neurons	12%	ChAT/TK/ENK/GABA	Dogiel type I
Inhibitory circular muscle motor neurons	16%	NOS/VIP/PACAP/ENK/NPY/GABA	Dogiel type I
Excitatory longitudinal muscle motor neurons	25%	ChAT/calretinin/TK	Dogiel type I
Inhibitory longitudinal muscle motor neurons	~2%	NOS/VIP/GABA	Dogiel type I
Ascending interneurons	5%	ChAT/calretinin/TK/ENK	Dogiel type I
Descending interneurons	5%	ChaT/NOS/VIP/BN/NPY	Dogiel type I
Myenteric IPANs	26%	ChAT/TK/NK_3 receptor/calbindin	Dogiel type II

Table 1.1: Types of neurons in the myenteric plexus. This table lists a subset of neurons found in the myenteric plexus of guinea-pig small intestine. The neurons types highlighted are involved in local control of the peristaltic reflex. Table modified from The Enteric Nervous System (Furness, 2006)[77].

Table 1.1 (cont'd)

ChAT (choline acetyltransferase), BN (bombesin/gastrin-releasing hormone/GRP), ENK (encephalin), GABA (gamma amino butyric acid), NK (neurokinin), NOS (nitric oxide synthase), NPY (neuropeptide Y), PACAP (pituitary adenylyl cyclase activating peptide), TK (tachykinin), VIP (vasoactive intestinal peptide)

inhibitory motor neurons are nitric oxide (NO), vasoactive intestinal peptide (VIP) and ATP. Thus, these neurons are identified by immunoreactivity for neuronal nitric oxide synthase (nNOS) and VIP. Ascending and descending interneurons, which form neuronal chains along the length of the gut, signal via nicotinic ACh receptors and these neurons are also immunoreactive for ChAT. However, co-staining for other proteins and neurotransmitters such as somatostatin, serotonin (5-HT), gastrin-releasing peptide (GRP or bombesin/BN) and GABA allow for the distinction between different classes of interneurons that are involved in different reflex pathways. The final class of neurons, sensory or intrinsic primary afferent neurons (IPANS), are immunoreactive for ChAT, tachykininins and the neurokinin 3 (NK3) receptor (***Table 1.1***).

The ENS also contains other classes of neurons that do not regulate GI motility but contribute to neural control of other GI functions. These include 1) viscerofugal neurons, which have cell bodies within the ENS but project out of the gut to sympathetic ganglia and do not synapse with any enteric neurons[78,79], 2) vasomotor and secretomotor neurons which control blood vessel vasodilation and epithelial cell secretions respectively[80-82] and 3) motor neurons that innervate enteroendocrine cells[77].

Enteric Neural Circuitry

The various classes of enteric neurons are arranged in an intricate circuit that controls the internal reflexes of the GI tract (***Figure 1.3***)[83]. The primary enteric reflex, peristalsis, is activated when a bolus of food increases intraluminal volume and activates intrinsic primary afferent neurons (IPANs) in the gut wall [73]. IPANs have cell bodies in the SMP and myenteric plexus with projections to the mucosal layer of the gut.

Individual subtypes of IPANs have axons that synapse onto other IPANs, motor neurons or onto ascending and descending interneurons[57]; the latter activates ascending excitatory and descending inhibitory pathways respectively. Neuron-to-neuron synapses between interneurons form ascending and descending neural chains oral and aboral to the site of distention, respectively. The ascending/excitatory tract culminates with cholinergic excitatory neurons that synapse onto longitudinal and circular smooth muscle and causes smooth muscle contraction oral to the food bolus. Simultaneously, the descending inhibitory pathway terminates with inhibitory nitregic motor neurons that cause smooth muscle relaxation aboral to the food bolus. Synchronized smooth muscle contraction and relaxation propels the food bolus in an aboral direction, as described by Bayliss and Sterling (1899) in their law of the intestine [47,84]: "Excitation at any point of the gut excites contraction above, inhibition below". The ENS is also responsible for controlling other motor patterns and behaviors of the gut including accommodation to increased fluid content [85] and the non-propulsive colonic migrating motor complexes, which are proposed to be important for mixing and promoting absorption [86-88].

In addition to enteric neurons, other non-neuronal components are important for regulation of GI motility. These include the interstitial cells of Cajal (ICCs) which function as pacemaker cells to regulate control GI smooth muscle contraction [89-91] and are hypothesized to at least partially mediate both excitatory and inhibitory smooth muscle neurotransmission [92-94]. The second non-neuronal cell type of interest are enteric glial cells, a unique class of peripheral glia that will be discussed in further detail later in this chapter [33-35].

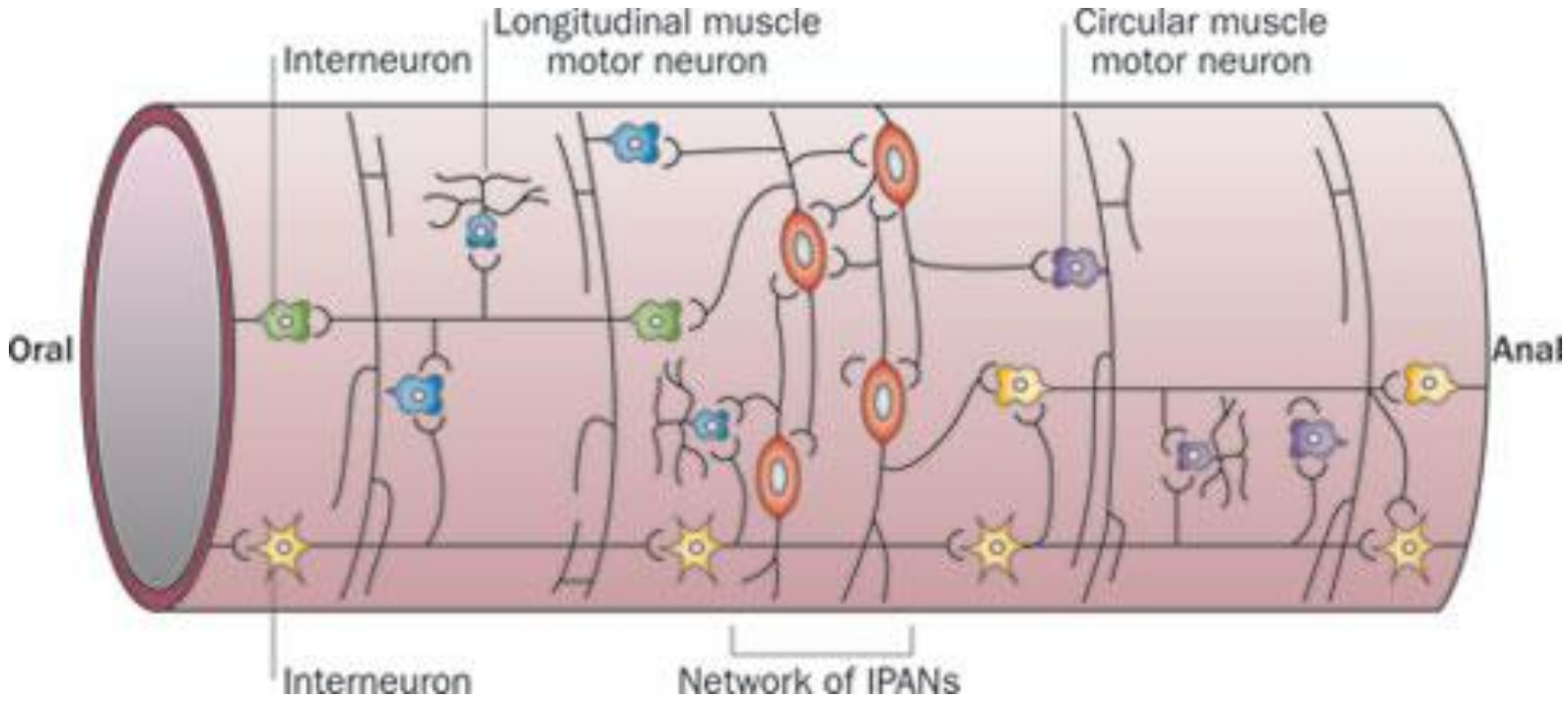

Figure 1.3: The enteric neural circuitry controlling peristalsis in the GI tract. Distention caused by a bolus of food in the GI tract activates IPANs, which have their projections in the mucosa. IPANs activated ascending and descending motor pathways by synapsing on ascending and descending interneurons, respectively or directly to excitatory or inhibitory motor neurons. Finally, motor neurons regulate smooth muscle contraction (oral to the food bolus) and relaxation (aboral to the bolus) to cause propulsion. Figure from The Enteric Nervous System (Furness, 2006)[77].

Enteric Neuropathies

Disruption of the enteric reflex circuit is a primary cause of gut dysfunction in GI motility disorders[31,95]. This disruption may be due to either a loss of the functional units of the circuit or functional impairment without cell death. Here, we focus on enteric neuropathies where an irreversible loss of enteric neurons contributes to GI motility dysfunction. Of note, enteric neuron death may be genetic/congenital or acquired, or present as a secondary symptom of another condition, and cause dysfunction of varied severity and pathologies[96].

Congenital Neuropathies

The most well understood congenital neuropathy, Hirschsprung's disease, is characterized by aganglionosis of the myenteric and submucosal plexuses in the distal colon and rectum. Hirschsprung's is estimated to affect about 1 in 5000 children[14] and about 20% of cases are defined by genetic abnormalities that prevent the proper migration of neural-crest derived enteric progenitor cells into the hindgut[97-99].

Inflammatory Neuropathies

A large percentage of enteric neuron death is associated with gut inflammation and the infiltration of immune cells into the GI tract. These aptly named inflammatory neuropathies include conditions such as the Inflammatory Bowel Diseases (IBD) Crohn's and Ulcerative Colitis[9,100], Chagas' disease[101] and Irritable Bowel Syndrome (IBS)[24,102,103]. Significant neuron death is reported in both animal models of IBD and in biopsies from human patients[13,104] and is widely shown to directly contribute to the motility dysfunction seen in these conditions. NOS-immunoreactive inhibitory motor neurons are selectively targeted in animal models of colitis, allowing us to speculate to

the mechanisms that cause motility dysfunction during colitis, as will be addressed in this dissertation.

Secondary Enteric Neuropathies

This terminology refers to diseases or syndromes where GI motility dysfunction is present but the gut is not a primary target of disease pathology. A common example is in diabetes mellitus patients where dysfunction throughout the length of the GI tract is present[105]. In an animal model of diabetes mellitus, altered colonic transit time is associated with a preferential loss of inhibitory nitregic myenteric neurons[104]. Constipation is frequently seen in Parkinson's disease patients[106] where enteric neuron death, and impairment of intrinsic and extrinsic enteric nerve innervation, are thought to contribute to GI motility dysfunction. Lastly, pronounced gut dysfunction, particularly constipation, is a symptom of general physiological aging and is associated with enteric neuropathy[107]. In opposition to neuron loss in diabetes mellitus and IBD, cholinergic neurons are particularly vulnerable in age-related neuron loss[24].

Recent work has begun to identify the cellular mechanisms that can contribute to enteric neuron loss, particularly during inflammation[24]. What is still unclear is how factors such as increased oxidative stress[108,109] and inflammatory mediators[110], which are associated with enteric neuropathy: 1) contribute to neuron loss and 2) are regulated by the cellular components of the ENS during inflammation.

Oxidative Stress in Enteric Neuropathies

Oxidative stress is a prominent feature associated with motility dysfunction in both primary and secondary GI disorders[17,25,27,28,111-116]. Evidence in support of this

includes the increased production of oxidants[117], and the concurrent depletion of antioxidants[118], in animal models of IBD. Mice with genetic disruptions in the antioxidant glutathione peroxidase genes (GPX-1 and GPX-2)[119,120] or the redox-sensitive transcription factor nuclear factor-erythroid 2–related factor 2 (Nrf2)[121] develop spontaneous ileocolitis and have an increased susceptibility to chemical models of colitis, respectively. Treatment with antioxidant L-glutathione was protective against enteric neuropathy in the jejunem of diabetic rats[122], further supporting a role for oxidative stress in enteric neuropathy in animal models.

Evidence also supports a role for oxidative stress in human IBD patients[27]. IBD patients have decreased mucosal content of antioxidants[123] and increased biomarkers of oxidative stress[124]. Genetic polymorphisms associated with decreased expression/ activity of antioxidant genes are also related to IBD incidence and considered an important genetic predictor for disease development[125,126]. Consistent with our animal model, genetic polymorphisms in Nrf2 gene were associated with ulcerative colitis in a human study[127]. Human trials investigating the efficacy of antioxidant treatment as a therapy for human IBD have yielded more inconclusive results than animal studies. In a subset of trials, while antioxidant treatment improved patient oxidative status and decreased overall oxidative stress, disease index and patient health were not significantly improved[128-130]. These findings demonstrate the need for further study to elucidate the potential causative relationship between oxidative stress and IBD, identify enteric mechanisms that contribute to increased oxidative stress, and determine the downstream pathways by which oxidative stress may lead to neuron death.

Inflammatory Mediators and Cytokines in Enteric Neuropathies

The infiltration of inflammatory mediators into the gut, and the disruption of immune balance within the gut, are also associated with enteric neuron loss and motility dysfunction of the GI tract[30-32]. Recent studies have begun to identify a role for the immune system in GI disorders such as IBS[131,132] and Chagas' disease[133], where inflammation was not thought to play a significant role. Conversely, a role for inflammatory cytokines and the immune system in the pathology of IBDs Crohn's disease and ulcerative colitis (UC) has been long established and much better studied. Both Crohn's and UC are characterized by persistanr GI inflammation but have key differences in their pathology and presentation[134]. For example, Crohn's disease presents with transmural inflammation anywhere along the GI tract, while in UC, inflammation is restricted to the colon and primarily mucosal.

The pro-inflammatory cytokines associated with Crohn's and UC also differ considerably[30,32,135]. UC is characterized as a Th2-mediated disease associated with increased interleukins 3, 4, 5 and 13[136]. Conversely, Crohn's Disease is traditionally classified as a Th1-mediated disease with increased IL-12, Interferon-γ, IL-1β and tumor necrosis factor α (TNF-α). However, recent studies propose a mixed Th1/Th17 profile[137]. Although enteric neurons have the ability to produce inflammatory cytokines and mediators[138,139], there has been little study of their ability to respond to directly respond to extracellular cytokines and the expression of such receptors. This suggests that an intermediate cell type mediates the immunomodulatory effects of pro-inflammatory cytokines on enteric neurons in IBD.

Enteric Glia

Enteric glial cells are a specialized type of peripheral glia found within enteric ganglia[33,34,140] and have the unique ability to both regulate ENS oxidative stress and mediate the immunomodulatory effects associated with enteric neuropathy. Like their CNS counterpart, enteric glia were historically considered to simply be passive support cells and the “glue” of the ENS. Increasing evidence supports a more dynamic role for enteric glia in not only regulating neuronal health, but participating in neuro- and gliotransmission and regulating GI motility[35,43].

The first detailed anatomical analysis of enteric glial cells was presented by Giorgio Gabella in 1972[141]. Here, Gabella noted important characteristics of enteric glia including cell and nucleus size and the ratio of enteric glia to neuronal cell bodies. Gabella also demonstrated that enteric glia were non-myelinating, differentiating them from other peripheral glia and Schwann cells[141]. These findings were confirmed by other investigators[142,143] and Gabella himself[144], who later coined the term “enteric glial cell”.

Enteric glial cells are morphologically and molecularly similar to astrocytes in central nervous system , further emphasizing the parallel of the ENS as the “brain of the gut”. Intraganglionic or Type I enteric glia are identified by their characteristic star-shaped morphology with prominent glial processes extending circumferentially from the glial cell body[35,145] (***Figure 1.4***). Type I enteric glia differ considerably in shape, and possible function, from interganglionic (Type II), mucosal (Type III) and intramuscular (Type IV) enteric glia, the detailed study of which is well reviewed by Gulbransen and Sharkey (2012)[140]. Enteric glial cells also have profound molecular similarities to CNS astrocytes. Immunohistochemical identification of enteric glia is achieved using

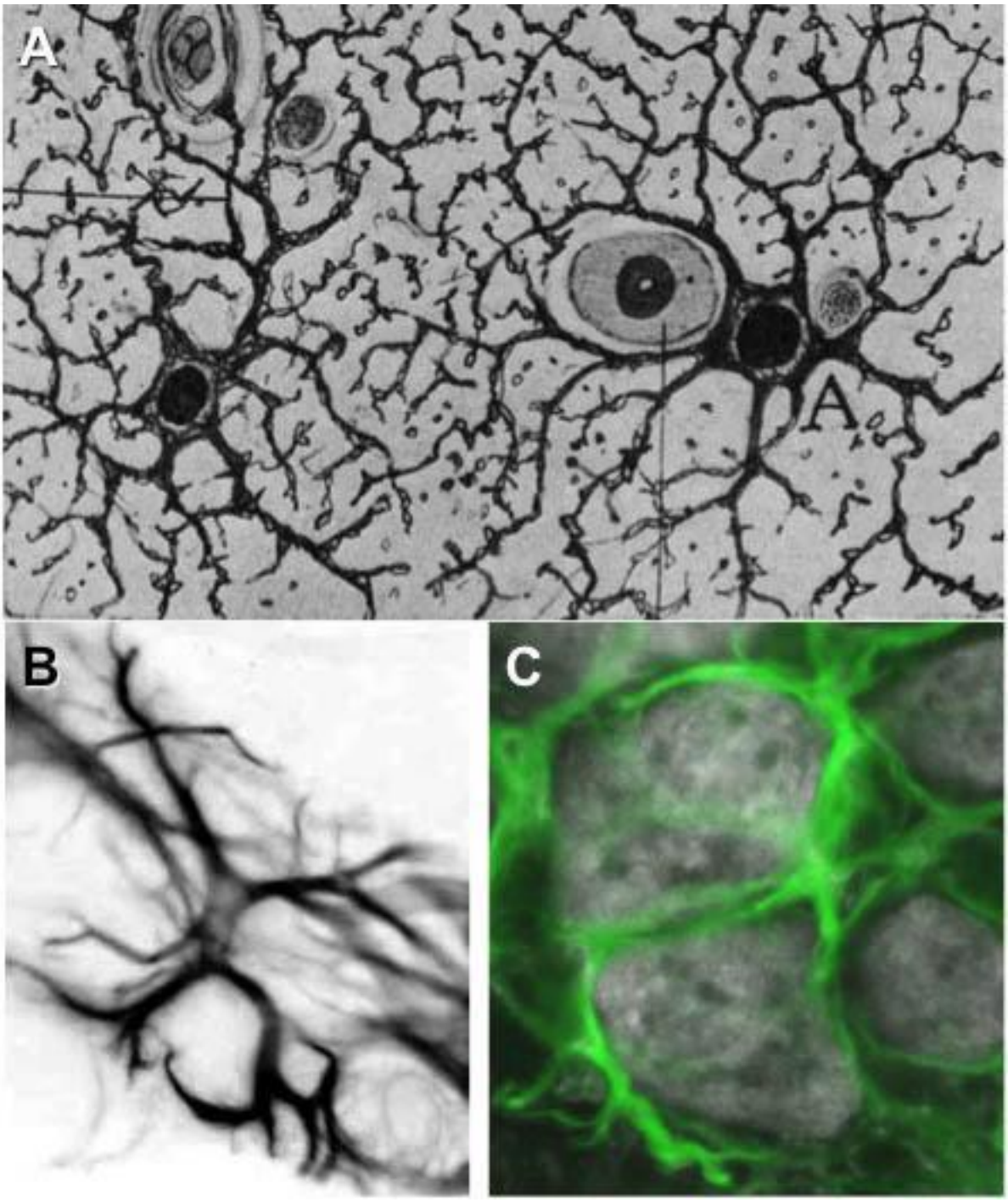

Figure 1.4: Enteric glial cells are morphologically similar to astrocytes. (A) A classic drawing of astrocyte surrounding a neuronal cell body. (B,C) Myenteric plexus glial cells stained with the prototypical astrocytic marker GFAP (*black* in B, *green* in C). Figure from Enteric Glia (Gulbransen, 2014)[146]**.**

prototypical astrocyte markers such as the filamentous proteins glial fibrillary acidic protein (GFAP)[147], vimentin[148] and the astrocyte marker S-100beta[149] (***Figure 1.4***). Enteric glia are also immunoreactive for the transcription factor Sox10 which is necessary for proper ENS development from neural crest cells[150].

The number of enteric glia, and hence the neuron to glia ratio, varies considerably in different species of animals and in the different plexuses[52,150]. In mice, the laboratory animal used in this dissertation, enteric neurons and glia present in equal numbers in the myenteric plexus[52]. Guinea pigs, which are a more widely studied rodent in gastroenterology, have a glia:neuron ratio closer to 2:1 in myenteric ganglia. This ratio continually increases with animal size, with humans having up to seven times more myenteric glia than neurons[150]. The heterogeneity of glia:neuron ratio may highlight increasingly complex roles of enteric glia in higher order mammals, and is worth considering when translating findings from animal models to human disease.

Recent work has begun to identify a bevy of functional roles for enteric glia based on 1) the histological expression of receptors, channels, enzymes and signaling molecules on enteric glia and 2) demonstration of the functional relevance of said receptor/channel/molecule by a secondary method. Below, I highlight key findings that verify the role of enteric glial cells as activate participants in the enteric neural circuitry.

Enteric glial receptors

Enteric glia express a number of neurotransmitter receptors allowing them to "listen to" neuron to neuron communication and dynamically respond to changes in extracellular neurotransmitter concentrations. Further, these receptors may mediate glia's ability to communicate with other glia via gliotransmission[151]. The class of

receptors most widely expressed on enteric glia are purinergic receptors including the P2Y1 receptors for ADP[24,39,151,152], P2Y4 receptors for ATP and UTP[102,153] and A2B receptors for adenosine[154,155]. Enteric glia also express adrenergic receptors[156] and receptors for glutamate[157], although their functional relevance is still unclear. Enteric glia also express receptors for other bioactive compounds besides neurotransmitters including protease-activate receptors (PAR1 and PAR2)[158], bradykinin receptors[159,160] and endothelin receptors[161].

Enteric glial regulation of neurotransmitters

Enteric glial cell can contribute to the concentrations of neurotransmitters in the ENS through two primary mechanism: 1) synthesis and production of neurotransmitters and necessary precursors and 2) degradation or sequestration of neurotransmitters. Enteric glia are immunoreactive for L-arginine[162], suggesting that enteric glia are capable of contributing to nitrergic signaling within the gut. Enteric glia also play a key role in the degradation and sequestration of neuroactive compounds, and in doing so contribute to the maintenance of neuronal health by preventing over-excitation of enteric neurons. Neurotransmitter sequestration occurs through the uptake of neurotransmitters via the PEPT2 transporter[163] and GABA transporter[164]. In coordination with their prominent expression of purinergic receptors, enteric glia express ectonucleotidases, which play an important role in the degradation on ATP to ADP and promotes neuronal-glial purinergic signaling in the ENS[24,39,165,166].

Enteric glial channels

Analogous to their CNS counterparts, enteric glia express Cx43 hemichannels, which play a key role in mediating glial purinergic signaling and neuron to glia

communication[39,151]. In addition, glia express both voltage-gated sodium and potassium channels, although the functional roles of these in glial signaling are still being elucidated[167,168].

Enteric glial regulation of oxidative stress

Enteric glial cells are ideally positioned to regulate the oxidant/antioxidant balance in the ENS through the production of both oxidants and antioxidants. Enteric glial produce and secrete antioxidant compounds such as reduced glutathione (GSH)[36,169] and the prostaglandin derivative 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2)[37,170], which aid in neuronal defense against oxidative stress and protect against neuron death. A loss of these antioxidants, and glial derived neurotrophic factors[171,172], may contribute to the neuron death associated with ablation of enteric glial cells[173]. In addition, enteric glia produce oxidants such as nitric oxide (NO) via inducible nitric oxide synthase (iNOS)[40,174,175]. During inflammation, glial cells (including enteric glia) convert to a reactive phenotype[176] which can increase iNOS activity[177-179]. Although increased iNOS activity and NO production are associated with IBD[180,181], studies inhibiting NO production in animal models of inflammation have produced conflicting results where depletion of NO is pathogenic while treatment with exogenous NO was both protective and pathogenic against different aspects of GI inflammatory damage[182]. These findings highlight the need for an improved understanding of the role and mechanism of action of NO during GI inflammation.

Enteric glial cells as immunomodulators

Enteric glia are also uniquely able to respond to, and directly modulate, the extracellular balance of inflammatory mediators such as pro and anti-inflammatory cytokines. Immunohistochemical staining shows the expression of receptors for the pro-inflammatory cytokines IL-1β and TNF-α on cultured rat enteric glia[183]. Further, these receptors are functional as treatment with IL-1β, IL-4 and TNF-α increased the expression of GFAP in cultured enteric glia, denoting their conversion to a "reactive" or "activated" state[184]. Activation of glia with IL-1β stimulates the production and release of additional cytokines or neuroprotective factors, further cementing an immunomodulatory role for enteric glial cells[185,186]. Enteric glia also express Toll-like receptors 2 and 4, which may be activated by lipopolysaccharide (LPS) or other bacterial components during inflammation[42,187,188]. Glial activation via the TLR4 receptor also induces cytokine synthesis and release[187]**.**

Enteric glia can also transform into antigen-presenting cells in response to inflammatory mediators during inflammation. As their name suggests, antigen-presenting cells (APCs) express of portion of a foreign antigen on their cell surface, coupled to one of two classes of major histocompatibility complex (MHC) proteins. The presentation of an antigen to a T-cell, along with the coupling of co-stimulatory molecules such as CD80 and CD86, causes T-cell activation and differentiation, and initiates an inflammatory response. In patients with chagastic megacolon, enteric glial cell upregulate expression of the MHC class II peptide and the costimulatory proteins CD80 and CD86[189].Increased glial MHC II expression in also seen in human Crohn's disease patients [190,191].

The regulation of anti-inflammatory cytokines is a key factor in the pathogenesis of GI inflammation and motility dysfunction. Animals with disrupted signaling of anti-inflammatory cytokines such as IL-10 [192]and TGFβ[193] develop spontaneous colitis. The specific cellular mechanisms contributing to colitis in these animal models on unclear, but it is possible that they are mediated through the immunomodulatory signaling pathways of enteric glia.

Summary and Aims of Dissertation

Normal function of the GI tract is controlled by the enteric nervous system (ENS), which is comprised of enteric neurons and glial cells organized into enteric ganglia[6]. Disruption of this neural network, due to loss of enteric neurons, contributes to motility dysfunction in a number of GI diseases. While some of the mechanisms that contribute to neuron loss during inflammation have been elucidated[24], our lack of knowledge of other key mechanisms involved is still incomplete and has led to a lack of therapeutic targets for disease relief.

Increased oxidative stress and immune dysregulation are two factors associated with enteric neuropathy and motility dysfunction in GI disorders. Enteric glial cells are ideally positioned to regulate ENS oxidative balance and mediate immunological signals. The objective of this dissertation is to investigate how ENS oxidative stress and immune dysfunction contribute to enteric neuropathy during inflammation, and the role of enteric glial cells in mediating those effects. Chapters 2 and 3 investigate how enteric glial cells contribute to oxidative stress regulation during inflammation. Chapter 2 studies glial production of the oxidant nitric oxide (NO) and its downstream signaling

pathways. Chapter 3 studies the other arm of oxidative balance, focusing on glial antioxidant production and how its (dys)regulation mediates enteric neuropathy and GI inflammation. Chapter 4 studies the effect of disrupted immune homeostasis on neuronal and glial structure and function. The final data chapter, Chapter 5, directly measures the effect of pro-inflammatory cytokines and oxidative mediators on a key glial cell function. Together, these chapters aim to improve the understanding of key glial mechanisms that contribute to enteric neuropathy and allow the development of novel therapeutics. Further, this dissertation serves to improve broader understanding of neuron-glia interactions in the ENS in health and disease.

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CHAPTER 2

Enteric glia mediate neuron death in colitis through purinergic pathways that require connexin-43 and nitric oxide

Work presented in this chapter has been published as Brown IAM, McClain JL, Watson RE, Patel BA, Gulbransen BD. Enteric glia mediate neuron death in colitis through purinergic pathways that require connexin-43 and nitric oxide. *Cellular and Molecular Gastroenterology and Hepatology*. 2016;2(1):77-91

Abstract

The concept of enteric glia as regulators of intestinal homeostasis is slowly gaining acceptance as a central concept in neurogastroenterology. Yet how glia contribute to intestinal disease is still poorly understood. Purines generated during inflammation drive enteric neuron death by activating neuronal P2X7 purine receptors (P2X7R), triggering ATP release via neuronal pannexin-1 channels that subsequently recruits intracellular Ca^{2+} ($[Ca^{2+}]_i$) in surrounding enteric glia. We tested the hypothesis that the activation of enteric glia contributes to neuron death during inflammation. We studied neuroinflammation *in vivo* using the 2,4-dinitrobenzenesulfonic acid model of colitis and *in situ* using whole-mount preparations of human and mouse intestine. Transgenic mice with a targeted deletion of glial connexin-43 (Cx43) [*GFAP::Cre*$^{ERT2+/-}$*/Cx43*$^{f/f}$] were used to specifically disrupt glial signaling pathways. Mice deficient in inducible nitric oxide (NO) synthase (*iNOS*$^{-/-}$) were used to study NO production. Protein expression and oxidative stress were measured using immunohistochemistry and *in situ* Ca^{2+} and NO imaging were used to monitor glial $[Ca^{2+}]_i$ and $[NO]_i$. Purinergic activation of enteric glia drove $[Ca^{2+}]_i$ responses and enteric neuron death through a Cx43-dependent mechanism. Neurotoxic Cx43 activity, driven by NO production from glial iNOS, was required for neuron death. Glial Cx43 opening liberated ATP and Cx43-dependent ATP release was potentiated by NO. Our results show that the activation of glial cells in the context of neuroinflammation kills enteric neurons. Mediators of inflammation that include ATP and NO activate neurotoxic pathways that converge on glial Cx43 hemichannels. The glial response to inflammatory mediators might contribute to the development of motility disorders.

Introduction

Reflex behaviors of the intestine, such as peristalsis, are orchestrated by the enteric nervous system (ENS); a complex network of neurons and glia embedded in the gut wall. The basic neural circuitry of the ENS is now well defined and it is generally accepted that the breakdown of ENS control is a major contributing factor in the development of functional bowel disorders[1]. However, it is only recently that we are beginning to appreciate the potential roles of enteric glial cells in the physiology and pathophysiology of the ENS[2]. Despite intense interest in enteric glia as regulators of enteric neurons, the precise functions of enteric glia remain poorly defined.

Enteric glia are a unique population of peripheral astroglial cells that surround enteric neurons and are thought to sustain neural signaling and survival. In support, enteric glia secrete neuroprotective factors[3] and the selective ablation of glial signaling alters the neural control of motility[4]. Likewise, *in vivo* models of glial ablation cause enteric neuron death[5,6]. Thus, the loss of glial supportive functions is postulated as a potential mechanism contributing to enteric neuropathy[2]. However, new data show that chronic astroglial activation, rather than glial cell loss, is responsible for driving neurodegeneration during neuroinflammation in the brain[7]. Indeed, the conversion of astroglia to reactive astrocytes can promote the secretion of factors that promote neuron death[8].

We recently discovered that enteric glia are activated by purines released from enteric neurons prior to neuronal death during colitis[9]. Specifically, the activation of neuronal P2X7 purine receptors (P2X7Rs) triggers the release of ATP from neurons through pannexin-1 (panx1) channels as a signal to enteric glia. In the brain, neuronal

ATP release through panx1 is considered a danger signal that glial cells interpret as a "search and destroy" message; causing glia to execute otherwise healthy neurons. Given that stimulation of P2Y1Rs is a potent stimulus for reactive astrogliosis in the central nervous system[10], we hypothesized that the activation of glial purine receptors contributes to neuropathy in the ENS. We tested our hypothesis using a combination of *in vivo* models of colitis with inducible and conditional transgenic mice and *ex vivo* intestinal preparations to address specific mechanisms. Our data show that glial activation is sufficient to cause enteric neuron death via a mechanism that depends on the activation of connexin-43 (Cx43) hemichannels and subsequent ATP release. Surprisingly, our data show that glial-driven neuron death requires the gating of glial Cx43 hemichannels by nitric oxide (NO). In all, our results suggest that the activation of enteric glial cells is a central mechanism in the development of enteric neuropathy.

Materials and Methods

Animals

All work involving animals was approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University. Male mice (8-10 weeks of age) were maintained on a 12-hour light/dark cycle with access to food and water *ad libitum*. C57Bl/6 mice were purchased from Charles River and inducible nitric oxide synthase (iNOS) null mice (B6.129S2-*Nos2*^{tm1Mrl}N12; Taconic Labs; RRID: MGI_4837857; hereafter referred to as *iNOS*$^{-/-}$) from Taconic[11]. Transgenic mice with an inducible and conditional deletion of Cx43 in GFAP-expressing glia (*GFAP::Cre*$^{ERT2+/-}$*/Cx43*$^{f/f}$; hereafter referred to as Cx43i-cKO) and their Cre negative littermate controls

(*GFAP::Cre*$^{ERT2+/+}$*/Cx43*$^{f/f}$) were generated in-house as previously described[4] by crossing *GFAP::Cre*$^{ERT2+/-}$ mice (GFAP-cre/ERT2)505Fmv/J; Jackson Labs; RRID: IMSR_JAX:012849) with *Cx43*$^{f/f}$ mice (B6.129S7-Gja1^{tm1Dlg}/J; Jackson Labs; RRID: IMSR_JAX:008039). Cre recombinase activity was induced by feeding animals tamoxifen citrate in chow (400 mg/kg) for two weeks. Animals were returned to normal chow for 1 week to clear tamoxifen prior to beginning experiments.

Human tissue

Work involving human tissue was approved by the Institutional Review Board of Michigan State University (IRB# 13-945M). Samples of live, full-thickness human jejunum were collected from a 57-year-old female with hypertension and type 2 diabetes who underwent elective laparoscopic bariatric surgery for weight loss, and placed in chilled DMEM/F12 media during transfer to the laboratory. Live longitudinal muscle myenteric plexus whole-mount preparations were prepared by microdissection for Ca^{2+} imaging.

Whole-mount Immunohistochemistry

Whole-mount preparations of mouse colonic longitudinal muscle and myenteric plexus (LMMP) were prepared by microdissection from tissue preserved in Zamboni's fixative. Processing of LMMPs via immunohistochemistry was conducted as described previously[4] with the primary and secondary antibodies listed in ***Tables 2.1*** *and* ***2.2***, respectively. Briefly, dissected LMMP preparations underwent three 10 min washes in 0.1% Triton X-100 in phosphate-buffered saline (PBST) followed by a 45 min block in

blocking solution containing 4% normal goat serum, 0.4% Triton X-100 and 1% bovine serum albumin (4% NGS). Preparations were incubated in primary antibodies (listed in ***Table 2.1, appendix***) for 48h at 4^{o}C and secondary antibodies (listed in ***Table 2.2, appendix***) for 2h at room temperature before mounting. Antibody specificity was confirmed by preadsorption with the corresponding control peptides or in knockout mice as previously described[9]. Fluorescent labeling was visualized using the 40X (PlanFluor, 0.75 numerical aperture) objective of an upright epifluorescence microscope (Nikon Eclipse Ni, Melville, NY) with a Retiga 2000R camera (QImaging, Surrey, BC, Canada) controlled by QCapture Pro 7.0 (QImaging) software or 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).

