HOMEOSTATIC RESPONSES OF THE ENTERIC CHOLINERGIC SYSTEM IN STRESS AND ENTERITIS

By

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ABSTRACT

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Stressful, traumatic events are a well-recognized trigger leading to acute and chronic gastrointestinal (GI) disease like Irritable Bowel Syndrome (IBS) or Inflammatory Bowel Disease (IBD). Two major types of stress which are pervasive within the world population are early life adversity and depression, both of which are strongly associated with GI disease throughout life. Though much of the underlying pathology in IBS and IBD has been described, the underlying mechanisms explain how stress may trigger onset or increased symptom severity in these chronic disease is poorly understood.

The enteric cholinergic nervous system is a major regulator of GI homeostasis with broad regulatory roles over epithelial barrier permeability, epithelial cell secretion, smooth muscle contraction and motility, and immune activation. Though a role of the enteric nervous system has been describe in acute stress induced GI dysfunction, little is known about the role of this system in chronic stress, early life adversity, or in infectious models.

The objective of this dissertation was to determine if the enteric cholinergic nervous system contributed to GI disease under different types of environmental challenges including early life adversity, chronic stress and pathogen challenge. To answer these questions, we utilized several different small and large animal models in combination with pharmacological agonists and antagonist of the cholinergic system.

The results presented here demonstrate that different types of stressors differentially impact the enteric cholinergic system. Following early life adversity, we observed a persistent upregulation of the enteric cholinergic system, which predisposes individuals to increased intestinal secretion, permeability, motility, and upregulation of stress related genes. In a pathogen challenge model, we observed an upregulation of a non-neuronal component of the enteric cholinergic system which correlated positively with disease severity. Finally we observed that chronic stress in adulthood results in a strong down regulation of the enteric cholinergic nervous system with reduced cholinergic mediated functional secretion. Combined these findings demonstrated that different modes of stress have dichotomous impacts the enteric cholinergic system, which differentially impact GI function. Future work should focus on the precise factors impacting the function and expression of the cholinergic system in order to develop better therapies to cope with stress induced GI disease.

For my all family

ACKNOWLEDGEMENTS

The body of work presented here is the product of excellent mentorship and support from my peers and family members. Only with time will I be able to more fully appreciate the sacrifices made by those who helped me through this journey; however, I will do my best to acknowledge their efforts below.

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Finally, I am indebted to my family for their support while I've pursued this degree. This career I've pursued would not have been possible without the sacrifice and support from the generations that came before me. I am particularly thankful for the hard work and sacrifice of my parents and grandparents whose hard work established all the opportunities I have within reach now. Thank-you to my wife Elaine, who has supported me whilel I've pursued a career path that has been interesting to me. I hope this dissertation work will provide a foundation for the success of our future family.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

GI – gastrointestinal

IBS – Irritable bowel syndrome

IBD - Inflammatory bowel disease

ELA – early life adversity

HPA – hypothalamic pituitary adrenal axis

CRF - corticotrophin releasing factor

ENS – enteric nervous system

ChAT – choline acetyltransferase

Ach- acetylcholine

CHT- high affinity choline transporter

VAChT – vesicular acetylcholine transporter

ACHE – acetylcholinesterase

CHRM1- cholinergic muscarinic receptor 1

CHRM2- cholinergic muscarinic receptor 2

CHRM3- cholinergic muscarinic receptor 3

CHRNA7 – cholinergic nicotinic receptor subunit 7

EWS – early weaning stress

LWC – late weaned controls

FGID – functional gastrointestinal disorders

SMP – submucosal plexus

MP – myenteric plexus

FD4 – fitc4kDa dextran

NMS – neonatal maternal separation

NH – normal handled controls

RS – restraint stress

HC-3 – hemicholinium-3

EFS – electrical field stimulation

Isc – short circuit current

ATR- Atropine

TTX – tetrodotoxin

Bument – bumetanide

CMMC – colonic migrating motor complex

CSDS – chronic social defeat stress

CHAPTER 1

Introduction

Some of the text presented in this introductory chapter was previously published as part of a review in Pohl CS, Medland JE, and Moeser AJ. Early-life stress origins of gastrointestinal disease: animal models, intestinal pathophysiology, and translational implications. *American journal of physiology Gastrointestinal and liver physiology* 309: G927-941, 2015.

Introduction

Stress and gastrointestinal disease: a crtical public health issue

Stressful, truamatic events are a well recongized trigger leading to onset or increased symptom severity in major chronic gastrointestinal (GI) diseases including irritable bowel syndrome (IBS) (22, 191) and inflammatory bowel disease (IBD) (63, 84), and is a subject that has been extensively reviewed.(130, 131) Of growing importance is the impact of early life adversity (ELA) on long term GI health, with several reports demonstrating that ELA is a risk factor for development of IBS (28, 38, 40, 62) and IBD (1, 2) later in life. Additionally, infectious enteritis in individuals with exisiting psychological distress, further predisposes them to developing of functional bowel disorders, (128) highlighting the compounding effects of stress on GI health. Not only is ELA a major risk factor for GI disease, but psychological conditions induced by chronic life stress such as depression and anxiety often preceed onset of IBS (118, 164) and IBD.(23, 91, 124) Together, these examples demonstrate that stress is a leading environmental factor contributing to GI disease in the human population.

Though stress is clearly a risk factor for increased suceptiblity to GI disease, it remains unclear if stress at different ages, or if different types of stress (acute versus chronic psychological stress or acute infectious challenge) all induce a conserved response aimed at regaining homestasis. Better understanding stress and mechanisms of influnece over GI health are an important public health issue, requiring further understanding. The aim of this chapter is to introduce the reader to the concepts of stress, the central nervous system response to stress, and the known underlying mechanisms of stress induced GI disease. Previous work from our group and others

demonstrates a central role for the cholinergic enteric nervous system in mediated GI reponses to different types of stress, so we will also introduce the reader to canonical cholinergic signaling pathways and known cellular sources of cholinergic function. We will also address different animals models of stress and their translational value. Finally, we will close this section with the aims of the work for this disseration.

Definition of stress

"Stress is defined as a state in which homeostasis is threatened or perceived to be so [and] homeostasis is re-established by a complex repertoire of behavioral and physiological adaptive responses of the organism" (48). The neonate and infant are exposed to tremendous, stressful changes in homeostasis at birth and weaning, and both immunological and hypothalamic system adaptation and plasticity are necessary for survival. Importantly, the outcome of reestablished homeostasis has major implications for long-term health. The organism returns to original homeostasis (or eustasis), or the adaptive response creates a new homeostasis. The new homeostatic parameters can be inappropriate (allostasis) or beneficial (hyperstasis). (41) Adaptive responses to these early-life challenges likely dictate health later in life (88) and are central to long-term GI disease susceptibility. A number of early-life stressors, including psychosocial (maternal deprivation, loss of caregiver, and physical and emotional abuse) and immunologic (allergy, infectious, or metabolic/nutritional) stress, have been implicated as risk factors of GI disease onset later in life. While these stressors are diverse, they fit the definition of stress, in that they threaten the host's homeostasis. If these stressors occur during a time of significant developmental plasticity, it is likely that new adaptations can reshape brain and gut function for the individual's lifetime.

Neuroendocrine response in stress

The hypothalamic pituitary adrenal (HPA) axis is a very well understood homeostatic mechanism by which host adapts to environmental challenges and stress, both psychological and inflammatory. The response of the HPA axis described here as well outlined by Francis and Meany (70) and by Soderholm (104) and is illustrated in **Figure 1.1**. The neuroendocrine cascade of the stress begins with perceived stress activating the parvocellular region of the paraventricular nucleus of the hypothalamus (PVNh) with norepinephrine. Activation of PVN neurons results in release if corticotrophin releasing factor (CRF) and arginine vasopressin (AVP) into the hypophyseal-portal system of the anterior pituitary, stimulating adrenocorticotropic hormone (ACTH) synthesis and release into systemic blood supply. Once ACTH reaches the adrenal cortex, glucocorticoid synthesis is initiated, which mediates many of the adaptive roles of different organ systems to the inciting environmental threat. Glucocorticoids circulating back to the hypothalamus negatively regulate the HPA axis, and resolve the stress response.

Peripheral stress responses are also mediated through the autonomic nervous system, which becomes activated through the periaqueductal gray (PAG) nucleus action on the pontomedullary nuclei facilitating peripheral cholinergic and sympathetic signaling **(Figure 1.1**).(104)

In healthy individuals, the neuroendocrine stress axis can negatively regulate itself and shut down following an environmental challenge. However, early life adversity has been shown to modulate HPA axis function, preventing negative regulation of the stress response and allowing persistent HPA action.(70) Importantly, individuals with

early life adversity develop poor health under conditions of stress later in life. (45, 71, 166)

Early life adversity and the impact of HPA axis function

During infancy, childhood, and early adolescence the HPA axis is mostly hyporesponsive with low basal glucocorticoid secretion until near puberty (111, 121, 158, 184). In humans and rodents, severe environmental stressors in the absences of parental care disrupt HPA axis maturation, and these early life stressors, characterized by inappropriate release of stress hormones such as cortisol during subsequent stressful conditions, are associated with development of adult psychological and gastrointestinal disease, such as IBS (58, 72, 170, 182). During the neonatal stage the adrenal gland is highly sensitive to small quantities of ACTH while the negative glucocorticoid receptor (GR) feedback system is poorly developed (49, 181), thus HPA axis activation induced by early life stress results in high and prolonged glucocorticoid production. Elevated glucocorticoids are detrimental to HPA axis and GR development and thus prepubescent individuals exposed to early life stress exhibit exaggerated CNS/behavioral and physiological responses to subsequent stressful episodes later in life (3, 39, 126).These phenomena support the hypothesis that early life stress stimulates the HPA axis during a period when the neuroendocrine system is intended to be hyporesponsive. Inappropriate stimulation of the HPA axis during the intended hyporesponsive period results in altered development and lifelong allostasis.

Concurrent with HPA-axis development in early life, the gut undergoes a similar extensive maturation period. Given the bidirectional communications between the HPA axis and the gut, aberrations in gut development due to environmental stress may

directly induce HPA axis dysfunction. Conversely, HPA axis allostasis may contribute to inappropriate development of the gut. In either case, interruptions in the development of either system are well associated with risk of developing GI disease.

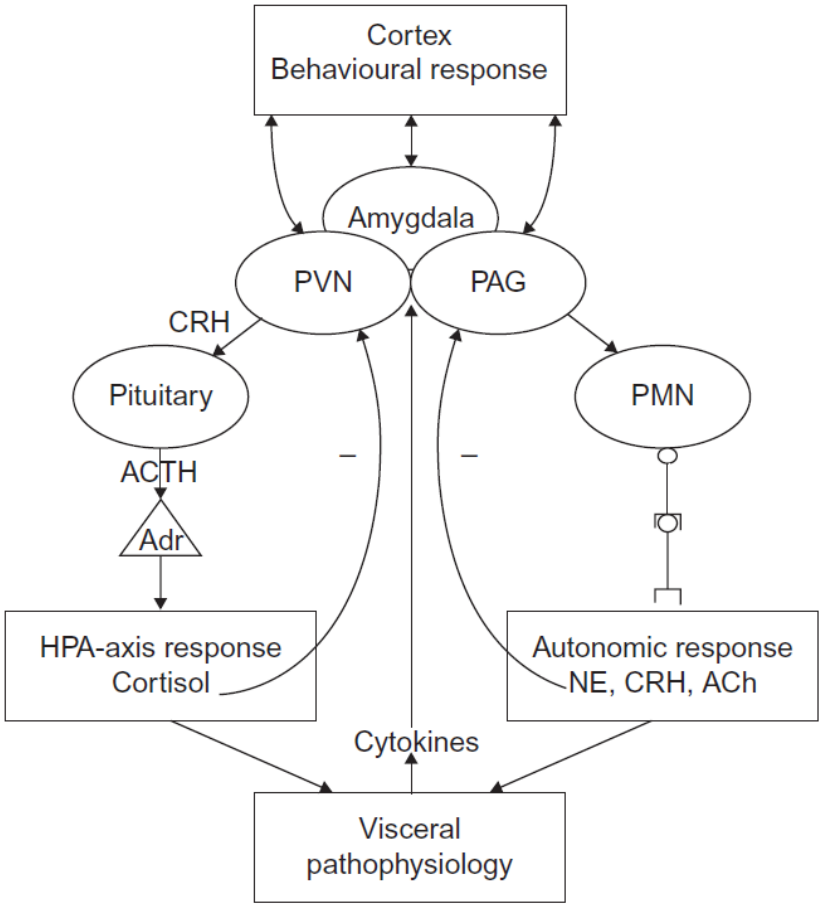

Figure 1.1. Neuroendocrine response of stress. *Soderholm, Chapter 74, Physiology of the Gastrointestinal Tract*

The developmental biology of the postnatal intestine

In order to understand how early life stress might influence long-term GI development and disease susceptibility , it is important to consider the major intestinal developmental changes and adaptations occurring across different animal models species in the immediate postnatal period. In all mammals, the early postnatal period is marked by major developmental changes in preparation for long-term survival. Some

major systems that undergo extensive development and programming during this time are the enteric immune and nervous systems, epithelial barrier function and microbiota colonization and composition (133). While the early life developmental changes occurring in these systems allow the host to survive and thrive in the extra-uterine environment; perturbations in normal developmental processes by stress during these plasticity periods, can lead to a deviation in long-term function of the GI system and increased disease susceptibility (**Figure 1.2**).