Quantification of neuronal thiol oxidation

We quantified neuronal thiol oxidation as a measure of oxidative stress as described previously[12]. Reduced (-SH) and oxidized (-SS) thiols were labeled in live LMMP preparations with 1µM Alexa Fluor 680 C_2 maleimide and 1µM Alexa Fluor 546 C_5 maleimide, respectively. Fluorescent dyes were dissolved in 4% paraformaldehyde, 0.02% Triton X-100 and 1mM N-ethylmaleimide (NEM) in PBS. Oxidized thiols were converted to reduced thiols for labeling by washing tissue in 5mM tris(2-carboxyethyl)phosphine hydrochloride in PBS for 20 minutes. Images were obtained by epifluorescence microscopy as described above and the ratio of 546-maleimide/680-maleimide (SS/SH) calculated with ImageJ software (NIH).

Dihydroethidium (DHE) staining

Superoxide levels were measured by quantifying fluorescence of the superoxide marker DHE[14] in live LMMP ganglia after a 1h incubation with 2 μM DHE (Life Technologies) dissolved in DMEM at 37^{o}C.

In situ model of neuroinflammation

Enteric neuron death was driven, as previously described[9], by incubating live LMMP preparations with the P2X7R agonist BzATP (300 μM) for 2h in 95% air: 5%CO_2 at 37^{o}C. Following this incubation, LMMP preparations were rinsed with fresh buffer, incubated for an additional 2h in Kreb's buffer only, and fixed in Zamboni's fixative overnight.

Ca^{2+} and NO imaging

Live LMMPs were loaded with 4 μM Fluo-4-AM or DAF-FM (Life Technologies) for 45 min at 37^{o}C as described previously[4] to measure intracellular Ca^{2+} and NO, respectively. Loaded tissue was continuously perfused with pre-warmed buffer (34°C; 3 mL/min) and drugs were bath applied. Images were acquired every 1-5s with a Neo sCMOS digital camera (Andor, South Windsor, CT) through the 40X water-immersion objective of an upright Olympus BX51W1 fixed-stage microscope (Olympus, Center Valley, PA) controlled by Andor IQ3 software.

Determination of Cell Viability

Cell viability was determined using Calcein-AM; a hydrophobic compound that is converted to hydrophilic, fluorescent Calcein in live, membrane-intact cells[13]. The Calcein-AM cell viability assay is a simple, rapid and accurate method to measure cytotoxicity that is not dependent upon the mode of cell death and is a true end-point assay for cell viability. Calcein-AM is a non-fluorescent, hydrophobic compound that easily permeates live, intact cells. The hydrolysis of the Calcein-AM by intracellular esterases produces Calcein, a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm. A loss of membrane integrity in dying cells permits the dye to escape from the cell and a loss of cellular fluorescence is indicative of neuron death. Live LMMPs were loaded with 4 μM Calcien-AM for 30 min at 37°C following *in situ* induction of neuroinflammation with BzATP (300 μM) or ADP (100 μM) or incubation with buffer control. Static images of 10 ganglia per whole mount were acquired as described for Ca^{2+} imaging and neuronal density calculated based on the number of live (fluorescent) cells per ganglionic area.

Detection of ATP release

ATP release was measured using an in-house-fabricated, selective ATP sensor[14,15]. LMMPs were continuously perfused with buffer (34°C; 6 mL/min) and ATP release was stimulated by bath application of either BzATP (300 μM) or ADPβS (100 μM) in the presence or absence of various pharmacological inhibitors. The ATP-selective sensor was placed directly on a ganglion and a superfusion pipette was located within 100 μm

of the sensor. Traces were analyzed by measuring the area under the curve and converting this to the concentration of ATP using pre-obtained calibration curves[14].

Induction of colitis

*C*olitis was induced in mice via an enema of dinitrobenzene sulfonic acid (DNBS, 5.5 mg/mouse in 0.1mL ethanol/saline administered via a gavage needle inserted 3 cm into the colon) as previously described[9]. Animal weight was recorded daily and tissue harvested 6h and 48h following DNBS treatment. Upon tissue collection, macroscopic damage was assessed to quantify acute inflammation[16]. Inflammation is classified as mild if damage scores were <1, moderate if scores were >1 but <5 and severe if > 5.

Chemicals/ Drugs

2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (BzATP), Adenosine 5'-diphosphate monopotassium salt dihydrate (ADP), Adenosine 5'-[β-thio]diphosphate trilithium salt] (ADPβS), N-ethylmaleimide (NEM) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO) and MRS2365 from Tocris Bioscience (Bristol, UK). 1400W, PTIO, L-NAME and Propylamine Propylamine NONOate (PAPA NONOate) were purchased from Cayman Chemical (Ann Arbor, MI). The Cx43 hemichannel mimetic peptide 43Gap26 and panx1 mimetic peptide ^{10}panx were purchased from Anaspec (Fremont, CA).

Solutions

Live tissue was maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12; Life Technologies) containing 3 μM nicardipine and 1 μM scopolamine during microdissection and incubations. LMMPs were perfused with 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) with 3 μM nicardipine and 1 μM scopolamine.

Data and Statistical Analysis

Average fluorescence intensity was quantified by measuring average fluorescence using the in-program measure function in ImageJ software, version 1.49 (NIH). Neuron packing density was determined by counting the number of HuC/D-immunoreactive neurons per ganglionic area in ten ganglia per LMMP preparation using the cell counter plug-in tool in ImageJ. All results are presented as mean ± standard error of the mean (SEM) and statistical differences determined using an ANOVA or t-test as appropriate with a P value of <0.05 considered significant (GraphPad Prism). For Ca^{2+} and NO imaging, traces represent the average change in fluorescence ($\Delta F/F$) over time for all glial cells within a single ganglion.

Results

Purinergic activation of enteric glial cells drives glial Ca^{2+} responses and enteric neuron death

Enteric neuron death during intestinal inflammation requires the activation of neuronal P2X7Rs and neuronal ATP release through panx1 channels[9]. Neuronal ATP released via panx1 is rapidly hydrolysed to ADP by eNTPDase2 and ADP recruits Ca^{2+} responses in the surrounding enteric glial cells following stimulation of P2Y1Rs (***Figure 2.1A***)[9]. In agreement with our previous work in mice, we find that activation of P2Y1Rs with the agonist ADP stimulates Ca^{2+} responses in enteric glial cells within the myenteric plexus of the human jejunum (***Figure 2.1B***). Likewise, activation of neuronal P2X7Rs with the agonist BzATP in the human myenteric plexus recruits Ca^{2+} responses in the surrounding enteric glial cells (***Figure 2.1B***). Previous reports suggest that P2Y1Rs are expressed by enteric neurons in the guinea pig ENS[17] and by enteric glia in the mouse ENS[9,18]. Our data support the conclusion that P2Y1Rs are primarily localized to enteric glia in the mouse myenteric plexus because P2Y1R agonists including ADP (100 μM), the non-hydrolysable ADP analog ADPβS (100 μM) or MRS2365 (1 μM) primarily drive cellular activity in enteric glial cells and not neurons (***Figure 2.1C-D***, data not shown for ADP and MRS2365). Likewise, immunohistochemistry with specific antibodies against an extracellular loop of the P2Y1R shows that P2Y1Rs are primarily localized to enteric glial cell processes in the myenteric plexus (***Figure 2.1E***).

We tested how direct activation of glial cells with P2Y1R agonists affects neuron survival in isolated preparations of the mouse ENS. Activation of glial P2Y1Rs with

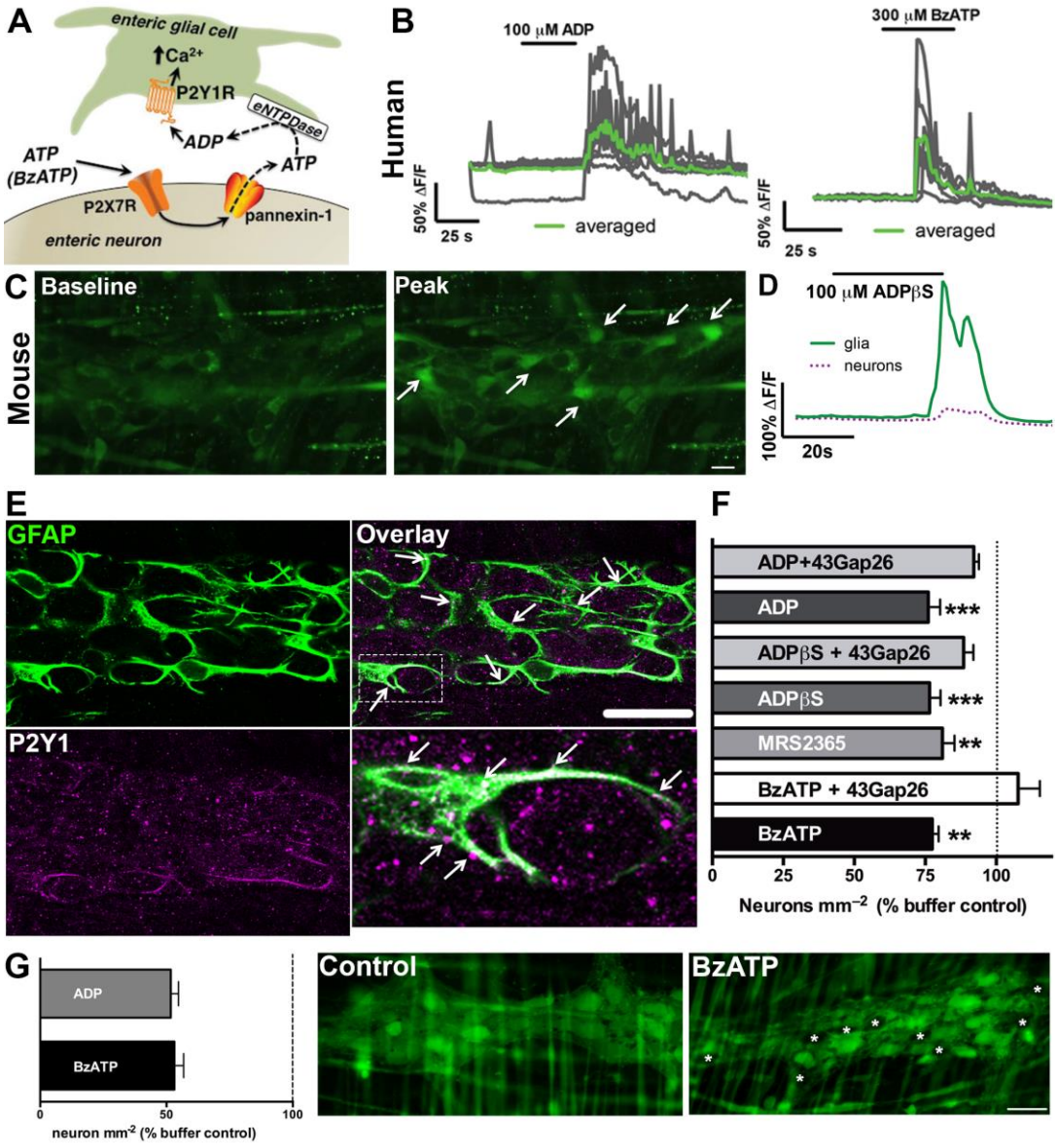

Figure 2.1: Activation of enteric glial P2Y1Rs drives neuron death through a Cx43-dependent mechanism. ***(A)*** Schematic depicting how activation of neuronal P2X7Rs elicits Ca^{2+} responses in enteric glia. ***(B)*** Representative traces of *in situ* Ca^{2+} imaging of human myenteric glia. As in mice, human glia respond to the P2Y1R agonist, ADP and to the neuronal release of ATP stimulated by the P2X7R agonist, BzATP. Gray traces show responses of individual glial cells within a ganglion and the averaged response of all glia within the ganglion is overlaid in green. Traces are representative of

Figure 2.1 (cont'd)

recordings from at least 4 myenteric ganglia from the human jejunum. ***(C-D)*** *In situ* Ca^{2+} imaging of the mouse myenteric plexus demonstrates that P2Y1R agonists primarily elicit Ca^{2+} responses in enteric glia. ***(C)*** Representative images of Fluo-4 fluorescence in a myenteric ganglion from the mouse colon at rest (baseline) and at peak stimulation (at time = 60s) with the non-hydrolysable P2Y1R agonist, ADPβS. Arrows point to representative enteric glia. ***(D)*** Averaged Ca^{2+} responses of glia (green line) and neurons (magenta dashed line) within a myenteric ganglion from the mouse colon in response to ADPβS. Traces are representative of responses in over 5 myenteric ganglia. ***(E)*** Representative mouse myenteric ganglion showing immunoreactivity for P2Y1Rs (magenta). Enteric glia are labeled with the glial cell marker glial fibrillary acidic protein (GFAP; green) and arrowheads highlight areas of co-localization (scale bar, 30 μM). The boxed region in the overlay image at top right is expanded in the bottom right panel to highlight a glial cell with dense P2Y1R expression. ***(F)*** Mean packing density of HuC/D-immunoreactive neurons in myenteric ganglia after *in situ* activation of P2Y1Rs with the agonists ADP, ADPβS and MRS2365 or activation of P2X7Rs with BzATP in the presence or absence of the Cx43 inhibitor 43Gap26 (n = 3-5 animals, **P≤0.01, ***P≤0.005 ANOVA). ***(G)*** Mean packing density of live enteric neurons in myenteric ganglia following *in situ* neuroinflammation with BzATP, ADP or buffer control, quantified by Calcein-AM fluorescence. Representative images show viable cells, labeled with fluorescent Calcien-AM (green), in mouse myenteric ganglia from control and BzATP-treated tissues. Dead (non-fluorescent) neurons are denoted by asterisks (scale bar, 30 μM), (n = 3-4 animals).

ADP, ADPβS or MRS2365 decreased the ganglionic density of HuC/D-immunoreactive neurons by 24 ± 4%, 23 ± 4% and 19 ± 4%, respectively (***Figure 2.1F***). Importantly, P2Y1R activation drove enteric neuron death to an equal extent as activation of neuronal P2X7Rs with BzATP (300 μM) (23 ± 2%, ***Figure 2.1F***). To confirm that the loss of HuC/D-immunoreactivity truly reflects a loss of neurons and not merely a loss of HuC/D-immunoreactivity in neurons, we assessed neuronal viability using the fluorescent dye Calcien-AM which labels live, membrane-intact cells[13]. Treatment with BzATP (300 μM) and ADP (100 μM) decreased neuronal density in myenteric ganglia by ~50% compared to buffer control (53 ± 4% and 52 ± 3% respectively, ***Figure 2.1G***), consistent with results determined by HuC/D-immunoreactivity. These results show that the activation of glial P2Y1Rs is sufficient to induce enteric neuron death driven through P2X7R activation.

In situ glial-driven neuron death requires Cx43 hemichannels

The activation of glial P2Y1Rs drives Ca^{2+} responses and triggers mechanisms that lead to Cx43 hemichannel opening in mice[4]. Astroglial Cx43 hemichannel opening in the context of neuroinflammation releases mediators that contribute to the development of neuropathic pain and neurodegeneration in the central nervous system (CNS)[19,20]. Thus, we tested if glial-driven enteric neuron death requires Cx43 hemichannel opening by activating glial P2Y1Rs in the presence of the specific Cx43 hemichannel mimetic peptide, 43Gap26 (100 μM) which inhibits Cx43 hemichannel opening[21]. The neurotoxic effects of both P2Y1R agonists (ADP and ADPβS) and P2X7R agonist (BzATP) were lost in the presence of 43Gap26, which protected against decreased ganglionic neuron

density (92 ± 2%, 89 ± 3% and 108 ± 8% for ADP, ADPβS and BzATP with 43Gap26 respectively, compared to buffer control, ***Figure 2.1F***). These results strongly suggest that glial cells are responsible for driving neuron death because Cx43 hemichannels are confined to enteric glial cells within the ENS[4].

Glial Cx43 hemichannels are required for in vivo neuron death during inflammation

To more accurately test the role of glial Cx43 hemichannels in inflammatory neuropathy, we combined *in vivo* DNBS-colitis with a targeted gene deletion approach to conditionally ablate Cx43 in GFAP-expressing glial cells following the administration of tamoxifen[4] (inducible and conditional Cx43 knockout; Cx43i-cKO; ***Figure 2.2***). In agreement with our previous work, the density of HuC/D-immunoreactive myenteric neurons was reduced by 24% (2057 ± 68 vs. 1565 ± 139 neurons mm^{-2}) at the peak of DNBS colitis in control mice (littermates treated with tamoxifen but lacking cre recombinase in glia, Cre-; ***Figure 2.2A***). However, mice with a conditional ablation of Cx43 hemichannels in glia (Cre+) were resistant to the neurotoxic effects of *in vivo* inflammation (2057 ± 68 neurons mm^{-2} in Cre-/Saline controls vs. 1850 ± 117 neurons mm^{-2} in Cre+/DNBS, ***Figure 2.2A***). This effect is not likely due to an alteration in the inflammation driven by DNBS because Cx43i-cKO (Cre+) mice exhibited the same pattern of weight loss and macroscopic damage scores as littermate controls (***Figure 2.2B-C***). Further, the ablation of Cx43 in glia did not affect normal neuron packing density in healthy animals (2057 ± 68 neurons mm^{-2} in Cre-/Saline animals vs. 2149 ± 101 neurons mm^{-2} in Cre+/Saline; ***Figure 2.2A***). Collectively, these data show that the

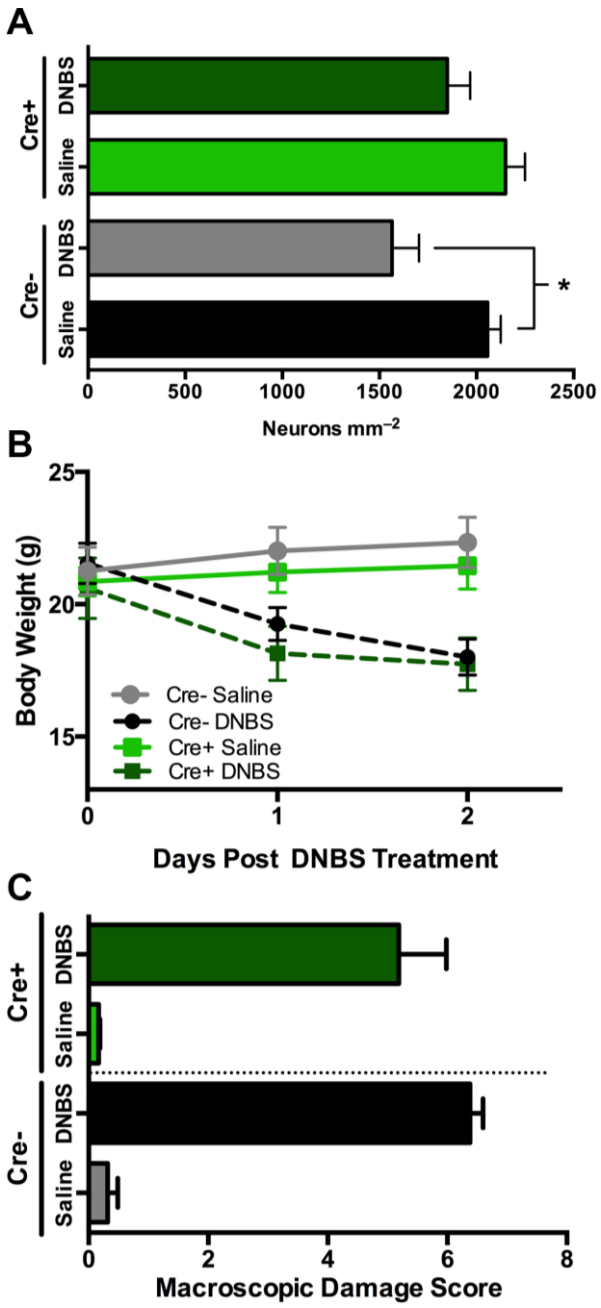

Figure 2.2: Genetic ablation of glial Cx43 limits neuropathy without modifying overt inflammation. (***A***) Mean packing density of myenteric neurons following DNBS-colitis in transgenic mice with an inducible ablation of Cx43 in glial cells (Cx43i-cKO, Cre+) and their littermate controls (Cre-). Weight loss pattern and macroscopic damage for experimental groups shown in (***B***) and (***C***), respectively (n = 5-7 animals, *P≤0.05, ANOVA).

expression of glial Cx43 hemichannels is a requirement for inflammatory neuropathy in the ENS.

Stimulation of enteric glial P2Y1Rs elicits Cx43-dependent ATP release

One possible mechanistic explanation for glial-driven neuron death is that glial Cx43 hemichannel opening modulates P2X7R activation threshold by augmenting levels of extracellular ATP. In support, astroglial Cx43 hemichannels are highly permeable to ATP[22,23] and neurotoxic activation of P2X7Rs requires a conformational change that only high concentrations of ATP are capable of inducing by occupying all four ATP binding sites[24]. We tested if purinergic activation of enteric glia drives Cx43-dependent ATP release by stimulating glial P2Y1Rs while monitoring extracellular ATP release with ATP-sensitive microelectrodes[14]. In these experiments, we either directly stimulated glial P2Y1Rs with the non-hydrolysable agonist ADPβS or indirectly generated endogenous ADP by activating neuronal P2X7R-dependent ATP release with the agonist BzATP. We found that stimulating glial P2Y1Rs with ADPβS elicits robust ATP release from enteric glia (***Figure 2.3A-B***). P2Y1R-driven glial ATP release was completely dependent upon Cx43 because ATP release was absent in the presence of the Cx43 mimetic peptide, 43Gap26 and in tissue from Cx43i-cKO (Cre+) mice (***Figure 2.3A-B***). Likewise, stimulation of P2X7R-dependent neuron-glia communication generated high levels of extracellular ATP (***Figure 2.3C-D***). Interestingly, panx1-dependent ATP release from enteric neurons accounted for only approximately 1/3 of total ATP release while Cx43-dependent release from glia accounted for the majority of

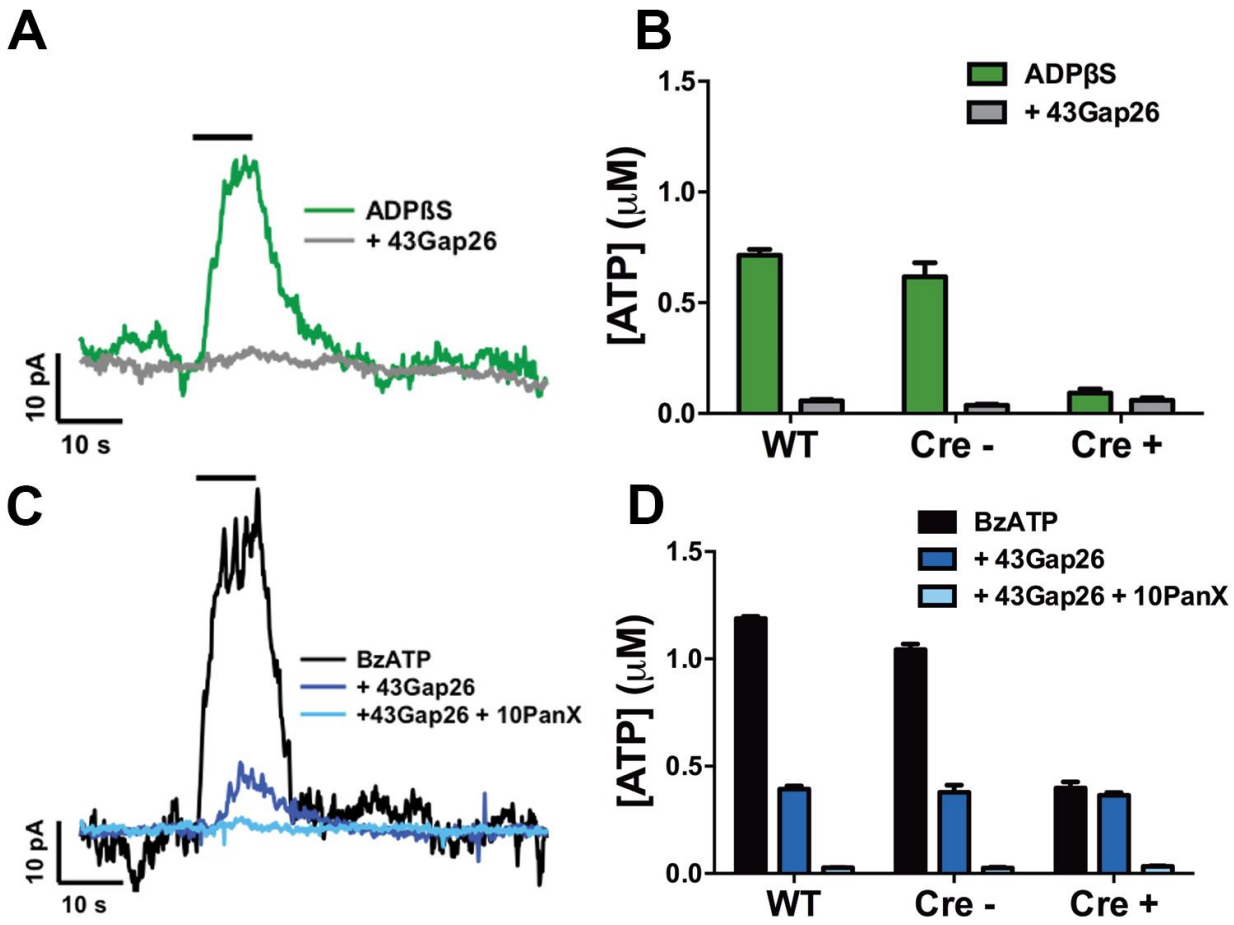

Figure 2.3: Stimulation of enteric glial P2Y1Rs elicits Cx43-dependent ATP release. (***A,C***) Representative traces and (***B,D***) quantified measurements of ATP release from the mouse myenteric plexus obtained with ATP-selective electrodes. Glial P2Y1Rs were directly stimulated with the P2Y1R agonist, ADPβS (***A,B***; 100μM), or by eliciting neuron-to-glia communication with the neuronal P2X7R agonist, BzATP (***C,D***; 300μM), in the presence or absence of the Cx43 mimetic peptide, 43gap26 (100μM), the pannexin-1 mimetic peptide, ^{10}Panx (100μM), or in tissue from Cx43i-cKO mice following the selective ablation of glial Cx43 (Cre+ KO or Cre- littermate controls). n=3 animals.

ATP release. These results indicate that enteric glia have the potential to release large quantities of ATP through Cx43 hemichannels following stimulation of P2Y1Rs.

Oxidative stress coincides with neuron death during in vivo inflammation and is required for P2X7R-driven neuron death in situ

Next, we asked whether the neurotoxic opening of glial Cx43 channels requires potentiation of channel function by other factors associated with inflammation. We initially focused on oxidative stressors because Cx43 hemichannel opening is potentiated by oxidative stress[25] and oxidative stress is considered a key mechanism in the pathogenesis of gut inflammation[1,26,27]. In support, we observed high levels of oxidative stress in myenteric ganglia during DNBS-colitis (***Figure 2.4A,C***). Measures of oxidative stress included neuronal thiol oxidation ratios (ratio of oxidized/reduced neuronal glutathione) (***Figure 2.4A****)* and ganglionic concentrations of superoxide measured by fluorescence of the superoxide-specific fluorescent indicator dihydroethidium (DHE) (***Figure 2.4C***). We confirmed that our measures truly reflected oxidative stress by administration of the antioxidant N-acetyl cysteine (NAC, 5g/L) *in vivo* prior to and during the induction of DNBS-colitis (***Figure 2.4A***). Treatment with NAC prevented increased oxidative stress during DNBS-colitis (***Figure 2.4A***) without altering the acute inflammatory insult, as there was no change in macroscopic damage score with NAC treatment (***Figure 2.4B***). Importantly, we investigated the mechanistic significance of oxidative stress and found that treatment with NAC prevented P2X7R-driven neuron death *in situ* (***Figure 2.4D-E***). Because our results above show that glial

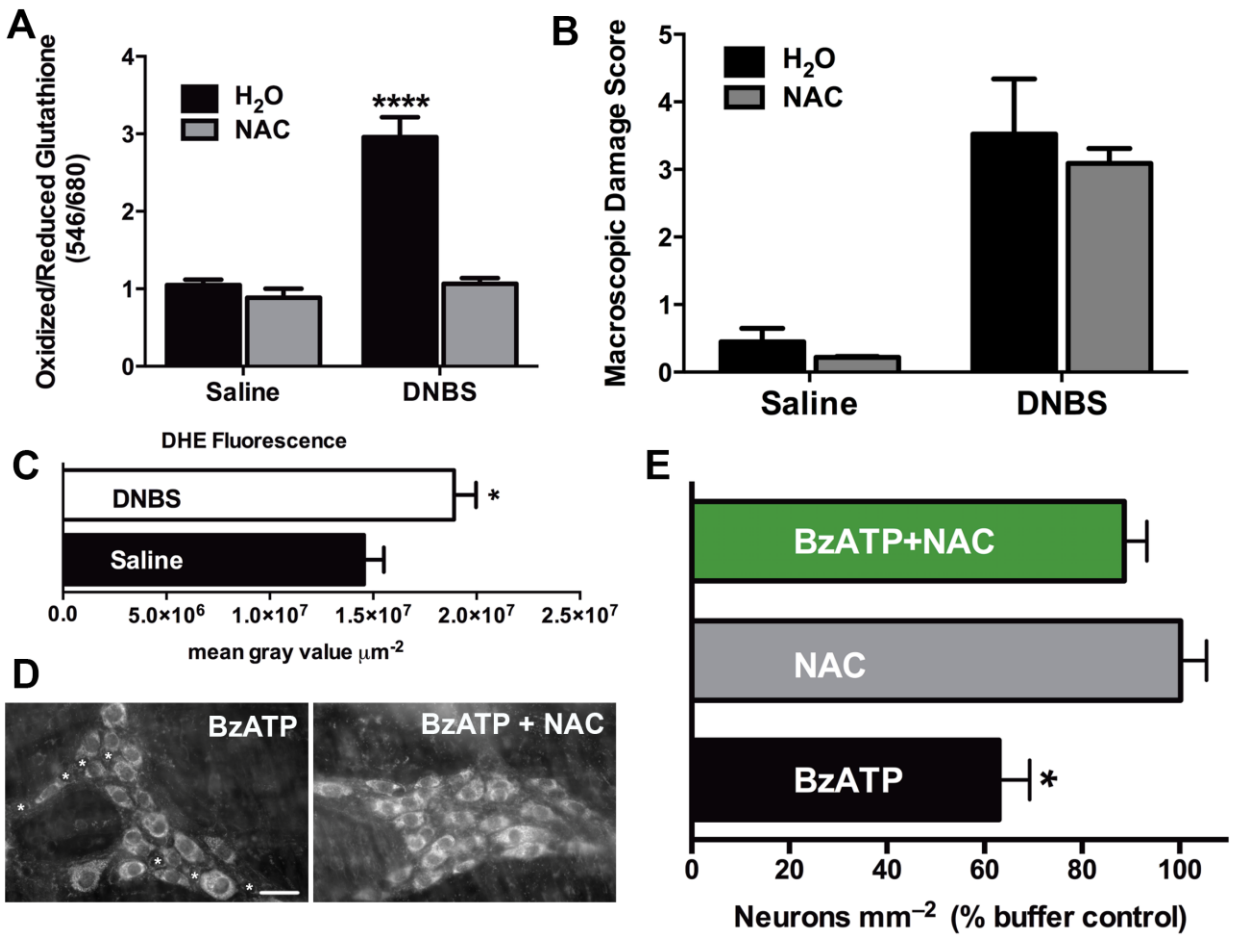

Figure 2.4: Oxidative stress coincides with neuron death during *in vivo* inflammation and is required for P2X7R-driven neuron death *in situ*. ***(A)*** Thiol oxidation measurements (ratio of oxidized/reduced glutathione) in myenteric neurons from healthy (saline) or inflamed (DNBS-colitis) animals drinking normal water (H_2O) or water containing the antioxidant N-Acetyl Cysteine (NAC, 5g/L) (n=5 ganglia, **** $P <$ 0.001, ANOVA). ***(B)*** Macroscopic damage score for animals treated in ***(A)***. ***(C)*** Ganglionic fluorescence of the superoxide indicator dihydroethidium (DHE, 2 μM) in the myenteric plexus of healthy (saline) or inflamed (DNBS-colitis) mice (n = 5-6 animals, * $P < 0.05$, unpaired t-test). ***(D)*** Representative myenteric ganglia from *in situ* preparations treated with BzATP in the presence or absence of the antioxidant NAC.

Figure 2.4 (cont'd)

Neurons are labeled with the neuronal marker HuC/D (scale bar = 30 μM). ***(E)*** Mean packing density of HuC/D-immunoreactive neurons in myenteric ganglia following *in situ* activation of P2X7Rs with BzATP (300 μM) in the presence of NAC (n=4 animals, * $P < 0.05$, ANOVA).

pathways mediate P2X7R-driven neuron death, these results indicate that oxidative stress contributes to the activation of neurotoxic glial mechanisms.

Enteric glia contribute to oxidative stress via local nitric oxide (NO) production during inflammation

Glial cells can directly contribute to the local generation of oxidative stressors by up-regulating the activity of iNOS[28,29]; an enzyme that produces large amounts of NO during inflammation[30]. We assessed the glial contribution to local NO levels within enteric ganglia from healthy and inflamed animals by measuring cellular NO concentrations with the NO-sensitive fluorescent dye DAF-FM (***Figure 2.5A***). DAF-FM fluorescence reliably reflected intracellular NO concentrations as treatment with the NO donor PAPA NONOate elevated fluorescence while treatment with the pan-NOS inhibitor L-NAME decreased fluorescence (***Figure 2.5A***). NO content was equally distributed between neurons and glia in non-inflamed animals and this distribution was not altered up to 6h following DNBS-colitis (***Figure 2.5B-C***). However, we observed an elevation of glial NO content during the peak of the inflammatory response 48hrs after initiation of DNBS-colitis (***Figure 2.5B-C***). Further, increases in immunoreactivity for nitrosylated proteins, another measure of NO concentrations, correlated with changes in glial NO content post DNBS-colitis treatment (***Figure 2.5D***). Nitrosylated protein immunoreactivity primarily co-localized with GFAP immunoreactivity within myenteric ganglia (***Figure 2.5E***), suggesting that glial cells are the main sites of nitrosylation in the myenteric plexus during inflammation. Importantly, the kinetics of glial NO production

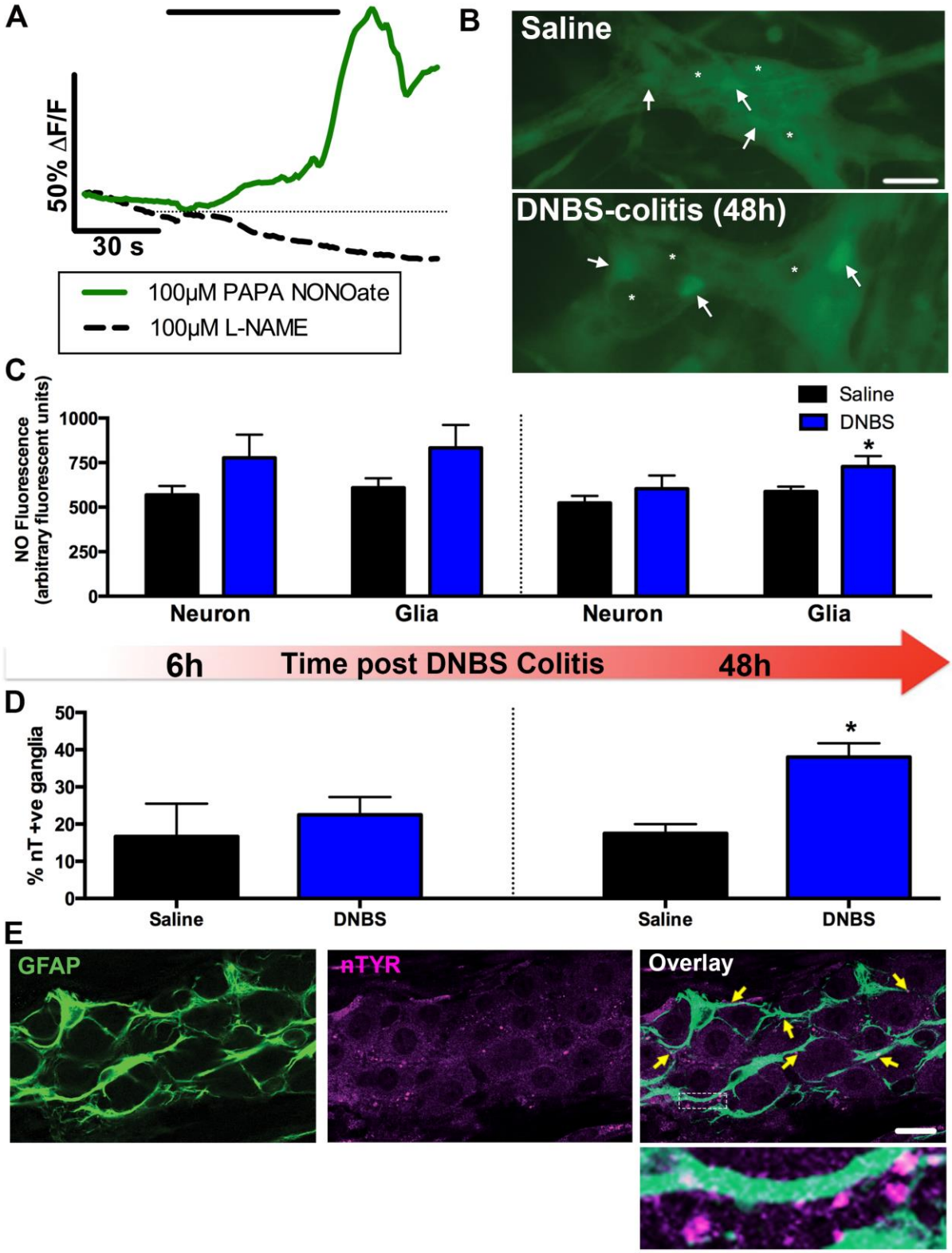

Figure 2.5: Enteric glia contribute to oxidative stress by producing nitric oxide (NO) during inflammation. ***(A-C)*** *In situ* NO imaging with the NO sensitive dye, DAF-

Figure 2.5 (cont'd)

FM. (***A***) Representative traces of mean glial NO responses following treatment with the NO donor PAPA NONOate (solid green line, 100 μM) or pan-nitric oxide synthase (NOS) inhibitor L-NAME (dashed black line, 100 μM). ***(B)*** Representative images of DAF-FM fluorescence in myenteric ganglia from healthy (saline) or inflamed (DNBS-colitis) mice. Arrows point to representative glial cells and representative neurons (or the lack thereof) are denoted by asterisks (scale bar, 30 μM). ***(C)*** Quantification of DAF-FM fluorescence in observable myenteric neurons and glia in the healthy (saline) or inflamed (DNBS) colon at 6h (left) and 48h (right) after the initiation of DNBS-colitis (n = 5-10 animals, **P* < 0.05, t-test compared to glia-saline*). (**D**)* Percentage of nitrotyrosine immunoreactive (nT +ve) ganglia in the myenteric plexus of saline DNBS-treated animals at 6h (left) and 48h (right) after the initiation of DNBS-colitis (n = 3-5 animals, * *P* < 0.05, unpaired t-test). ***(E)*** Representative myenteric ganglion showing immunoreactivity for nitrosylated proteins (nTYR; magenta). Enteric glia are labeled with the glial cell marker GFAP (green) and yellow arrowheads highlight areas of co-localization (scale bar, 20 μM).

and protein nitrosylation coincide with the appearance of neuron death during colitis in mice[9] and guinea pigs[31].

NO potentiates glial ATP release and promotes enteric neuron death through a Cx43-dependent mechanism

Given that nitrosylation of Cx43 hemichannels is associated with increased Cx43 channel opening in astrocytes[25], we hypothesized that NO would potentiate the Cx43-dependent release of ATP driven by glial P2Y1R activation. In support, we found that the Cx43-dependent release of ATP from glia stimulated by the P2Y1R agonist ADPβS was potentiated in the presence of the NO donor PAPA NONOate (***Figure 2.6A***). This suggests that NO production during intestinal inflammation can contribute to the activation of the neurotoxic Cx43 pathway in enteric glia.

In situ, we observed an equal extent of neuron death in whole-mounts of myenteric plexus incubated with the NO donor PAPA NONOate as in preparations exposed to the neuronal P2X7R agonist BzATP (24 ± 5% vs. 21 ± 4%, ***Figure 2.6C***), further supporting a for pathogenic role for NO in the context of neuron death. Importantly, the neurotoxic effects of PAPA NONOate and BzATP were not additive. This finding suggests that NO and P2X7R agonists drive neuron death through a common mechanism. Indeed, BzATP and PAPA NONOate-driven neuron death were both entirely dependent upon Cx43 hemichannel opening because the neurotoxic effects of these compounds were abolished in the presence of 43Gap26. Likewise, neurons were protected against BzATP-induced death in tissue from iNOS null (*iNOS*$^{-/-}$) mice and by blocking NO production from iNOS with the inhibitor, 1400W (***Figure 2.6C***).

Collectively, these data show that the production of NO by iNOS during inflammation is essential for the activation of neurotoxic pathways mediated through glial Cx43 hemichannels and strongly suggest that NO contributes to neuropathy by potentiating glial ATP release via Cx43 hemichannels.

Mechanisms of neuron death downstream of glial P2Y1R activation involve glial iNOS and neuronal P2X7Rs

To determine the sequence of signaling events downstream of glial P2Y1R activation that lead to neuron death, we activated glial P2Y1Rs *in situ* with the specific agonist ADP (100 μM) in the presence of drugs to inhibit either iNOS or P2X7Rs. The inhibition of glial iNOS with 1400W completely abolished the neurotoxic effect of glial P2Y1R activation (118 ± 13% vs. 72 ± 7%; ***Figure 2.6B***). Likewise, the inhibition of neuronal P2X7Rs with A740003 protected against neuron death driven by glial P2Y1R activation (90 ± 5%, vs. 72 ± 7%; ***Figure 2.6B***). Taken together, these results suggest a cyclical signaling mechanism where neuronal P2X7R-panx1-mediated ATP release activates glial P2Y1Rs and intracellular signaling mechanisms that lead to glial NO production and the potentiation of ATP release from glial Cx43 hemichannels which causes neuron death through actions on neuronal P2X7Rs.

NO positively modulates glial Cx43 hemichannels and negatively modulates neuronal panx1 hemichannels

NO could directly modulate a number of receptors and channels involved in the P2X7R-panx1 neuron death pathway including glial Cx43 hemichannels and/or neuronal panx1

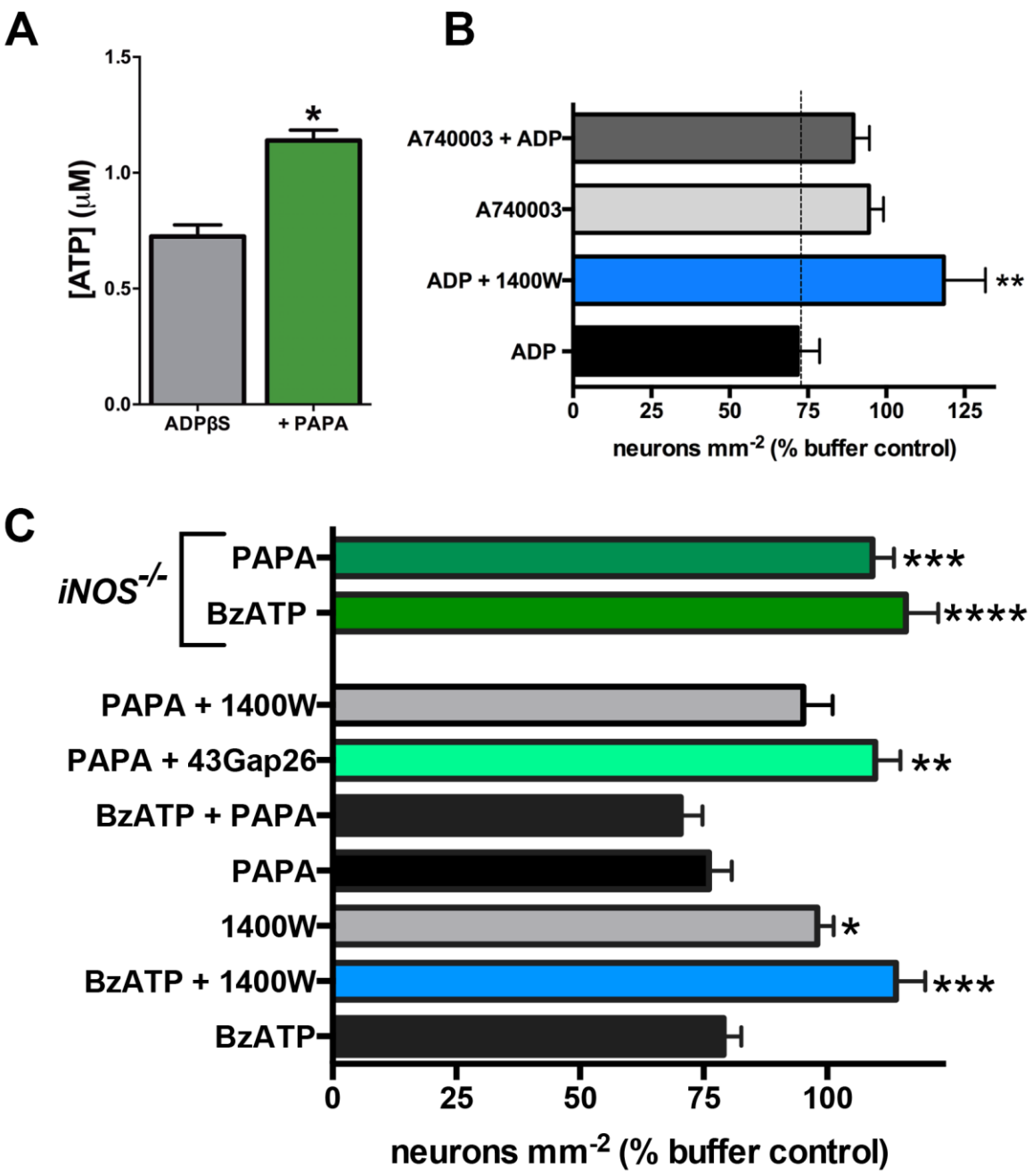

Figure 2.6: Nitric oxide (NO) potentiates ATP release from glia and promotes neuron death *in situ* through a mechanism that involves glial Cx43 hemichannels. (***A***) ATP release from myenteric ganglia following stimulation of glial P2Y1Rs with ADPβS (100 μM) alone or in the presence of the NO donor PAPA NONOate (n=4, * $P < 0.05$, unpaired t-test). (***B***) Mean packing density of Hu-immunoreactive myenteric

Figure 2.6 (cont'd)

neurons following direct glial P2Y1R stimulation with ADP and inhibition of iNOS (1400W; 10 μM) or P2X7Rs (A740003; 10 μM). Inhibition of iNOS or P2X7Rs protects against P2Y1R-driven neuron death (*P≤0.01, ANOVA as compared to ADP, n=3-4 animals). (***C***) Mean packing density of myenteric neurons following application of BzATP or NO-modifying drugs in wild type (bottom bars) or iNOS-knockout mice (*iNOS*$^{-/-}$, top two green bars). Inhibition (1400W; 10 μM) or ablation (*iNOS*$^{-/-}$) of iNOS protects against P2X7R-driven neuron death. The NO donor, PAPA NONOate (100 μM), drives neuron death to an equal extent as BzATP but the combination is not additive. Like BzATP, PAPA NONOate–driven neuron death requires iNOS (blocked by 1400W and in *iNOS*$^{-/-}$ mice) and Cx43 hemichannel opening (blocked by 43Gap26). *P≤0.05,**P≤0.01,***P≤0.001,****P≤0.0001, ANOVA as compared to BzATP, n=3-11 animals.

hemichannels and P2X7Rs[32]. To determine whether the neurotoxic effect of NO we observe is due to the potentiation of neuronal (P2X7R-panx1) or glial (Cx43) signaling mechanisms, we measured glial activity induced indirectly by the activation of neuronal P2X7R-panx1 or directly via the activation of glial P2Y1Rs in the presence of the NO donor PAPA NONOate (100 μM). We previously demonstrated that enteric glia can be used as endogenous "sniffer cells" to measure the activity of the neuronal P2X7R-panx1 pathway[9] and that glial network activity in response to the P2Y1R agonist ADP reflects glial Cx43 activity[4]. Our results show that glial Ca^{2+} responses downstream of neuronal P2X7R-panx1 activation are significantly blunted in the presence of PAPA NONOate (72% decrease in peak ΔF/F vs. control, ***Figure 2.7A-B***). Glial Ca^{2+} responses downstream of neuronal P2X7R activation are entirely dependent on the neuronal release of ATP through panx1[9] and blunted glial responses suggest that NO negatively regulates P2X7R-panx1-dependent neuron-to-glia communication. This results is in agreement with other studies showing that NO inhibits panx1 channel activity[32,33]. An alternate explanation for this result is that NO decreased the ability of glial to respond to neuronal activation. We tested this possibility by directly activating glial cells with ADP. Instead of decreasing glial responsiveness, we find that NO significantly potentiates glial Ca^{2+} responses to ADP (35% increase in peak ΔF/F vs. control, ***Figure 2.7C-D***). This outcome suggests that glial Cx43 hemichannel opening is facilitated by NO because Ca^{2+} responses through the enteric glial network are mediated by Cx43[4]. Our other data supports this conclusion by showing that NO potentiates glial Cx43-dependent ATP release (***Figure 2.6A***). Together, these results strongly support the conclusion that the

sensitization of glial release mechanisms, rather than neuronal signaling components, is the primary cause of neuron death.

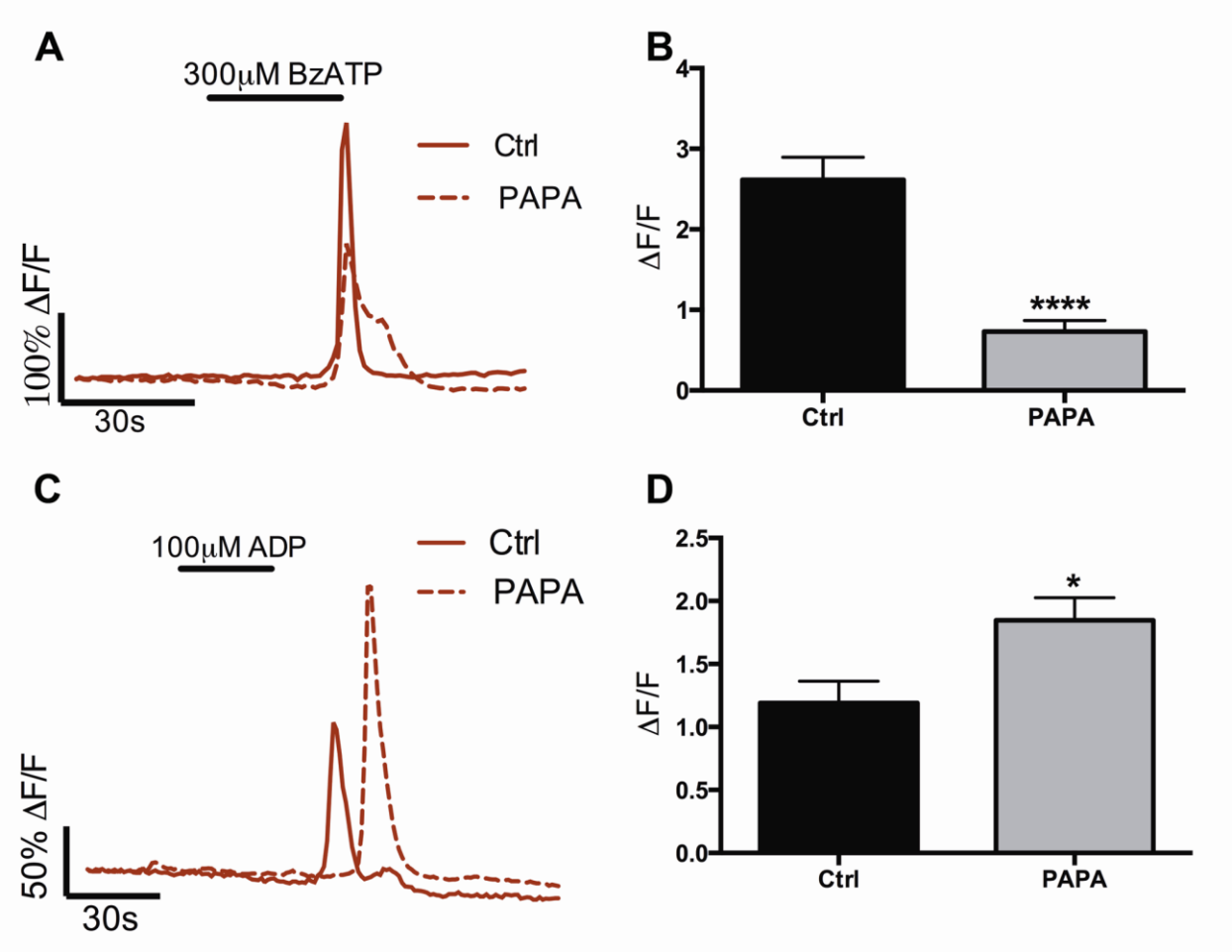

Figure 2.7: Nitric Oxide positively modulates glial Cx43 hemichannels and negatively modulates neuronal panx1 hemichannels. ***(A-B)*** Representative Ca^{2+} imaging traces ***(A)*** and averaged peak ΔF/F responses ***(B)*** show that NO (NO donor PAPA NONOate) blunts glial Ca^{2+} responses downstream of neuronal P2X7R-panx1 stimulation with BzATP (300 μM). ***(C-D)*** Representative Ca^{2+} imaging traces ***(C)*** and averaged peak ΔF/F responses ***(D)*** of glial Ca^{2+} responses during direct glial P2Y1R activation with ADP (100 μM) in the presence or absence of the NO donor PAPA NONOate. Note that NO potentiates glial network responses driven by the direct agonist ADP. *P<0.05, ****P<0.001, T-test compared to control, n= 51-139 individual cells in 3-7 ganglia.

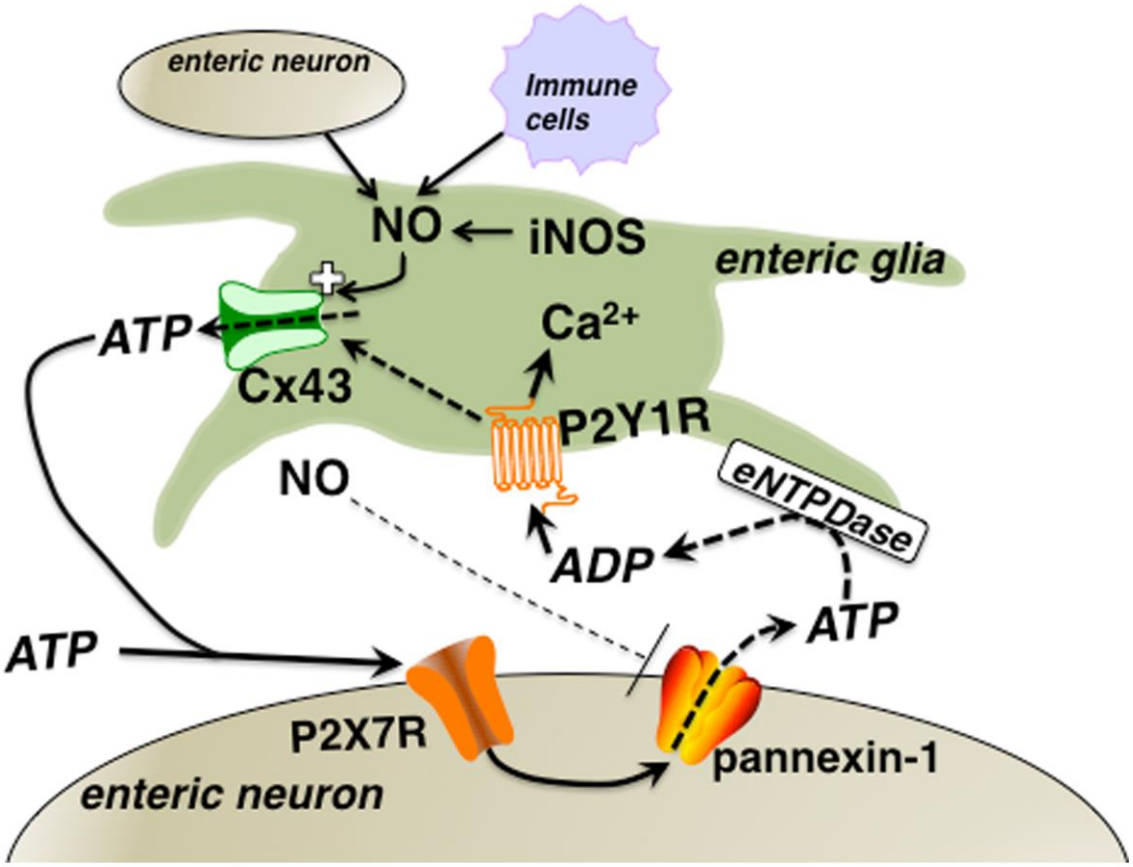

Figure 2.8: Proposed model of the mechanisms involved in glial cell-driven enteric neuron death during acute inflammation. The activation of neuronal P2X7Rs leads to ATP release through panx1 channels that recruits activity in the surrounding glia by activating glial P2Y1Rs. Intracellular signaling pathways downstream of P2Y1R activation drive glial iNOS production and glial NO potentiates Cx43 hemichannel-dependent ATP release. Glial NO may also feed back on neurons to block panx1 channel activity. Large quantities of ATP are released through glial Cx43 and act on neuronal P2X7Rs to drive neuron death.

Discussion

Our observations provide the first evidence that enteric glial cells play an active role in the death of enteric neurons during gut inflammation. Specifically, our data show that mediators of inflammation such as NO potentiate the gating of glial Cx43 hemichannels and subsequently, neuron death. Based on these data, we propose a model where neuronal P2X7-panx1-mediated purine release during inflammation or neuron stress, drives glial activation leading to pathogenic Cx43-dependent ATP release by glial cells and enteric neuron death by activation of neuronal P2X7Rs (***Figure 2.8***).

Enteric glial cells are generally thought to support the function and survival of enteric neurons and models of glial ablation support this conclusion as glial ablation produces a rapid loss of enteric neurons[5,6]. Thus, a loss of the supportive roles of glial cells is postulated as a potential mechanism for the development of ENS dysfunction in inflammatory bowel disease[2,5]. In contrast, newer data show that the chronic activation of astroglia in the context of neuroinflammation leads to the release of neurotoxic mediators from glial cells[7]. Our results support a similar scenario in the ENS during inflammation where a gain, rather than a loss, of glial cell function leads to the death of enteric neurons. These findings highlight the dichotomous roles of enteric glial cells during health and disease and suggest that either a loss of protective glial function or gain of pathological glial functions can drive ENS dysfunction but presumably through very different mechanisms.

Physiologically, enteric glial cells possess neuroprotective properties and are integral in maintaining enteric neuron populations. Enteric glia secrete neuroprotective compounds such as reduced glutathione and the prostaglandin derivative 15-deoxy-

PGJ2[3,34]. Further, intraganglionic enteric glia express a number of receptors and enzymes that can respond to and degrade neuroactive compounds, thus preventing aberrant neuronal activation[35,36]. Finally, glial cells can directly participate in neuron signaling by responding to and releasing neurotransmitters as previously shown for Cx43 hemichannels in hippocampal astrocytes in the CNS[37]. In support of a functional role for Cx43 hemichannels in the enteric nervous system, we observed that ablation of Cx43 in enteric glial cells blunts glial network activity and disrupts neuronal regulations of gastrointestinal transit under physiological conditions[4].

Here, we find that glial Cx43 hemichannel opening is required for neuron death during inflammation and that the selective ablation of glial Cx43 is neuroprotective in gut pathology. We postulate that these observations reflect the necessity for Cx43 hemichannels in both physiological and pathophysiological functions of enteric glia, where the pathological potential of glial Cx43 is evident when potentiated by inflammatory mediators such as NO. Indeed, here, NO potentiated Cx43-mediated ATP release from enteric glia, driving neuron death. Although inhibition of Cx43 was neuroprotective, the therapeutic potential of CX43 inhibition may be limited given the essential role of glial Cx43 in normal gut function. However, selective inhibition of pathological channel opening by NO has the potential to disrupt pathology without affecting physiological functions. Thus, drugs such as GW274150 that display excellent iNOS selectivity and little toxicity[38] may represent important new therapeutics in the treatment of functional gastrointestinal disorders.

Cx43 hemichannels are subject to post-translational modifications including S-nitrosylation[25] by increased oxidant concentrations. Here, we show an increase in

nitrosylated protein immunoreactivity in enteric glia that correlates with increased glial NO following *in vivo* inflammation. Importantly, the pattern of nitrosylation mirrors Cx43 immunoreactivity previously shown in enteric glia[4]. This strongly supports our hypothesis that Cx43 is a primary candidate of post-translational nitrosylation as a result of increased NO production during *in vivo* inflammation. We observed glial NO production during *in vivo* inflammation using a fluorescent NO marker during the initiation (6h) and peak (48h) of inflammation. Although we did not observe a significant increase in glial NO content at 6h after initiating inflammation, this could be due to the high variability between the inflammatory stages of individual ganglia at this early stage. However, our nitrosylation data show a similar timeline of nitrosylation in glia so it is likely that glial NO production occurs in response to active inflammation and is not necessarily responsible for the initiation of inflammation.

One alternate interpretation of our findings is that NO production by glia and oxidative stress in neurons directly affects the P2X7Rs and panx1 hemichannels on enteric neurons; leading to neuron death independent of glial mechanisms[9]. Indeed, NO modulates the activity of hemichannels including those composed of panx1[32,39,40] and Cx43[25,41]. However, we do not believe that our data support this interpretation because of several reasons. Firstly, we show that NO significantly blunts glial activity initiated by the neuronal release of ATP through P2X7R-panx1. In contrast, NO potentiated Cx43-dependent intercellular communication between enteric glia in response to direct P2Y1R stimulation. Likewise, the neurotoxic effect of the NO donor was completely abolished by the Cx43 mimetic peptide or the genetic ablation of glial iNOS. Further, our data show that glial cell release of ATP through Cx43 is the primary contributor to

extracellular ATP levels following stimulation of either neuronal P2X7Rs or glial P2Y1Rs while neuronal panx1 opening contributes only minor amounts of ATP. Finally, the selective ablation of glial Cx43 alone was able to protect enteric neurons during inflammation *in vivo*. We interpret these findings as indicating that the potentiation of neurotoxic mechanisms is primarily occurring in glial cells although we recognize that a combination of effects on neuronal and glial pathways could be required for neuron death.

Our *in vivo* model of Cx43 ablation utilizes cre/lox technology and requires expression of cre recombinase, driven by the GFAP promoter, in response to tamoxifen administration. Although enteric glial cells are a heterogeneous population, the majority of glia in the myenteric plexus are GFAP positive[42] making this a suitable promoter for our study. Importantly, our model is dependent on the presence of the GFAP promoter and thus variability in GFAP protein expression with inflammation and time would not likely have a major effect on cre activity and Cx43 excision[42]. In our experimental paradigm, inflammation was induced after the ablation of Cx43 was induced. However, enteric glia are subject to gliogenesis in response to injury and inflammation[43]. Thus, any newly generated glia following inflammation would have been exposed to tamoxifen and would express functional Cx43 hemichannels. Notably, the timeline for gliogenesis[43] is substantially longer than that of our current inflammatory model[31] so we do not believe that glial turnover was a major confounding factor in these experiments.

GFAP is also expressed by astrocytes in the CNS[44,45] and tamoxifen treatment in our mouse model induces the ablation of Cx43 hemichannels in enteric glia and CNS astrocytes. Disruption of astrocytic Cx43 gap junction and hemichannel function results

in CNS dysfunction and this may have exerted confounding effects in our disease model. However, we do not observe unusual weight loss or gut inflammation in these animals in the absence of inflammatory stimuli and we observed comparable weight loss and inflammation to wild type animals during inflammation (***Figure 2.2***). Thus, dysfunction in the brain-gut axis does not seem to be a significant contributor to disease outcome in our *in vivo* model.

Purinergic activation of enteric glial cells is a central component of our proposed signaling mechanism (***Figure 2.8***). We show glial purinergic responses are mediated through P2Y1Rs that are primarily localized to enteric glial cells in the mouse myenteric plexus. This supports previous work demonstrating glial P2Y1R expression and activity in cultured glial cells[18] and in whole mount preparations in mouse and guinea pig[9,46,47]. Previous work suggests P2Y1R expression on enteric neurons[17,48]. However, we demonstrate that activation with the P2Y1R agonist ADP elicits Ca^{2+} responses in glial cells, a phenomenon observed in the CNS notwithstanding P2Y1R expression on both neurons and astrocytes[49]. Given the present data, it is tempting to speculate that the P2Y1R-mediated slow excitatory postsynaptic potentials recorded in enteric neurons[48] are actually downstream of glial activation and this will be an interesting hypothesis to test in future experiments.

In conclusion, our findings have uncovered a novel role for enteric glia in the pathogenesis of enteric neuropathies. Enteric neuropathy is increasingly recognized as a key pathological finding in functional gastrointestinal disorders including irritable bowel syndrome[50] and slow transit constipation[51]. Inflammation is thought to produce persistent gut dysfunction through effects on the ENS including enteric neuron death

and the functional remodeling of enteric circuitry. Indeed, our prior results show that an acute inflammatory event triggers enteric neuron death and leads to gut motor dysfunction that persists despite resolution of active inflammation[9]. Our current observations suggest that the activation of enteric glial cells is necessary for at least a portion of these permanent effects on the ENS. Thus, new therapies that modulate the pathophysiological functions of enteric glial cells could lead to the development of more effective treatments for functional bowel disorders.

APPENDIX

Antibody	Source	Dilution	Catalog #
Rabbit anti-iNOS	Abcam, Cmabridge, MA	1:200	ab15323
Human anti HuC/D	Molecular Probes, Grand Island, NY	1:200	A-21272
Chicken anti-GFAP	Abcam, Cambridge, MA	1:1000	ab4674
Rabbit anti-nitrotyrosine	Millipore, Billerica, MA	1:100	06-284
Rabbit anti-P2Y1R	Alomone Labs, Jerusalem, Israel	1:200	APR-021

Table 2.1: Primary Antibodies used in Chapter 2.

Antibody	Source	Dilution	Catalog #
Alexa Fluor 488 Goat anti-rabbit	Invitrogen, Carlsbad, CA	1:200	A-11034
Alexa Fluor 488 Goat anti-chicken	Invitrogen, Carlsbad, CA	1:200	A-11039
Alexa Fluor 568 Goat anti-chicken	Invitrogen, Carlsbad, CA	1:200	A-11041
Alexa Fluor 594-conjugated Streptavidin	Jackson ImmunoResearch, West Grove, PA	1:200	016-580-084

Table 2.2: Secondary Antibodies used in Chapter 2.

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CHAPTER 3

The antioxidant glutathione protects against enteric neuron death *in situ,* but its depletion is protective during colitis

Abstract

Enteric glia play an important neuroprotective role in the enteric nervous system (ENS) by producing neuroprotective compounds such as the antioxidant reduced glutathione (GSH). The specific cellular pathways that regulate glial production of GSH, and how these pathways are altered during, or contribute to, neuroinflammation *in situ* and *in vivo* are not fully understood. We investigated this issue using immunohistochemistry to localize GSH synthesis enzymes within the myenteric plexus and tested how the inhibition of GSH synthesis with the selective inhibitor L-Buthionine Sulfoximine (BSO) impacts neuronal survival and inflammation. Both enteric glia and neurons express the cellular machinery necessary for GSH synthesis. Further, glial GSH synthesis is necessary for neuronal survival in isolated preparations of the ENS. *In vivo* depletion of GSH does not induce colitis but alters myenteric plexus neuronal phenotype and survival. Importantly, global depletion of glutathione is protective against some macro and microscopic measures of colonic inflammation. Together, our data highlights the heterogeneous roles of GSH in the ENS and during GI inflammation.

Introduction

The enteric nervous system (ENS) is a network of neurons and glial cells that controls the reflexes and behaviors of the gastrointestinal (GI) tract. Inflammation, among many other insults, can disrupt 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[1-4]. Recently, enteric glial cells have received increased attention for their active roles in ENS function and mechanisms of neuron death[5,6]. The activation of glia contributes to neuron death via pathways that involve connexin-43 hemichannels and oxidants such as nitric oxide[7]. However, glia also serve an important neuroprotective role and the targeted ablation of glial cells results in enteric neurodegeneration and gut inflammation[8,9]. The specific cellular mechanisms that regulate the production and secretion of glial neuroprotective factors, and how these are altered in (patho)physiology, are poorly understood.

Enteric glia synthesize and release a number of neuroprotective compounds such as the prostaglandin derivative 15-deoxy-prostaglandin-J2[10], the neurotrophic factor glial-derived neurotrophic factor (GDNF)[11], the endogenous antioxidant reduced glutathione [12] and the glutathione derivative s-nitrosoglutathione[13]. Reduced glutathione (GSH) is the primary endogenous antioxidant in the body and promotes neuronal survival in the central and peripheral nervous systems [14-18]. In the central nervous system, astroglia are the primary cell type responsible for the production of GSH and contain large quantities of GSH *in vivo* and *in vitro*[19]*.* Further, astrocytes have the ability to secrete GSH, and glial-derived GSH can be uptaken by neurons or elicit its antioxidant functions in the extracellular space[20-22]. Given the similarities between

astrocytes and enteric glia, we hypothesized that enteric glia play a similar role in the production and regulation of GSH in the ENS.

We tested our hypothesis by studying the cellular localization of glutathione synthesis enzymes in the ENS and the impact of GSH depletion on neuron survival and GI inflammation *in situ* and *in vivo*. Our data show that both enteric neurons and glia express the cellular machinery necessary for the production of GSH. GSH production is necessary for enteric neuron survival *in situ* but, surprisingly, *in vivo* depletion of GSH was protective against some measures of GI inflammation. Together, our results provide novel insight into the cellular regulation of a key antioxidant in the intestine. GSH plays complex roles in the context of GI inflammation and its cytoprotective effects may contribute to disease pathogenesis.