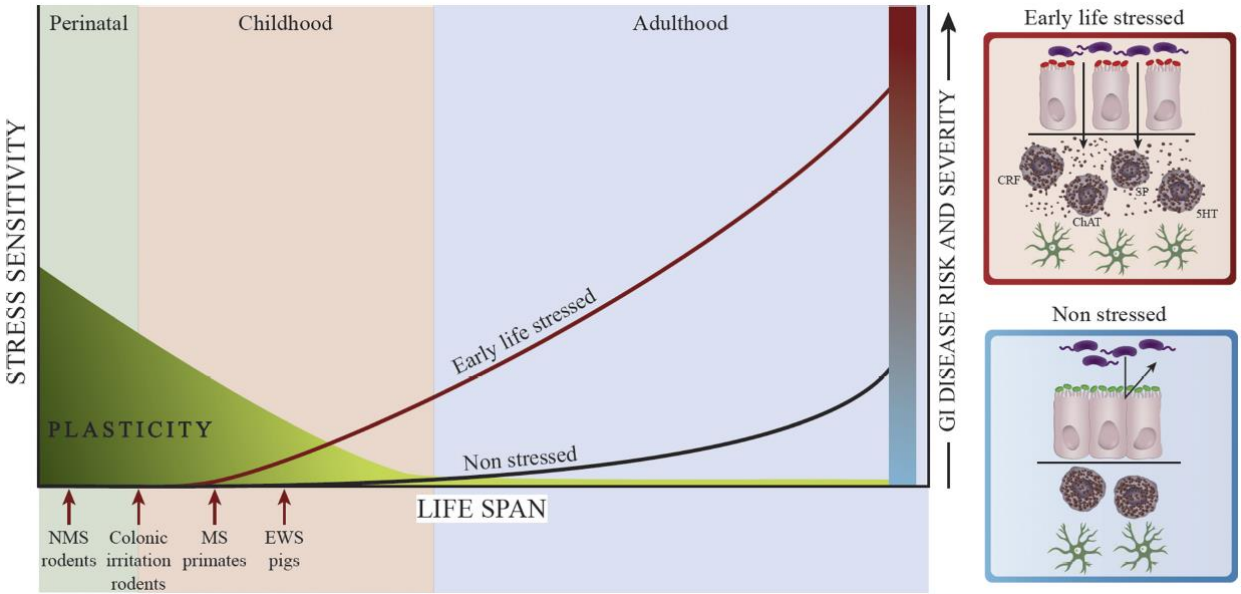

Figure 1.2. Proposed Paradigm of Early Life Stress and GI Disease Development. Evidence from rodent and porcine models and human data demonstrate that early life stress is a major risk factor in later life GI disease development and severity. Early life psychosocial stressors occur during times of high developmental plasticity (green) and initiates a trajectory towards increased GI disease susceptibility (red line) in later life. Animal models such as neonatal maternal separation (NMS) and colonic irritation in rodents, maternal separation (MS) in non-human primates, and early weaning stress (EWS) in pigs occur during early life during times of intestinal developmental periods (comparable to human perinatal and childhood intestinal developmental periods) and enhance susceptibility to development of GI dysfunction later in life (adulthood). Common mechanisms of early life stress-induced disease between animal models (boxes to the right) are increased intestinal permeability, altered microbiota, increased enteric nervous system activity, heightened mast cell numbers and activation, CRF, cholinergic nervous system (ChAT), substance P (SP), and serotonin (5HT).

Figure 1.2. (cont'd):
Collectively, these mechanisms can result in clinical signs of GI disease including abdominal pain, diarrhea, constipation, and increased susceptibility to enteric infections.

Postnatal development of the enteric nervous system

Although the ENS can operate independently of CNS input, the ENS is an essential system integrating signaling between the peripheral neurons of the CNS and the brain. Major developmental processes and adaptations take place in the ENS during postnatal life which exhibit a high degree of plasticity. Given the plasticity of this system, stressful or harmful stimuli in early postnatal ENS development have the potential to deviate normal ENS development and lifelong function. Key ENS postnatal processes include the formation of functional neurocircuits, gangliogenesis, differentiation of neuron phenotypes and neuron cell death (120, 160). After neurogenesis and gangliogenesis, the ENS undergoes a normal decline in neuronal numbers via apoptosis as demonstrated in laboratory animals and humans (6, 78, 162, 190). Although the mechanisms of "ENS pruning" in the postnatal gut are incompletely defined, it is thought that loss of appropriate survival factors (32, 37), possibly including nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF) (69, 116, 183, 186) may contribute to these changes. In addition, throughout the postnatal period, the neurochemical composition of the ENS changes significantly. Of particular importance are the alterations seen in the cholinergic innervation to the gut where the proportion of neurons expressing acetylcholine (Ach), the major excitatory neurotransmitter in the GI tract, increases (52) dramatically, often doubling, and accounting for approximately 44% of all neurons in the submucosal plexus, and 62% in the myenteric plexus by maturity (75, 95). ENS neurite outgrowth is another important postnatal event (74). In summary,

the ENS undergoes significant development and maturation during the early postnatal period and exhibits a high degree of plasticity. Therefore, an understanding of how early life stress influences normal ENS development could be critical to the understanding of early life stress-induced GI disease.

Postnatal development of the GI immune system

In childhood, both birth and weaning represent major challenges for early life host immunity. At birth, the host must adapt to microbial colonization of the lungs, intestine, and skin as well as consumption of milk antigen, without inducing massive inflammation. Likewise, at weaning, the host must cope with the psychological stress of maternal separation (MS) or deprivation while also adapting to a sudden exposure to a large amount of food antigen and changes in microbial community, without the support of maternal immunity. Massive change in the gut transcriptional profile at birth and weaning indicate the adaptive effort of the host during disruptions in homeostasis (153). Ontologically, the early life immune system has been described as suppressed, yet active (46, 73). It is hypothesized that the perinatal period is a ‘window of opportunity’ for tolerogenic induction, and excessive inflammatory interruption during this period may lead to maladaptive responses with long term health consequences (11, 157). Research over the last 20 years has described multiple layers of both exogenous and endogenous immunosuppressive mediators that modulate infant immunology in order to promote an active, yet tolerogenic immune system. Exogenously, maternal milk provides immune-supportive factors such as sIgA, maternal leukocytes, and milk glycans, all of which modulate and neutralize intestinal microbes. Additionally, breastmilk provides massive amounts of anti-inflammatory cytokines and peptides, which negatively regulate

neonatal TLR and inflammatory cytokine expression (142). Endogenously, several pathways inhibit the innate immune system, which in turn, leads to polarization toward a tolerogenic lymphocyte population in early life (11, 46, 60, 73). However, the neonatal immune system is not inherently unresponsive or defective. Presence of the commensal microbes induces neonatal immune activity represented by developmental of secondary lymphoid organs in both mice, swine, and humans during the postnatal period (11, 17, 157). Clinically, neonates can respond to vaccination, albeit the response is weak. Additionally, various types of leukocytes from the neonate can be stimulated to induce inflammation. Finally, in early and abruptly weaned pigs, antibodies to new feedstuffs can be detected (11, 46, 60). These observations highlight the *inflammatory capability* of the neonate, and reinforce that there may be consequences of immune-overstimulation during this quiescent period.

At weaning, the immunosuppressive dominance gives way to a spike in inflammation. Mast cell degranulation and proliferation, intraepithelial lymphocyte proliferation, mucosal inflammatory cytokine induction, and T-cell stimulation coordinate the homeostatic adjustment to weaning (46, 150). Host inflammatory and metabolic pathways are also upregulated with weaning to cope with a dynamic microbiota and an introduction to novel food antigens, factors which are likely controlled by TLR and IL-1 pathways (26, 153). Weaning can be abrupt or gradual, can occur at different stages of development, and weaning is associated with an inflammatory response; thus, the time and (or) developmental stage at which the weaning event occurs can interrupt the tolerogenic period. This becomes particularly important in stressful situations such as

early weaning where premature immune-stimulation during the tolerogenic 'window of opportunity', can have serious health implications later in life.

Postnatal development of the intestinal epithelial barrier

The intestinal epithelium undergoes rapid maturation during the postnatal period. While certain postnatal epithelial changes are thought to be genetically "hard wired", many epithelial changes are driven by environmental, microbial and endocrine cues. One of earliest and most critical epithelial changes in the postnatal period is the establishment of intestinal epithelial barrier function. Intestinal epithelial barrier function refers to the ability of the epithelium to form a selectively permeable barrier, regulated predominantly by tight junction proteins and mucus, which prevent the vast amounts of luminal antigens, pathogens, and toxins from gaining excessive entry into the underlying tissues and systemic circulation. Impairment of the epithelial barrier results in exposure of luminal constituents to the underlying immune, circulatory, and nervous system inciting local neuro-inflammatory events and systemic inflammation. Disturbances in this epithelial barrier, characterized by heightened intestinal permeability or "leaky gut", is a hallmark in the pathogenesis of major GI diseases including IBD, IBS, Celiac disease and food allergy/intolerance (34, 102, 136). The postnatal development of intestinal barrier properties has been investigated in multiple animal species and humans. At birth, the neonatal intestinal barrier is highly permeable and then matures during the postnatal period indicated by a progressive decline in permeability with age; however, species-specific variations exist. In term human infants, it was shown that GI permeability (measured as urinary lactulose: mannitol ratios) remained stable during the first 4 d of postnatal life (187). Catassi et al (1995) demonstrated that GI permeability,

also measured via urinary: mannitol ratios, declined significantly by ~ 3.7-fold, between d 1 and d7 of life, indicating a rapid postnatal decline in GI permeability (36). It was also shown in this study that breast feeding accelerated the decline in GI permeability (36). In pre-term human infants, GI permeability was shown to be markedly higher compared with term infants and then declined rapidly with age (178, 187). In mice, GI permeability (measured by *in vivo* FITC Dextran 4kDa permeability) is high at birth and declines with age; however, the most pronounced reductions in GI permeability in mice occurs later than in humans, at 2-3 weeks of age (147). Similarly, in neonatal rabbits, small intestinal permeability was shown to be high at birth and then declined progressively into adulthood (>120 d)(175). In term piglets, GI permeability (measured by *in vivo* lactulose: mannitol ratio sugar absorption tests) remained stable after birth with little changes between birth and d 10 of postnatal life (101). However, utilizing *ex vivo* jejunal preparations on Ussing chambers, De Quelen et al. (2011) reported that intestinal permeability increased between postnatal days 0 and 14, and then declined thereafter (36, 50). Together, these findings indicate that while many species exhibit a postnatal maturational decline in intestinal permeability, the time course is very different. The implications for these species and therefore model differences, relative to human clinical relevance are discussed later in this review. In addition to the developmental aspects of intestinal epithelial permeability, other key barrier and innate epithelial cell changes also occur in the postnatal period including marked changes in the expression and repertoire of antimicrobial peptides, pattern recognition receptors and immune signaling pathways(152), nutrient transporters (117, 173), and crypt-epithelial regenerative complexes (51, 96). Furthermore, as with the other GI system changes described

above, postnatal epithelial development is modulated and shaped extensively by dietary, microbial, neuro-endocrine and environmental influences and differ by species.

Postnatal establishment of the enteric microbiota

The microbiota exerts a large influence on GI function and health throughout life, but its composition is determined largely during the postnatal period (67, 93, 140). While the majority of literature available suggests that colonization of the GI tract occurs at birth, with first exposure occurring in the vaginal canal (93, 185), there is also evidence to suggest that colonization occurs *in utero* (103). Given that this is the founding group of bacteria, any abnormal stress or inflammatory state of the mother can influence the microbiota of the offspring (149, 185). Breast milk has a profound impact upon the microbiota, and further aids in colonization, with breastfed individuals having a higher proportion of Bifidobacteria compared to those who are formula fed (67, 185, 195). At weaning, the microbiota is subject to great change with the transition from breast milk to a solid diet (67, 153, 155), and this transition coincides with a period of gut maturation (155). The effect of diet (high fat, carbohydrate availability etc.) on the microbiota continues after the transition from breast milk to solid food at weaning, and may play a role in diseases such as IBD (5, 48, 193). One of the key roles of the microbiota in the neonate is to establish oral tolerance to commensal microorganisms and food (108, 168). The microbiota has additional roles in the developing mucosal immune system in development of gut associated lymphoid tissue (GALT), intestinal lymphocytes and antimicrobial peptide secretion to the lumen (106). Neonatal colonization is also required for normal neurological development, including development of the HPA axis (57, 141, 167), which further highlights this period as a critical window in development.