Material and Methods

Animals

All work was approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC). All experiments used male C57Bl/6 mice (Charles River Laboratories, Hollister, CA) aged 6-10 weeks and maintained on a 12-hour light/dark cycle with *ad libitum* access to food and water.

In Vivo Colitis

Colitis was induced in male C57Bl/6 as previously described [4,7]. An enema of 2,4-dinitrobenzene sulfonic acid (DNBS, 5.5 mg/mouse in 0.1 mL 50:50 ethanol/saline) was administered via a gavage needle inserted 3 cm into the colon. Experimental controls

were given an enema of saline only. Animals were sacrificed 48 hours after DNBS/saline treatment and macroscopic damage to the colon was assessed with a well established scoring system [23]. Tissue was then fixed overnight in Zamboni's fixative for use in immunohistochemical assays. Animal body weight and appearance were recorded daily following the induction of colitis.

In Vivo Glutathione Depletion

Colonic glutathione was depleted as described by Watanabe et al. [24]. Briefly, mice were given *ad libitum* access to drinking water containing 20 mM L-Buthionine-Sulfoximine (BSO) or normal drinking water (H_2O) for a period of 14 days. Mice were observed daily and changes in appearance, body weight and fluid consumption were recorded.

In Situ Model of Neuroinflammation

Enteric neuron death was driven in live longitudinal muscle myenteric plexus (LMMP) preparations *in situ* by the P2X7 receptor agonist 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate triethylammonium salt (BzATP) (300 mM) [4,7]. Live LMMPs were prepared from segments of mouse distal colon tissue in sylgard-coated 35 mm^2 dishes containing DMEM F12 media as described previously[7]. LMMPs were incubated with BzATP (300 mM) or other drugs for 2 hours in 95% air: 5% CO_2 at 37°C. LMMPs were then rinsed three times with fresh Kreb's Buffer and incubated for an additional 2 hours in Kreb's Buffer. LMMPs were fixed overnight at 4°C in Zamboni's fixative for IHC analysis.

Whole-mount Immunohistochemistry (IHC)

Whole-mount LMMP preparations were prepared from colonic tissue fixed overnight in Zamboni's fixative at 4°C. Briefly, preparations were permeabilized by three 10 minute washes in 0.1% Triton X-100 in phosphate buffered saline (PBS). Following a 45 minute block in 4% normal goat or donkey serum, LMMP preparations were incubated in primary antibody (see ***Table 3.1, appendix***) either overnight at room temperature (RT) or for 48 hours at 4°C. Finally, preparations were incubated in secondary antibodies (see ***Table 2, appendix***) for 2 hours at RT before mounting and imaging.

Images were acquired using the 40X objective (0.75 numerical aperture; Plan Fluor, Nikon, Melville, NY) of an upright epifluorescence microscope (Nikon Eclipse Ni) with a Retiga 2000R camera (QImaging, Surrey, BC, Canada) controlled by QCapture Pro 7.0 (QImaging) software or 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).

Dual-Labeling with Primary Antibodies Raised in the Same Host

Dual-labeling with primary antibodies raised in the same host was achieved using a Tyramide Signal Amplification Kit #14 (T20924) containing HRP conjugated-Goat Anti-Rabbit IgG and Alexa Fluor 568 Tyramide from Life Technologies (Carlsbad, CA), using a modification of the protocol described by Gulbransen et al.[25]. The first 1° (rabbit anti-synaptophysin) was used at a 1:2000 dilution and detected via TSA reaction while the second primary (rabbit anti-GCLC) was detected via normal IHC staining protocols. Preparations were incubated in the first 1° overnight at RT after endogenous

peroxidases were quenched by a 60-minute pre-incubation in 3% H_2O_2 diluted in PBS. Next, preparations were incubated with HRP-Goat Anti-Rabbit IgG for 2 hours at RT followed by a 10 minute incubation in Alexa Fluor 568 Tyramide and an overnight incubation with unlabeled Goat anti-Rabbit Fab fragments (1:50) at RT. Finally, preparations were labeled with the second 1° and 2° pair as described above (whole-mount IHC).

Quantification of Neuronal Thiol Oxidation

Cellular oxidative stress was quantified using a neuronal thiol oxidation ratiometric analysis as previously described[7,26]. Reduced (-SH) and oxidized (GSSG) thiols were labeled with 1μM Alexa Fluor 680 C_2 maleimide and 1μM Alexa Fluor 546 C_5 maleimide, respectively (Life Technologies). Oxidized thiols were reduced to free thiols for labeling using 5mM tris(2-carboxyethyl)phosphine hydrochloride (Sigma) in PBS. Images were obtained by epifluorescence microscopy as described above and the ratio of 546-maleimide/680-maleimide (SS/SH) calculated with ImageJ software (http://imagej.nih.gov/ij/).

Chemicals/Drugs

BzATP, N-ethylmaleimide, tris (2-carboxyethyl)phosphine hydrochloride (TCEP), L-Buthione-sulfoximine (BSO) and γ-glutamyl-cysteine (γ-GC) were purchased from Sigma-Aldrich (St. Louis, MO).

Solutions and Media

Live tissue was collected in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Life Technologies) supplemented with 3 μM nicardipine and 1 μM scopolamine. Normal goat/donkey serum contained 4% normal goat/donkey serum, 0.4% Triton X-100 and 1% bovine serum albumin in PBS.

Data and Statistical Analysis

Average ganglionic neuronal density was determined by calculating the number of HuC/D-immunoreactive neurons per ganglionic area using the cell counter tool in ImageJ, in at least 10 ganglia/animal. Average ganglionic fluorescence was quantified using the measure tool in ImageJ. Thiol oxidation ratios were calculated using the Region of Interest (ROI) tool in ImageJ. Fluorescence of 546-maleimide (oxidized thiols) and 680-maleimide (reduced thiols) were measured in 15-20 neurons/ganglia in a minimum 10 ganglia/animal. SS/SH (546/680) ratios were calculated per neuron and averaged - first per ganglia and then per mouse.

Subpopulations of excitatory (calretinin positive) and inhibitory (nNOS positive) neurons were calculated by expressing the number of calretinin or nNOS positive neurons as a percent of HuC/D (total) neurons in a minimum of 10 ganglia/mouse. Density of infiltrating neutrophils was calculated using the cell counter tool in ImageJ to quantify the number of MPO-positive neutrophils in a 76,800 μm^2 field of view surrounding a single myenteric ganglion. Similarly, activation of MHC-II muscularis macrophages was quantified by measuring average fluorescence (AFU) of a 76,800

μm^2 field of view surrounding a single myenteric ganglion. Both measurements were performed in a minimum of 10 ganglia/mouse.

Colonic macroscopic damage was quantified using the well characterized scoring system described by Storr et al.[23]. The presence of fecal blood, diarrhea and/or hemorrhage received 1 point/feature present. Adhesion of the colon to other organs and peritoneal space was scored 0,1 or 2 based on the severity of adhesions. Colon length and thickness were also scored with greater points for a shortened and/or thickened colon. Data were analyzed by student's t-test, One-Way or Two-Way ANOVA (with Dunnett's or Tukey's post-hoc test) using Graphpad Prism 7 (Graphpad Software, San Francisco, CA).

Results

Glutathione synthesis enzymes are expressed in myenteric ganglia

Enteric glia are thought to serve important neuroprotective roles in the enteric nervous system because the ablation of glia leads to widespread neuron death[8,27]. How glia protect neurons in the gut is not clear. Reduced glutathione (GSH) is a neuroprotective antioxidant that is secreted by enteric glia in culture[12] and mice lacking the regulatory glutathione peroxidase enzymes develop spontaneous colitis[28]. However, whether enteric glia express the enzymes necessary for glutathione synthesis and contribute to the protection against colitis *in vivo* is not known.

We tested whether glia express the machinery required for glutathione synthesis *in vivo* by labeling for two key enzymes: glutamate-cysteine ligase and glutathione

synthetase. Glutathione is synthesized in a two-step pathway[29]. In the first and rate-limiting step, glutamate-cysteine ligase (GCL) catalyzes the formation of γ-glutamylcysteine (γ-GC) from glutamate and cysteine. γ-GC then combines with glycine in a step catalyzed by glutathione synthetase (GS) to form reduced GSH (γ-glutamyl-cysteinyl-glycine). Our data show that the catalytic subunit of GCL (GCLC) is expressed in mouse myenteric ganglia and co-localizes with the glial cell marker, glial fibrillary acidic protein (GFAP; white arrows, ***Figure 3.1A***). Further, GCLC is also expressed in neuronal varicosities and its labeling co-localizes well with the pre-synaptic vesicle marker synpatophysin (***Figure 3.1B***). The second synthesis enzyme, GS, is also expressed by enteric glia but, interestingly, the majority of GS labeling was localized to HuC/D-immunoreactive myenteric neurons (***Figure 3.1C***). This pattern of expression is consistent with the known cellular distribution in the central nervous system, where neurons express GSH synthesis enzymes and can synthesize GSH when supplied with the dipeptide precursors, such as CysGly, from astrocytes[21,30]. These results show that enteric glia express the enzymes necessary for GSH synthesis and further, suggest a novel pathway where enteric neurons are capable of producing GSH.

In situ inhibition of GSH synthesis with L-Buthionine Sulfoximine decreases myenteric neuronal density

Next, we tested the role of glial glutathione production in the maintenance of enteric neuron survival by selectively blocking GCL activity with the specific inhibitor, L-Buthionine Sulfoximine (BSO, 100 μM)[31]. GCL is the rate-limiting enzyme in GSH synthesis and the inhibition of GCL with BSO is sufficient to deplete GSH *in vitro* and *in*

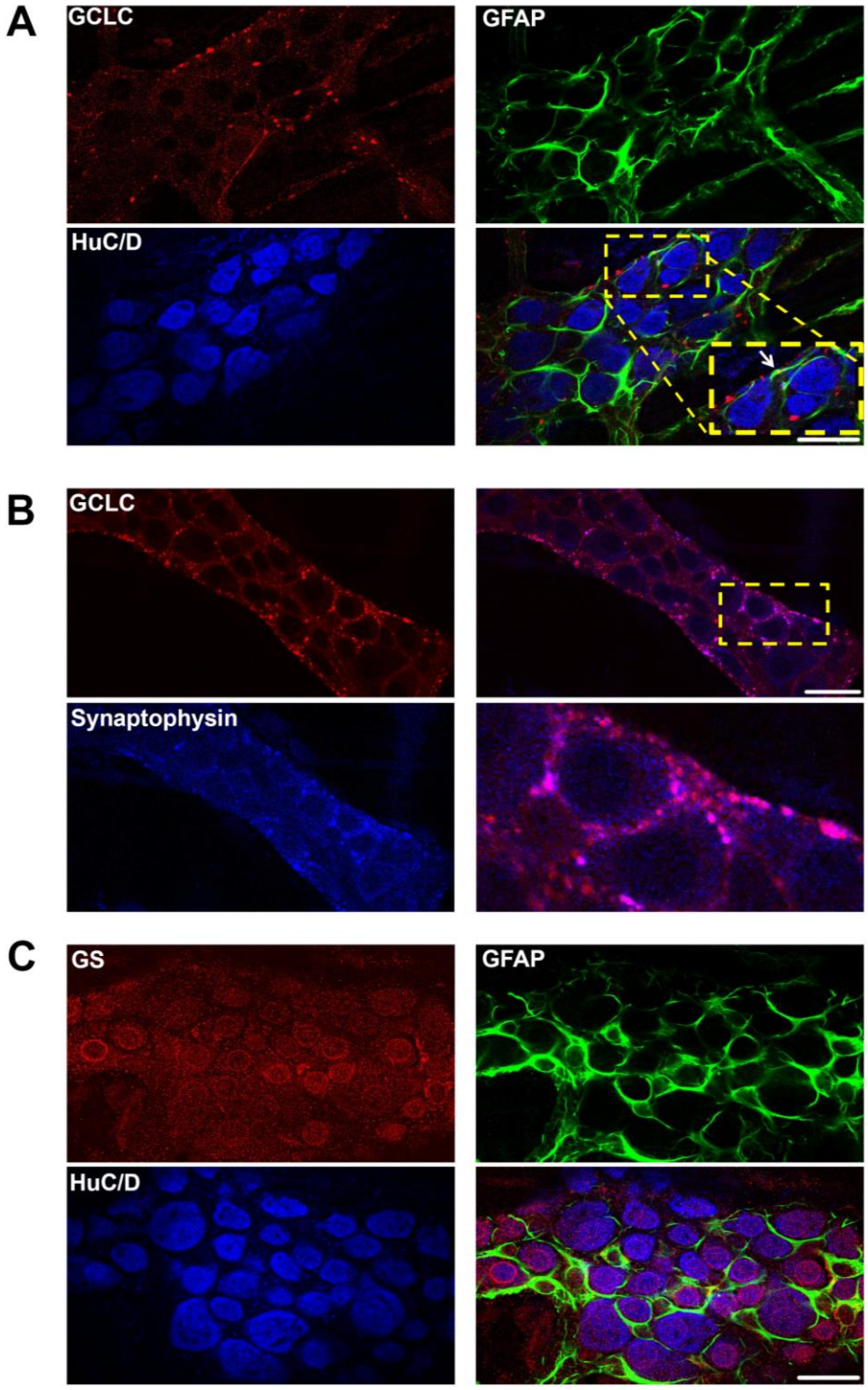

Figure 3.1: Expression of the glutathione synthesis enzymes, glutamate-cysteine ligase and glutathione synthetase, in the enteric nervous system. Representative

Figure 3.1 (cont'd)

mouse myenteric ganglia showing immunoreactivity for (***A,B***) the catalytic subunit of glutamate cysteine ligase (GCLC; *red*) and (***C***) glutathione synthetase (GS, *red*). Enteric neurons are labeled with the neuronal marker Hu C/D (*blue*), enteric glia with the glial marker GFAP (*green*) and pre-synaptic vesicles with the vesicular marker synaptophysin (*blue)*. White arrowheads in the overlay image panel of ***A*** highlight areas of co-localization between glutathione synthesis enzymes and GFAP. (scale bars = 20 μm).

vivo [18,24,32]. In LMMP whole mount preparations, the inhibition of GCL decreased myenteric neuron packing density by 14.1 ± 4% (1849 ± 75 neurons mm^{-2} in control vs. 1532 ± 62 neurons mm^{-2} in BSO-treated tissue; ***Figure 3.2A and 3.2B***) within four hours. Glutathione depletion drove neuron death to a similar extent as the activation of P2X7 receptors (P2X7R) on enteric neurons with BzATP, a known mechanism of enteric neuron death during inflammation (1532 ± 62 neurons mm^{-2} with BSO vs. 1559 ± 74 neurons mm^{-2} with BzATP) [4,7]. Importantly, the effects of BSO and BzATP were not additive (1334 ± 54 neurons mm^{-2}), suggesting either that BSO and BzATP drive neuron death through the same pathways or that they act through different neurotoxic mechanisms in the same subpopulation of neurons.

We hypothesized that increased oxidative stress following BSO-inhibition of GCL is the most likely mechanistic explanation for neuron death in our experiments. However, BSO could also drive neuron death through direct neurotoxic effects on enteric neurons. To differentiate between these two possibilities, we treated tissue with γ-glutamyl-cysteine (γ-GC), the product of GCL, to "rescue" the depletion of GSH by BSO. Treatment with γ-GC was protective against BSO-mediated neuron death (1889 ± 118 neurons mm^{-2} with γ-GC + BSO vs. 1532 ± 62 neurons mm^{-2} with BSO only, ***Figure 3.2A and 3.2B***), indicating that BSO-mediated neuron death is primarily through direct inhibition of GSH synthesis.

Treatment with BSO *in vitro* depletes cellular GSH, increases free radicals and drives cellular oxidative stress [32,33]. We measured neuronal oxidative stress in myenteric preparations after *in situ* treatment with BSO and BzATP using a thiol oxidation ratiometric analysis that quantifies the ratio of oxidized/reduced thiols.

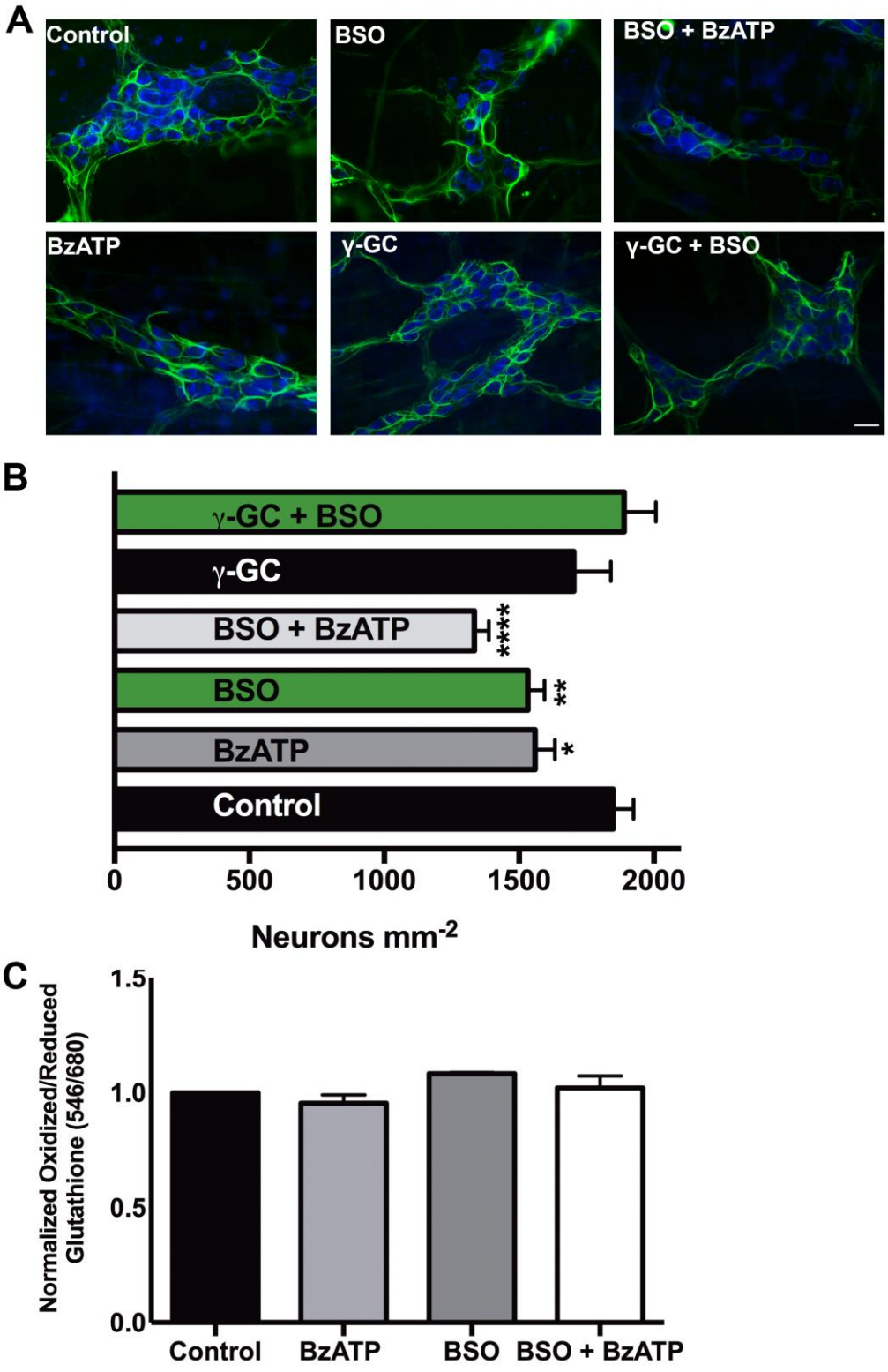

Figure 3.2: Glutathione (GSH) synthesis is required for enteric neuron survival.

(***A***) Representative images and ***(B)*** quantification of mean neuronal packing density in

Figure 3.2 (cont'd)

mouse myenteric ganglia following inhibition of GSH synthesis with the GCLC inhibitor L-Buthionine-sulfoximine (BSO, 100 μM). Whole mount preparations of myenteric plexus were incubated with BSO in the presence, or absence, of the GCLC product γ-glutamyl-cysteine (γ-GC, 100 μM) or the P2X7 receptor (PRX7R) agonist BzATP (300 μM). Inhibition of BSO decreases neuronal density to the same extent as P2X7R activation, while simultaneous treatment with γ-GC protects against BSO-mediated cell death. (* P≤0.05, ** P≤0.005, **** P≤0.0001, One-Way ANOVA with Dunnett's multiple comparison test vs. control, n=4-16 myenteric preparations/group from 9 animals.) Enteric neurons are labeled with HuC/D (*blue*) and enteric glia with GFAP (*green*) in all panels. (scale bar = 30 μm). ***(C)*** Neuronal thiol oxidation measurements (ratio of fluorescently labeled oxidized/reduced glutathione) from *in situ* myenteric preparations treated with BSO (100 μM) in the presence or absence of BzATP (300 μm) (n=3-6 ganglia/group).

Surprisingly, *in situ* oxidative stress was not significantly increased despite the cell death associated with BSO and BzATP treatment (***Figure 3.2C***). These results demonstrate that *in situ* inhibition of glial GSH synthesis with BSO is neurotoxic to myenteric neurons but does not alter neuronal thiol state.

In vivo inhibition of glutathione synthesis does not cause overt inflammation but is protective against macroscopic measures of inflammation

To better understand the role of glial GSH during GI pathology, we treated mice *ad libitum* with BSO in drinking water (20mM) for 14 days prior to induction of *in vivo* inflammation with DNBS-colitis. This treatment paradigm decreases colonic GSH content by 84% in adult mice [24], and liver GSH by 86% in pregnant rats [34], without any toxic effects. In our experiments, BSO treatment produced no significant adverse effects as evaluated by daily body weight measurements and observations of behavior and appearance (***Figure 3.3A***). Half of BSO-treated mice developed preputial gland abscesses around Day 7 of treatment (white and black arrows; ***Figure 3.3E***). However, all abscesses self-ruptured by Day 10 and mice were alert and in healthy condition prior to DNBS treatment on Day 14. To determine whether there was an aversion to water containing BSO, we measured daily fluid consumption in both treatment groups. Mice drinking water containing BSO, consumed similar volumes of fluid daily, compared to mice drinking normal H_2O (***Figure 3.3B***). In fact, mice drinking BSO consumed slightly more fluid (2.9mL H_2O vs. 3.5 mL BSO in H_2O /mouse/day).

Next, we induced inflammation with an enema of 2,4-dinitrobenzene sulfonic acid (DNBS), while control mice received a saline enema. BSO consumption without

exposure to an inflammatory insult (***Figure 3.3C***, BSO-Saline, *green solid line*) did not induce colonic inflammation as determined by weight loss (Body weight 2.6 ± 0.4 % and 2.4 ± 0.5% of control at days one and two, respectively; ***Figure 3.3C***). Mice that consumed normal drinking water prior to inflammation (H_2O-DNBS, *black dashed line*) lost significant body weight one and two days post-DNBS compared to H_2O-Saline controls (11.8 ± 1% at day one and 15.4 ± 0.9% at day two; ***Figure 3.3C***). Similarly, mice drinking BSO that received a DNBS enema (BSO-DNBS, *green dashed line*) also lost significant body weight compared to H_2O-Saline controls (8.5 ± 1.3% at day one and 7.9 ± 2.4% at day two, ***Figure 3.3C***). Importantly, BSO consumption prior to and during inflammation was protective against inflammatory damage, as BSO-DNBS mice lost significantly less weight than H_2O-DNBS mice two-days post DNBS (7.9 ± 2.4% vs. 15.4 ± 0.9%; ***Figure 3.3C***).

At sacrifice, mouse colons were collected and macroscopic damage score assessed as an additional measure of inflammatory damage [23]. BSO consumption in drinking water was not sufficient to increase macroscopic markers of inflammation (***Figure 3.3D***; BSO-Saline). Macroscopic damage score was increased in DNBS-treated mice, regardless of H_2O or BSO consumption prior to DNBS treatment (*gray bars*, ***Figure 3.3D***). In agreement with the trend seen in body weight, BSO treatment prior to DNBS was protective against increased colonic macroscopic damage (4.5 ± 0.7 and 2.5 ± 0.5 in H_2O-DNBS and BSO-DNBS respectively; ***Figure 3.3D***). Collectively, these results show whole body glutathione depletion does not induce overt colonic inflammation. Rather, BSO treatment is protective against some key features of colitis in mice.

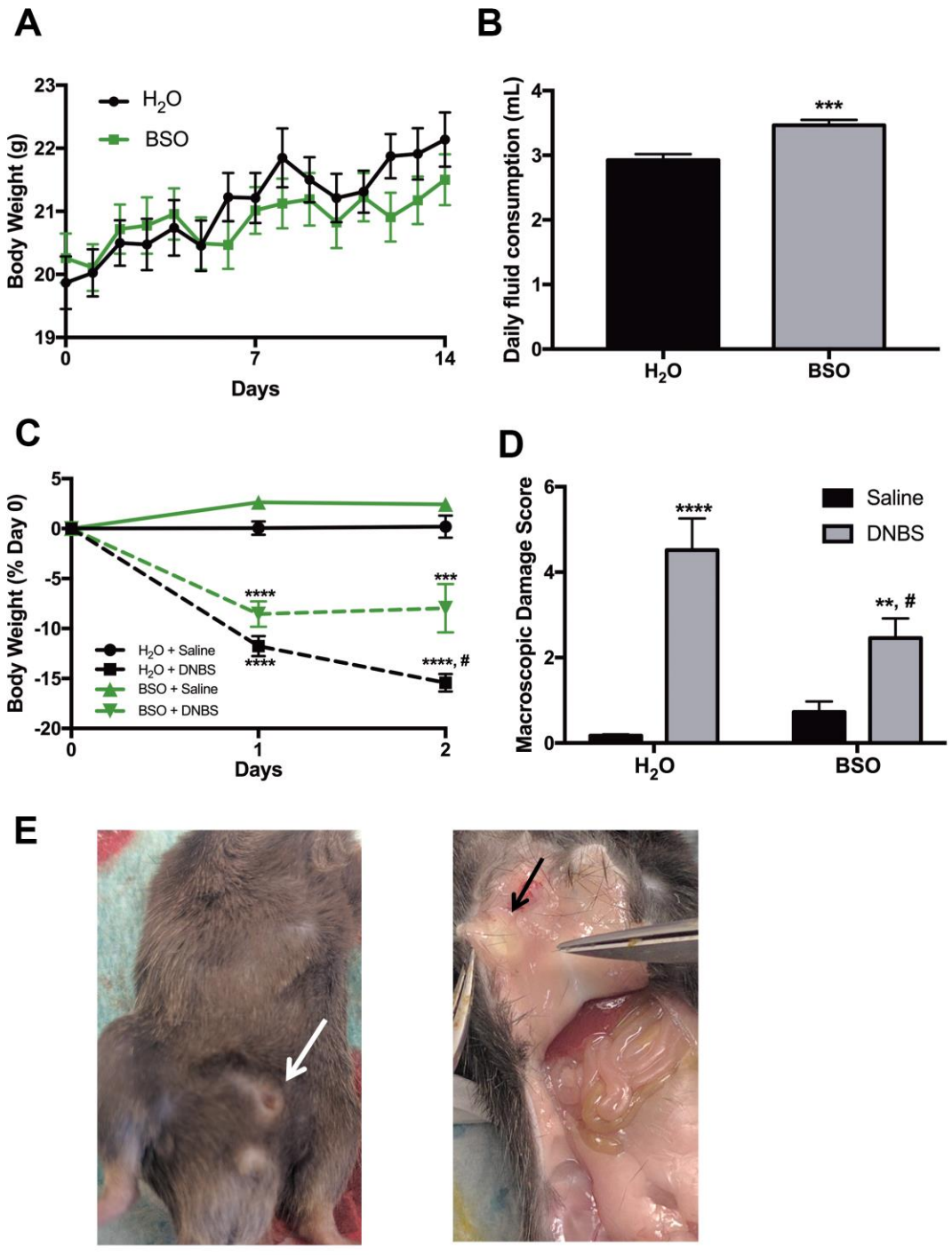

Figure 3.3: Inhibition of glutathione synthesis alone is not sufficient to cause overt inflammation but does alter susceptibility to inflammatory insults.

*(**A**)* Daily body weight of mice consuming either normal drinking water (H_2O, *black*) or

Figure 3.3 (cont'd)

water containing the GCLC inhibitor L-Buthionine Sulfoximine (BSO at 20 mM , *green*) for 2 weeks. ***(B)*** Average daily *ad libitum* fluid consumption for mice drinking normal water (H_2O) or water containing 20 mM BSO (*** p< 0.001, Student's t-test). ***(C)*** Daily percent weight loss and ***(D)*** macroscopic damage score of BSO treated mice (*green lines and gray bars*) or their normal drinking water controls (H_2O, *black lines and bars*) after DNBS colitis (dashed lines in ***C***, gray bars in ***D***) or saline control treatment (solid lines in ***C***, black bars in ***D***) (** P≤0.01, *** P≤0.005 and **** P≤0.0001 compared to H_2O Saline, # P≤0.05 compared to H_2O DNBS via Two-way ANOVA with Tukey's post-hoc test; n= 6-9 animals/group). ***(E)*** External (white arrow) and internal (black arrow) view of preputial gland abscess in BSO-treated mice on day of sacrifice.

Glutathione depletion decreases neutrophil infiltration during DNBS-colitis but does not alter reactivity of MHC-II expressing macrophages or neuronal thiol oxidation levels

Next, we investigated how *in vivo* glutathione depletion affects microscopic markers of inflammation. Increased immune cell infiltration is a key feature of many animal models of colitis, including DNBS [1]. In agreement with prior reports, we observed increased infiltration of myeloperoxidase (MPO)-positive neutrophils into a 76,800 μm^2 field-of-view area in the myenteric plexus in water drinking-inflamed animals (23.3 ± 10 neutrophils in H_2O-DNBS vs. 0.1 ± 0.04 neutrophils in H_2O-Saline, ***Figure 3.4A and 3.4B***). The frequency of neutrophils within the myenteric plexus of healthy animals drinking water supplemented with BSO was comparable to that of healthy animals drinking water alone (0.2 ± 0.05 neutrophils in BSO-Saline, ***Figure 3.4A and 3.4B***). However, mice drinking BSO during the course of colitis exhibited significantly attenuated myenteric plexus neutrophil infiltration compared to inflamed animals that did not receive BSO (5.8 ± 2.5 neutrophils in BSO-DNBS vs. 23.3 ± 10 neutrophils in H_2O-DNBS; ***Figure 3.4A and 3.4B***).

Next, we measured changes in major histocompatibility complex class II (MHC II) immunoreactivity to quantify the reactivity of antigen-presenting cells in a 76,800 μm^2 area surrounding individual myenteric ganglia. This population of cells predominantly consists of muscularis macrophages which reside at the level of the myenteric plexus in the colonic wall [35,36]. Colonic inflammation increased the immunoreactivity of MHC II-expressing muscularis macrophages in the myenteric plexus (***Figure 3.4C***). MHC-II expression was also induced by glutathione depletion as both inflamed (DNBS) and

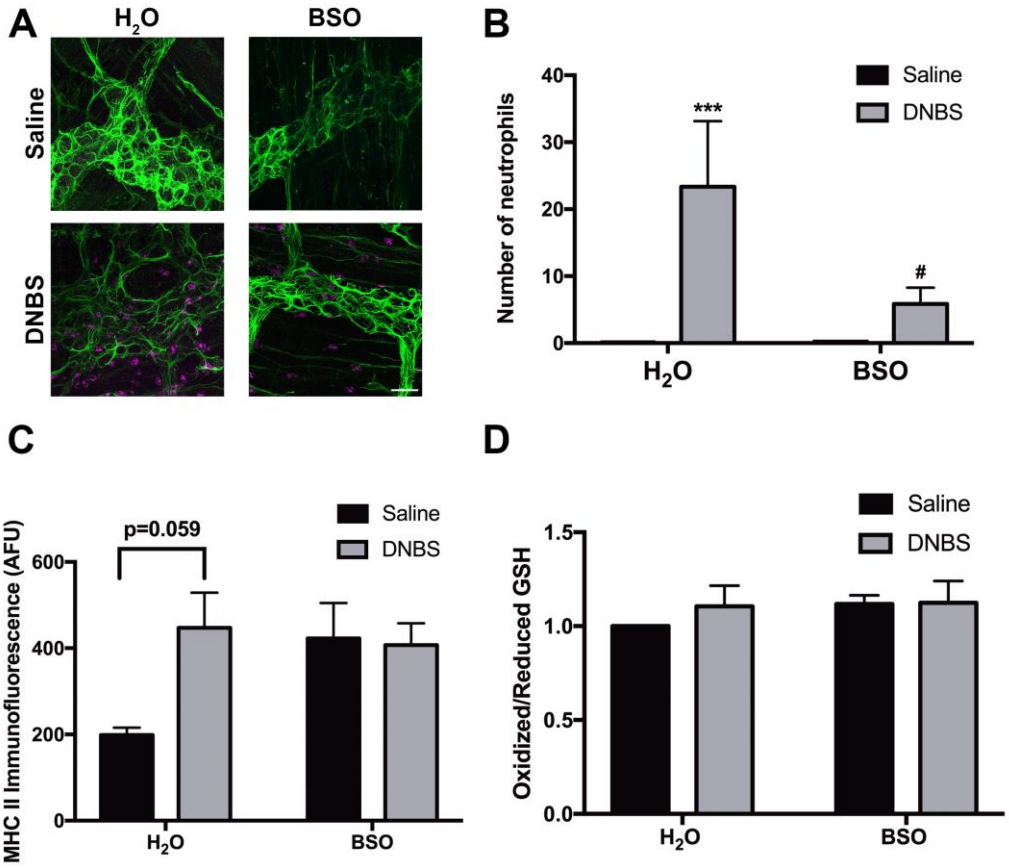

Figure 3.4: Effects of glutathione depletion on neutrophil infiltration into the myenteric plexus, MHC II-positive muscularis macrophage infiltration and neuronal thiol oxidation levels during DNBS colitis in mice.

*(**A**)* Representative images and *(**B**)* quantification of the infiltration of myeloperoxidase-positive neutrophils (MPO, *magenta*) in myenteric ganglia of mice drinking normal water (H_2O) or water with 20mM BSO prior to induction of colitis with DNBS (*gray bars*) or saline control (*black bars*). Enteric glia are labeled with GFAP (*green*). (*** P≤0.005 compared to H_2O Saline, $^{\#}$ P≤0.05 compared to H_2O DNBS via Two-Way ANOVA with Tukey's post-hoc test; n=3-6 animals/group). *(**C**)* Fluorescence of major histocompatibility complex class II (MHC-II)-immunoreactive muscularis macrophages in

Figure 3.4 (cont'd)

mouse myenteric plexus after DNBS-colitis (*gray bar*) or saline control animals (*black*), drinking either normal H_2O or water containing 20mM BSO. (Two-Way ANOVA with Dunnett's multiple comparison test, n=3 animals/group). ***(D)*** Neuronal ganglionic oxidation measurements (ratio of fluorescently labeled oxidized/reduced glutathione) (n=3-6 ganglia/group).

control (Saline) animals that consumed BSO showed a non-significant increase in ganglionic MHC II expression (***Figure 3.4C***). In agreement with our *in situ* data (***Figure 3.2C***), neuronal thiol oxidation state was not significantly altered following induction of colonic inflammation (H_2O-DNBS) or depletion of cellular glutathione in normal and inflamed animals (BSO-Saline and BSO-DNBS; ***Figure 3.4D***). Together, these results show that BSO treatment attenuates the infiltration of neutrophils during DNBS-colitis but does not significantly affect the infiltration or reactive state of MHC II-positive cells or neuronal thiol oxidation levels.