In summary, there are tremendous and complex developmental changes that occur in the GI tract during early postnatal life. During this time, enteric neuronal, immune, epithelial, and microbial signaling act in concert to prepare the host for adaptation and survival to the immediate and long-term postnatal environment. During this time, the enteric systems exhibit a high degree of plasticity and thus disturbances in the normal developmental windows, such as early life stress/adversity, can lead to long-lasting changes in intestinal function and disease susceptibility. Therefore, there is great potential for early life adversity to disrupt both the HPA axis in the gut, make for a severity dysfunction Brain-Gut-Axis.

Enteric nervous system at the center of the brain gut axis

Figure 1.3 illustrates how signals from the central nervous system are integrated into the GI tract. Neuroanatomical attachment of the central nervous system to the GI tract is mediate through parasympathetic, vagal/sacral innervation and sympathetic spinal nerve innervation (**Figure 1.3**). The vagus and sacral nerves represent the major extrinsic cholinergic input to the GI tract (44); however these parasympathetic neurons only superficially innervate the intestines and ramifies immediately on the myenteric plexus of the enteric nervous system (ENS).(24, 114) (**Figure 1.3**). On the other hand, sympathetic neurons more extensively innervate all levels of the GI tract as autonomic sympathetic fibers can be found in the mucosa and in muscle layers.(77) The sympathetic system also extensively innervates the enteric nervous system, making the ENS a central hub for central nervous system signals to the gastrointestinal tract. Functionally, the ENS exerts regulatory control over numerous GI functions including

motility, visceral sensation, secretion and absorption, and immune and epithelial barrier function (74, 76, 129).

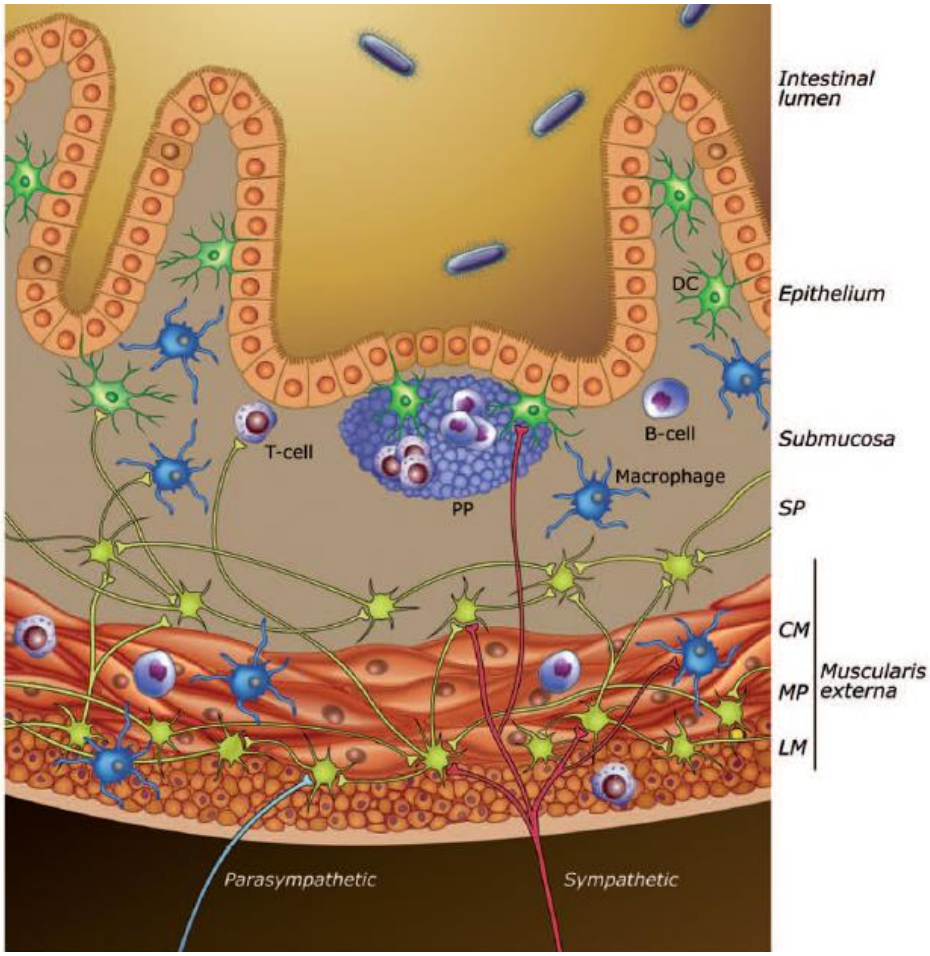

Figure 1.3. Neuroanatomical innervation of the GI tract. Adapted from Costes et al. 2013.(44) Parasympathetic innervation by vagal and sacral neurons. Sympathetic innervation from post ganglion neurons of the para- and prevertebral neurons. Note parasympathetic neurons do not innervate gut as extensively as sympathetic neurons. Enteric neurons make up majority of intrinsic neuronal activity synapsing on epithelium, muscle, and immune cells.

Physiological impact of stress on the gastrointestinal tract

The impact of stress on the GI tract can be debilitating in otherwise healthy people, but particularly in patients suffering with IBS (191), and the interaction between stress and GI health illustrates a connection between the brain and the gut, known as the Brain-Gut-Axis (**Figure 1.1 and 1.3**). In health and disease, the HPA axis is known

to play a role in regulation of gut function, thus the HPA axis has been established as an important component in the Brain-Gut Axis (68, 182).

Even in healthy individuals, acute stress significantly changes major GI physiology. For example, acute psychological stress and pain in healthy humans resulted in reduced fluid/ion absorption and increased fluid/ion secretion, factors that could predisposed an individual to diarrhea.(15, 16) Stress also alters GI motility in health humans and animals by accelerated colonic motility.(154, 192) Public speaking in healthy individuals and water avoidance stress in healthy rats also significant increased intestinal permeability (109, 179), a process which may trigger mucosal inflammation by allowing foreign food and microbial antigens to interact with immune cells in the gut wall. Stress induced fluid secretion, motility, and intestinal permeability are all factors that may contribute to clinical manifestation of diarrhea, particularly in individuals that may have a hyperresponsive HPA axis. Understanding the molecular and cellular pathways contributing to stress induce GI pathophysiology has been the focus of over 20 years of research; however, questions remain, particularly as to how acute stress may compound GI disease in individuals with prior early life stressful experiences.

Known underlying mechanism contributing to altered physiology following acute stress

Increased fluid/ion secretion, colonic hypermotility, and intestinal permeability are all hallmarks of stress induced GI dysfunction and serve as potential pharmacological targets. Since the cholinergic nervous system mediates intestinal secretion (43, 98, 119) and motility (174) under non-stressed condition, it was hypothesized that cholinergic

nerves may become hyperactive during stress and contribute to the underlying pathology.

Indeed, the first studies demonstrating that stress induced increased fluid/ion secretion demonstrated a role of cholinergic muscarinic receptor signaling as IV injection of atropine, a muscarinic receptor blocker, prevented the stress induced fluid secretion.(16) These findings were supported in animal models, were acute stress increased fluid and ion transport, which was inhibited with pretreatment of atropine.(161)

By assessing the impact of stress on colonic motility *in vivo*, Gourcerol et al demonstrated that stress induced hypermotility could be blocked by pretreating individuals with atropine. Furthermore, the group demonstrated that restraint stress results in myenteric nerve activation, determined by increase immunoreactivity of Fos in enteric neurons.(90) Simulating stress, with parental IP injection of CRF, Fos expression was upregulated in cholinergic nerves of the colon, highlighting, that stress may promote GI dysfunction by activation of enteric cholinergic neurons.(196) Supporting intestinal cholinergic nerve hyperfunction during stress, *ex vivo* preparations of rat colon demonstrated hypermotily in response to CRF, which was subsequently blocked by the neuronal blocker, tetrodotoxin and atropine administration.(113) These results indicate a local function of cholinergic nerves and muscarinic receptors in mediating stress induced hypermotility. Together, these reports resemble the responses observed in cholinergic mediated fluid secretion and suggests that stress may active enteric neurons, resulting in hypermotility and secretion via acetylcholine signaling on muscarinic receptors.

Similar to both secretion and motility, intestinal permeability was found to be mediated by cholinergic muscarinic signaling. For example, increased intestinal permeability in response to 4 hour long restraint stress could be inhibited by pretreating animals with atropine.(112, 161) Follow-up studies again demonstrated that muscarinic receptor signaling contributed to stress induced intestinal permeability.(109) In this study the authors demonstrate muscarinic receptor expression on epithelium and mast cells, and argue that acetylcholine binding to muscarinic receptors on these cells mediates the increase in permeability. However, specific depletion of muscarinic receptors on these cells types would be required to demonstrate this functional role under stress.

To summarize, stress induced GI dysfunction is, in large part, mediated by cholinergic muscarinic receptor signaling. Evidence suggests, at least for stress induced hypermotlity, that stress induced release of CRF results in activation of cholinergic enteric neurons. Activated cholinergic nerves go on to increase intestinal fluid transport, permeability and motility by releasing acetylcholine on muscarinic receptors (**Figure 1.4**). Though not discussed at length here, we have demonstrated a role of mast cell in mediated stress induced GI disease. Specifically, application of exogenous CRF induced mast cell activation and subsequent barrier permeability, (145) and lack of CRF receptor 1 on mast cells prevented stress induces GI permeability.(8)

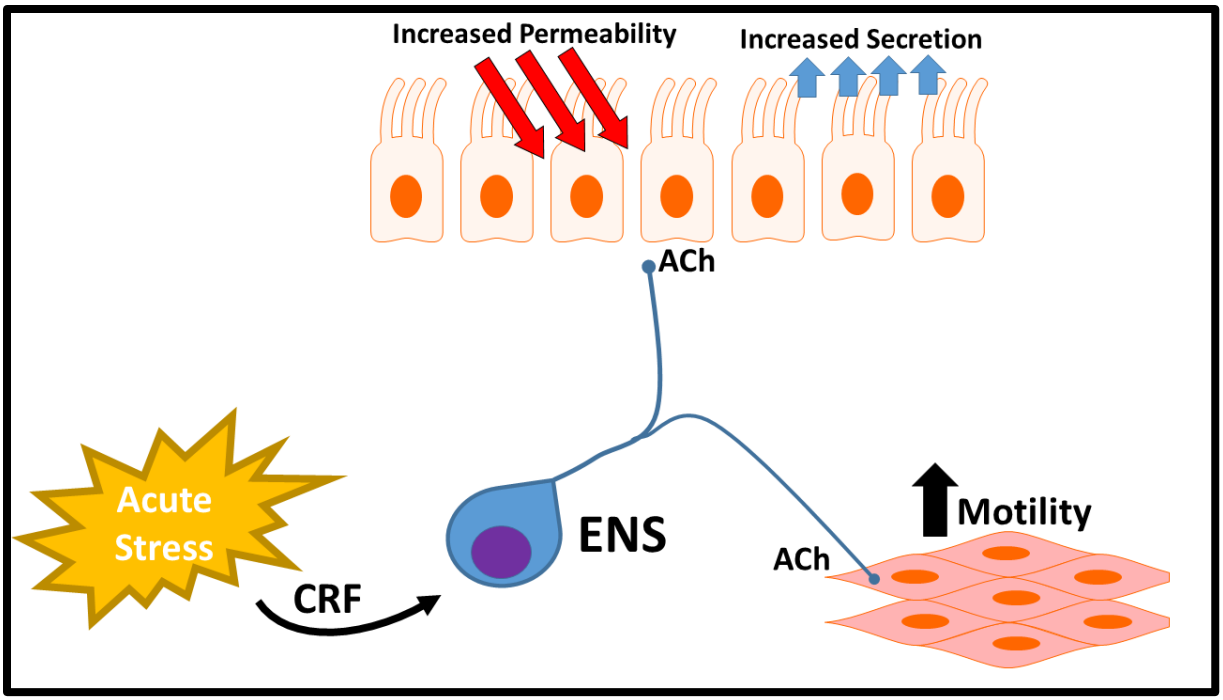

Figure 1.4. Proposed mechanism of stress induced GI dysfunction based off available literature. In healthy individuals, stress induces the release of CRF, which activated enteric cholinergic nerves. Activation of cholinergic nerves results in the release of acetylcholine onto muscarinic receptors of the epithelium and smooth muscle. Muscarinic signaling at epithelium drive increased secretion and permeability while muscarinic activation on smooth muscle induces hypermobility.