In vivo inhibition of glutathione synthesis decreases neuronal density and alters myenteric plexus neurochemical coding

Next, we assessed how *in vivo* depletion of glutathione affects myenteric neuron density and neurochemical coding. In agreement with our prior published findings [4,7], DNBS-colitis decreased neuronal density by 19 ± 7% in animals drinking normal water (1751 ± 155 neurons mm^{-2} in H_2O-Saline vs. 1421 ± 127 neurons mm^{-2} in H_2O-DNBS animals; ***Figure 3.5A and 3.5B***). *In vivo* glutathione depletion with BSO decreased HuC/D-immunoreactive neuron density to a similar extent as *in vivo* inflammation (1409 ± 78 neurons mm^{-2} in BSO-Saline animals). Consistent with our *in situ* results (***Figure 3.2B***), glutathione depletion during DNBS administration does not potentiate neuron loss during colitis (1647 ± 111 neurons mm^{-2} in BSO-DNBS mice; ***Figure 3.5A and 3.5B***).

Glutathione depletion is known to reduce levels of inhibitory neurochemicals in gastrointestinal tissue [37]. However, how glutathione depletion specifically alters neurochemical coding within myenteric ganglia is not known. We used

immunohistochemical markers for specific classes of myenteric neurons to investigate how neurochemical coding is altered in myenteric ganglia after glutathione depletion. Neither glutathione depletion nor inflammation altered the percentage of calretinin-positive excitatory neurons in healthy animals (19 ± 1.5% in H_2O-Saline, 21 ± 2.8% in H_2O-DNBS and 25.5 ± 1.7% in BSO-Saline; ***Figure 3.5C and 3.5D***). In contrast, glutathione depletion caused the percentage of calretinin-positive neurons to decrease by approximately 10% during inflammation (25.5 ± 1.7 % in BSO-Saline vs. 14.4 ± 1.2 % in BSO-DNBS; ***Figure 3.5C and 3.5D***). Conversely, glutathione depletion did not significantly affect the percentage of nNOS-positive inhibitory neurons in healthy or inflamed animals (33 ± 3% in H_2O-Saline vs. 38 ± 2.5% in BSO-Saline vs. 39 ± 2% in BSO-DNBS; ***Figure 3.5E and 3.5F***). Together, these results show that glutathione is necessary to maintain both the phenotype and survival of neurons in the myenteric plexus of the colon.

Inflammation alters the expression of the glutathione synthesis enzymes, glutamate-cysteine ligase and glutathione synthetase, in the ENS

Finally, we investigated how *in vivo* inflammation affects the localization and expression of glutathione synthesis enzymes. DNBS-colitis did not alter the localization of either GCLC or GS. GCLC expression remained localized to enteric glia and pre-synaptic neuronal vesicles (***Figure 3.6A***) and GS expression remained localized to glial cells and myenteric neuron cell bodies (***Figure 3.6C***). However, both *in vivo* inflammation and BSO treatment altered the expression levels of GCLC and GS. *In vivo*

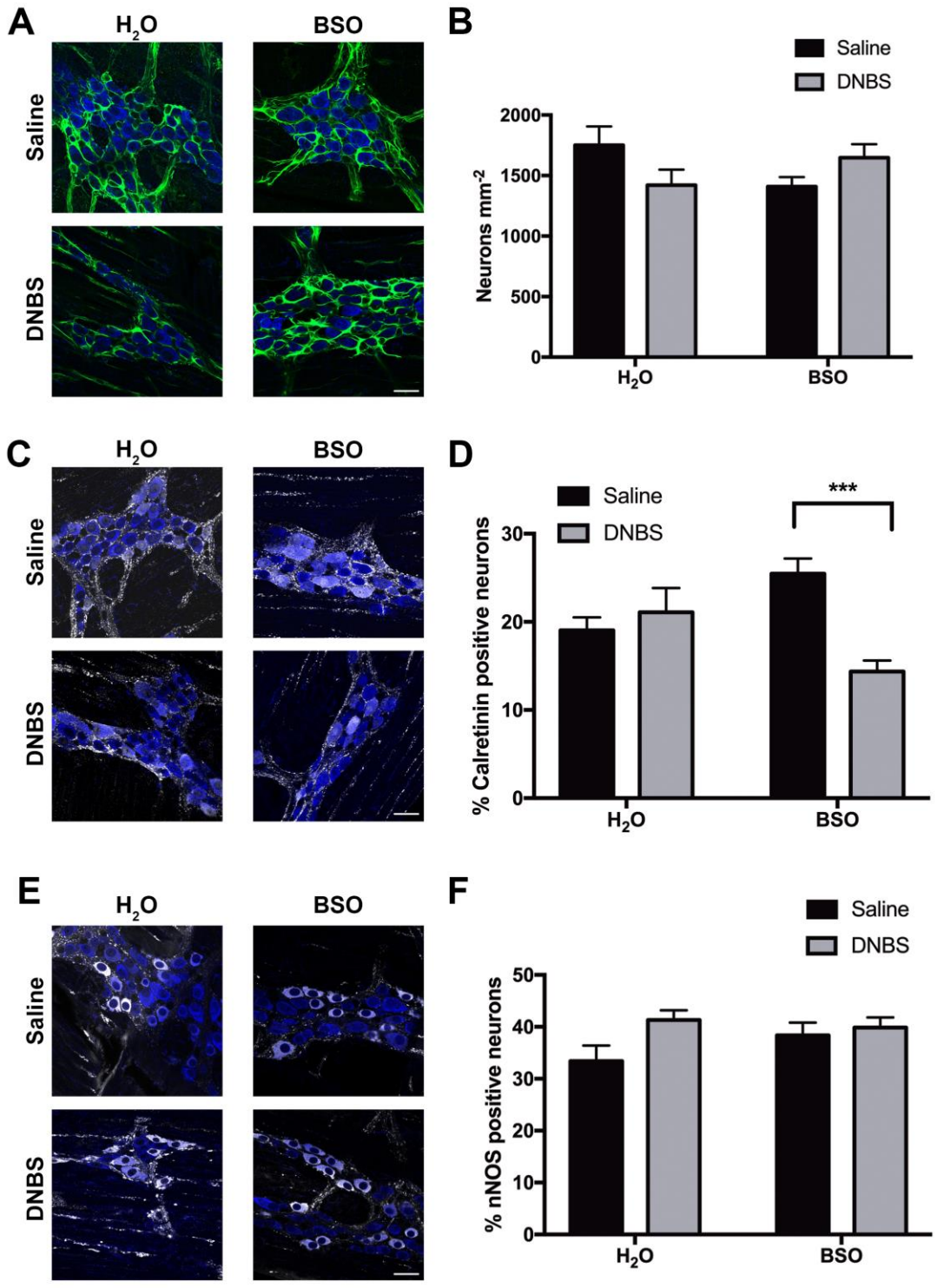

Figure 3.5: Inhibition of glutathione synthesis *in vivo* decreases enteric neuron survival and alters myenteric plexus neurochemical coding.

*(**A**)* Representative images and *(**B**)* mean-packing density of HuC/D-immunoreactive

Figure 3.5 (cont'd)

myenteric neurons (*blue)* from mice consuming normal drinking water (H_2O) or water with 20mM BSO prior to DNBS (*gray bars*) or saline (*black bars*) enema administration. Enteric glia are labeled with GFAP (*green*). ***(C, E)*** Representative images showing excitatory (Calretinin, *gray;* ***C***) and inhibitory (nNOS – neuronal nitric oxide synthase, *gray;* ***E***) neuronal sub-populations in myenteric ganglia. ***(D, F)*** Percentage of HuC/D-positive neurons that are immunoreactive for Calretinin (***D***) and nNOS (***F***) in mice drinking water (H_2O) or water with 20mM BSO prior to DNBS (*gray bars*) or saline (*black bars*) enema administration. Myenteric neurons are labeled with the pan-neuronal marker Hu-C/D *(blue*), excitatory neurons with Calretinin (*gray*) and inhibitory neurons with nNOS (*gray*). (n = 4-9 animals/ group, ***P≤0.05 Two-Way ANOVA with Tukey's post-hoc test), (scale bars = 20 μm).

inhibition of glutathione synthesis with the GCLC-inhibitor BSO (20 mM) decreased ganglionic GCLC expression by 36% (Mean fluorescence: 267 ± 18 in H_2O-Saline vs. 170 ± 46 in BSO-Saline; ***Figure 3.6B***). This result supports well-established data showing that cellular GSH concentrations regulate GCLC activity and expression via a negative feedback loop [38,39]. BSO-treated healthy animals also show a ~40% decrease in expression of the second glutathione synthesis enzyme GS (Mean fluorescence: 284 ± 19 in H_2O-Saline animals vs. 168 ± 48 in BSO-Saline animals; ***Figure 3.6D***).

In vivo inflammation doubled the ganglionic expression of GCLC in both normal and BSO-treated animals (Mean fluorescence: 267 ± 18 vs. 555 ± 65 in H_2O Saline vs. DNBS, 170 ± 46 vs. 386 ± 79 in BSO Saline vs. DNBS; ***Figure 3.6A and 3.6B***). Similarly, GS expression increased ~200% following inflammation in both normal animals and animals with depleted glutathione (Mean fluorescence: 285 ± 19 vs. 581 ± 46 in H_2O Saline vs. DNBS animals, 169 ± 48 vs. 372 ± 83 in BSO Saline vs. DNBS animals; ***Figure 3.6D***). These results demonstrate a dynamic relationship between *in vivo* inflammation and regulation of glutathione synthesis within the ENS.

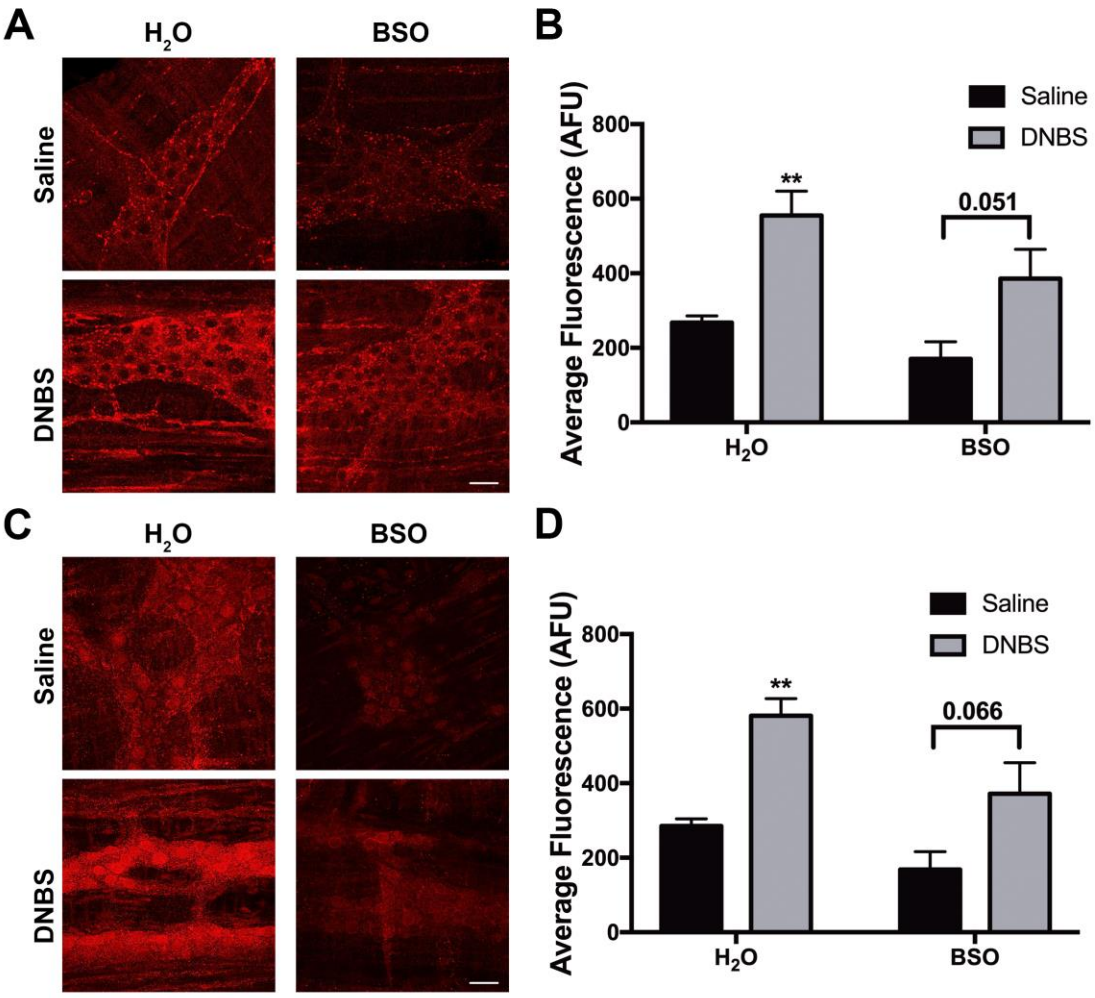

Figure 3.6: Inflammation alters the expression of the glutathione synthesis enzymes, glutamate-cysteine ligase and glutathione synthetase, in the enteric nervous system. Representative images ***(A,C)*** and corresponding quantification ***(B,D)*** of immunoreactivity for the catalytic subunit of glutamate-cysteine ligase (***A,B***; GCLC, *red*), the rate-limiting enzyme in glutathione (GSH) synthesis, or glutathione synthetase (***C,D***; GS, *red*) in mouse myenteric ganglia from healthy (saline) or inflamed (DNBS) mice drinking either normal water (H_2O) or water supplemented with BSO (20 mM; BSO). (* P≤0.05 compared to H_2O-Saline via Two-Way ANOVA with Tukey's post-hoc analysis), (n=7-9 animals/group).

Discussion

Our study investigates the role of the endogenous antioxidant reduced glutathione (GSH) in the ENS and probes how enteric glial GSH contributes to neuroprotection during *in situ* and *in vivo* neuroinflammation. Our findings show that glutathione is necessary to support neuronal survival and phenotype in the gut. However, the systemic inhibition of glutathione synthesis *in vivo* is neuroprotective during colitis, possibly by limiting the infiltration of immune cells.

Glutathione and its derivatives are well known for their neuroprotective effects in the ENS. Cultured enteric glia produce and secrete reduced glutathione, which is neuroprotective to enteric neurons in the myenteric plexus[12]. In the mucosa, the glial derived s-nitrosoglutathione (GSNO) helps maintain mucosal barrier function and promotes tight junction formation[13]. However, no work to date has directly shown that enteric glia express the enzymes necessary for glutathione synthesis. Our data here show that the enteric glia express the molecular machinery necessary for glutathione synthesis *in vivo*. These findings provide important support for previous work that measured the release of glutathione and its derivatives from cultured enteric glia[12,13].

The expression pattern of the glutathione synthesis enzymes in the ENS is similar to that in the central nervous system, where astroglia are primarily responsible for the production and secretion of neuroprotective GSH[19,40]. An unexplored hypothesis in the ENS was the ability of enteric neurons to also produce GSH. In the brain, neurons express the GSH synthesis enzymes, albeit at much lower levels and are capable of producing limited quantities of GSH[30]. In addition, neurons rely heavily on astrocytes to provide key precursor molecules necessary for GSH synthesis[16,21]. Our findings show a

similar relationship in the ENS where enteric neurons express GCLC and GS in neuronal varicosities and cell bodies, respectively. This suggests that enteric neurons have the ability to produce GSH and we hypothesize a novel neuroglia relationship similar to that in the CNS in regards to GSH synthesis and release.

Our data show that glutathione is necessary to support neuronal survival because the inhibition of glial GSH synthesis with the selective GCL inhibitor BSO decreased myenteric neuron density in acutely isolated tissues. One possibility is that BSO could produce direct neurotoxic effects on enteric neurons independent on its inhibition of glutathione synthesis. However, this is unlikely because other reports of *in vitro* BSO treatment show decreased GSH content in concurrence with cell death[18,33]. Further, treatment with GSH[18] or its precursors was sufficient to protect against BSO-mediated cell death[14] (***Figure 3.2B***), suggesting that the cytotoxic effects of BSO are directly related to its inhibition of GSH synthesis.

Glutathione depletion is reported to increase cellular oxidative stress and cellular susceptibility to oxidative damage[32]. However, neuronal thiol oxidation (a measure of cellular oxidative stress) was not increased in our live whole-mount preparations following treatment with BSO, suggesting that *in situ* inhibition of GSH synthesis does not affect cellular thiol state. A number of factors may explain this finding. Firstly, the thiol oxidation ratiometric analysis quantifies the oxidation states of all cellular thiols inclusive of, but not limited to, cellular glutathione. GSH depletion, while decreasing total thiol levels, may not alter the state of other proteinaceous thiols. In our treatment paradigm, live whole-mounts are incubated with BSO for 2h followed by a 2h incubation in buffer only. BSO has been shown to dissociate from 1-3% of bound GCL enzyme/

hour when a BSO treatment solution is replaced with normal buffer [38]. Thus, it is also possible that dissociation of BSO during the second 2h incubation is sufficient to return cellular thiol levels to normal, but not to reverse the cytotoxicity and cell death induced by the 2h incubation with BSO.

GSH is synthesized in two-step process catalyzed by the rate-limiting GCL and GS. Regulation of GCL and GS activity and expression are important mechanisms in the regulation of cellular glutathione content[41]. In fact, GSH synthesis is controlled by a negative feedback loop where GSH acts as a non-allosteric inhibitor on the rate-limiting GCL enzyme[38,39]. Similarly, GCL transcription and protein expression are induced by increased oxidative stress[42,43]. We observed this in our studies where increased oxidative stress during colitis increased expression of both GCL and GS. Increased expression of both enzymes is reported to significantly increase cellular GSH capacity and is thus an expected result in response to GI inflammation[44,45].

A primary goal of our study was to investigate the effects of *in* vivo inhibition of GSH synthesis and how GSH depletion affects macro and microscopic damage during inflammation. To this end, we administered 20 mM BSO *in vivo* via drinking water for 14 days prior to the induction of inflammation. This treatment paradigm has been previously well characterized as a suitable animal model for tissue GSH depletion without cytotoxicity[24]. In support, we observed few adverse effects in healthy BSO-treated animals throughout the treatment period or at the time of sacrifice. A proportion of animals did develop preputial gland abscesses, a common condition seen in non-breeder male mice[46]. If severe, this ailment requires surgical rupturing and antibiotic treatment. However, our mice developed mild abscesses that self-ruptured and did not

significantly affect animal health and welfare. Specifically, depletion of GSH did not decrease body weight, increase macroscopic colon damage or increase the infiltration of neutrophils in otherwise healthy animals.

Reduced glutathione is the primary endogenous antioxidant in the body and is an important cellular tool in protection against oxidative stress[47]. Typically, antioxidant depletion increases cellular oxidative stress and increases cell susceptibility to exogenous toxicants including oxidative stressors[41]. However, our results show that decreased glutathione is protective during *in vivo* inflammation. Specifically, GSH depletion prior to inflammation decreases colonic macroscopic damage score and neutrophil infiltration, partially protects against colitis-associated weight loss and prevents additional loss of myenteric neurons. One explanation of our findings is that GSH supports glial cells in a pathogenic state and that decreasing glial GSH is protective against glial activation and the subsequent neuron death. GSH serves a similar unexpected function in central nervous system gliomas. In this disease, increased glioma GSH synthesis is associated with the glutamate excitotoxicity responsible for many glioma symptoms[48]. In support, inhibition of the cysteine/glutamate System x_c^- transporter on glioma cells with sulphasalazine, a system x_c^- inhibitor currently used to treat Crohn's disease patients[49], decreases cellular GSH synthesis and protects against symptoms[50]. Thus, decreased glial synthesis of the normally protective GSH may have therapeutic benefits in some pathogenic conditions.

The infiltration of immune cells is an important aspect of colitis pathology[1]. An alternate hypothesis for our findings is that the protective effects of GSH depletion are mediated via altered immune cell behavior. In some bacterial infections, decreased

neutrophil GSH content is associated with reduced neutrophil cytokine production and decrease migration[51]. This aligns with our own finding showing decreased neutrophil infiltration into the myenteric plexus of animals with depleted GSH prior to inflammation.

In conclusion, our findings show evidence for both enteric glial and neuronal production of GSH in the ENS. Further, we demonstrate that *in situ* GSH synthesis is necessary for neuroprotection and present a novel hypothesis where *in vivo* GSH depletion is protective against inflammation. These observations present a potential therapeutic target for improved GI pathology during inflammation.

APPENDIX

Antibody	Dilution	Source	Catalog No.
Chicken anti-GFAP	1:1000	Abcam, Cambridge, MA	ab4674
Biotinylated mouse anti-human HuC/D	1:200	Invitrogen	A21272
Rabbit anti-GCLC	1:200	Abcam	ab190685
Rabbit anti-GS	1:200	Abcam	ab133592
Rabbit anti-MPO	1:200	Abcam	ab9353
Rat anti-MHC II	1:200	Novus	NBP2-21789
Goat anti-Calretinin	1:1000	Swant, Switzerland	CG1
Sheep anti-nNOS	1:500	Millipore, Darmstadt, Germany	ab1529
Rabbit anti-synaptophysin	1:2000	Abcam	ab32127

Table 3.1: Primary Antibodies used in Chapter 3.

** GFAP, glial fibrillary acidic protein; GCLC, glutamate cysteine ligase catalytic subunit; GS, glutathione synthetase; MPO, myeloperoxidase; MHC II, major histocompatibility complex II; nNOS, neuronal nitric oxide synthase

Antibody	Source	Catalog No.
Streptavidin Dylight 405	Jackson	016-470-084
Goat anti-Chicken Alexa 488	Invitrogen	A-11039
Donkey anti-Goat Alexa 488	Jackson	705-545-003
Donkey anti-Sheep Alexa 488	Jackson	713-545-003
Goat anti-Rabbit Alexa 568	Invitrogen	A-11036
Streptavidin Alexa 594	Jackson	016-580-084

Table 3.2: Secondary Antibodies used in Chapter 3.

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CHAPTER 4

The effect of disrupted T cell TGFβ signaling on myenteric plexus structure and function

Abstract

Transforming growth factor beta (TGFβ) is a key anti-inflammatory cytokine that is important for the regulation of immune homeostasis in a variety of tissues. Mice with disrupted TGFβ signaling in $CD4^+$ and $CD8^+$ T cells (CD4-dnTGFβRII) develop severe mucosal inflammation in the gastrointestinal (GI) tract. However, the alterations within the myenteric plexus, which controls GI motility, are less understood. We used immunohistochemistry and *in vivo* colonic motility assays to measure changes in female CD4-dnTGFβRII mice. Disruption of T cell TGFβ signaling delayed colonic motility and induced “reactive gliosis” in enteric glia, but did not alter myenteric plexus neuronal density or neurochemical coding. Innate, but not adaptive, immune homeostasis was altered in the myenteric plexus of CD4-dnTGFβRII mice. In a novel result, we find that enteric neurons express the type II TGFβ receptor. Together, these findings demonstrate that TGFβ signaling in $CD4^+$ and $CD8^+$ T cells regulates immune homeostasis and function within the myenteric plexus.

Introduction

Transforming growth factor beta (TGFβ) is a critical regulatory cytokine that maintains immune homeostasis in a majority of tissues. TGFβ signaling requires activation of the TFGβ receptor (TGFβR), a tetramer consisting of homodimers of the Type I (TGFβRI) and type II (TGFβRII) receptor subtypes. Binding of TGFβ to TGFβRII catalyzes the formation of the receptor tetramer, which then functions as a serine/threonine kinase. TGFβ signaling ultimately results in the phosphorylation of Smad proteins that then translocate to the nucleus, altering gene transcription and resulting in the characteristic features associated with TGFβ signaling.

TGFβ consists of three isoforms (TGFβ1, 2, and 3) whose signaling is crucial for normal fetal development. TGFβ2 knockout mice are embryonic lethal while TGFβ1 null mice have a mixed phenotype where ~ 50% die by embryonic day 14[1,2]. The remaining 50% surviving mice develop multi-organ autoimmune syndrome[2]. A similar phenotype is also observed when TGFβ signaling is disrupted in CD4 and CD8 positive T cells by the expression of a dominant-negative form of TGFβRII (dnTGFβRII)[3]. Specifically, CD4-dnTGFβRII mice have prominent gastrointestinal (GI) inflammation characterized by immune cell infiltration and disruption of the normal architecture of intestinal crypts[3]. Here, we investigate how disrupted TGFβ signaling in CD4-dnTGFβRII mice affects GI motility and immune homeostasis at the level of the enteric nervous system – the intrinsic neural network that controls GI function.

Using female CD4-dnTGFβRII mice and their wild-type (WT) littermates, we show that disrupted T cell TGFβ signaling alters distal colon function without changes to myenteric plexus neuronal density or neurochemical coding. CD4-dnTGFβRII mice have

altered myenteric plexus immune homeostasis with increased infiltration/ activation of monocytes and macrophages, but no changes to B cell expression or immunoreactivity of the key pro-inflammatory cytokine IL-1β. Disrupted TGFβ signaling in T cells resulted in "reactive gliosis" of enteric glia, a hallmark phenotypical change observed in response to inflammation or cellular injury. Finally, we show that enteric neurons express the TGFβRII, the first demonstration of TGFβ receptor expression in the ENS. In conclusion, we demonstrate that disrupted TGFβ signaling in $CD4^+$ and $CD8^+$ T cells is sufficient to alter immune homeostasis and function within the myenteric plexus.

Materials and Methods

Animals

Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. Transgenic dominant negative transforming growth factor beta receptor type II mice (dnTGFβRII) expressing a truncated TGFβRII in $CD4^+$ and $CD8^+$ positive T cells were 1) generated in house by crossing C57Bl/6 x CD4-dnTGFBRII$^{+/-}$ or 2) purchased as hemizygotes from The Jackson Laboratory (Bar Harbor, ME) (B6.Cg-Tg(Cd4-TGFBR2)16Flv/J a.k.a. CD4-dnTGFBRII; Stock No. 005551). Wild-type (WT) littermates were used as experimental controls and all experiments used female mice aged 30-32 weeks. Mice were maintained in a temperature controlled 12 h light: 12 h dark cycle environment with *ad libitum* access to food and water.

Distal Colon Motility (Colon Bead) Assay

Mice were lightly anesthetized using isoflurane and a 2mm glass bead inserted 2cm into the distal colon. Mice were then individually housed and the time to bead expulsion recorded as a measure of distal colon transit time.

Whole-mount Immunohistochemistry (IHC)

Whole-mount longitudinal muscle myenteric plexus (LMMP) preparations were dissected from colon tissue fixed overnight in Zamboni's fixative. LMMP preps were permeabilized by 3x10 minute washes in 0.1% Triton X-100 in phosphate buffered saline (PBS) followed by a 45 minute block at room temperature (RT) in 4% normal serum containing 4% goat/donkey serum, 0.4% Triton X-100, and 1% bovine serum albumin. Preparations were incubated in primary antibodies (***Table 4.1, appendix***) overnight at RT, followed by a 2 hour incubation at RT in secondary antibodies (***Table 4.2, appendix***). Images were acquired using the 40X objective (Plan Fluor, 0.75 numerical aperture) or 20X objective (Plan Apo, 0.75 numerical aperture) of an upright epifluorescence microscope (Nikon Eclipse Ni, Melville, NY, USA) with a Retiga 2000R camera (QImaging, Surrey, BC, Canada) controlled by QCapture Pro 7.0 software (QImaging) or 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).

Scoring of Colonic Macroscopic Damage

Colonic macroscopic damage was quantified using a well established scoring system as described by Storr et al.[4]. Colon length and thickness were scored with greater points for a shortened and/or thickened colon. The severity of adhesion of the colon to other organs was scored as 0,1, or 2 based on the severity of adhesion and the presence of fecal blood, diarrhea and/or hemorrhage scored at 1 point/feature.

Data and Statistical Analysis

Ganglionic neuronal density was calculated by quantifying the number of HuC/D-immunoreactive neurons per GFAP-positive myenteric ganglia in at least 10 ganglia/ animal using the cell counter tool in ImageJ. Average ganglionic fluorescence was calculated using the measure tool in ImageJ and expressed in arbitrary fluorescence units (AFU). Subpopulations of calretinin-positive (excitatory) and nNOS-positive (inhibitory) neurons were calculated by quantifying the number of calretinin/ nNOS positive neurons as a percentage of total HuC/D myenteric neuronal population.
All data was analyzed by a student's t-test using Graphpad Prism 7 software (La Jolla, CA, USA). All data are presented as a mean ± the standard error of the mean (SEM).

Results

Mice with disrupted T cell TGFβRII signaling have increased distal colon transit but no overt markers of colonic inflammation

Transforming growth factor beta (TGFβ) and its downstream signaling pathways have long been recognized for their role in regulating a variety of cellular behaviors including

cell proliferation and differentiation[5,6]. In addition, TGFβ signaling is necessary for the proper maintenance of immune homeostasis[7] and animals with disrupted TGFβ signaling develop diseases with a key autoimmune component[8-10]. Here, we investigate how disrupted TGFβ signaling in T cells affects immune homeostasis and inflammation within the gastrointestinal (GI) tract and specifically, within the enteric nervous system (ENS) - the neural network that controls GI function.

To this end, we used CD4-dnTGFβRII mice (see Methods) that express a truncated dominant-negative form of the human TGFβ type II receptor (dnTGFβRII) in $CD4^+$ and $CD8^+$ T-cells (***Figure 4.1***). This expression is sufficient to disrupt TGFβ signaling in only T cells, while maintaining normal TGFβ signaling in other immune cells such as B cells[3]. dnTGFβRII mice develop an inflammatory bowel disease (IBD)-like phenotype characterized by immune cell infiltration at the level of the mucosa, and disruption of normal intestinal crypt morphology[3]. However, how colonic motility and gross macroscopic damage were altered in these mice was not investigated.

We investigated changes in colonic motility in dnTGFβRII by measuring distal colon transit time using the colon bead assay, which records the time to expulsion of a glass bead from the distal colon. Transgenic dnTGFβRII mice had increased distal colon transport time (49 ± 9 seconds in WT vs. 154 ± 39 seconds in dnTGFβRII mice; ***Figure 4.2A***), suggesting that disruption of T cell TGFβ signaling impairs normal colonic function. Next, we measured changes in gross colonic macroscopic damage using a scoring system described by Storr et al.[4]. Disruption of TGFβ signaling in T cells did not significantly increase total colonic macroscopic damage (0.18 ± 0.005 in WT vs. 1.33 ± 0.55 in dnTGFβRII mice; ***Figure 4.2B***). Because of the anti-proliferative effects of TGFβ

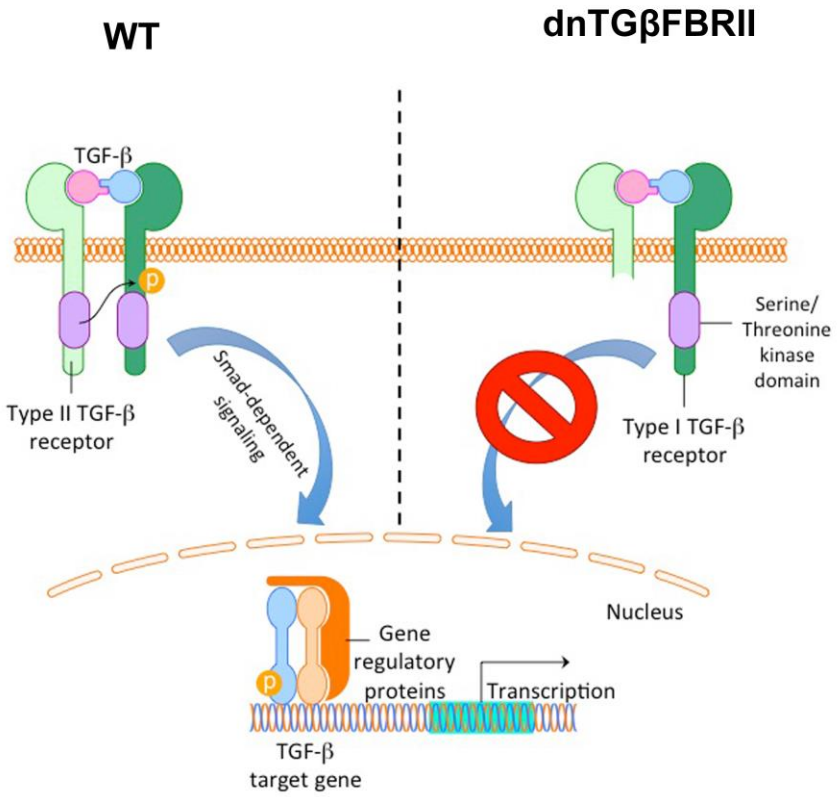

Figure 4.1: dnTGFβRII mice have disrupted TGFβ signaling in CD4 and CD8 positive T cells compared to wild-type (WT) mice. Schematic representing downstream signaling after activation of TGFβRI(I) receptors in wild-type (WT) and dominant negative transgenic mice (dnTGFβRII) which feature a truncated TGFβ type II receptor in $CD4^+$ and $CD8^+$ T cells.