Features of cholinergic signaling

Considering the significant role played by the cholinergic system in stress induced GI dysfunction, a fundamental understanding of cholinergic signaling seems appropriate. The primary ligand of the cholinergic system is acetylcholine (ACh). Below, I will briefly review the synthesis, degradation, and different methods of signaling. Finally I will cover different intestinal sources of acetylcholine and potential differences in classical signaling.

Acetylcholine is synthesized by the catalytic combination of choline and acetyl coenzyme A by the enzyme choline acetyltransferase (ChAT). The rate limiting step in ACh synthesis is the uptake of the precursor choline. While all tissue have a low affinity

choline uptake apparatus, neurons express a high affinity choline transporter (CHT-1), which allows sodium dependent recycling of choline from the synapse. Synthesized acetylcholine is transported into nerve vesicles at synapses or varicosities by vesicular ACh transporters (VAChT). Depolarization of a nerve varicosity or synapse results in a calcium mediated release of ACh. Once in the extracellular space, ACh is rapidly degraded by acetylcholinesterase (AChE). See **Figure 1.5** for illustration. (98, 171) Neurons expressing ChAT, VAChT, and CHT-1 are identified as being cholinergic and have frequently been localized within enteric nervous system.(97)

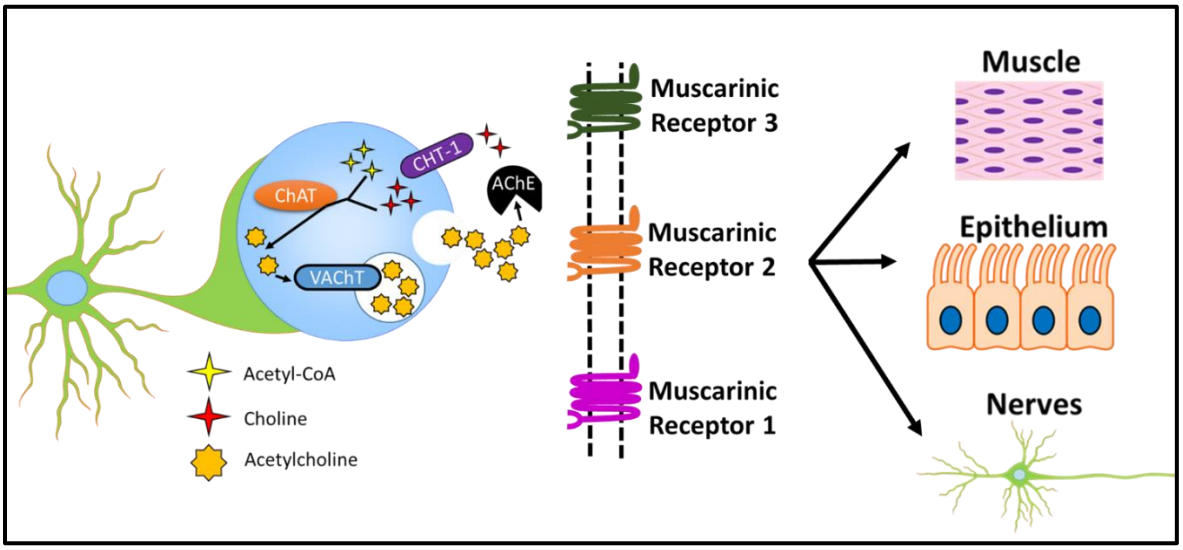

Figure 1.5. Canonical cholinergic signaling in the gastrointestinal tract.

Cholinergic transmission is achieved by acetylcholine binding cholinergic muscarinic or nicotinic receptors. Nicotinic receptors are pentameric ligand gated ion channels, made of heterogeneous or homogenous assembly of five different subunits that rapidly transmit signals once activated. Muscarinic receptors are g-coupled proteins and exists in five different subtypes, cholinergic muscarinic receptors 1-5. Both cholinergic receptors can be found throughout the GI tract on enteric neurons and on effector cells.(97) Stimulation of fluid secretion from epithelium is driven mostly by

cholinergic muscarinic receptor 1 and 3.(97, 98) Muscarinic receptors 1-3 have been identified on enteric nerve fibers and appear to have diverse functional roles, with muscarinic receptor 1 activity facilitating additional acetylcholine release, and muscarininc receptor 2 functioning to inhibit cholinergic nerves.(97) Cholinergic neurotransmission on GI muscle appears to occur predominately through muscarinic receptor 2 and muscarinic receptor 3, however, expression of either receptors varies with species.(87, 89, 97)

Rapid cholinergic signaling is mediated almost exclusively by neurons; however it is important to mention that there are non-neuronal sources of acetylcholine in the gut, which have functional consequences. For example, short chain fatty acids were shown to induced fluid secretion in a cholinergic muscarinic receptor manner independent of neurotransmission.(194) Additional investigation revealed that colonic epithelium can synthesize ACh through expression of ChAT,(10, 194) however, other aspects of ACh biosynthesis in epithelium appear to occur in a non-classical route. For example, ACh release from epithelium is mediated through organic cation transporters rather than VAChT.(10) Interestingly, immediately following early life stress, rat pups demonstrated an upregulation in mucosal ChAT expression, which was localized to the epithelium.(81) Additionally, increased epithelial ChAT expression was observed in patients suffering with IBD. These findings suggest a non-neuronal role of cholinergic signaling in stress and inflammation.(105) ChAT positive lymphocytes were also recently identified in the intestinal mucosa, demonstrating another non-neuronal source of ACh, although the density of this cell population in the intestinal mucosa is relatively sparse (56), and may not constitute a significant source of ACh. Chronic stress or enteritis may result in an

expansion in these cells and an increased contribution to gut cholinergic signaling; however, this has yet to be shown.

Use of animal models and comparative physiology

In this dissertation, both murine and porcine models were utilized to study the impacts of stress on gastrointestinal function. Here I evaluate the comparative physiology of both species and consider the benefits and deficits of either model used.

Neonatal Maternal Separation

The most commonly utilized animal model of early life stress is the neonatal maternal separation (NMS) model in rodents. There are several different types of NMS, including short handling, where the pups are handled 15 minutes daily during the postnatal period, and long maternal separation, where they are separated from the dam for 3 hours daily. This review will however focus mainly on long NMS model as because there are few studies using the short NMS model that address GI outcomes. Moreover, short NMS (handling), has been shown to be protective (decreased anxiety responses) (127, 134), and does not result in long-term GI dysfunction, compared with long NMS (144). In long NMS, rat or mouse pups are separated daily from their dam for 3 hour periods during postnatal days, in rats commonly between 2 -14 or 4 – 20 days of age (21, 82) and in mice commonly between 1-14 or 1-18 days of age (7, 123). While the majority of NMS research has occurred in mice, much of the NMS research concerning GI function has been performed in rats. The NMS model is based on the premise of disrupting the development of the HPA axis by inducing stress and HPA axis activation during the hypo-responsive period (4-14 in rodents) (143, 151) as discussed previously in this review. One of the most studied GI-related effects of the NMS in rodents is long-

term changes in GI motor function (e.g. motility) and sensory function (e.g. visceral hypersensitivity). Adult rodents that were previously exposed to NMS exhibited delayed gastric emptying and accelerated colonic transit (9, 30). Increased fecal pellet output (an indirect measure of increased motility) was observed in NMS rats that were subjected to later-life psychological stressors (25, 45, 163) indicating heightened or exaggerated motility responses to later life adversity. Fecal output and altered colonic transit, while not identical, are comparable to symptoms of altered bowel frequency, such as diarrhea or constipation, observed in IBS in humans (61, 65). The effects of NMS on visceral sensitivity, a surrogate marker for abdominal pain, has also been well-documented. Because abdominal pain of the GI tract is a key symptom of stress-related GI disorders such as IBS (4), studying the effects of NMS on visceral sensation is highly relevant to human GI disorders. A number of investigations have demonstrated that rats subjected to NMS exhibit lasting visceral hypersensitivity, as determined by increased sensitivity (lower threshold) to colonic distention (42, 45, 54, 92, 159). Several papers have discussed the differences between males and females exposed to NMS, with the most prominent differences in HPA function, where females appear to have a more reactive HPA response (55). Rosztoczy et al (2003) demonstrated that females exhibited greater visceral hypersensitivity than males in response to two MS protocols (removal of all pups from home cage or separation of half the littermates) (159). The increased visceral hypersensitivity observed in female rodents is in line with the paradigm of human IBS where disease is more prevalent in females. However, there is a paucity of data concerning the mechanistic differences in the GI system between male and female rats exposed to NMS.

Further investigations into the mechanisms of NMS-induced visceral hypersensitivity in rodents, showed that mast cell degranulation, CRF receptor activation, transient receptor potential cation channel subfamily V member 1 (TRPV1) and increased intestinal permeability were central mechanisms to the visceral hypersensitivity observed in this model (163, 176). These mechanisms appear to be similar to those proposed in human IBS pathophysiology and thus mast cells, CRF receptors, and voltage-gated sodium channels are currently targets for drug development for human GI disorders associated with abdominal pain (4, 39)**.**

In addition to motility and visceral hypersensitivity changes induced by NMS, changes in neurochemical phenotype of enteric neurons have been reported. Gareau et al (2007) demonstrated that NMS resulted in increased numbers of cholinergic (choline acetyltransferase-positive) epithelial cells observed in 20-day-old weanling pups (82). Furthermore, the authors also reported that *ex vivo* application of muscarinic receptor antagonist, atropine, reduced the elevated permeability observed in intestinal tissues from NMS rats (82), indicating a functional role of cholinergic signaling in NMS-induced permeability changes. The role of cholinergic function in the early life stress GI disorders has not been further investigated in rodents, but could be a major target in human GI disease. Serotonin, a major neurotransmitter involved in motility, secretion, and visceral hypersensitivity, was shown to be elevated in the colon of NMS rats following water avoidance stress or colonic distention (25, 156). Increased serotonergic signaling observed in the NMS model has important implications to human IBS, in which dysregulated serotonin signaling is thought to play a significant role in GI symptoms (33). NMS rats were also shown to exhibit increased intestinal mucosal nerve fiber

density and synaptogenesis in the GI tract, which was prevented via the administration of an antibody against NGF (21). These findings are in line with Dothel et al. (2015) who showed increased nerve fiber outgrowth and NGF in adult human IBS biopsies (59).

Another consistent GI pathology observed in the NMS model is persistent elevations in GI permeability. Numerous investigations with the NMS model have demonstrated both transcellular and (or) paracellular permeability defects in adult rats that were previously subjected to NMS (20, 82, 83, 123, 166). Increased intestinal permeability observed in this model is highly relevant to human GI disorders as increased intestinal permeability is a well-established pathophysiology in diseases including as IBS, IBD and food allergy (35), and is linked with abdominal pain, inflammation, and disease susceptibility. The mechanism by which early life stress induces persistent defects in intestinal permeability in humans and animals remain to be fully elucidated; however, studies utilizing the NMS model have shown that activation of multiple signaling pathways are involved. CRF receptor signaling pathways have been shown to mediate NMS-induced intestinal permeability as peripheral administration of CRF receptor antagonists reduced intestinal permeability in NMS rats (82, 83, 166). In addition, as described previously, enteric muscarinic receptor blockade also inhibited intestinal permeability in NMS rats, suggesting an interplay between enteric cholinergic nerves and CRF receptors in regulating NMS-induced intestinal permeability.

There have been several investigations studying the impact of NMS on subsequent GI inflammatory and (or) psychological stress responses in later life. Barreau et al., (2006) have shown that rats exposed to NMS were more susceptible to infection by *Nippostrongylus brasiliensis* (19). Additionally, Barreau et al., (2004)

demonstrated that rats that were previously subjected to NMS exhibited increased colonic myeloperoxidase, mast cell numbers, and expression of cytokines (20). Furthermore, administration of TNBS induced higher inflammatory and intestinal permeability responses in NMS rats compared with normal-reared controls (20). Similarly, both adult rats and mice that were previously exposed to NMS exhibited worsened colitis induced by dextran sulphate sodium (DSS) (137, 180). In a study using NMS in WT mice and $IL10^{-/-}$ mice, Lennon et al. (2013) demonstrated that while NMS had no effects on colonic cytokine levels and colitis histologic scores in adult WT mice, $IL10^{-/-}$ adult mice exposed to NMS exhibited an early onset and increased severity of colonic cytokine levels (increased IL-12 p40 and IFN gamma mRNA) and colitis scores and intestinal permeability(123). It was also shown in this study that NMS and IL10 deficiency both contributed to increased intestinal permeability, in the absence of marked inflammation, suggesting that both IL10 deficiency and NMS were required to induce the onset of severe colitis. This is consistent with a “two-hit” theory (NMS and IL10 deficiency) that has been associated with human IBD, as well as other GI diseases. Findings from the NMS model and colitis susceptibility are in line with human evidence of early life adversity leading to increased chance of developing IBD in humans (1, 2, 66). Maternally separated animals also show greater reactivity to later life psychological stressors, such as water avoidance or restraint stress. In response to these acute stressors, animals subjected to NMS display increased visceral hypersensitivity (25, 45, 159, 163, 176, 177), increased intestinal short circuit current and macromolecular permeability (166), increased numbers of mast cells (176), increased motility (25, 163) and increased fecal output (45). While there is limited

human data in the responses of IBS patients to acute stressors, trials have shown that IBS patients show increased cortisol, gastrointestinal symptoms, and skin conductance in response to an acute stress test (110). Together, these studies highlight that NMS in rodents predisposes them to subsequent susceptibility to inflammatory and psychological stressors, leading to exacerbated intestinal injury.

As described previously, the microbiota undergoes significant development in early life and has emerged as a key player in a number of GI diseases in humans. A few investigations have shown that NMS in rodents alters the microbiota composition (18, 79, 80). Specifically, studies report decreased populations of lactobacillus species (80), and increased total numbers of adherent bacteria (80), with specific increases in clostridia, enterococci and *E. coli* species (18). Additionally, administration of probiotics was shown to reduce transcellular intestinal permeability and ion transport in NMS rodents (80).

Early weaning stress (EWS) porcine model

A large animal model that has emerged as a valid model to study early life stress-induced GI disease is the porcine EWS model. The EWS model is based upon the interruption of the natural weaning process with an abrupt and stressful early weaning. Weaning in mammals is defined as the transition from maternal breast milk to solid food or infant formula (99). In nature, weaning is a prolonged, gradual process as the nursing animal or infant transitions to food and social independence. In the EWS model, piglets are removed from their dam at an early age (15-18 d of age), compared with a gradual weaning over 3 months as in nature (11). Weaned piglets are moved to a nursery environment which encompasses a number of additional stressors including

psychosocial (e.g. maternal and sibling separation, transport and transition to a new environment, fighting and establishment of new social hierarchy) and immunological (e.g. exposure to new dietary antigens and pathogens) stressors. Moreover, weaning stressors in this model are occurring during a period of significant postnatal GI development as mentioned previously. Given the complex stressful events associated with early weaning, the EWS model provides a novel model of adverse early life events such as loss of caregiver, abandonment, or early, abrupt transition to formula or solid food. Furthermore, early weaning is a routine practice in animal agriculture systems; therefore, studying the mechanisms of EWS provides an opportunity to understand disease mechanisms and improve the health and well-being of agricultural animals.

Under normal, unstressed housing conditions, EWS pigs are generally healthy and exhibit growth rates similar to late weaned (> 23 d weaning age) control pigs. In response to weaning, both EWS pigs and late-weaned control pigs exhibit marked elevations in serum CRF and cortisol (139), indicating that, regardless of the age of weaning, the perceived stress is comparable. However, baseline GI pathophysiology and stress reactivity is markedly different between EWS pigs and controls. EWS pigs exhibit a chronic, relapsing functional diarrhea that persists into adulthood (manuscript in preparation). Compared with rodent models, the chronic relapsing diarrhea is a unique clinical feature in the EWS porcine model and is a relevant and translatable finding to human chronic stress diarrheal conditions such as diarrhea-predominant IBS. When faced with a later-life enteric pathogenic challenge (Enterotoxigenic *E. coli*), EWS pigs exhibit a more rapid onset and severity of diarrhea (135). In comparison, in humans, early or premature weaning (<4-6 mo of age) in infants was shown to be a risk

factor for developing subsequent gastroenteritis (64, 122). Therefore, the EWS pig could be a valid model of specific weaning-related GI disorders in humans. The mechanisms of diarrhea that is observed in the EWS model are not completely understood, but are likely due to heightened secretory mechanisms. Increased intestinal short circuit current (I_{sc}), a measure of net electrogenic ion transport has been observed in the EWS pig intestine, compared with unweaned or late-weaned controls (138, 165). The increased I_{sc} tone in the EWS intestine was shown to be inhibited by pharmacologic blockade of CRF receptors, mast cell degranulation, and enteric nerves (138, 139, 165) suggesting an interplay between the ENS and mast cells in this model.

Compared with the rodent NMS model, EWS in pigs induces both immediate (within 24 h) and long-lasting increases in intestinal permeability (138, 139, 165). Intestinal permeability in EWS pigs is associated with disruption in the expression and localization of tight junction proteins, including Occludin, Claudins, and ZO-1 (100, 148). Intestinal barrier defects in EWS pigs are most pronounced within 24 hours post-weaning but persist throughout life. Comparably, human infants weaned directly onto formula after parturition exhibited increased GI permeability compared with age-matched, breast-fed infants, and this increased permeability persisted for at least one week during the postnatal period(36, 188). Additionally, early weaning onto formula in infants has been correlated with increased risk for IBD and Celiac Disease (115, 169). The mechanisms for increased GI permeability and disease susceptibility in early weaned infants is not known, but could be similar mechanisms as defined in the EWS pig model (e.g. CRF and mast cell dependent pathways). Dietary factors (e.g. milk protein, fat, growth factors, etc.), immunologic stimuli (e.g. introduction to novel antigen)

and psychological factors likely also plays a role in intestinal permeability in EWS pigs and early-weaned infants. In the pig, diet (milk vs. cereal-based-diet)(27) and dietary soy antigens(125) have been demonstrated to influence intestinal inflammation and function in the newly-weaned pig, therefore, could also play a role in intestinal permeability disturbances observed at weaning.

A predominant histopathological feature of the EWS pig model is the presence of increased intestinal mast cell numbers and their active degranulation status (139, 165). As mentioned previously, mast cells are critical stress effector cells within the Brain-Gut axis and have been shown to be increased in many stress-related and allergic GI disorders. For example, IBS is associated with increased mucosal mast cell numbers and activated mast cells have been correlated with clinical symptoms of abdominal pain and diarrhea in humans (12-14). Mast cells are capable of releasing numerous pre-stored granule mediators (e.g. histamine, TNF, and proteases) as well as synthesized mediators (e.g. cytokines, chemokines, lipid-derived mediators). Released mast cell products can act upon numerous cell types triggering increases in secretion, permeability (epithelial and endothelial), recruitment of immune cells, and enteric nerve depolarization. The functional significance of heightened mast cell activity in the EWS pig model was demonstrated in studies by Smith et al. (2010) and Moeser et al. (2006) where intraperitoneal administration of the mast cell stabilizing agent, sodium cromolyn, to EWS pigs, reduced intestinal permeability in EWS pigs (139, 165), demonstrating that persistent mast cell activation is a central mechanism in intestinal permeability defects in the EWS model. Interestingly, despite the intestinal permeability defects and persistent mast cell activation observed in EWS pigs, histological lesions and

inflammatory responses are limited. This is in contrast to NMS in rats which have been shown to exhibit increased baseline inflammation(20). The lack of significant histological inflammation in the pigs, along with the significant functional and clinical GI abnormalities is in line with biopsy findings in human IBS. The precise mechanisms by which mast cells are regulated by EWS remain to be elucidated; however, EWS and mast cell-mediated intestinal permeability in the EWS porcine model was shown to involve activation of the intestinal CRF receptor system (138, 139, 145, 165). As with rodent models of early life stress and the human GI diseases discussed, the interplay between the ENS, CRF system and mast cells is clearly evident in the porcine EWS model. Together, this suggests that the ENS-CRF-mast cell axis represents a conserved pathophysiologic response to early life stress across different orders of species and different types of early life stressors. In addition to providing a model of early life stress/adversity, the EWS porcine model also provides a model to study the effects of weaning age and formula in infants on long-term GI development and later life health outcomes. In summary, EWS in pigs and humans involves multiple stressors, including psychological, immunological, and dietary stressors, which together induce GI injury during a highly developmental and plastic period in GI development (Figure 1). Given the similar pathophysiology and clinical GI outcomes induced by EWS in pigs compared with humans, the EWS pig model holds promise as a valuable translational model for human GI disease linked with early life adversity.

Translation consideration for animal models of early life stress-induced GI disease

While there are some common pathophysiology and mechanisms that mimic clinical GI disease states in humans, there are also inherent differences with regard to species and models that present advantages and(or) limitations within the context of translatability to humans. These advantages and limitations are discussed below.

Regardless of the early life stressor used in animal models of early life adversity (e.g. NMS or EWS), it is evident that there are common pathophysiologies conserved across species including increased intestinal permeability, altered ENS development, up-regulation of the CRF system, and mast cell activation (**Figure 1.2**). While it is essential that valid animal models possess similar mechanisms to human disease, the nature of the stress and comparative genetic and biological differences that exists across different animal models and species (compared with humans) is significant. Therefore, the species used in the model could have a significant role in the translatability and eventual therapeutic efficacy in humans. For example, rodent models employ stressors such as intermittent MS, water avoidance stress and restraint stress, which may not be directly relevant to the complexity of life stressors in humans. In the EWS pig model, piglets undergo significant psychosocial trauma as they are removed permanently from their mom and siblings and are forced to adapt to a new environment and social hierarchy with unfamiliar pigs. Therefore, the design of the EWS pig model may more closely resemble human conditions where children are forced to adapt to strenuous conditions without proper parental care.

In addition to the nature of the stress, species differences (genetic, anatomical, and clinical) present both strengths and limitations that should be considered in the selection of the model and the potential translational value. Rodent models have been, and will likely continue to be critical to the understanding of GI diseases associated with stress. Some obvious strengths of laboratory rodents are that techniques and reagents for genetic manipulation and molecular biology are readily available. There are a number of genetic animal models and approaches available (knockout, knock-in, transgenic, conditional knockdown, etc.) which are powerful tools to study the precise contribution *in vitro and vivo* role of specific mediators. However, genetic manipulation of large animal species such as the pig is now possible and becoming more common. Due to their small body size, rodent colonies are easy to manage and the inbreeding of rodent genetic lines reduces animal-to-animal variation in biologic responses. However, human populations are heterogeneous and variation between individual animals better reflects human disease conditions and provides a framework for personalized medicine. Based from the EWS studies and years of research using the pig as a biomedical model, the pig also is valid model for studying stress-related GI disease in humans. Moreover, the pig possesses distinct species-specific translational advantages compared with rodent. It is well-accepted that, compared to rodents, the GI anatomy, physiology, biochemistry, and evolution is more similar between humans and pigs (29, 94, 107, 189). At term, there are common GI developmental pathways shared by all species, but human and porcine neonatal intestine are considered to both be in advanced stages of development compared to rodents. Considering histological morphology and epithelial ontogeny, humans and swine have fully developed villi and

crypts in the small intestine at birth, whereas the rodent develops intestinal crypts in the post weaning period (53). In addition, as discussed above, the postnatal development of intestinal barrier function (decline in intestinal permeability) occurs largely in the first 7 days of life, compared with 14-21 d of life in mice. Therefore, with regards to the morphological ontogeny described above, and the delayed decline in intestinal permeability in mice, this suggests that during the first 7-14 days of life, the murine intestine is more comparable to a pre-term human intestine. This could have significant implications for interpreting clinical relevance of murine models of early life GI stress/injury that occur between 1-14 d of age (NMS) as the GI injury is induced during a relatively underdeveloped state compared with humans. Ontogeny studies of the ENS suggests that rodents, swine, and humans all have a semi-mature ENS in place at birth, which undergoes significant postnatal modification.(31, 78, 146, 190) However, the complexity of the humans ENS is more closely modeled in the porcine intestine than the rodent. For example, the human and porcine gut have additional inter-neuronal networks and plexi that are absent in the rodents (172). Furthermore, the co-localization of neurotransmitters to certain neuronal subtypes is more common between humans and pigs than humans and rodents (29). Considering the neuronal nature of psychological stress, the interconnection between the brain and the gut, and the ENS as a clinical drug target, the added complexity of the porcine ENS may more similarly mimic human stress induced gastrointestinal syndromes and better predict therapeutic efficacy in humans. Similar to the ENS, the neonatal immune system is present, but rudimentary in humans, swine, and rodents. Considering the mechanistic role played by the immune system in early life stress, it is important to note that the frequency of

preserved immunologically related genes between humans and pigs is nearly 80% while the number of orthologous immunological genes between mice and humans is only 6% (132). Immunologically, mast cells are a major component of gut-mediated stress responses, and play a major role in the weaning process in humans, swine, and rodents. Mast cell degranulation at weaning is similar across these three mammalian species; however, the mediators released from mast cells are species-dependent. For example, humans and pigs only have 17-18 genes in the Granzyme/Mast Cell Tryptase/Serine protease super family while mice have up to 26 genes in this family (47). Release of these additional and different proteases in rodents compared to humans and pigs may contribute to different mechanisms of pathology and disease. Clinically, in the EWS model, pigs develop a functional, relapsing diarrhea, which has not been reported in rodents. This unique clinical presentation for an animal model of early life stress mirrors stress-related diarrhea in humans can thus serve as clinical readout, especially when evaluation therapies directed at diarrhea symptoms.