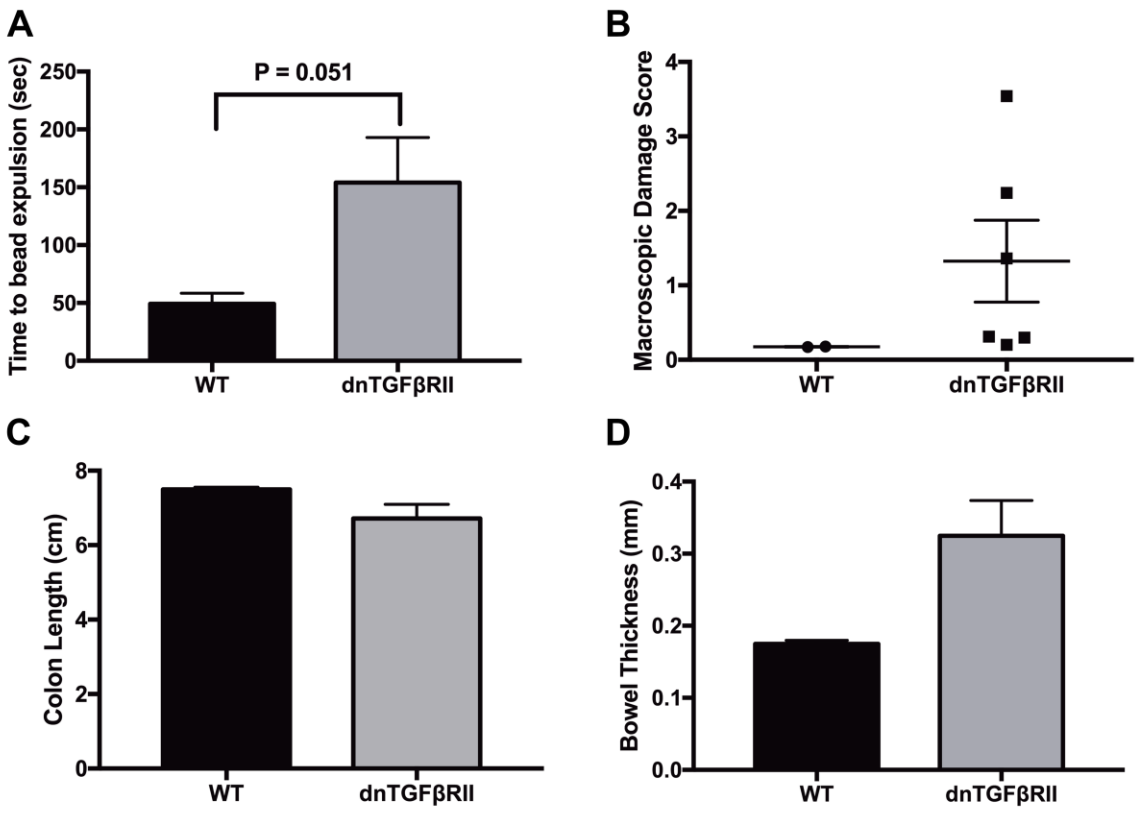

Figure 4.2: **dnTGFβRII mice have increased distal colon transport time but no significant changes in macroscopic measures of inflammatory damage.** (***A***) Distal colon transport time, (***B***) total macroscopic damage score, (***C***) total colon length and (***D***) distal colon thickness in wild-type (WT, *black bars*) and transgenic dnTGFβRII (*gray bars*) mice. (n = 2-6 mice per group, analyzed by student's t-test).

signaling[5,11,12], we hypothesized that disrupted T cell TGFβ signaling may alter macroscopic features that are dependent on changes in cell proliferation, such as colon length and thickness. Disrupted T cell TGFβ signaling did not significantly alter colon length (7.5 ± 0.07 cm in WT vs. 6.7 ± 0.4 cm in dnTGFβRII mice; ***Figure 4.2C***). Distal colon bowel thickness was doubled in dnTGFβRII mice doubled compared to WT mice (0.18 ± 0.005 mm in WT vs. 0.32 ± 0.05 mm in dnTGFβRII mice; ***Figure 4.2D***). However, this measure was variable and did not reach statistical significance. Together, these results show that disrupted T cell TGFβ signaling alters distal colon motility without driving gross macroscopic inflammation of the colon.

Expression of a dominant negative TGFβRII on $CD4^+$ and $CD8^+$ T cells does not alter myenteric plexus neuronal density or neurochemical coding

Colonic motility is controlled by the myenteric plexus of the enteric nervous system (ENS)[13]. Inflammation drives gut dysfunction, in part, by altering the survival, phenotype, and functions of enteric neurons[14,15]. Therefore, we hypothesized that changes in neuronal density and/or neurochemical composition contribute to altered motility in dnTGFβRII mice. We investigated this using immunohistochemistry to quantify the changes in myenteric plexus neuronal density and neurochemical coding caused by disrupted T cell TGFβ signaling. The packing density of HuC/D-immunoreactive myenteric neurons was not significantly different in dnTGFβRII mice (1809 ± 141 neurons mm^{-2} in WT vs. 1954 ± 49 neurons mm^{-2} in dnTGFβRII mice; ***Figure 4.3A'-A'''***). Similarly, disruption of T cell TGFβ signaling in our dnTGFβRII mice did not significantly alter neurochemical coding in myenteric neurons (***Figure 4.3B'-***

C'''). Myenteric ganglia in WT and dnTGFβRII mice contained similar percentages of calretinin-positive excitatory neurons (36 ± 8 % in WT vs. 37 ± 2 % in dnTGFβRII mice; ***Figure 4.3B'-B'''***) and nNOS-positive inhibitory neurons (52 ± 4 % in WT vs. 46 ± 2 % in dnTGFβRII mice; ***Figure 4.3C'-C'''***). These results show that disrupted T cell TGFβ signaling causes motility dysfunction without significantly altering myenteric neuronal survival or neurochemical composition.

Disrupted TGFβRII signaling induces reactive gliosis in the myenteric plexus enteric glia

Next, we investigated how disrupted T cell TGFβ signaling affects other aspects of ganglionic structure and function in the myenteric plexus. In addition to enteric neurons, myenteric ganglia also contain enteric glial cells, a unique class of peripheral glia that are being increasingly recognized for their active roles in regulating neuronal survival and function, and GI motility[16,17]. Like their central nervous system counterparts, peripheral glia undergo cellular and morphological changes, a process known as "reactive gliosis", in response to environmental changes and stressors[18]. We used immunohistochemistry to measure whether enteric glia became "reactive" in response to disrupted TGFβ signaling in T cells. Disrupted TGFβ signaling in dnTGFβRII mice increased ganglionic expression of the glial cell marker glial fibrillary acidic protein (GFAP) in enteric glia (Average fluorescence: 663 ± 55 AFU in WT vs. 918 ± 61 in dnTGFβRII; ***Figure 4.4A'-A'''***). Increased GFAP fluorescence is a hallmark of reactive gliosis in the central and peripheral nervous systems[19,20] and thus, these results highlight a relationship between T cell TGFβ signaling and glial reactivity state.

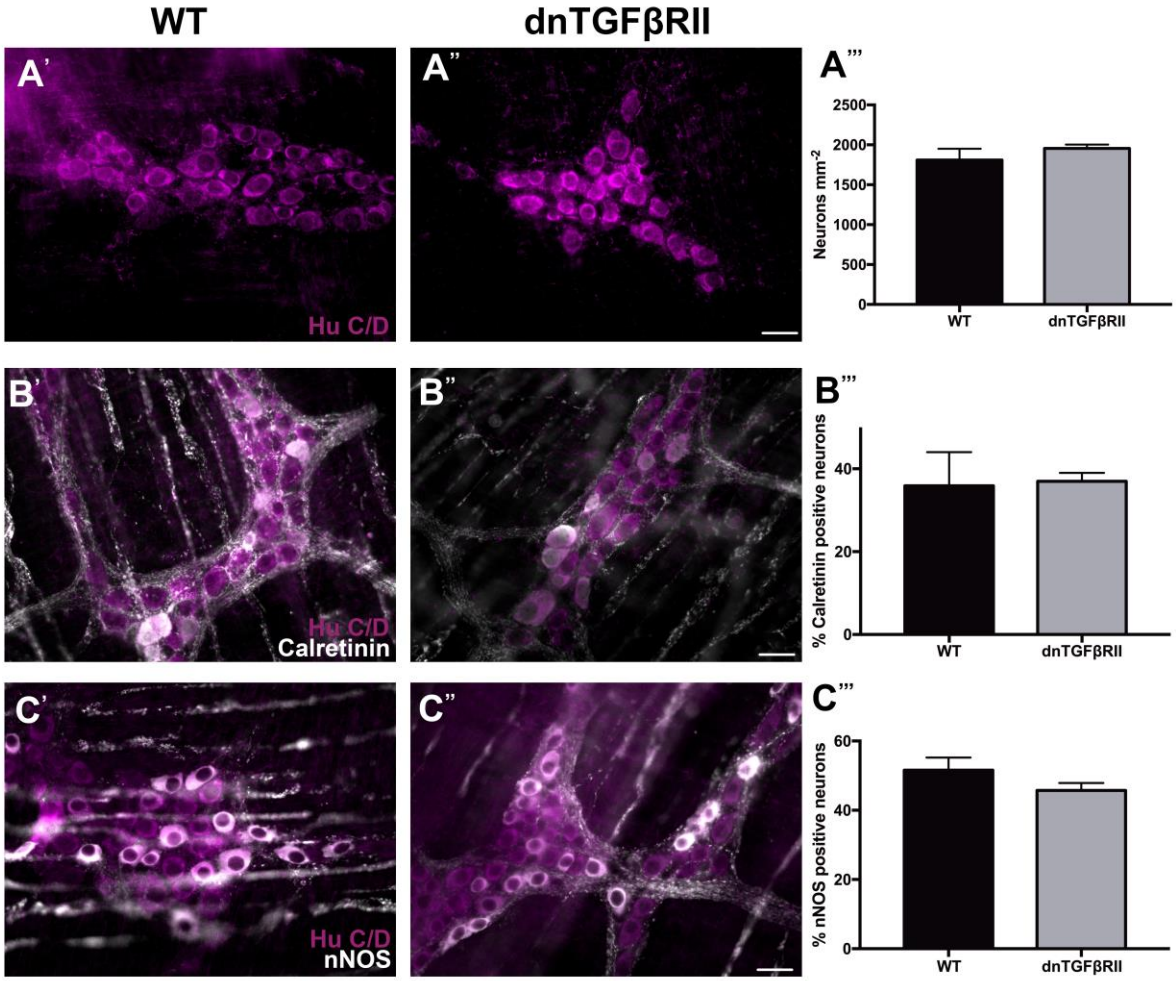

Figure 4.3: Disrupted TGFβRII signaling does not alter myenteric neuronal density or neurochemical coding. (***A'-A'''***) Representative images (***A'-A''***) and mean packing density (***A'''***) of Hu C/D-immunoreactive neurons (*magenta*) in myenteric ganglia in WT (*black bars*) and dnTGFβRII (*gray bars*) mice. (***B'-C'''***) Representative images (***B'-B''*** and ***C'-C''***) and quantification (***B'''*** and ***C'''***) of calretinin-positive excitatory neurons (B'-B'', *gray*) and nNOS-positive inhibitory neurons (***C'-C''***, *gray*) in myenteric ganglia. Total neuronal populations are labeled with the pan-neuronal marker Hu C/D (*magenta*). (n=10 ganglia in 5-9 animals/group, analyzed by student's t-test, scale bar = 30 μm).

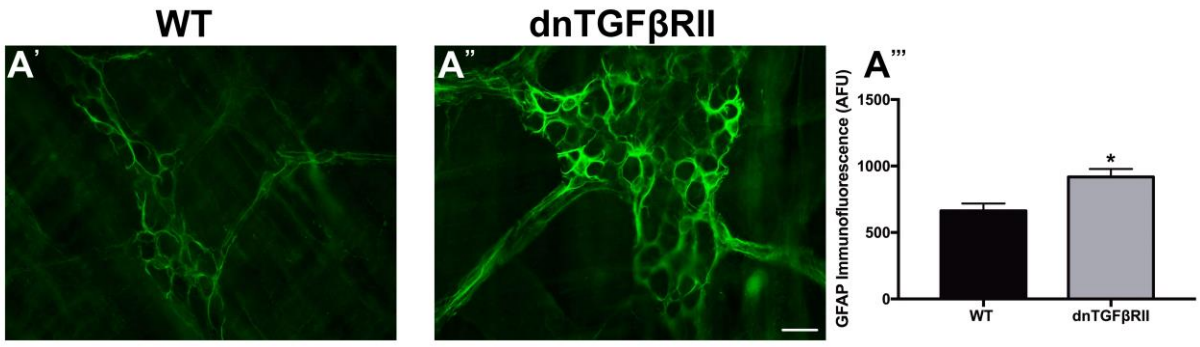

Figure 4.4: Disrupted TGFβRII signaling induces reactive gliosis in the myenteric plexus. Representative images (***A' and A''***) and quantification (***A'''***) of ganglionic glial fibrillary acidic protein (GFAP; *green*) fluorescence in WT (*black bar*) and dnTGFβRII (*gray bar*) mice. (n=3 animal/group, *P<0.05 by student's t-test, scale bar = 30 μm).

dnTGFβRII mice have increased activation of MHC-II positive muscularis macrophages but no change in ganglionic IL-1β expression

T cells play an important role in maintaining immune homeostasis as the appropriate activation and differentiation of T cells is a key step in our body's immune response[21,22]. Specifically, T cell mediated cytokine release is instrumental in determining the type and extent of immune response that is initiated after detection of an antigen. Thus, we next investigated whether disruption of T cell TGFβ signaling affected the expression and activation of immune cells in the ENS and the cytokine expression profile within the myenteric plexus.

We measured changes in the immunoreactivity of major histocompatibility complex class II (MHC-II)-expressing antigen presenting cells in a total of ten regions of interest (area = 307,200 μm^2) surrounding 2-3 GFAP-positive myenteric ganglia per mouse. This population of cells includes resident muscularis macrophages that inhabit the muscle layer adjacent to the myenteric plexus[23], but may also include infiltrating macrophages from outside the plexus[24]. Macrophages at the level of the myenteric plexus of transgenic dnTGFβRII mice had increased MHC-II immunoreactivity (Average fluorescence: 80 ± 28 AFU in WT vs. 375 ± 86 AFU in dnTGFβRII mice; ***Figure 4.5A'-A'''***). However, it is unknown whether this increased expression is due to increased infiltration of macrophages from outside the plexus or increased activation of resident muscularis macrophages.

TGFβ plays a key role in regulating the production of key pro- and anti-inflammatory cytokines[7]. Thus, we next investigated how disrupted T cell TGFβ signaling affects myenteric plexus expression of the pro-inflammatory cytokine

interleukin 1β (IL-1β). WT mice with normal TGFβ signaling express IL-1β primarily in enteric neurons (***Figure 4.5B'***). Disrupted T cell TGFβ signaling in transgenic dnTGFβRII mice did not significantly alter ganglionic IL-1β expression levels or localization (Average fluorescence: 228 ± 40 AFU in WT vs. 206 ± 53 AFU in dnTGFβRII mice; ***Figure 4.5B'-B'''***).

We also used immunohistochemistry to investigate changes in the infiltration of myeloperoxidase (MPO)-positive neutrophils in dnTGFβRII mice. However, neither WT nor dnTGFβRII mice were immunoreactive for MPO within the myenteric plexus (data not shown), suggesting that disrupted TGFβ does not significantly increase neutrophil infiltration, a hallmark of colonic inflammation[14]. Together, these results show that disrupted T cell TGFβ signaling increased myenteric plexus MHC-II expression but does not alter the expression or localization of IL-1β or the infiltration of MPO-positive neutrophils.

Mice with disrupted TGFβRII have dysregulated innate and adaptive immune responses at the level of the myenteric plexus

Cluster of different (CD) markers are commonly used to identify particular subclasses of immune cells. We used immunoreactivity for two such markers to investigate how disrupted TGFβ signaling altered the infiltration of immune cell subtypes into the myenteric plexus. We first measured changes in CD43 expression in a total of ten regions of interest/mouse (area = 307,200 μm^2), each containing 2-3 GFAP-positive myenteric ganglia. CD43, the common lymphocyte antigen, is a surface protein expressed on multiple immune cells and its expression is commonly used to identify

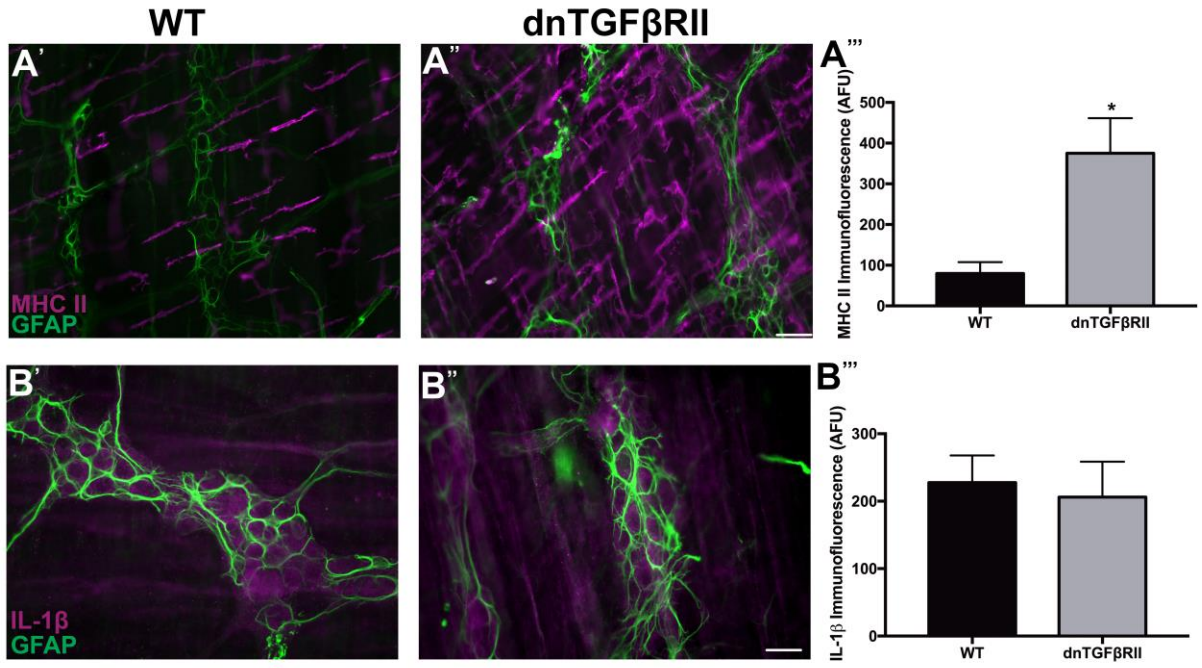

Figure 4.5: dnTGFβRII mice have increased activation of MHC-II positive muscularis macrophages but no changes in IL-1β immunoreactivity.

(***A'-A''***) Expression and (***A'''***) quantification of major histocompatibility complex class II (MHC-II) fluorescence (*magenta*) in a 307,200 μm^2 field of view surrounding myenteric ganglia labeled with GFAP (*green*) in WT (***A'***, *black bar*) and dnTGFβRII (***A''***; *gray bar)* mice. (***B'-B'''***) Ganglionic IL-1β expression (***B'-B''***; *magenta*) and quantification (***B'''***) in WT (*black bar)* and transgenic (*gray bar*) mice. (n=3-9 animals/group, *P<0.05 by student's t-test). (scale bar = 60 μm for ***A'-A''*** and 30 μm for ***B'-B''***).

populations of T and B lymphocytes[25]. WT mice had negligible CD43 expression in the myenteric plexus (***Figure 4.6A'***). In dnTGFβRII mice, mean CD43 immunoreactivity increased by almost 500% (Average fluorescence: 6 ± 3.4 AFU in WT vs. 29 ± 10 in dnTGFβRII mice; p=0.2086; ***Figure 4.6A'-A'''***). However, this increase did not achieve statistical significance (p=0.2086), likely due to the wide variability of expression levels measured in individual animals (***Figure 4.6A'''***).

Secondly, we investigated how disrupted TGFβ altered the expression and/or infiltration of CD68-positive monocytes and macrophages into the myenteric plexus. WT mice with normal TGFβ signaling had a small population of CD68-positive monocytes/macrophages surrounding GFAP-positive enteric glia (***Figure 4.6B'***). Disruption of T cell TGFβ signaling increased myenteric plexus CD68 immunoreactivity, indicative of increased infiltration of CD68-positive macrophages and/or increased activation of resident CD68-positive macrophages (Average fluorescence: 131 ± 16 AFU in WT vs. 213 ± 21 in in dnTGFβRII mice; ***Figure 4.6B'-B'''***). Further, in WT mice CD68 expression was restricted to the extraganglionic space while dnTGFβRII mice had CD68 positive cells both surrounding and within enteric ganglia. Importantly, this increase in CD68 immunoreactivity supports our earlier result of increased macrophage infiltration/activation as measured by immunoreactivity of MHC-II positive macrophages (***Figure 4.5A'''***). Taken together, our study of CD markers in the myenteric plexus suggests that disrupted TGFβ signaling causes a variable adaptive immune response, as measured by CD43 expression, but consistent innate immune activation in this model.

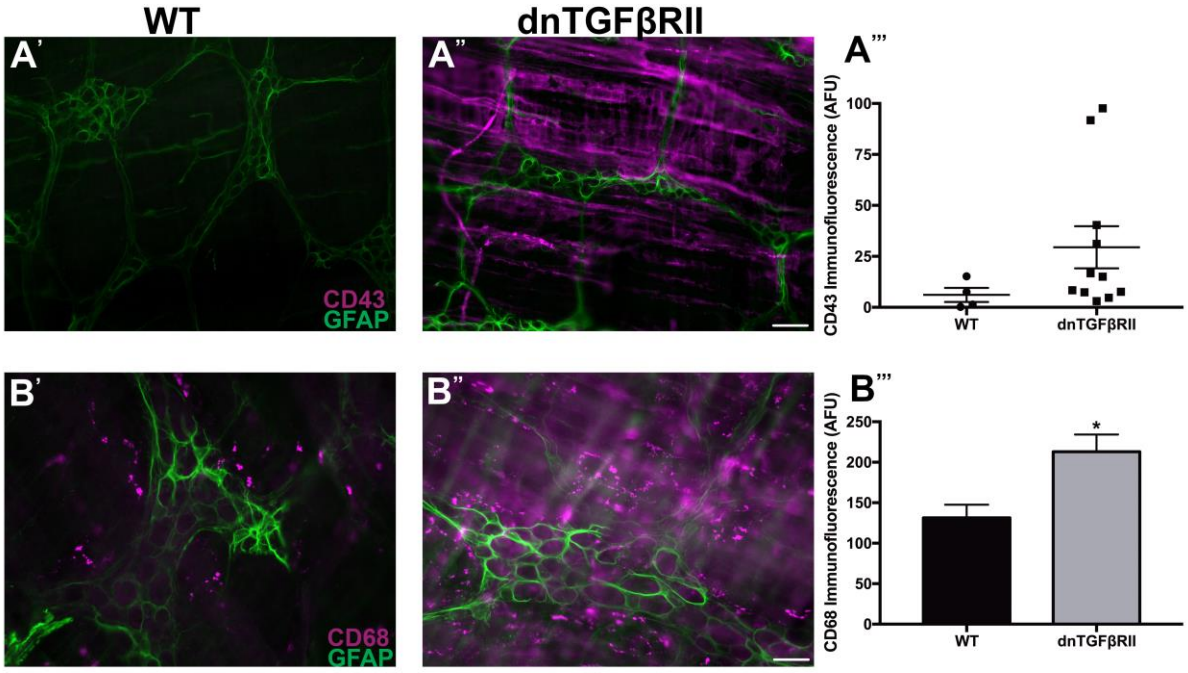

Figure 4.6: Mice with disrupted TGFβRII have dysregulated innate and adaptive immune responses at the level of the myenteric plexus.

(***A'-A'''***) Representative images (***A'-A''***) and quantification (***A'''***) of immune cell CD43 immunoreactivity (*magenta*) in a 307,200 μm^2 area surrounding GFAP-positive myenteric ganglia (*green*) in WT (*black bar*) and dnTGFβRII (*gray bar*) mice. (***B'-B'''***) Immunofluorescence (***B'-B''***) and quantification (***B'''***) of CD68-positive macrophages (*magenta*) surrounding a single GFAP-positive myenteric ganglion (*green*) in WT (*black bar*) and dnTGFβRII (*gray bar*) mice. (n=4-11 animals/group, *P<0.05 by student's t-test). (scale bar = 60 μm for A'-A'' and 30 μm for B'-B'').

The transforming growth factor beta receptor type II (TGFβRII) is expressed in enteric neurons, but not enteric glial cells, in mouse distal colon myenteric plexus

TGFβ is a wide reaching cytokine and it is hypothesized that almost every cell type expresses a subtype of the TGFβ receptor, and is sensitive to activation by one of three isoforms of TGFβ. To this end, we investigated whether the TGFβ type II receptor (TGFβRII) was expressed within the neural components of the ENS. We performed immunohistochemistry using antibodies against the TGFβRII (*magenta*), enteric neurons (HuC/D, *blue*) and enteric glial cells (GFAP, *green*). We found that TGFβRII is expressed in the myenteric plexus of mice (***Figure 4.7***) and that receptor localization is restricted to the cytoplasm and extracellular membrane of enteric neurons, as receptor expression patterns co-localized well with the pan-neuronal marker HuC/D but not with the glial marker GFAP (***Figure 4.7***). This finding supports the idea of indiscriminate expression of the TGFβ receptor and presents the possibility for para-cellular TGFβ-dependent signaling between immune cells and the neural components of the enteric nervous system.

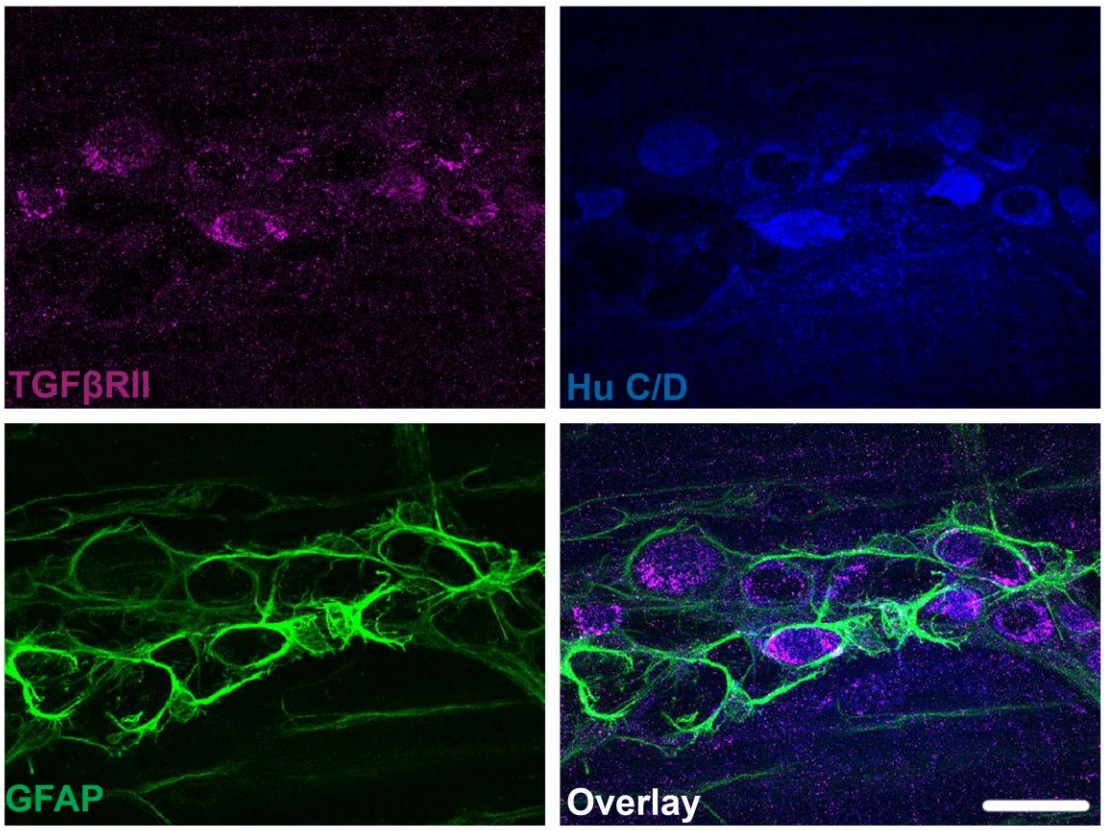

Figure 4.7: TGFβRII is expressed in murine myenteric neurons.

(***A-D***) A representative mouse myenteric ganglion showing immunoreactivity for TGFβRII (*magenta*), Hu C/D (enteric neurons; *blue*) and GFAP (enteric glia; *green*). The overlay image shows that TGFβRII is expressed in enteric neurons, but not enteric glial cells. (scale bar = 40 µm).

Discussion

Transforming growth factor beta (TGFβ) is an important signaling molecule with a wide range of physiological functions. In early studies, TGFβ was primarily recognized for its ability to maintain tissue homeostasis through the regulation of cell proliferation and death[12]. In addition, TGFβ is also well recognized for its role in regulating immune homeostasis[7,26,27]. In fact, disrupted TGFβ signaling produces diseases that feature a key autoimmune component in many organ systems [3,8,28]. In this manuscript, we investigated how disruption of TGFβ signaling in $CD4^+$ and $CD8^+$ T cells affects immune homeostasis and inflammation in the gut using CD4-dnTGFβRII mice, which express a dominant negative form of the human type II TGFβ receptor driven by a CD4 promoter[3].

dnTGFβRII mice with disrupted TGFβ signaling have increased distal colon transit time (a measure of motility function) without changes in macroscopic colonic damage or the density or neurochemical phenotype of myenteric neurons. This result was unexpected as changes in motility are typically accompanied by increased macroscopic colon damage and decreased neuronal density[15,17]. GI motility is controlled by a neuromuscular circuit where enteric neuronal signaling ultimately synapses to a smooth muscle cell, causing tissue contraction or relaxation. One possibility is that disrupted T cell TGFβ signaling acts downstream in the motility pathway, acting directly on smooth muscle cells or disrupting the culminating neuromuscular communication. Additionally, it is possible that neuronal function, rather than survival or neurochemical coding, are altered in these mice. In fact, Linden et al. show that the electrophysiological properties of surviving neurons are altered following colitis, in the absence of persistent macroscopic damage[29]. These hypotheses may explain the GI motility dysfunction

present in these mice in the absence of macroscopic changes to the intestine or enteric neurons.

TGFβ is classically recognized for its role as an anti-proliferative signaling molecule[30] and as a “master regulator” of tissue fibrosis. Increased TGFβ signaling is associated with increased deposition of extracellular matrix proteins, and cellular and tissue injury are associated with increased TGFβ mRNA and protein expression[31]. Intestinal fibrosis is a serious and common complication associated with GI inflammation and is a primary cause for bowel resections in Crohn’s disease patients[32,33]. dnTGFβRII mice did not present with significant colon shortening or thickening, indicating a lack of significant tissue fibrosis or cell proliferation. This suggests that disrupted T cell TGFβ signaling may be sufficient to ameliorate the pro-fibrotic effects of TGFβ and that the shift to a proinflammatory T cell phenotype in dnTGFβRII mice might be insufficient to drive fibrosis and smooth muscle proliferation.

$CD4^+$ T cells (T helper cells) play an important role in the regulation of our host immune response. The interaction of $CD4^+$ T cells with MHCII-expressing antigen presenting cells activates the adaptive immune response and the resulting cytokine release directly controls the type (T_h1 vs. T_h2 vs. T_h17) of response generated[34]. In our study, we investigated how disrupted T cell TGFβ signaling altered the infiltration and activation of immune cells and the expression of key regulatory cytokines within the myenteric plexus of the ENS. Aberrant T cell TGFβ signaling increased the expression of major histocompatibility complex class II (MHC II) at the level of the myenteric plexus. dnTGFβRII mice also had increased intraganglionic immunoreactivity for CD68-positive monocyte and macrophages. These combined results suggest that disrupted T cell

TGFβ signaling increases the activation and/or infiltration of macrophages into/at the level of myenteric plexus. Unfortunately, the hallmark immunological markers used do not allow us to determine if these changes are due to increased activation of resident macrophages[23] or increased infiltration of activated macrophages from outside of the plexus.

Our findings are consistent with previous work showing a relationship between TGFβ signaling and MHC-II expression in multiple cell types. Disruption of TGFβ signaling in TGFβ1 null mice increased tissue MHC II expression[35], while treatment with TGFβ suppresses MHC II expression *in vitro*[36,37]. In further support, a MHC-II knockout background was sufficient to rescue TGFβ-1 mice from their the characteristic autoimmune features[38]. In light of these studies, increased MHC II expression in our dnTGFβRII mice was not surprising and the validity of these results is further supported by increased immunoreactivity of CD68, another marker for macrophages.

The anti-inflammatory effects of TGFβ are thought to be due in part, to their ability to regulate the production of pro and anti-inflammatory cytokines[7,27] such as interleukin 1-beta (IL-1β). It is thought that the autoimmune features of disrupted TGFβ signaling are due to disturbance of the sensitive immune balance by increased production of pro-inflammatory cytokines and/or decreased production of anti-inflammatory cytokines. In contrast, we observed that both WT and dnTGFβRII mice had similar expression levels and localization of the pro-inflammatory cytokine IL-1β within myenteric ganglia. One explanation of these finding is that our experiments, which disrupt T cell TGFβ signaling and shift T cells to a proinflammatory state, are not suitable for directly assessing the effects of TGFβ on cytokine production in the ENS. In addition, studies investigating the

specific relationship between TGFβ and IL-1β show that while TGFβ does alters IL-1β dependent effects, it is through the production of an IL-1R antagonist[39] or down regulation of the IL-1 receptor[40], rather than directly altering IL-1β levels. A worthy future study would thus be to investigate the expression levels of cytokine receptors, as well as investigating a broader panel of pro- and anti-inflammatory cytokines.

Infiltration of myeloperoxidase (MPO)-positive neutrophils into the myenteric plexus is a hallmark of GI inflammation[14] and represents the response of the innate immune system to GI pathogens. dnTGFβRII mice did not show any changes in myenteric plexus MPO expression and MPO-positive cells were undetectable in both WT and dnTGFβRII mice via immunohistochemistry. Typically, the infiltration of macrophages is associated with acute phase of inflammation and dissipates within 24 hours in chemical models of colitis [14]. Given the considerable age of our dnTGFβRII mice, it is likely that any infiltrating neutrophils would no longer be present. Alternately, is it also possible that this model of inflammation is simply not associated with the influx of neutrophils into the myenteric plexus.

Reactive gliosis in the central nervous system is well associated with increased cellular injury and inflammation. A hallmark molecular feature of said “reactivity” is increased expression of the glial cell marker glial fibrillary acidic protein (GFAP)[41]. Disrupting T cell TGFβ signaling in our dnTGFβRII mice transformed enteric glia to a reactive phenotype as evidenced by increased glial branching and increased GFAP expression (***Figure 4.4***). This demonstrates that disrupted TGFβ signaling in T cells can impact ENS structure, and likely function. In fact, TGFβ is known to potentiate astrocyte production of nitric oxide[42], a signaling molecule that we have previously shown to be

important for regulating enteric neuron survival and thus gut function[17]. One hypothesis is that this increased glial reactivity modulates glial communication and control of GI function[43], and may explain the observed changes in GI motility in the absence of accompanying neuron death or changes in myenteric neurochemical coding.

Previous studies have primarily focused on understanding TGFβ signaling in immune cells, due to its significant roles in maintaining immune homeostasis. However, it is hypothesized that almost every cell type expresses some form of the TGFβ receptor due to its other roles in regulating cell proliferation and apoptosis. We found that the type II TGFβR is expressed in the myenteric plexus of the mouse and that antibodies against TGFβRII co-localize well with the pan-neuronal marker HuC/D. One potential area of future study would be to investigate the role TGFβ signaling in neural crest derived cells (enteric neurons and glia), through the production of nestin-dnTGFβRII mice. As our immunohistochemistry shows that TGFβRII expression is restricted to enteric neurons, these transgenic mice would allow us to specifically investigate the role of TGFβ signaling in enteric neurons.

In conclusion, we show that disruption of TGFβ signaling in $CD4^+$ and $CD8^+$ T cells disrupts distal colon motility without accompanying changes in macroscopic colonic damage or enteric neuronal density and neurochemical composition. On the microscopic level, dnTGFβRII mice have increased infiltration and activation of MHC-II and CD68 positive macrophages but not CD43 positive T and B lymphocytes or MPO-positive neutrophils. We show that altered TGFβ signaling in $CD4^+$ and $CD8^+$ T cells in dnTGFβRII mice disturbs GI immune homeostasis and alters normal GI motility function.