Neurobiological comparison between humans, swine, and rodents reveals several differences between species; however, the HPA axis demonstrates a conserved neurological pathway for mediating homeostasis between the brain and the gut during stress across all species. The complexity of the human brain maybe better modeled in the pig as they both possess advanced gyrencephalic neuroanatomy (86). In line with the anatomy, several lines of behavioral evidence indicate that pigs are highly intelligent, and capable of performing several cognitive tasks. For example, pigs have been shown to be able to pass the ‘mirror test’ indicating that they are self-aware animals, a property which only primates and a handful of other mammals are known to

possess (85). Therefore, pigs likely have a more complex cognition of psychological stress, and may have a more complicated brain-gut integration of stress compared to rodents.

Finally, inherent differences in diet separate humans and pigs from mice because pigs and humans are naturally omnivores whereas rodents are naturally granivorous. This important distinction explains differences in metabolism and digestive biochemistry between rodents and humans, and these differences in digestion could influence developmental mechanisms and subsequent clinical outcomes.

Summary and Objectives

Stress is a major public health challenge due to its ubiquity and associated risk factors with many different chronic diseases. There is a clear association between the brain and the gut in stress induce GI disease as stress hormones clearly potentiate actions of the enteric cholinergic system, leading to GI dysfunction. Several other modes of stress negatively impact GI health, particular ELA as well as chronic stress in adulthood, and it is unclear if these stressors translate to GI dysfunction through similar mechanisms detailed in acute stress. Finally, given the central role of the cholinergic enteric nervous system in mediating acute stress, it is surprising to find that little is known about the impact of stress on expression of key cholinergic enzymes, transporters, and receptors. Understanding the expression of the cholinergic system under different modes of stress or in stress at different ages may better help to develop targeted therapies aimed at maintaining cholinergic homeostasis. The objective of this dissertation was to fill the knowledge gaps highlighted above.

In Chapter 2, I further characterized our large animal porcine model of early life adversity, early weaning stress. Functional gastrointestinal disorders, like IBS, are sex biased toward females and are commonly associated with underlying pathophysiology including increased intestinal permeability and an increase in mast cell number and mast cell activity. Additionally, there is a link that early life adversity predisposes females, more often than males, to IBS development later in life. Therefore, we hypothesized that early weaning stress would result in long term GI disease resembling the pathophysiology of IBS biased toward females. Here we demonstrate the long term impact of ELA on GI health, establishing the importance of further understanding underlying mechanisms which may be contributing to long term GI disease.

In Chapter 3, we focus on the immediate and long term impacts of ELA on GI health. Previously, we demonstrated the ELA leads to long term upregulation in expression of the cholinergic system, contributing to elevated ion secretion in adult animals. In Chapter 3, we seek to compare these results to a rodent model, and also determine if elevated cholinergic function contributes to other GI dysfunction observed in adults with prior exposure to ELA. Since the action of the cholinergic system induces secretion, permeability, and motility in acute stress responses, we hypothesized that the rodent model of early life adversity, NMS, would result in persistent GI disease mediated by hyperactivity of the enteric cholinergic nervous system.

In Chapter 4, we explore the impact of infectious enteritis on expression of the enteric cholinergic system. The goal in Chapter 4 was to determine if pathogen challenge induced enteric cholinergic dysregulation similarly observe during psychological stress. Additionally, we sought to determine if there was a relationship

between enteric cholinergic expression and status of GI inflammation, in order to outline an additional regulator role of the enteric cholinergic system. Our hypothesis was that pathogen challenge would result in upregulation of the cholinergic system and that this upregulation would positively correlate to the level of tissue injury.

In Chapter 5 we seek to determine the impact of chronic stress and depression on the enteric cholinergic system. Psychological disease is often associated with GI inflammation. Other groups have demonstrated that animal models of depression result in reduced cholinergic tone and increased pathology and inflammation in response to DSS colitis. Little is known about the colon's intrinsic expression of the cholinergic system during depression, so we sought to determine the impact of depression on this system. We hypothesized that a depressive phenotype would result in down regulation of cholinergic genes and subsequent down regulation in functional responses of this tissue to cholinergic stimulation. We also hypothesized that this down regulation may come from reduced expression of the cholinergic system in the ENS.

In summary, the work presented here demonstrates that mode and age at which stress occurs differentially impacts enteric cholinergic expression and function. Considering the central role of the enteric cholinergic system in mediating homeostatic responses to environmental challenges, the new knowledge generated here can be used to help develop more targeted therapeutics. Furthermore, these results serve as a foundation for future exploration into understand mediators that may regulate cholinergic expression, and how stress contributes to long and short term changes in cholinergic expression and function.

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CHAPTER 2

Early Weaning Stress Induces Chronic Functional Diarrhea, Intestinal Barrier Defects, and Increased Mast Cell Activity in a Porcine Model of Early Life Adversity.

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Abstract

Background: Early life adversity (ELA) is a risk factor for development of gastrointestinal disorders later in life. The underlying mechanisms through which ELA and sex interact to influence disease susceptibility remains poorly understood.

Methods: Utilizing a porcine early weaning stress (EWS) model to mimic ELA, we investigated the long-term effects of EWS on functional diarrhea, ileal permeability, mast cell activity and relationship to enteric ganglia.

Key Results: Juvenile and adult EWS pigs exhibited chronic, functional diarrhea (EWS 43.6% vs LWC 4.8%, $p<0.0001$), increased intestinal permeability (2 fold increase EWS vs LWC, $p<0.0001$), and mast cell numbers (at 7 weeks and 20 weeks ~1.6 fold increase EWS vs LWC, $p<0.05$). Compared with EWS male castrates (Male-C), females EWS pigs exhibited more frequent diarrhea (58.8% vs 29.9%, $p=0.0016$), and increased intestinal permeability (1-2 fold higher in EWS females, $p<0.001$). Increased mast cell numbers and their enhanced co-localization with neuronal ganglia were observed in both Male-C and female EWS pigs; however, female pigs exhibited greater release of mast cell tryptase upon activation with c48/80 (~1.5 fold increase, $p<0.05$), compared with Male-C pigs.

Conclusions and Inferences: These data demonstrate that pigs exposed to ELA exhibit increased vulnerability to functional diarrhea, intestinal permeability and mast cell activity. Further, these studies also showed that EWS female and Male-C pigs exhibited dimorphic responses to EWS with female piglets exhibited greater susceptibility and severity of diarrhea, intestinal permeability and mast cell tryptase release. Together, these findings mimic some of the key pathophysiologic findings in

human functional GI disorders (FGIDs) suggesting that the EWS porcine model could be a valuable preclinical translational model for FGID research associated with ELA.

Key Points:

- Early life adversity (ELA) is risk factor for functional gastrointestinal disorders (FGID) later in life.
- Using a porcine early weaning stress (EWS) model of ELA, female and Male-C pigs exposed to EWS exhibited a greater incidence of functional diarrhea, increased intestinal permeability, and increased mast cell numbers and localization to enteric ganglia.
- Compared with Male-C pigs, female pigs exhibited a more chronic and severe diarrhea, and heightened ileal permeability and mast cell tryptase release.
- Results here highlight the clinical and pathophysiologic responses to ELA in a large animal model which could be relevant to human FGID's

Introduction

Early life adversity (ELA) is a risk factor in the susceptibility to chronic gastrointestinal (GI) diseases later in life, including functional gastrointestinal disorders (FGIDs) such as irritable bowel syndrome (IBS).(10, 14, 15, 20) ELA encompasses many risk factors including physical, emotional, and sexual abuse, which are all known to contribute to development of FGIDs.(15) Further supporting the connection between early life experiences and adult GI health, patients with both FGIDs and a history of ELA experience increased GI symptom severity compared to patients with FGIDs lacking a history of ELA.(28)

The underlying mechanisms linking ELA with increased vulnerability to FGIDs such as IBS remain poorly understood. The primary underlying pathophysiology reported in IBS patients, particularly diarrheal predominant IBS (IBS-D), include increased gut epithelial permeability (21, 23, 45, 51, 53, 56) and increased intestinal mast cell numbers and degranulation.(5, 6, 17, 37, 38, 50, 61) Furthermore, the association of mast cells with enteric nerves has correlated with increased FGID symptom severity.(5, 19, 49) Increased mast cell numbers and their release of mediators such as histamine and proteases are thought to be responsible for increased intestinal permeability and a central mechanism in abdominal pain reported in IBS patients.(13, 30) Rodent models have been critical to understanding the GI pathophysiology induced by ELA; however, translation of this research to effective therapies in conditions such as human FGID has been slow and unpredictable.(25) In order to facilitate bench to bedside science for FGIDs, a new animal model may be necessary to compliment treatments researched and developed in rodents.

Pigs are recognized as an important translational model for humans, especially for GI research. The ontogeny and at birth developmental stage of the porcine intestinal epithelium, immune system, and enteric nervous system are more similar to human infants than neonatal mouse pups thus making pigs an ideal model to study ELA-associated diseases. (52) Early weaning stress (EWS) has been used previously by our group to mimic ELA in humans and to study the mechanisms of ELA-associated disease. Similar to cases of human early life adversity, EWS in piglets imposes significant psychosocial and environmental stress during a critical window of post-natal development. The stressors associated with EWS in pigs include the abrupt loss of

maternal care, littermate separation and commingling with unfamiliar pigs, changes in social status, and introduction to a novel unfamiliar environment. Previously, we have shown that EWS induces activation of the hypothalamic-pituitary-adrenal (HPA) axis, upregulation of intestinal corticotropin releasing factor (CRF), and increased mast cell numbers and activation leading to elevated intestinal permeability (43, 44, 58) in pigs at least 9 weeks old. While previous studies have focused on early intestinal barrier responses to EWS up to juvenile stages, the long-term effects of EWS on clinical disease, intestinal permeability, and mast cell activity and localization, and potential sex differences have not been investigated. The objective of the present experiment was to define the influence of EWS on the incidence of diarrhea, intestinal permeability, and intestinal mast cell numbers, activity, and localization in female and Male-C pigs.

Methods

Animals

The University Institutional Animal Care and Use Committee (IACUC) approved all studies (Protocol #09-047-B). Animal protocols were performed as in our previous studies.(41) Yorkshire-duroc cross, female and Male-C piglets (castrated at 9 days of age) were weaned from their sow at 15 days of age (EWS) or 28 days of age (LWC). Weaned pigs were housed in pens with Tenderfoot® flooring (3.7 m x 2.7 m, n = 6 pigs per pen) in the same environmentally controlled room. All pigs were offered *ad libitum* access to the same diet and water. The diets were formulated to meet or exceed the nutrient requirements of all pigs in the study.(46) Upon arrival, all pigs were evaluated by a licensed veterinarian and no clinical evidence consistent with common enteric

diseases (e.g. reduced feed intake, depressed activity, hypothermia/huddling) were observed.

At 7 and 20 weeks of age, representing juvenile and adulthood stages, respectively, n = 12 pigs/weaning age group (6 females, 6 Male-C) were euthanized via captive bolt penetration and intestinal tissues were immediately harvested for experiments, and prepared for Ussing chambers analysis or stored at -80°C for subsequent biochemical analyses.

Fecal Scoring

Fecal scores were recorded on 12 early weaned and 12 late weaned pigs (equal number of Male-C and females between groups) for a 4 week period between 16-20 weeks of age at 1500-1700 h. To evaluate stool form, pigs were rectally palpated every 2-3 days at the same time of day to stimulate defecation. The resulting bowel movements from each pig following rectal palpation were scored by a trained individual blinded to experimental treatments using The Bristol Stool Form Scale. Scores ≥6 were considered diarrhea based on the description of the scoring system and what has been used to determine diarrhea in humans (48). The percentage of days in diarrhea were calculated by counting the number of times a pig was scored with ≥6 divided by the total number of days scored. Bowel movements rated as 7 on the Bristol Stool Form Scale were considered severe diarrhea.

Histology evaluation of intestinal tissues

Distal ileum sections were collected at euthanasia and placed directly in 10% neutral buffered formalin. After 24 hours, samples were removed and placed in 70% ethanol for long-term storage. Transverse sections of ileum were embedded in paraffin

for and stained with hematoxylin and eosin. A board certified veterinary pathologist (KJW) read the slides (n=6 for each weaning age group and balanced by sex) to evaluate differences in inflammatory cell infiltrate or epithelial cell morphology between EWS and LWC pigs.