APPENDIX

Antibody	Dilution	Source	Product No.
Chicken anti-GFAP	1:1000	Abcam	ab4674
Goat anti-calretinin	1:200	Millipore	AB1550
Mouse anti-HuC/D	1:200	Invitrogen	A21272
Rabbit anti-IL-1β	1:200	Abcam	ab9722
Rabbit anti-MPO	1:200	Abcam	ab9353
Rabbit anti-TGFβRII	1:500	Abcam	ab61213
Rat anti-CD43	1:200	AbD Serotec	MCA4702GA
Rat anti-CD68	1:200	AbD Serotec	MCA1957
Rat anti-MHC II	1:200	Novus	NBP2-21789
Sheep anti-nNOS	1:500	BD Labs	610332

Table 4.1: Primary Antibodies used in Chapter 4.

** GFAP = glial fibrillary acidic protein, IL-1β = interleukin 1 beta, MPO = myeloperoxidase, TGFβRII = transforming growth factor beta receptor type II, CD43 = cluster of differentiation 43, CD68 = cluster of differentiation 68, MHCII = major histocompatibility complex class II, nNOS = neuronal nitric oxide synthase

Antibody	Dilution	Source	Product No.
Streptavidin Dylight 405	1:400	Jackson	016-470-084
Donkey anti-Goat Alexa 488	1:400	Jackson	705-545-003
Donkey anti-sheep Alexa 488	1:400	Jackson	713-545-003
Goat anti-chicken Alexa 488	1:400	Invitrogen	A-11039
Goat anti-rabbit Alexa 488	1:400	Invitrogen	A-11034
Goat anti-chicken Alexa 568	1:400	Invitrogen	A-11041
Goat anti-rabbit Alexa 568	1:400	Invitrogen	A-11036
Goat anti-rat Alexa 594	1:400	Jackson	112-585-003
Streptavidin Alexa 594	1:400	Jackson	016-580-084

Table 4.2: Secondary Antibodies used in Chapter 4.

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CHAPTER 5

The effects of pro-inflammatory mediators on glial hemichannel activity

Abstract

Connexin-43 hemichannels are expressed on enteric glia and are important for glial communication and regulation of gastrointestinal (GI) function. Increased channel opening is also associated with pathogenic glial activity in GI inflammation. Yet, how pro-inflammatory mediators affect (basal or activated) Cx43 channel opening is unknown. We used uptake of the dye ethidium bromide (EtBr) to measure channel activity and found that the pro-inflammatory mediators IL-1β, IL-17, IFN-γ and NO increase glial dye uptake under basal conditions. IL-1β potentiated ADP-stimulated dye uptake in a Cx43-dependent manner. IL-17 and IFN-γ did not change EtBr uptake in activated glia and were insensitive to the effects of the Cx43 mimetic peptide. NO decreased dye uptake in activated glia. In summary, our results demonstrate the differential effects of pro-inflammatory mediators on basal or activated Cx43 hemichannel opening in enteric glia.

Introduction

Connexin-43 gap junctions and hemichannels are important for cell-to-cell signaling and communication and are a key conduit for transport between the intra and extracellular spaces in many types of central and peripheral glia[1,2]. In the enteric nervous system (ENS), Cx43 hemichannels are expressed by enteric glia and are necessary for the maintenance of normal glia-to-glia communication[3] and contribute to pathogenic glial activation and neuron death during inflammation[4]. Cx43 activity and expression on astrocytes can be modulated by a number of factors including reactive oxygen/nitrogen species, pro- and anti-inflammatory cytokines, growth factors and neurotransmitters[5-7]. However, it is unknown how pro-inflammatory cytokines and mediators affect the activity of Cx43 hemichannel on enteric glia and whether cytokines/mediators affect channel opening differently under basal or stimulated conditions.

Here, we tested the effects of key pro-inflammatory mediators on glial Cx43 hemichannel opening by measuring uptake of the cell impermeable intercalating agent ethidium bromide (EtBr) [8,9]. Our results show that the pro-inflammatory mediators IL-1β, IL-17, IFN-γ and NO all increase glial dye uptake under basal conditions, albeit to different extents. In the presence of the purinergic glial agonist ADP, which stimulates Cx43 opening [4], NO decreased dye uptake. IL-1β potentiated dye uptake in stimulated glia and this effect was blunted by the Cx43 mimetic peptide 43Gap26. IL-17 and IFN-γ did not alter dye uptake in activated glia and, surprisingly, diminished the inhibitory effects of the Cx43 mimetic peptide. Together, our findings demonstrate differential effects of pro-inflammatory cytokines and mediators on enteric glial Cx43 hemichannel opening under basal or activated conditions.

Materials and Methods

Animals

Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Michigan State University. All experiments used either 1) C57Bl/6 mice from Charles River (Wilmington, MA) or 2) wild-type (WT) mice on a C57Bl/6 background bred in house as genetic WT littermates for transgenic mouse lines. Mice used in experiments were male and aged 8-12 weeks. Mice were maintained in a temperature controlled 12 h light: 12 h dark cycle environment with *ad libitum* access to food and water.

Murine Model of Colitis

We induced colitis in mice using the 2,4-dinitrobenzene (DNBS) model of colitis, as previously described[4,10]. Mice were briefly anesthetized with isoflurane and an enema of DNBS (5.5mg/mouse in 0.1 mL of 50:50 ethanol/saline) was administered via a gavage need inserted 3 cm into the colon. Control mice received an enema of saline. Groups of mice were sacrificed at the peak of inflammation (48 hours after treatment) and after the resolution of inflammation (3 weeks after treatment). Colonic macroscopic damage score was assessed using the scoring matrix described by Storr et al.[11]. Colon tissue was collected and either 1) fixed overnight in Zamboni's fixative for analysis by immunohistochemistry or 2) immediately frozen on dry ice and stored at -80°C for multiplex protein analysis.

Whole-mount Immunohistochemistry (IHC) for Interleukin 1 beta (IL-1β)

Ganglionic expression of the pro-inflammatory cytokine IL-1β was detected by IHC in whole-mount longitudinal muscle myenteric plexus (LMMP) preparations that were dissected from colon tissue fixed overnight in Zamboni's fixative, as previously described[3,4,10]. Tissue preparations were permeabilized by three 10 minute washes in 0.1% Triton X-100 in phosphate buffered saline (PBS), followed by a 45 minute block in 4% normal goat serum at room temperature (RT). LMMP preps were incubated in rabbit anti-IL-1β *(*Abcam, ab9722, 1:200) overnight at RT and then in goat anti-rabbit Alexa 568 (Invitrogen, A-11036, 1:200) for 2 hours at RT. Images were acquired using the 40X objective (0.75 numerical aperture; Plan Fluor, Nikon, Melville, NY) of an upright epifluorescence microscope (Nikon Eclipse Ni) with a Retiga 2000R camera (QImaging, Surrey, BC, Canada) controlled by QCapture Pro 7.0 (QImaging) software and analyzed using ImageJ software (https://imagej.nih.gov/ij/)

Multiplex Protein Analysis

Tissue samples were prepared for multiplex analysis from distal colon samples that were frozen in dry ice immediately after collection and stored at -80°C until processing. Tissue samples were homogenized in homogenizing buffer using a tissue grinder and sonicator. Samples were then centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant was collected and transferred to a new tube and protein concentration measured. For this assay, sample concentrations were adjusted so that total protein/ sample was the same across all treatment groups. Samples from healthy and inflamed

mice were analyzed to detect changes in pro-inflammatory cytokines by Eve Technologies (Calgary, Alberta, Canada) using a Multiplex Assay.

Ethidium Bromide Dye Uptake Assay

Connexin-43 hemichannel activity in enteric glial cells was measured by ethidium bromide (EtBr) dye uptake as previously described by McClain et al. in enteric glia [9] and Orellana et al.[12] in cultured astrocytes . Live whole-mount LMMP preparations were dissected from the distal colon in $35mm^2$ sylgard-coated dishes and treated with an enzyme mix containing 1 U/mLDispase and 150 U/mL of Collagenase Type II for 15 minutes at RT. Tissue was titurated and repined in fresh DMEM. Preparations were then incubated in a two-step process. In the first step, preps were incubated for 30 minutes at 37°C in 5% CO_2/95% air in either Kreb's buffer only (Control) or pro-inflammatory cytokines and mediators (see ***Table 5.1, appendix***) in the presence or absence of the Cx43 mimetic peptide 43Gap26 (100 μM). After 30 minutes, the first incubation mixture was removed and preps incubated with EtBr (5 μM in Kreb's) in the presence or absence of the purinergic glial agonist ADP (100 μM) for 10 minutes at 37°C. Extracellular EtBr was removed by three washes in fresh Kreb's and EtBr fluorescence immediately recorded using an upright Olympus BX51WI fixed-stage microscope (Olympus, Center Valley, PA) fitted with a 40X water-immersion objective (LUMPlan N, 0.8 n.a.) and a Lambda DG-4 Plus xenon light source (Sutter, Novato, CA). Fluorescence of EtBr was detected using a TRITC filter set with a 535 nm, 20 nm band-pass excitation filter and a 610 nm, 75 nm band-pass emission filter. We acquired images of individual ganglia using Andor iQ3 software (Andor, Belfast, UK) and

identified ganglia based on morphology, based on our expertise and previous experiments with cell-permeable dyes[4].

Chemicals, Reagents and Solutions

All tissue was collected and dissected in DMEM/F12 media buffer (ThermoFisher, Carlsbad, CA) supplemented with 3uM Nicardipine and 1uM Scopolamine. Kreb's buffer contained (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) with 3uM Nicardipine and 1uM Scopolamine. 4% normal serum contains 4% goat serum, 0.4% Triton X-100, and 1% bovine serum albumin. 100 mL of homogenizing buffer contains: 90 mL of dI H_2O, 10 mL of 10x Tris-buffered Saline (TBS; 1X concentration = Tris/Tris-HCl 25mM, NaCl 130 mM, KCl 2.7 mM), 0.5 mL of Tween 20, one tablet of protease inhibitor cocktail (Sigma Aldrich, Cat No. S8820) and was pH adjusted to pH = 7.5. Adenosine 5'-diphosphate sodium salt (ADP) and Ethidium Bromide (15585011) were purchased from ThermoFisher and the Connexin-43 mimetic peptide 43Gap26 (Cat. No. AS-62644) from AnaSpec (Fremont, CA).

Data and Statistical Analysis

Images of individual ganglia were exported from Andor iQ3 software as tiff image files and analyzed using ImageJ software. Individual glial cells were highlighted using the region of interest (ROI) tool and fluorescence measured in a minimum of 10 glia/ ganglia. Mean fluorescence was then averaged per ganglion and n values represent the number of individual ganglia analyzed. EtBr measurements were made in an average of

15 ganglia/ animal in 3-6 animals per treatment group. Ganglionic immunoreactivity for IL-1β was calculated using the measure tool in ImageJ. Data were analyzed using by student's t-test, One-Way or Two-Way ANOVA using Graphpad 7 software (La Jolla, CA) and are presented as mean ± the standard error of the mean (SEM).

Results

Activation of glial P2Y1 receptors leads to connexin-43 (Cx43) hemichannel opening

Recent data show that the activation of enteric glial cells contributes to enteric neurodegeneration during inflammation[4]. Increased extracellular purines activate glial P2Y1 receptors during colitis[10], producing robust glial calcium responses and increasing connexin-43 (Cx43) hemichannel opening (***Figure 5.1***)[4]. Astroglial Cx43 activity is altered in the central nervous system (CNS) by a number of signaling molecules including cytokines, neurotransmitters and reactive oxygen/nitrogen compounds [5]. Ganglionic concentrations of a number these compounds are modified during colitis and other gastrointestinal (GI) diseases[13]. However, it is not known how these pro-inflammatory cytokines and mediators specifically affect enteric glial Cx43 activity. We used glial uptake of the fluorescent intercalating agent ethidium bromide (EtBr)[9,14] to investigate if/ how the activity of Cx43 on enteric glia is modulated by key pro-inflammatory cytokines and signaling molecules.

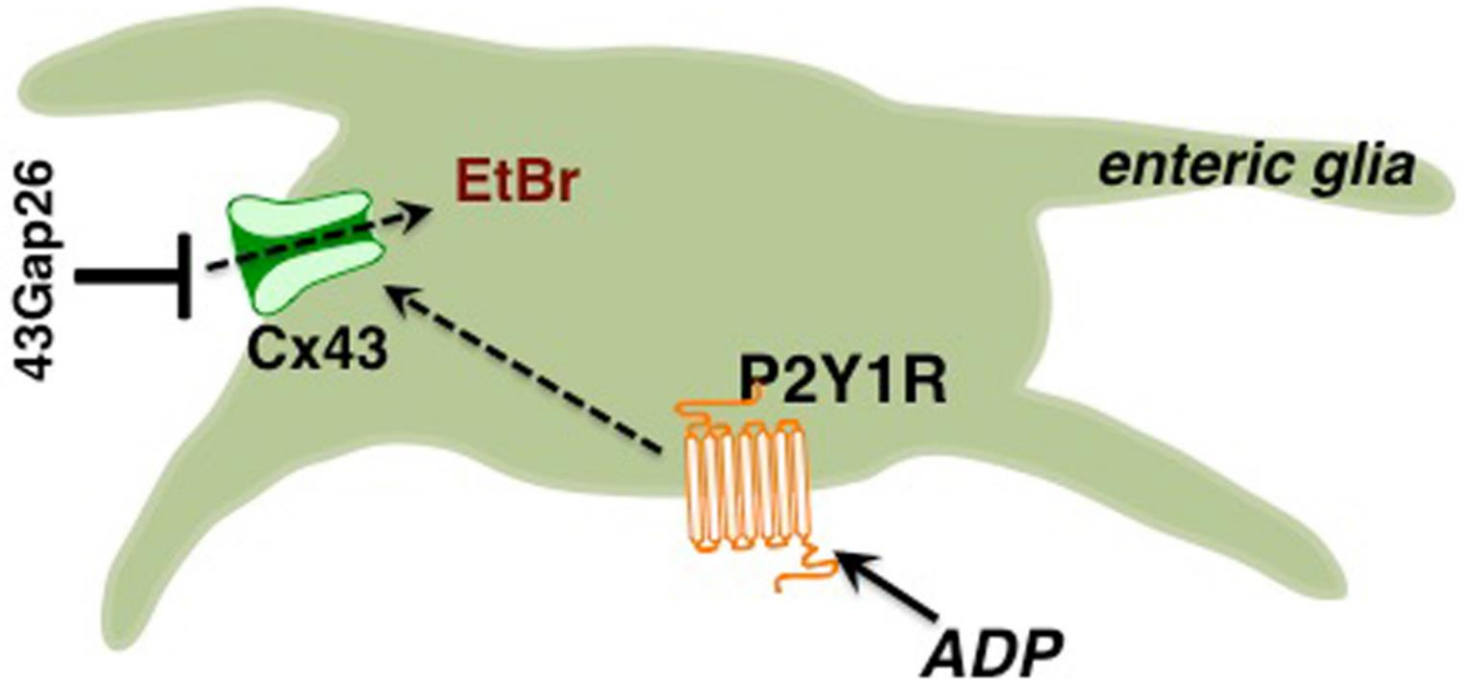

Figure 5.1: Purinergic glial activation leads to connexin-43 (Cx43) hemichannel opening. We measured Cx43 hemichannel opening by uptake of the intercalating agent ethidium bromide (EtBr) and blocked channel opening with the mimetic peptide 43Gap26.

Pro-inflammatory cytokine levels are altered in the distal colon during GI inflammation

Disruption of the balance between pro- and anti-inflammatory cytokines is a hallmark of inflammatory bowel disease (IBD) in both animal models and human disease[13,15,16]. We measured tissue concentrations of three (3) pro-inflammatory cytokines in the distal colon following the induction of DNBS colitis in mice. Protein levels of interferon-γ (IFN-γ) were negligible in healthy animals and were not significantly altered during colitis (0.0 pg/mL in Saline vs. 0.73 ± 0.43 pg/mL in DNBS animals; p=0.1054; ***Figure 5.2A***). Healthy animals had similar baseline colonic levels of the pro-inflammatory cytokines IL-1β and IL-17 (IL-1β = 1.0 ± 0.13 pg/mL, IL-17 = 1.0 ± 0.19 pg/mL; ***Figure 5.2B and 5.2C***), but IL-1β and IL-17 were differentially altered during DNBS-colitis. Inflammation decreased IL-17 protein content by 43% (1.0 ± 0.19 pg/mL in Saline vs. 0.57 ± 0.09 pg/mL in DNBS; ***Figure 5.2B***) and increased GI IL-1β protein content by over 400% (1.0 ± 0.13 pg/mL in Saline vs. 4.4 ± 0.93 pg/mL in DNBS; ***Figure 5.2C***).

We next used immunohistochemistry to investigate whether IL-1β expression within myenteric plexus ganglia mirrored changes to whole tissue protein levels. In the myenteric plexus, IL-1β signaling is primarily mediated through enteric glial cells, which secrete IL-1β [17] and express IL-1β receptors[18]. IL-1β expression is associated with GI inflammation[19,20] and we hypothesized that ganglionic, and specifically enteric glial, IL-1β content would be increased during inflammation. We induced colitis using DNBS and measured immunoreactivity for IL-1β in myenteric ganglia from healthy and inflamed animals at peak inflammation (48 hours) and following resolution (3 weeks). In support of our hypothesis, ganglionic IL-1β expression was doubled at the peak of inflammation

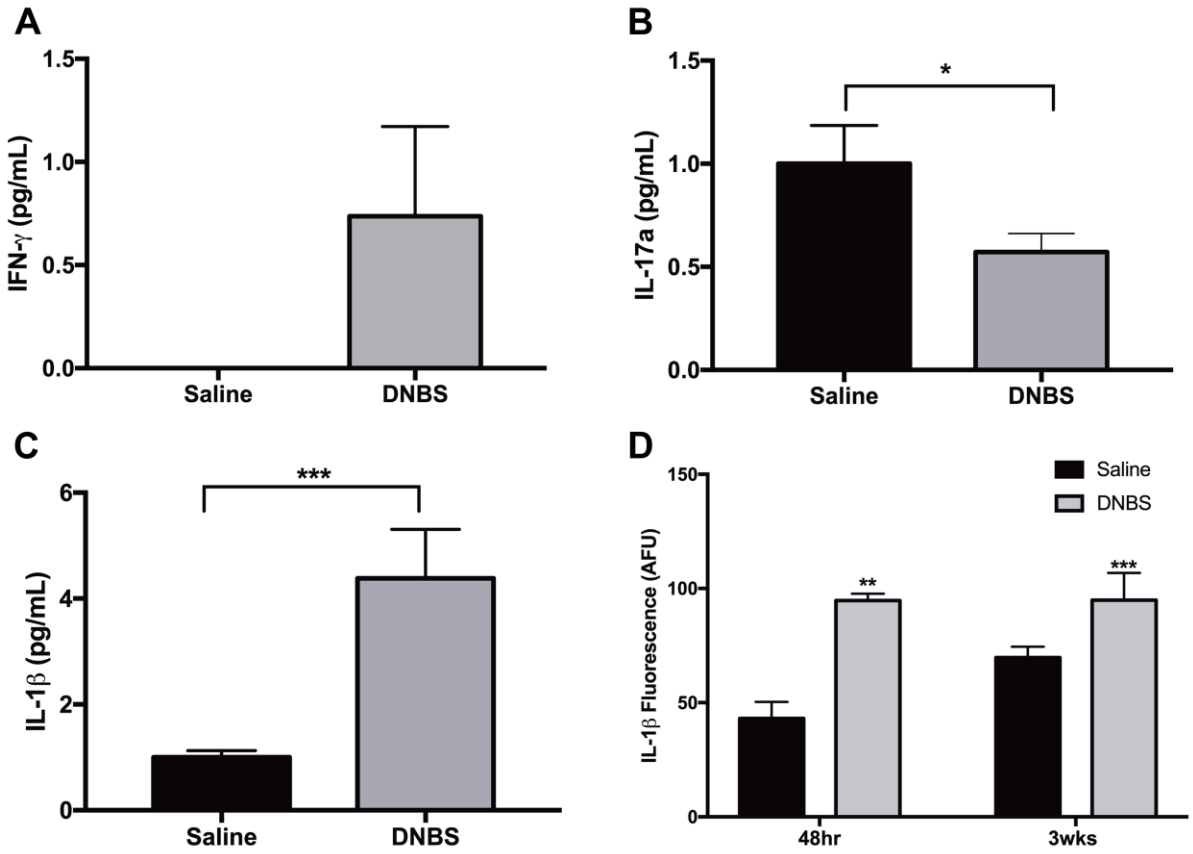

Figure 5.2: Pro-inflammatory cytokine levels are altered in the distal colon during GI inflammation. (***A-C***) Whole tissue protein levels of the pro-inflammatory cytokines (***A***) Interferon-γ (IFN-γ), (***B)*** Interleukin-17a (IL-17) and (***C***) Interleukin-1β (IL-1β) in healthy (Saline; *black bars*) and inflamed (DNBS; *gray bars*) animals. (*P<0.05, ***P<0.005 by Student's t-test, n= 11-12 animals/group). (***D***) Immunofluorescence of the pro-inflammatory cytokine IL-1β in murine myenteric plexus at the peak (48hr) and resolution (3wks) of inflammation (DNBS; *gray bars*) and in healthy controls (Saline; *black bars*). (**P<0.01, ***P<0.005 vs. 48hr-Saline by Two-Way ANOVA with Dunnett's multiple comparison test, n=3-5 animals/group).

(Arbitrary Fluorescence: 43 ± 7.2 in 48hr saline vs. 95 ± 3 in 48hr-DNBS; ***Figure 5.2D***) and remained elevated following resolution of colitis (Arbitrary Fluorescence: 95 ± 3 in 48hr-DNBS vs. 95 ± 12 in 3 week DNBS animals; ***Figure 5.2D***). Basal ganglionic expression of IL-1β was not significantly altered in saline-treated animals 3 weeks after saline enema (Arbitrary Fluorescence: 43 ± 7 in 48hr saline vs. 70 ± 5 in 3 week saline; ***Figure 5.2D***). Our results support the previously suggested role for IL-1β as a key pro-inflammatory cytokine in the pathogenesis of colitis[21-23].

Pro-inflammatory mediators increase glial connexin-43 (Cx43) hemichannel opening in the absence of purinergic glial stimulation

Cellular uptake of the intercalating agent ethidium bromide (EtBr) is a selective method to measure Cx43 hemichannel opening in astroglia[24-26]. Cx43-dependent EtBr uptake in enteric glia is increased following purinergic stimulation with the P2Y1 agonist ADP[9], which also increases Cx43-dependent ATP release [4]. EtBr uptake by enteric glia is minimal in the absence of ADP[9], demonstrating differential Cx43 activity in stimulated and unstimulated myenteric plexus preparations. Thus, we investigated how pro-inflammatory cytokines and mediators altered Cx43 activity in both the presence and absence of purinergic activators of enteric glia.

First, we tested the singular effects of pro-inflammatory cytokines and mediators on Cx43-mediated dye uptake in the absence of purinergic stimulation. Cx43 hemichannel opening in unstimulated glia was increased by incubation with the pro-inflammatory cytokines IL-1β, IL-17 and IFN-γ and the enteric signaling molecule and inflammatory mediator nitric oxide (NO)[4,27] (***Figure 5.3***). However, the extent of channel

opening varied by cytokine/mediator. NO and IL-17 had modest effects and both increased EtBr uptake by 14% (Arbitrary Fluorescence: 479 ± 15 in control vs. 540 ± 16 in NO vs. 547 ± 16 in IL-17 treated preparations; ***Figure 5.3A-B, 5.3D, 5.3F***). IL-1β and IFN-γ produced much more robust increases in channel opening. Glial cell EtBr fluorescence was increased by ~38% in tissue treated with IFN-γ (Arbitrary Fluorescence: 479 ± 15 in control vs. 661 ± 31 in IFN-γ treated preparations; ***Figure 5.3E-F***). Incubation with IL-1β increased EtBr uptake by ~70% compared to control (Arbitrary Fluorescence: 479 ± 15 in control vs. 798 ± 21 in IL-1β treated preparations; ***Figure 5.3C and 5.3F***). Thus, the pro-inflammatory cytokines/ mediators IL-1β, IL-17, IFN-γ and NO can increase Cx43 hemichannel activity in otherwise unstimulated enteric glial cells.

Stimulation of glial P2Y1 receptors increase glial EtBr uptake

Next, we investigated how pro-inflammatory cytokines/mediators affect Cx43 opening in enteric glia where channel opening was induced by purinergic activation. We stimulated Cx43 channel opening by treating LMMP preparations with the P2Y1 agonist ADP (100 μM) for 10 minutes in the presence of EtBr. In agreement with McClain et al. (2016) [9], treatment with ADP increased EtBr uptake by ~27% (Arbitrary Fluorescence: 479 ± 15 in control vs. 610 ± 18 in ADP treated preparations; ***Figure 5.4C***), demonstrating that this treatment paradigm was a suitable method for activating enteric glia and stimulating Cx43 hemichannel opening in subsequent experiments.

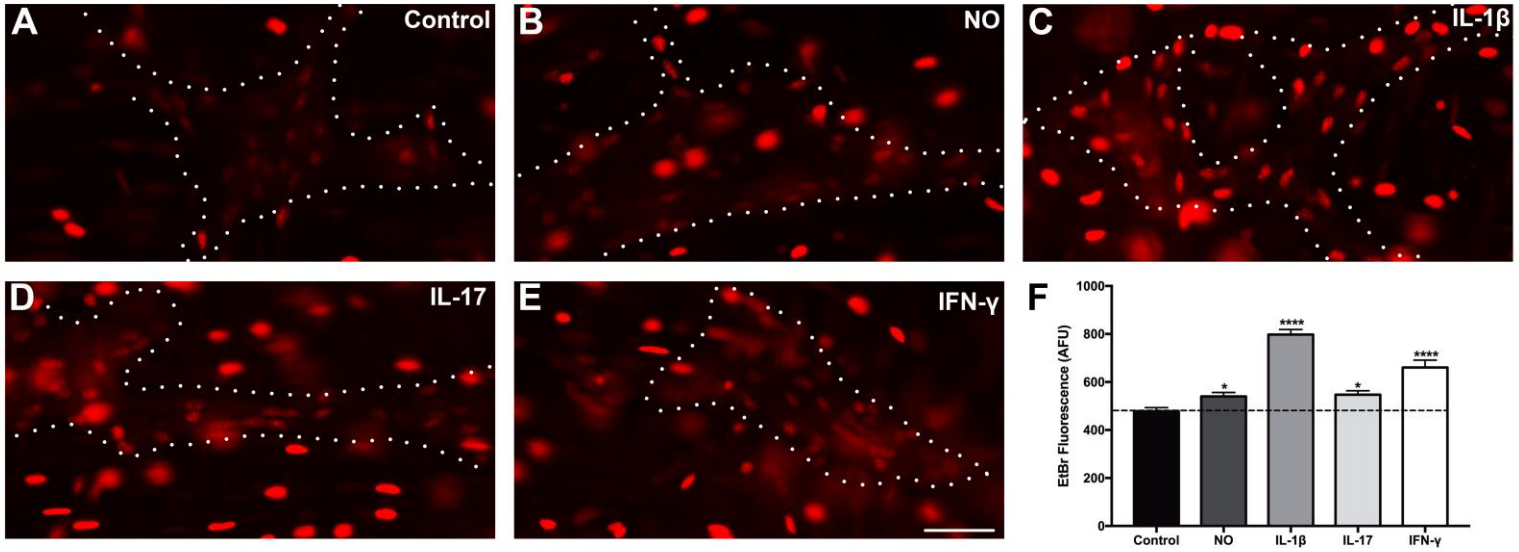

Figure 5.3: Effects of the pro-inflammatory mediators NO, IL-1β, IL-17 and IFN-γ on Cx43 hemichannel opening in unstimulated enteric glia. (***A-E***) Representative images and (***F***) quantification of glial ethidium bromide (EtBr) fluorescence in live LMMP preps treated with (***A***) buffer control, (***B***) 100 μM PAPANONOate (Nitric Oxide donor), (***C***) IL-1β (10 ng/mL), (***D***) IL-17 (10 ng/mL) and (***E***) IFN-γ (10 ng/mL), in the absence of the purinergic agonist ADP. (Scale bar = 50 μm) (*P<0.05, ****P<0.0001 vs. Control via One-Way ANOVA with Dunnett's post-hoc test) (n= a minimum of 10 individual glia, from 7-24 ganglia/treatment group).

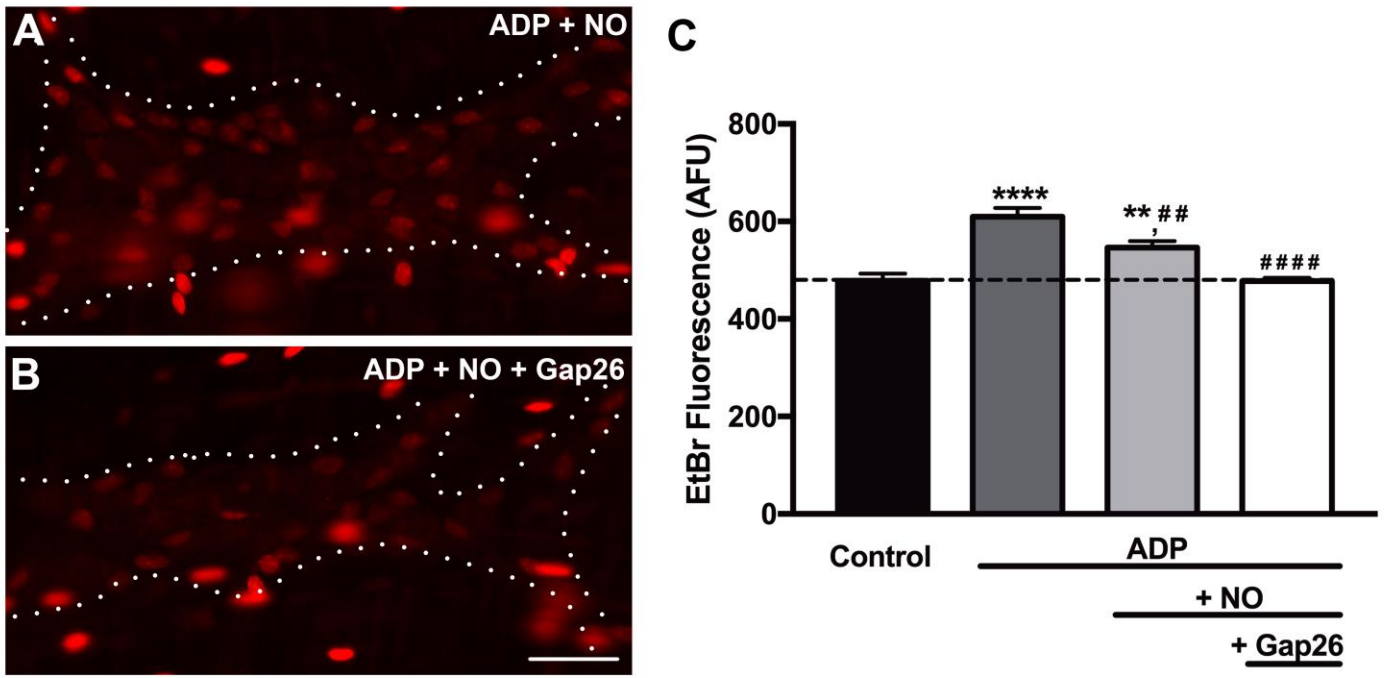

Figure 5.4: Effects of Nitric Oxide (NO) on EtBr dye uptake in ADP-stimulated enteric glia. (***A, B***) Representative images and (***C***) quantification of Cx-43 mediated EtBr dye uptake in enteric glia stimulated with the purinergic agonist ADP (100 μM) in the presence or absence of the nitric oxide donor PAPANONOate (NO; ***A***) and the Cx43 mimetic peptide 43Gap26 (***B***). Control preps were treated with buffer only. (Scale bar = 50 μm) (**P<0.01, ****P<0.0001 vs. Control, $^{\#\#}$P<0.01, $^{\#\#}$P<0.0001 vs. ADP via One-Way ANOVA with Dunnett's post-hoc test.) (n=a minimum of 10 individual glia, from 7-33 ganglia/mouse and 3-6 mice/treatment group).

Nitric Oxide (NO) decreases glial EtBr uptake through a Cx43-independent pathway

Increased NO is frequently associated with tissue inflammation, in part due to the increased activities of inducible nitric oxide synthase (iNOS)[28,29]. Cx43 hemi-channels are susceptible to NO-mediated post-translational modifications [6,14,30] and we have previously shown that NO increases Cx43-mediated release of ATP from enteric glial cells[4]. Unexpectedly, NO slightly (~10%) diminished EtBr uptake in stimulated enteric glia (Arbitrary Fluorescence: 610 ± 18 in ADP vs. 547 ± 13 in NO + ADP treated preps; ***Figure 5.4A and 5.4C***). Co-treatment with the Cx43 hemichannel mimetic peptide 43Gap26 (100 μM) decreased glial EtBr uptake by an additional 12% and returned EtBr fluorescence to basal levels (Arbitrary Fluorescence: 547 ± 13 in NO + ADP vs. 478 ± 6 in NO + ADP + 43Gap26 treated preps; ***Figure 5.4B-C***). These data show that the NO inhibits EtBr uptake.

IL-1β potentiation of glial EtBr uptake is sensitive to connexin-43 blockade with 43Gap26

Next, we investigated the effect of IL-1β on Cx43 opening in activated glia. IL-1β concentrations were increased in distal colon tissue, and importantly in myenteric plexus ganglia, following colitis (***Figure 5.2C-D***). Due to its expression within enteric ganglia, IL-1β is spatially primed to alter Cx43 activity and opening. In the presence of 20 ng of IL-1β protein, EtBr uptake in stimulated enteric glia was increased by ~42% (Arbitrary Fluorescence: 610 ± 18 in ADP vs. 869 ± 16 in IL-1β + ADP treated preps; ***Figure 5.5A and 5.5C***). Treatment with the Cx43 mimetic peptide 43Gap26 (100 μM) decreased dye uptake to levels similar to that in tissue treated with ADP only (Arbitrary

Fluorescence: 610 ± 18 in ADP vs. 685 ± 15 in IL-1β + ADP + 43Gap26 treated preps; ***Figure 5.5B-C***). These results suggest that the IL-1β-mediated increase in EtBr dye uptake is dependent on Cx43 hemichannel activity.

Interleukin 17 (IL-17) does not alter ADP-stimulated dye uptake and in the presence or absence of the Cx43 mimetic peptide 43Gap26

Colonic concentrations of the T_h17-associated pro-inflammatory cytokine IL-17 were decreased during colitis. Thus, we hypothesized that IL-17 would not significantly alter Cx43 opening during inflammation. EtBr uptake in activated glia was not altered in the presence of the T_h17-associated cytokine IL-17 (Arbitrary Fluorescence 610 ± 18 in ADP vs. 640 ± 23 in IL-17 + ADP treated preps; ***Figure 5.6B-C***). Further, the inhibitory effects of the mimetic peptide 43Gap26 were lost in the presence of IL-17 (Arbitrary Fluorescence: 640 ± 23 in IL-17 + ADP vs. 653 ± 21 in IL-17 + ADP + 43Gap26 treated preps; ***Figure 5.6B-C***). Together, these results show that IL-17 does not potentiate EtBr uptake, and further alters the inhibitory effect of the Cx43 mimetic peptide.

Interferon-γ (IFN-γ) does not potentiate ADP-stimulated dye uptake and is insensitive to blockade with the connexin-43 mimetic peptide

During inflammation, protein levels of IFN-γ in the distal colon were not significantly changed and both healthy and inflamed animals had minimal colonic IFN-γ. Thus, we hypothesized that IFN-γ would not significantly affect glial Cx43 activity, which we have shown to be important in GI inflammation[4]. Treatment with 20 ng of IFN-γ did not

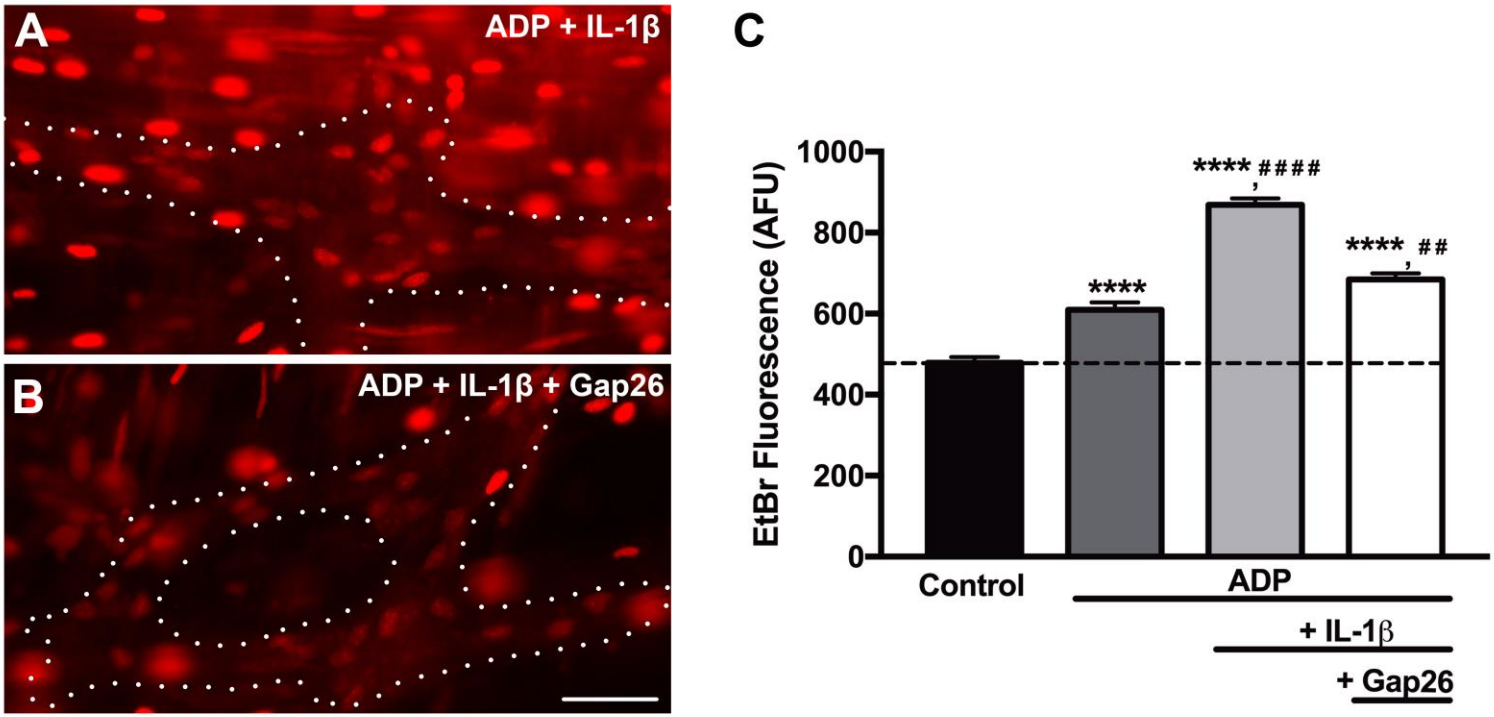

Figure 5.5: Effects of Interleukin 1-β (IL-1β) on dye uptake in ADP-stimulated enteric glia. ***(A, B)*** Representative images and ***(C)*** quantification of Cx43-mediated EtBr dye uptake in enteric glia in LMMP preps treated with buffer (*Control*) or the purinergic agonist ADP (100 μM) in the presence or absence of the pro-inflammatory cytokine (IL-1β; **A**) and the Cx43 mimetic peptide 43Gap26 (**B**). (Scale bar = 50 μm) (**P<0.01, ****P<0.0001 vs. Control, $^{\#\#}$P<0.01, $^{\#\#}$P<0.0001 vs. ADP via One-Way ANOVA with Dunnett's post-hoc test.) (n=a minimum of 10 individual glia, from 7-23 ganglia/mouse and 3-6 mice/treatment group).

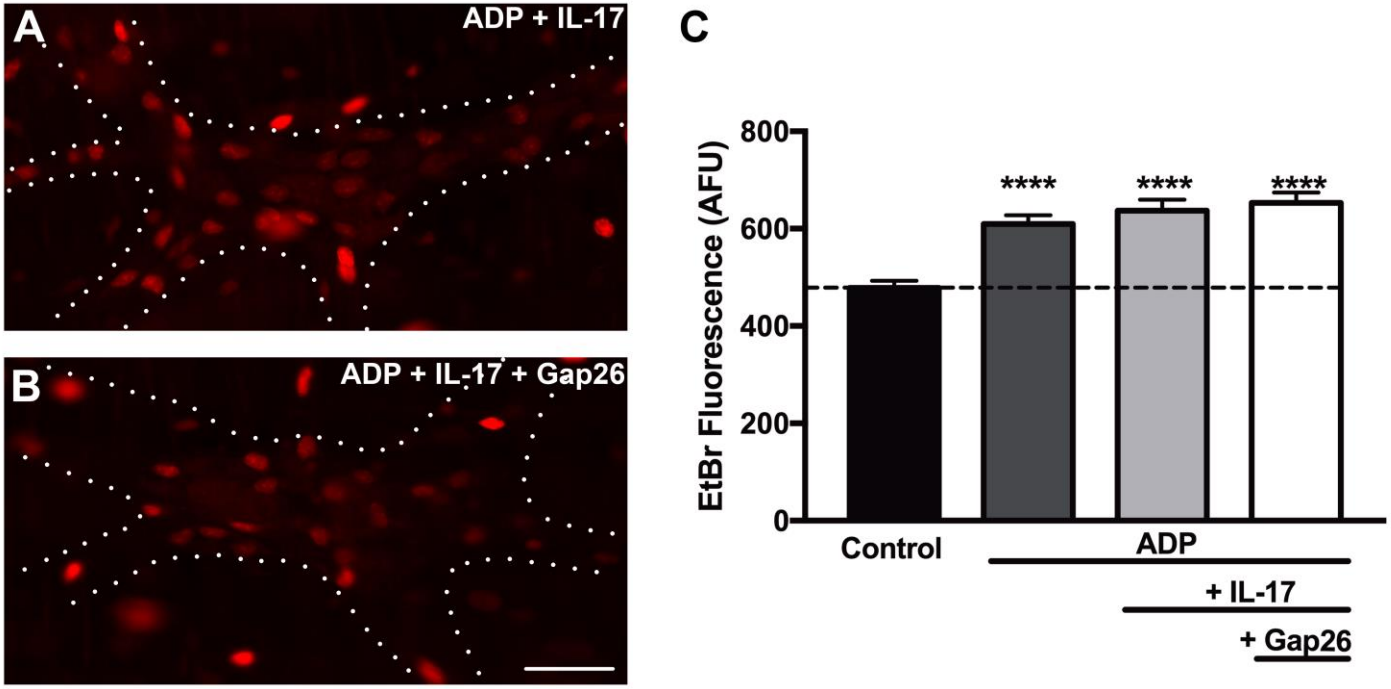

Figure 5.6: Effect of Interleukin 17 (IL-17) on ADP-stimulated dye uptake in enteric glia. ***(A, B)*** Representative images and ***(C)*** quantification of Cx-43 mediated EtBr dye uptake in enteric glia in treated with buffer (*Control*) or stimulated with the purinergic agonist ADP (100 μM) in the presence or absence of (**A**) the pro-inflammatory cytokine IL-17 and (**B**) the Cx43 mimetic peptide 43Gap26. (Scale bar = 50 μm) (****P<0.0001 vs. Control via One-Way ANOVA with Dunnett's post-hoc test.) (n=a minimum of 10 individual glia, from 4-20 ganglia/mouse and 3-6 mice/treatment group).

significantly change glial EtBr uptake in tissue stimulated with ADP (Arbitrary Fluorescence: 610 ± 18 in ADP vs. 593 ± 12 in IFN-γ + ADP treated preps; ***Figure 5.7A and 5.7C***). The inhibitory effects of 43Gap26 were lost in the presence of IFN-γ (Arbitrary Fluorescence: 593 ± 12 in IFN-γ + ADP vs. 592 ± 9 in IFN-γ + ADP + 43Gap26 treated preps. Consistent with the effects seen with IL-17, IFN-γ does not potentiate EtBr uptake, and negates the inhibitory effects of the Cx43 mimetic peptide 43Gap26.

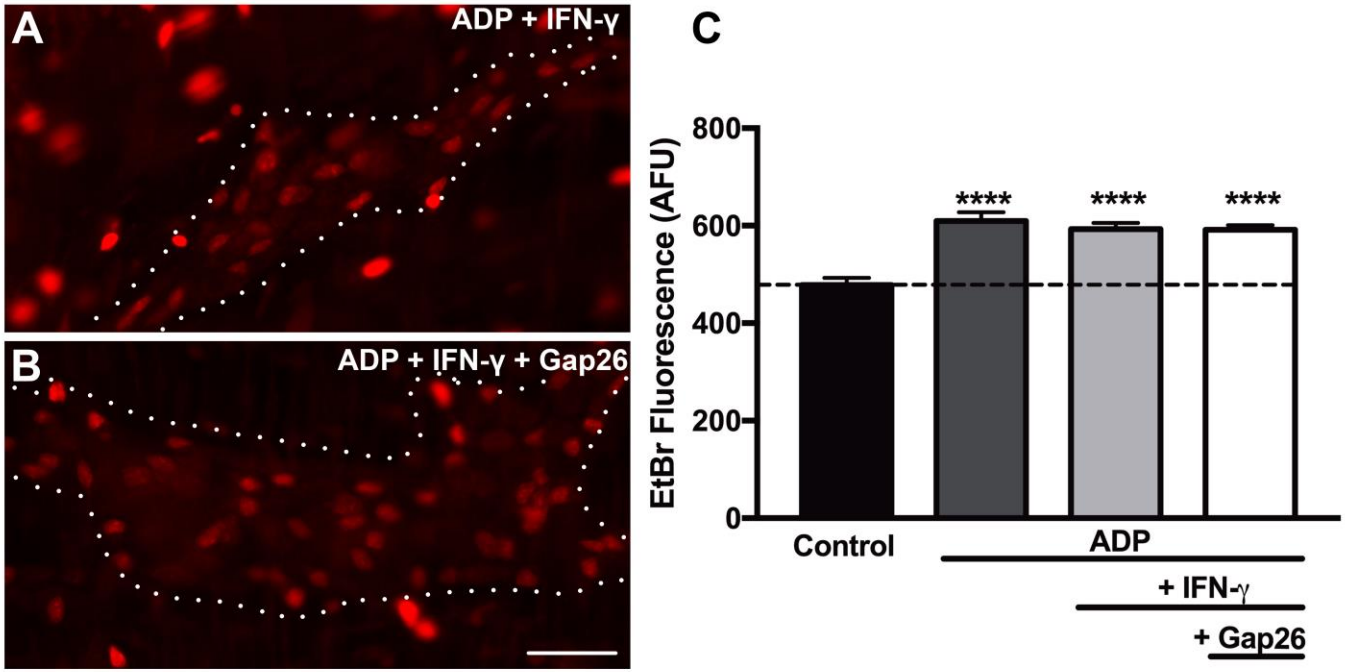

Figure 5.7:Effects of the pro-inflammatory cytokine Interferon-γ (IFN-γ) of Cx43-mediated dye uptake in stimulated enteric glia. (A, B) Representative images and ***(C)*** quantification of Cx-43 mediated EtBr dye uptake in enteric glia treated with the purinergic agonist ADP (100 μM) in the presence or absence of the pro-inflammatory cytokine Interferon-γ (IFN-γ; **A**) and the Cx43 mimetic peptide 43Gap26 (**B**). Control preps were treated buffer only. (Scale bar = 50 μm) (****P<0.0001 vs. Control via One-Way ANOVA with Dunnett's post-hoc test.) (n=a minimum of 10 individual glia, from 7-22 ganglia/mouse and minimum 3-6 mice/treatment group).

Discussion

Glial hemichannels composed of Cx43 are important mediators of intercellular signaling during health and disease in the ENS. Here, we investigated how glial hemichannels are affected by key pro-inflammatory mediators such as IL-1β, IL-17, IFN-γ and NO. Our key results show that IL-1β, IL-17, IFN-γ and NO differentially affect the baseline and activity-dependent uptake of EtBr through Cx43. All pro-inflammatory mediators tested had the potential to increase the glial uptake of EtBr, albeit to differing extents. However, their effects on activity dependent uptake of EtBr were vastly different. IL-1β significantly increased Cx43-dependent dye uptake in activated glia, while IL-17 and IFN-γ did not change EtBr uptake following glial activation and interfered with the inhibitory effects of the Cx43 mimetic peptide 43Gap26. Surprisingly, nitric oxide decreased activity-dependent EtBr uptake.

Immune homeostasis in healthy animals involves maintenance of the sensitive balance between pro- and anti-inflammatory cytokines. As such, disruption of this balance is a characteristic component in the pathology of inflammatory bowel disease (IBD) and other autoimmune disorders[13,31,32]. At the peak of inflammation during colitis, distal colon concentrations of the pro-inflammatory cytokines IL-1β, IL-17 and IFN-γ are changed. Inflammation increased IL-1β protein concentrations while simultaneously decreasing IL-17 levels. IFN-γ levels were slightly increased, however the effect did not reach statistical significance (P=0.1). One possibility is that 48 hours post DNBS colitis is too late to measure the peak colonic expression of IFN-γ protein.

The DNBS model used in these experiments produces a T_h1 cytokine response[33,34], which is associated with increased production of the effector cytokines IFN-γ, TNF-α, IL-

2 and IL-10, and mimics some of the features of human Crohn's disease. Thus, although colonic IFN-γ levels were not increased to a statistically significant level, this finding may have some functional relevance and be indicative of the T_h1 immune response caused by DNBS. IL-17 is traditionally associated with a T_h17-type immune response. Thus, an increase in IL-17 would not be expected following induction of T_h1-associated DNBS colitis and decreased IL-17 may represent an suppression of T_h17 T cells in inflamed mice. The final cytokine studied was IL-1β, which is not traditionally associated with a particular (T_h1, T_h2 or T_h17) type of immune response. Rather, it is up-regulated in a variety of autoimmune diseases, including IBD, where it can contribute broadly to T Cell activation[20,35-37]. Consistent with our findings, IL-1β is associated with animal models of colitis[18,22], and more importantly, with human disease[38-40].

The pro-inflammatory mediators IL-1β, IL-17, IFN-γ and NO all increased Cx43 hemichannel opening under control conditions. Our finding supports the long-standing hypothesis that Cx43 hemichannel activity is modulated by pro-inflammatory cytokines during inflammation[5,7]. These mediators altered Cx43 opening to different extents, with IL-1β and IFN-γ robustly increasing channel opening by more that 40% and NO and IL-17 producing a more moderate 14% increase in dye uptake. One explanation for these differential effects is that Cx43 hemichannels are alternately modulated by different pro-inflammatory mediators. Cx43 is subject to post-translational modification by NO where cysteine residues undergo S-nitrosylation in the presence of increased NO[30,41]. Conversely, the mechanisms of cytokine-induced hemichannel opening are not as clearly understood. In fact, Bennett et al. (2012)[42] propose that the pro-inflammatory cytokines TNF-α and IL-1β induce increased Cx43 opening through action on "stressed"

astrocytes, rather than directly modifying the channel itself. We hypothesize that differential mechanisms of action likely explains the variability in extent of channel opening produced by treatment with IL-1β, IL-17, IFN-γ and NO.

Hemichannel activity is the CNS is differentially regulated under normal and pathological (inflamed) conditions[43,44]. We found that the effects of IL-1β, IL-17, IFN-γ and NO on Cx43 hemichannel opening were activity dependent. IL-1β increased dye uptake by an additional 42%, compared to the 27% increase in channel opening following purinergic stimulation with ADP. Interestingly, the combined effect of ADP + IL-1β (i.e. a 69% increase in dye uptake compared to control) was similar to that achieved with incubation with IL-1β only (70%; ***Figure 5.3C and 5.3F***). This highlights the presence of a potential maximal degree of channel opening (70% above basal opening) since the effects of ADP and IL-1β were additive solely to a maximal level. Importantly, incubation with the mimetic peptide 43Gap26 demonstrated that the effects of IL-1β were Cx43 sensitive. These findings, in conjunction with glial expression of the IL-1 receptor[18] and secretion of IL-1β[17], present the possibility of that IL-1β can function as an autocrine or paracrine signaling molecule, and modulate glial Cx43-dependent communication[3].

Consistent with our findings, Retamal et al.[8] report that IL-1β increases Cx43-mediated dye uptake in astrocytes[8]. Two potential mechanisms are that 1) IL-1β increases total cellular Cx43 expression but not individual rate of channel opening and/or 2) IL-1β increasing channel opening of individual hemichannels. IL-1β has been shown to increase cell surface Cx43 hemichannel expression[45] but because of the short treatment time in our experiments, we hypothesize IL-1β-mediated effects are not due

to a change in Cx43 expression. Rather, as proposed by Retamal et al.[8], we hypothesize that IL-1β alters cellular redox state[46] which then alters Cx43 channel state and opening[6,14,44].

IL-17 and IFN-γ had no effect on EtBr dye uptake in stimulated enteric glia cells (***Figures 5.6 and 5.7***). The effects of these cytokines specifically on Cx43 hemichannels have not been thoroughly investigated in the literature. IFN-γ treatment alters Cx43 expression and the formation of functional astroglial gap junctions[47,48]. Similarly, IL-17 treatment also leads to decreased Cx43 expression and decreased gap junction formation in mixed astrocyte/microglia cultures[48]. However, cytokines have different effects on Cx43 hemichannels vs. gap junctions[8]. Thus, the findings of these studies cannot be easily translated from the study of Cx43 gap junctions in astroglia, to that of Cx43 hemichannels in enteric glia.

An unexpected finding in our study was that NO decreased EtBr dye uptake in activated enteric glia. This finding contradicts the well-established report that NO increases Cx43 hemichannel through S-nitrosylation[4,14,30].

In summary, our findings show that the pro-inflammatory cytokines/mediators IL-1β, IL-17, IFN-γ and NO are capable of modulating glial uptake of the dye EtBr, as a measure of enteric glial cell Cx43 activity. The differential effects of the pro-inflammatory cytokines/ mediators on Cx43 channel activity, under basal and activated conditions, provide important insight into our understanding of activity-dependent hemichannel function. Cx43-insensitive effects on dye uptake potentially highlight novel mechanisms through which inflammatory mediators may alter cell permeability and warrants further study.

APPENDIX

Cytokine/Compound	Concentration	Source	Catalog Number
Interleukin 1β (IL-1β)	10 ng/mL	R + D Catalog	401-ML-005
Interleukin 17 (IL-17)	10 ng/mL	eBioscience	14-8171-62
Interferon- γ (IFN-γ)	10 ng/mL	eBioscience	BMD326
PAPANONOate (NO Donor)	100 μM	Cayman Chemical	82140

Table 5.1: Cytokines and Chemicals used in the Ethidium Bromide Dye Uptake Assay.

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CHAPTER 6

Summary and Future Directions

Summary

The enteric nervous system is an intrinsic neuronal network comprised of enteric neurons and glial cells embedded within the walls of the GI tract, and provides local and moment-to-moment control of gastrointestinal (GI) functions and motility[1]. Loss of enteric neurons disrupts this network and is associated with motility dysfunction in a host of disorders[2-6]. Despite the worldwide prevalence of motility dysfunction[7], drug treatments for motility disorders are limited and primarily focus on providing symptomatic relief. The lack of improved therapeutics is due to the gaps in our understanding of the key factors and cellular mechanisms that contribute to motility dysfunction. This dissertation investigated the cellular mechanisms by which oxidative stress and immune dysregulation, two factors associated with GI inflammation, contribute to enteric neuropathy and motility dysfunction. In particular, we were interested in the role of enteric glial cells in mediating these effects.

In Chapter 2, we investigated how glial nitric oxide (NO) production contributes to enteric neuron death during inflammation, and found that the activation of enteric glia by extracellular purines contributes to neuron death through the pathogenic release of ATP[8]. This release is mediated by increased opening of glial Cx43 hemichannels, which are gated by glial NO concentrations, as previously shown [9]. Pharmacological and genetic inhibition of glial inducible nitric oxide synthase (iNOS) activity was protective against neuron death *in situ*, highlighting a potential novel therapeutic target for prevention of enteric neuropathy in inflammation.

Chapter 3 explored the antioxidant arm of ENS regulation of oxidative balance. Enteric glia are hypothesized to be the primary producers of the endogenous

antioxidant reduced glutathione (GSH) in the ENS [10]. In support of this, we show that enteric glia express the enzymes necessary for GSH synthesis. In an additional finding, these enzymes are also expressed by enteric neurons. *In situ* inhibition of GSH synthesis caused enteric neuron death, supporting a neuroprotective role for GSH. Surprisingly, i*n vivo* depletion of whole body GSH was protective against some features of GI inflammation, suggesting a novel cellular role for this key antioxidant.

Chapter 4 studied how disrupted immune homeostasis in circulating T cells affected myenteric plexus structure and GI motility. CD4-dnTGFβRII mice with disrupted T cell TGFβ signaling have disrupted distal colon motility, without alterations in myenteric neuronal density or neurochemical coding. Myenteric glia were impacted in our animal model, with conversion to a “reactive” or “activated” state in CD4-dnTGFβRII mice. Disrupted TGFβ signaling was sufficient to dysregulate immune homeostasis within the innate, but not adaptive, immune system at the level of the myenteric plexus. Lastly, we show that enteric neurons express the TGFβ type II receptor, the first demonstration of TGFβ receptor expression in the ENS.

In the final data chapter (Chapter 5), we measured how key pro-inflammatory mediators and cytokines affect glial Cx43 hemichannel function. The pro-inflammatory cytokines IL-1β, IL-17, IFN-γ and NO all increase glial uptake of the intercalating agent ethidium bromide (EtBr) under baseline conditions, but have differential effects in glia stimulated with ADP. IL-1β potentiates dye uptake in ADP-stimulated glia in a Cx43-dependent manner while NO decreases dye uptake. IL-17 and IFN-γ did not alter dye uptake in activated glia and surprisingly, diminished the inhibitory effects of the Cx43 mimetic peptide 43Gap26.

Together, these studies add to our knowledge of key glial mechanisms that contribute to enteric neuropathy and improve our understanding of ENS neuron-glia interactions. These findings identified novel therapeutic targets (discussed below), which will allow for the development of new and improved drugs for treatment of GI motility dysfunction.

Novel Findings

Enteric neurons express the cellular machinery necessary for glutathione synthesis

Enteric glial cells help maintain neuronal health through the synthesis and release of reduced glutathione (GSH) [10,11]. This is analogous to the central nervous system (CNS) where astrocytic neuroprotection is mediated in part by GSH[12]. In addition, CNS neurons are capable of producing small amounts of GSH when provided with the necessary precursors from astrocytes[13]. To date, whether this mechanism occurs in the ENS had not been investigated. We showed that enteric neurons express the catalytic subunit of glutamate-cysteine ligase (GCLC) and glutathione synthesis (GS), the two enzymes necessary for glutathione synthesis. With this novel finding, we propose a mechanism where enteric neurons may contribute to their own neuroprotection through GSH synthesis.

Protective role of in vivo depletion of glutathione during inflammation

Reduced glutathione (GSH) is the primary endogenous cellular antioxidant in the body and is traditionally associated with its cytoprotective features. We hypothesized that depletion of glutathione would promote the enteric neuropathy, tissue damage and

motility dysfunction associated with GI inflammation, given the important role of oxidative stress in motility disorders. Instead, we found that GSH depletion is protective against some features of *in vivo* colitis, suggesting a novel role for GSH in the patho(physiology) of GI inflammation and motility dysfunction.

The TGF-beta type II receptor is expressed in enteric neurons

The anti-inflammatory and pro-fibrotic effects of the regulatory cytokine transforming growth factor β (TGFβ) are primarily mediated through TGFβ signaling in immune cells. Thus, the primary literature focuses on those signaling pathways and TGF-β signaling in other cell types has not been as thoroughly studied. In the CNS, there is evidence for neuronal expression of TGFβ isoforms and their receptors [14,15]. Here, we show novel expression of the type II TGFβ receptor in the enteric nervous system, specifically on enteric neurons. This finding presents the possibility for differential effects of immunological vs. neural TGFβ signaling in the ENS.

Study Limitations

DNBS Model of Colitis

Our studies primarily used the 2,4-dinitrobenzene (DNBS) model to induce murine colitis. This chemical model is characterized by a Th_1-mediated immune response and colonic fibrosis; features observed in human Crohn's disease[16]. Thus, it is important to interpret findings within the context of the features of this animal model and consider that they may not be broadly applicable to all GI inflammatory conditions. The validity of

our findings would benefit from repetition of our some studies in other chemical and genetic models of colitis[17-19].

Use of Pharmacological Modulators

The studies in this dissertation used a number of drugs to modulate the behavior and/or concentrations of enzymes, channels and signaling molecules of interest. Specifically, studies in Chapter 3 and 5 relied heavily on the pharmacological properties of BSO (GSH synthesis inhibitor) and 43Gap26 (Cx43 mimetic peptide). Thus, the conclusions of these chapters are dependent on the assumption that these inhibitors perform as intended and have little to no off target effects.

Sex of Experimental Animals

Recent research findings have increasingly emphasized the role of sex as a biological variable in animal models. Studies in this dissertation were done in a single sex of research animal (male mice for Chapters 2,3 and 5 and female mice for Chapter 4). This deficiency should be considered when interpreting these data and may limit the translation of findings to broader populations.

Future Directions

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

Cell-specific Inhibition of Glial NO Production

Findings in Chapter 2 show a pathogenic role for glial iNOS activity and NO production in enteric neuropathy during inflammation. This suggests that glial iNOS is a potential therapeutic target for the prevention of cell death during inflammation. Preliminary studies have begun to test this hypothesis and would be continued in future work. Working with Dr. Fredric Manfredsson (Michigan State University), we designed an AAV-FLEX-iNOS viral construct that will theoretically target iNOS expression in enteric glia.

In this viral construct, microRNA (miRNA) against iNOS is packaged into an adeno-associated virus (AAV) containing the flip excision (FLEX) system and a GFP reporter. The FLEX construct restricts expression of miRNA to cre-recombinase expressing cells. Thus, injecting this virus into Sox10-Cre^{ERT2} mice will result in inhibition of iNOS in enteric glia only. Target specificity of our miRNA will be confirmed by the use of a control scrambled miRNA packaged in the same manner. Pilot studies using cre-positive animals and cre-negative controls, both injected with control and iNOS mRNA, shows non-specific labeling for GFP in enteric neurons (***Figure 6.1***), highlighting the need for further troubleshooting and future work.

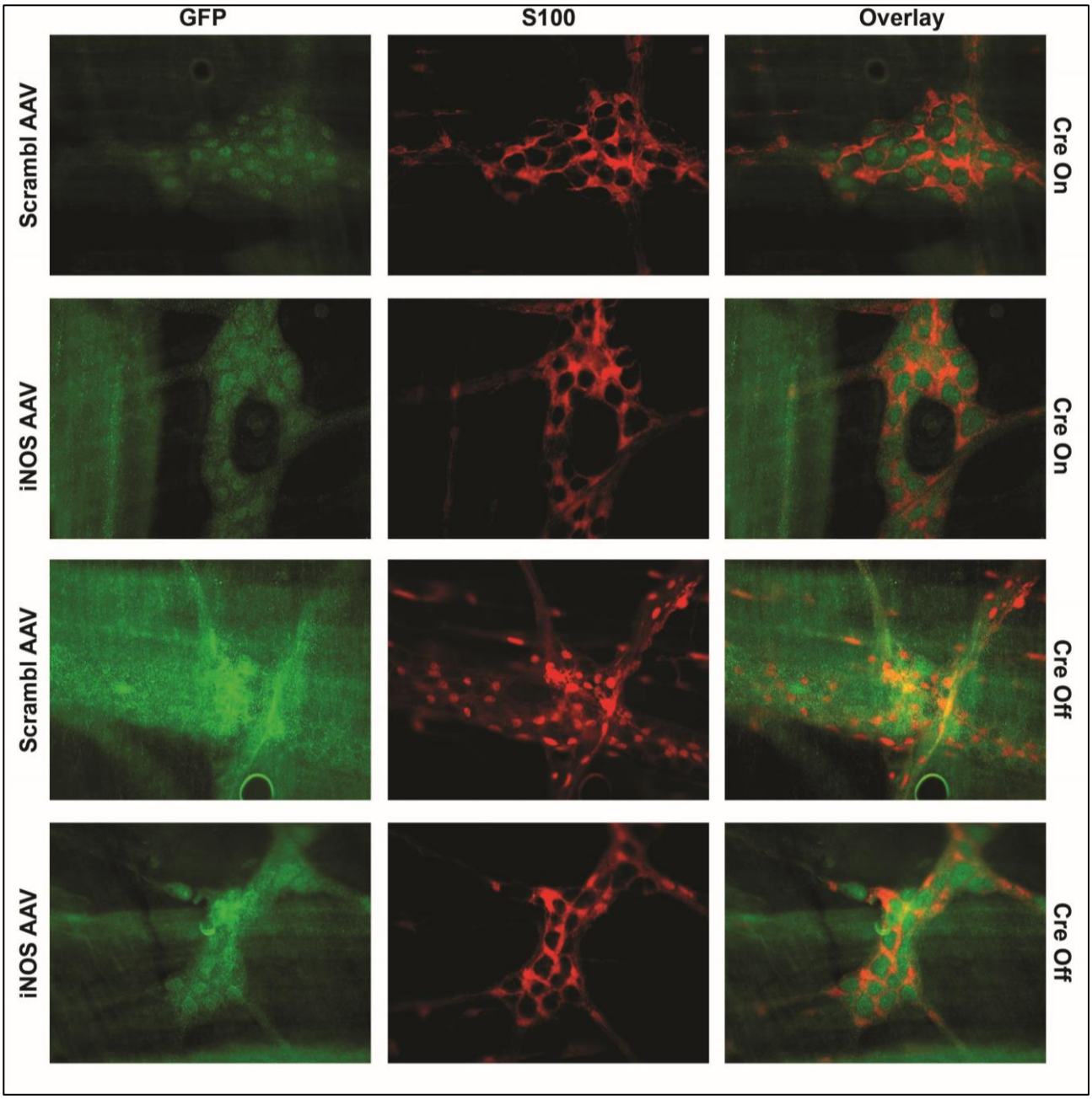

Figure 6.1: Myenteric ganglia in Sox10-Cre^{ERT2} mice and Cre^{-} controls.

Immunohistochemistry (IHC) for GFP (*green*) and the glial cell marker (*S100*β) in Cre^{+} and Cre^{-} mice injected with iNOS-AAV or scrambl-AAV

Cell-specific Inhibition of Glial GSH Production

Novel findings in chapter 3 highlight a potential heterogenous role for the antioxidant GSH. Our treatment paradigm involved whole body depletion of cellular GSH via consumption of the GCL inhibitor L-Buthionine Sulfoximine in drinking water. This treatment is sufficient to significantly deplete GSH in major organ systems including the liver, kidney, heart, lung, intestines and in plasma and blood cells [20]. Thus, it is challenging to elucidate what mechanisms are contributing to the protective effects of GSH depletion during inflammation.

To specifically investigate the role of enteric glial GSH production during *in vivo* inflammation, I would propose the development of a glial specific GCL inhibitor. This transgenic mouse would be generated by crossing *Sox10::Cre^{ERT2}* mice with a floxed GCL (*GCL$^{f/f}$*) mouse. In this transgenic animal, the excision of GCL (and hence inhibition of glial GSH synthesis) would by inducible and cre-recombinase activated by treating animals with tamoxifen citrate in chow. Induction of colitis in these animals would allow us to specifically determine role of enteric glial GSH production during *in vivo* inflammation. *Nestin::Cre/GCL$^{f/f}$* mice would lack GCL in both enteric neurons and glia, and their use in the DNBS colitis model would allow distinction between the roles of glial-derived and neuronal-derived GSH in the ENS.

The Role of Neuronal TGF-beta Signaling in Colonic Inflammation

We show that the type II TGFβ receptor is expressed in enteric neurons, but not enteric glia, in the myenteric plexus. In the CNS, neurons express TGFβ receptors [14,15] and disrupted TGFβ signaling is associated with increased neurodegeneration [21]. An

important future study would be to investigate if TGFβ signaling plays a similar role in enteric neurons. *Nestin::Cre* transgenic mice would express cre-recombinase in neural crest derived cells including enteric neurons and glia. Crossing these mice with B6;129-*Tgfbr2*^{tm1Karl}/J (*TGFβRII*$^{f/f}$; from Jackson Labs) would generate *Nestin::Cre*/*TGFβ*$^{f/f}$ mice. Cre expression would be constitutive (as in our CD4-dnTGFβRII mice) and would allow us to determine the effect of disrupted TGFβ signaling in enteric neurons. Although *Nestin::Cre* mice would have cre expression in both enteric neurons and glia, we have no evidence of TGFβRII expression in enteric glia.

Modulation of Cx43 Channel Opening by Pro-Inflammatory Cytokines and Mediators

In Chapter 5, we investigated the effect of key pro-inflammatory cytokines on Cx43 hemichannel opening and EtBr dye uptake. These studies used a small sample of key cytokines associated with GI inflammation. A key follow-up study would be to investigate the effects of a wider battery of cytokines. In particular, it would be interesting to determine if Th_1/Th_2/Th_{17} families of cytokines produce any differential effects on dye uptake. Secondly, these studies relied on the Cx43 mimetic peptide to determine whether observed effects were Cx43-dependent or independent. Repeating studies in transgenic Cx43 null mice (*Sox10::Cre*^{ERT2}*/Cx43*$^{f/f}$) would validate the findings produced by pharmacological methods.

Mechanisms of Glial GSH Release and Regulation

Our *in situ* experiments in Chapter 3 demonstrate that glial GSH synthesis is necessary for enteric neuron survival *in situ*. However, key questions regarding glial release and

metabolism of GSH still remain unanswered. Cx43-mediated glutathione release has been reported in astrocytes [22,23] so a key experiment would question whether enteric glial GSH is released by similar mechanisms. Secondly, the metabolism of GSH is an important mechanism for modulation of cellular glutathione content[12]. Immunohistochemistry for key GSH metabolism enzymes, such as glutathione peroxidase and glutathione reductase, within the enteric nervous system, would add considerably to our knowledge of GSH regulation in the ENS.

The data presented in this dissertation begins to address the important role of enteric glia cells, and glial regulation of oxidative stress and immune mediators, in regulating enteric neuropathy during inflammation. In combination with proposed future experiments, these findings will aid in the identification of novel therapeutic targets to treat GI motility dysfunction.

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