Mast Cell Staining and Counting

Ileum was collected from EWS and LWC pigs 7 at 20 weeks of age and fixed in neutral buffered formalin and processed as mentioned above. Slides were immunohistochemically labeled by Michigan State University's Investigative Histopathology Laboratory (East Lansing, MI) with Mast Cell Tryptase Antibody (FL-275) (sc-32889, Santa Cruz Biotechnology, Dallas, TX) at 1:300 dilution. Detection of tryptase was performed using secondary anti-rabbit-on-Farma HRP-Polymer for 30min at RT. Toluidine blue staining was performed on 4% PFA fixed sections that had been embedded in Tissue Tek OCT compound. 10 µm sections were stained in 0.5% Toluidine blue at 0.5 pH for 30 min. Mucosal mast cells were counted in 10 random fields per subject and corrected for lamina propria area using ImageJ (U.S. NIH, Bethesda, MD). Submucosal (SMP) and myenteric plexi (MP) were identified by morphology (collection of cells with large nucleus and large nucleolus) and confirmed with S100 immunohistochemical labeling. Plexus-associated mast cells were defined as mast cells that were adjacent to enteric plexi without any other cell or cell structure in between. For the SMP counts, the outer and inner SMP of the pig were included in the total SMP counts. Counts were performed on *n*=12 EWS and LWC pigs with 5-6 Male-C and females per group (for tryptase staining) and confirmed on n=6 early and late

weaned pigs with 3 males and 3 females per group with Toluidine ble staining. Images and quantification was performed by an individual blinded to experimental treatments.

Ileal mucosal permeability measurements on Ussing Chambers

Ileum was harvested from each animal at 7 and 20 weeks of age. Following euthanasia, ileal segments were opened lengthwise along the anti-mesenteric border. In oxygenated (95% O_2 , 5% CO_2) porcine ringer solution (154 Na^+ mM, 6.3 K^+ mM, 137 Cl^- mM, 0.3 H_2PO_3 mM, 1.2 Ca^{2+} mM, 0.7 Mg^{2+} mM, 24 HCO_3^- at pH 7.4) at 37°C, the seromuscular layer was removed from the tissue by blunt dissection. Tissue free of Peyer's patches was then mounted in a 0.3cm^2 aperture on Ussing chambers (Physiologic Instruments, Inc., Sand Diego, CA or World Precision Instruments, Sarasota, FL) as described in previous studies.(41, 57) The tissue was bathed in porcine ringer's solution on each side of the tissue. The serosal bathing solution contained 10 mM glucose that was balanced with 10 mM mannitol on the mucosal side. Bathing solutions were oxygenated (95% O_2 , 5% CO_2) and maintained at 37°C. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Tissues were maintained in the short-circuited state, except for brief intervals to record the open-circuit PD. Transepithelial electrical resistance (TER; Ω•cm^2) was calculated from the spontaneous PD and short-circuit current (I_{sc}). After a 30-min equilibration period on Ussing chambers, TER was recorded at 1-min intervals over a 60-minute period and then averaged to derive the basal TER values for a given animal.

Mucosal-to-serosal fluxes of FITC Dextran and ^{3}H-labeled mannitol

Permeability studies were performed as described in previous experiments.(31, 57) Mucosal-to-serosal fluxes of (FITC)-dextran 4kDa (FD4) and ^{3}H mannitol (180 Da) (Sigma, St. Louis, MO) were used to assess ileal permeability. After tissue was equilibrated for 15 minutes on Ussing chambers, 0.25mM FD4 and 0.2 mCi ml^{-1} of ^{3}H mannitol were added to the mucosal side of Ussing chamber-mounted tissues. FD4 flux was run on Physiologic Instruments Ussing Chambers and radioisotope flux was performed on WPI Ussing Chamber system. The probes were allowed to equilibrate for 15 min after which standards were taken from the mucosal and serosal side of each chamber and a 90-min flux period was established by taking 50 µl samples for FD4 and 500 µl samples for ^{3}H mannitol from the serosal compartment at the beginning and end of the 90-min flux period. Presence of FD4 was measuring using Ex/Em readings at 488/525 on a fluorescent plate reader. The presence of and ^{3}H was established by measuring β-emission in a liquid-scintillation counter (model 1219 Rack Beta, LKB Wallac, Perkin Elmer Life and Analytical Sciences, Boston, MA). Unidirectional FD4 and ^{3}H mannitol mucosal-to-serosal fluxes were evaluated by determining the net appearance of and FD4 and ^{3}H over time in the serosal bathing solution on a chamber over time and presented as µmol (cm^{2}) $^{-1}$ h^{-1} unit area basis. Data are presented as the Fold change in EWS pigs relative to their respective, sex matched LWC pigs, such that EWS Male-C paracellular probe flux was measured relative to LWC Male-C and EWS females paracellular probe flux was measured relative to LWC female.

Ex vivo ileal mast cell activation experiments

Mast cell activation experiments were performed with ileal mucosal explants from 20 week old EWS and LWC pigs Ileum. Tissues were pinned out and stripped of seromuscular layer as in Ussing chamber experiment. An approximately, 1cm x 3cm strip of mucosa was cut from an area free of Peyer's patch and placed in 3mL room air bubble RPMI and allowed to equilibrate for 15 minutes. At 15 minutes, 10ug mL^{-1} of the mast cell degranulating drug c48/80 (Sigma Aldrich, St. Louis, MO) was added, and the samples were allowed to incubate for 30 minutes. Tissues were removed, blotted dry, and weighed. Supernatants were set on ice and spun at 300xg at 4^{o}C for 15 minutes. Supernatant was aliquoted and stored at -70^{o}C, and sample were thawed on ice prior to tryptase activity quantification. Tryptase activity was quantified using a kinetic assay with a thiobenzyl ester substrate, and activity was standardized based on tissue weight.(27)

Clinical pathology and enteric infectious disease panel

Following euthanasia, ileum and colon samples were collected from pigs at 20 weeks and flash frozen on liquid nitrogen and stored at -80^{o}C. Three EWS female and two LWC females samples of ileum and colon were packaged on dry ice and shipped to the Veterinary Diagnostic Laboratory at Iowa State University's College of Veterinary Medicine in Ames, Iowa to screen for diarrheal enteric pathogens, including *Brachyspira hampsonii, Brachyspira hyodysenteriae, Lawsonia intracellularis,* and *Salmonella spp* using qPCR. Samples were considered negative if the cycle threshold exceeded 40.

Statistical Analysis

Data was analyzed with a Two Way ANOVA with post hoc Fisher's Least Significant Difference test where appropriate to determine effects of weaning, sex, or interaction and any specific difference between groups using GraphPad Prism version 6 for Windows, (GraphPad Software, San Diego CA USA). Fecal Scoring data was analyzed using SAS University Edition for Windows, SAS Institute, Cary NC USA, and a generalized linear mixed model with logistical regression and least square means was employed to determine interactions between weaning and sex.

Results

Early weaned pigs exhibit chronic, relapsing functional diarrhea

In the present study we used the Bristol Stool Form Scale to assess the consistency of stool produced by pigs. Our use of the Bristol Stool Form Scale was not to directly compare the nature of pig feces with that of human feces, but to provide a more detailed description and comparison of the stools produced by EWS and LW pigs. Analysis of fecal scores conducted over 4-week period, showed that EWS pigs exhibiting diarrhea 43.6%±4.0% of the time whereas LWS pigs exhibited diarrhea 4.80%±1.7% of the time (**Figure 2.1a)** (*p<0.0001*). When combining LWC and EWS data, there were no statistical differences in fecal scores between Female and Male-C groups (All Male-C, 14.4%±3.2%, All Females 18.8%±4.8%, *p=0.441*, **(Figure 2.1B***)* indicating that there were no inherent differences in fecal scores between Male-C and females pigs. However, 2-Way ANOVA analysis revealed that EWS female pigs exhibited diarrhea more frequently compared with EWS Male-C pigs (EWS-female, 58.8% ± 5.31% vs. EWS-Male-C 29.9% ± 5.0%, *p=0.0016*, **Figure 2.1C**). In addition,

EWS female pigs had significantly more days with fecal scores equal to 7 (extremely liquid diarrhea), compared to EWS Male-C pigs (**Figure 2.1D**). Representative bi-daily fecal scores for EWS Male-C pigs (**Figure 2.1E)** and females (**Figure 2.1F)** showed the episodic nature of fecal score patterns. Together these data demonstrated that while female and Male-C pigs both exhibited increased fecal scores in response to EWS, EWS female pigs exhibited more frequent and severe diarrhea compared with EWS Male-C pigs.

Histopathological analysis of ileal and colonic mucosa evaluated by a board certified veterinary pathologist (KJW) blinded to experimental groups, revealed no histopathological differences associated with early weaning or sex with regards to lamina propria cellularity (inflammation), presence of epithelial abnormalities, or edema. (**Figure 2.1G-J**). Intestinal samples were determined to be negative (CT values >40), for major porcine bacterial enteric pathogens (*Brachyspira hyodysenteriae, Brachyspira hampsonii, Salmonella spp,* and *Lawsonia intracellularis) (***Supplemental Table 2.1)**. No differences in rectal temperature, or peripheral blood leukocyte counts as determined by complete blood count analysis were detected between the groups (**Supplemental Figure 2.1**). Together, these findings provide evidence that the diarrhea in EWS pigs was functional in nature rather than from infectious or inflammatory causes.

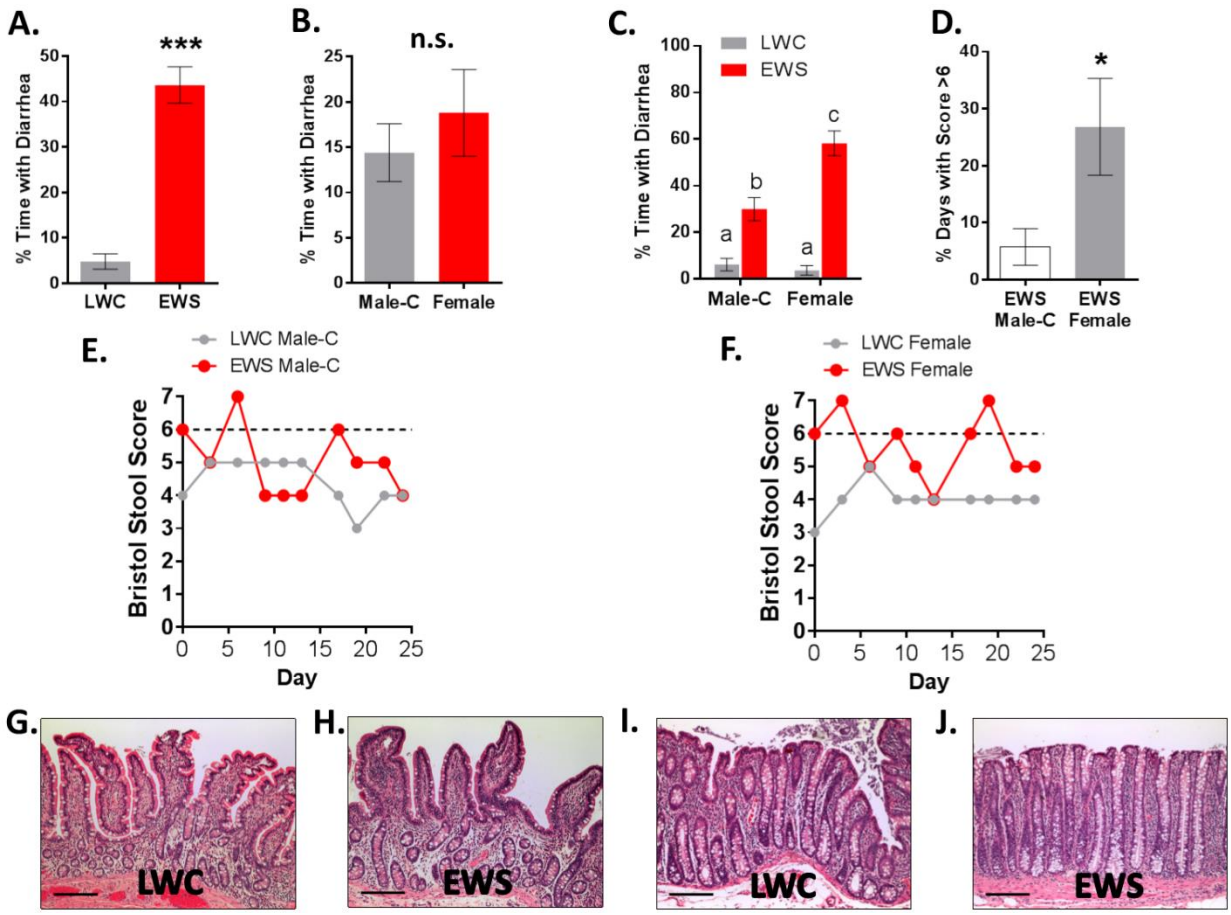

Figure 2.1. Influence of early weaning stress in pigs on diarrhea frequency and intestinal histopathology. Stool scores were conducted in pigs between 16-20 weeks of age using the Bristol Stool Scale Scoring System. A) % of days with diarrhea (stool score ≥ 6) in LWC and EWS pigs, B) Comparison of Male-C and female pigs (combined LWC and EWS pig data) C) % of days with diarrhea for Male-C and female pigs; D) % of days with severe diarrhea (stool score ≥ 7) in EWS Male-C and females. E&F) Representative Bristol Stool Form Scores over a 25-d period (dotted line indicating threshold for diarrhea). G-H) Representative H&E stained sections of ileum mucosa. I-J) Representative H&E stained sections of colonic mucosa. Scale bar = 100 µM. Data (A-D) are means ± standard error. Student's T-test (A,B, and D) *** $p<0.001$, * $p<0.05$; Letters "a, b, c" (Panel C) indicate statistical significance between groups by Two way ANOVA with Fisher's LSD post hoc test. LWC = Late Weaned Control, EWS = Early Weaning Stress, Male-C = male castrated pigs. LWC=Late.

EWS induces heightened and persistent elevations in ileal permeability in female pigs compared with Male-C pigs

In the present study, both Male-C and female EWS pigs exhibited increased ileal permeability (measured as increases in FD4 flux rate) relative to LWC pigs at 7 weeks

of age. Female EWS pigs exhibited significantly greater FD4 flux rates (2 fold increase over female LWC) compared with Male-C pigs (1.5 fold increase over Male-C LWC) (*p<0.0001*; **Figure 2.2A).** At 20 weeks of age, there were no significant differences between EWS and LWC pigs with regards to FD4 flux rate (**Figure 2.2B**); however, measurements using the smaller paracellular probe, ^{3}H mannitol, demonstrated an increased ileal permeability in female EWS pigs but not Male-C pigs (*p<0.05*; **Figure 2.2C).** Transepithelial electrical resistance (TER), a measurement predominantly reflecting paracellular ion permeability, showed that EWS pigs exhibited reduced TER at 7 weeks of age (*p=0.018)* compared with LWC pigs; with a significant difference between EWS Male-C and LWC Male-C. (**Figure 2.2D**). While TER differences between LWC and EWS pigs were not different at 20 weeks of age (2-WAY ANOVA, *p=0.15*), Female EWS pigs exhibited a lower ileal TER compared with Male-C EWS pigs (**Figure 2.2E**).

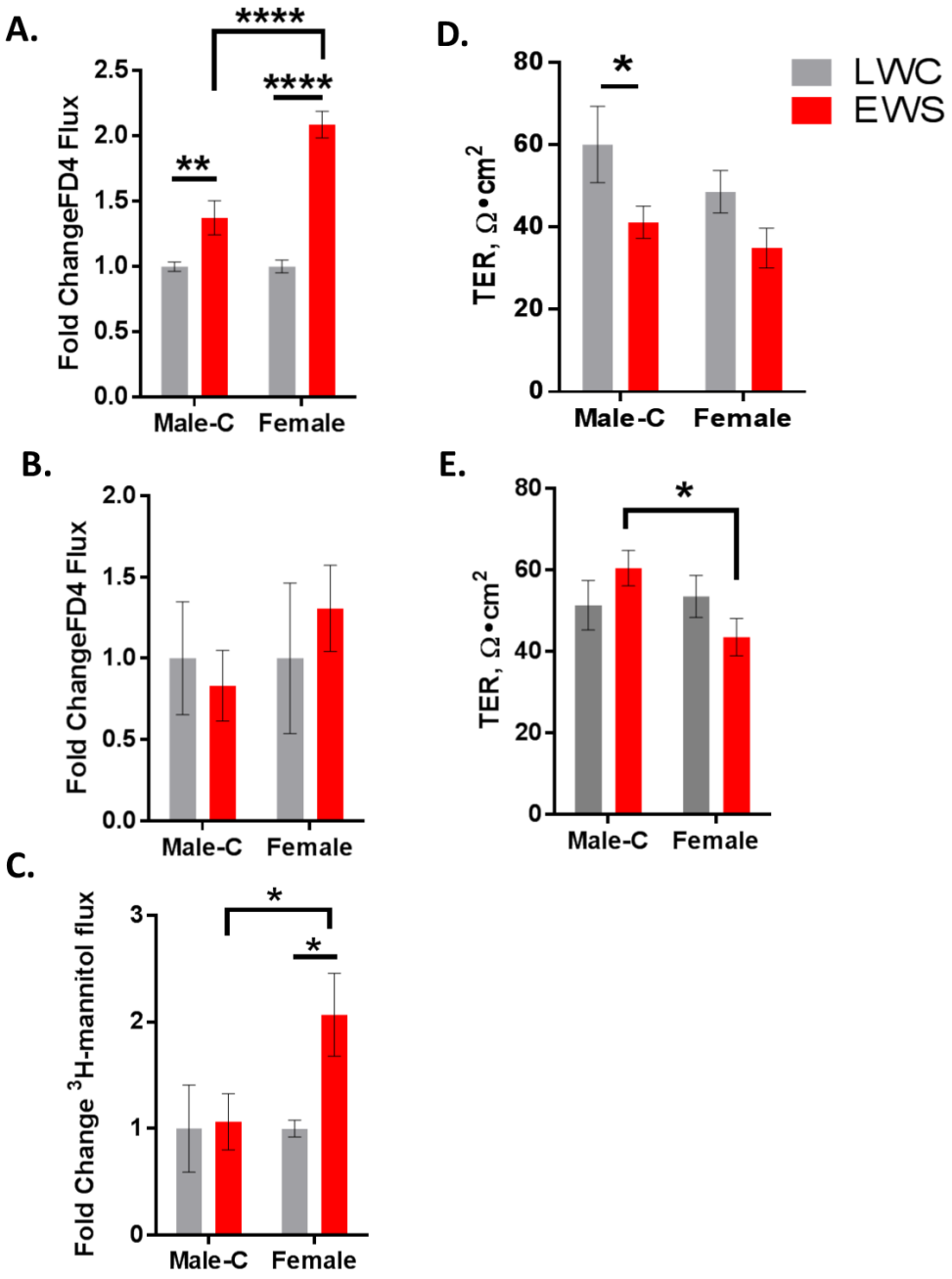

Figure 2.2. Influence of early weaning stress in pigs on ileal permeability. Ileal mucosa from pigs were mounted on Ussing Chambers for evaluation of permeability. A) FD4 flux in 7 week old Male-C and female pigs, data presented as fold change relative to respective to LWC control. B) FD4 flux in 20 week old Male-C and female pigs, C) ^{3}H-mannitol flux in 20 week old Male-C and female pigs. D) Ileal transepithelial resistance (TER) in 7 week old Male-C and female pigs. E) Ileal TER in 20 week old Male-C and female pigs. Data presented are means ± standard error (n=6/experimental group). Two way ANOVA with Fisher's LSD post hoc test for panels A-E. ****p<0.0001, **p<0.01,*p<0.05. LWC=Late Weaned Control. EWS=Early Weaning Stress, Male-C = male castrated pigs.

EWS induces increased ileal and colonic mast cell numbers and tryptase release

We examined the ileum mucosa of both LWC and EWS pigs at 7 and 20 weeks of age for numbers of tryptase-positive mast cells. A significant increase in tryptase-positive mast cells was observed in the ileal mucosa of EWS pigs, compared with LWC pigs at both 7 (**Figure 2.3A, 2.3Aii-2.3Aiii**; by 1.8 fold) and 20 weeks (**Figure 2.3B, 2.3Bii-2.3Biii** by 1.6 fold) of age. Comparisons between Male-C pigs and Female groups revealed that EWS Male-C pigs had a greater number of tryptase-positive mast cells relative to respective LWC controls while no differences were observed between LWC and EWS Female pigs (**Figure 2.3Ai and 2.3Bi**). Because tryptase is an intracellular MC mediator which the cellular composition can be influence by activation or regulation by external stimuli such as LPS,(29) we wanted to confirm increased mast cell numbers in EWS pigs using Toluidine blue (T. blue) staining which identifies all mast cell granules. In line with tryptase cell counts, the number of T. blue-positive mast cells was elevated in EWS pig mucosa, with a significant difference between EWS Male-C and LWC Male-C pigs (**Fig 2.3C-Ci**). A significant increase in mast cell numbers in colonic mucosa of EWS pigs was also observed at 7 weeks of age Male-C EWS pigs exhibiting the highest tryptase-positive cell counts (**Fig 2.3D**, **Di.**). At 20 weeks of age, there was a trend (*p=0.08*) for higher numbers of colonic mast cells in EWS pigs (**Supplemental Figure 2.2**).

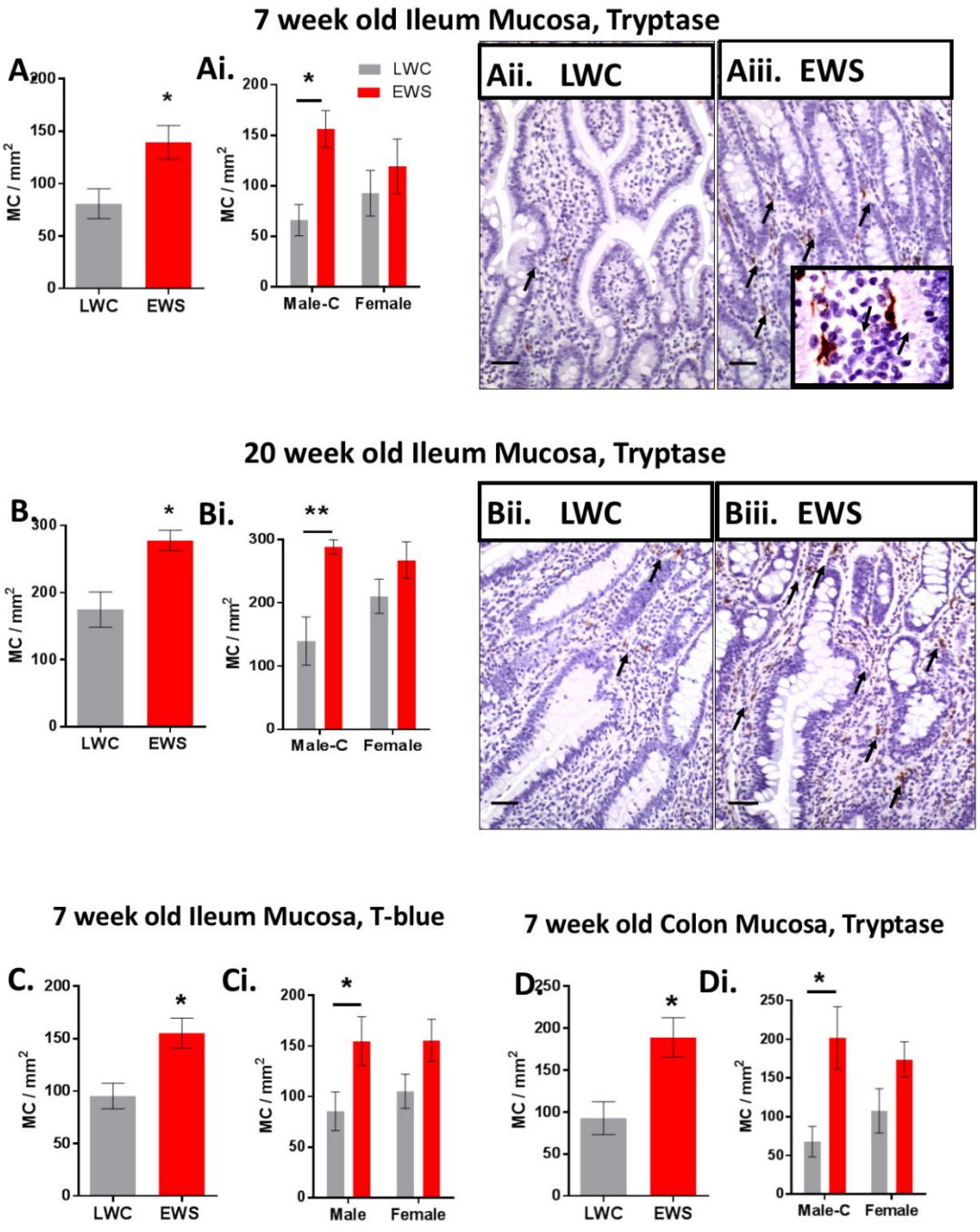

Figure 2.3. Influence of early weaning stress in pigs on ileal and colonic mast cell numbers. Ileum sections from pigs were stained for mast cell tryptase and tryptase stained cells were quantified to determine mast cell numbers. A) Ileal mast cell (MC) numbers in 7 week old LWC and EWS pigs. Ai) Ileal mast cell numbers in 7 week old Male-C and female pigs. Aii) LWC ileum mucosa and Aiii) EWS ileum mucosa (inset is 100x image demonstrating tryptase-positive mast cell morphology). Arrows indicate red stained tryptase positive mast cells. B) Ileal mast cell numbers in 20 week old pigs. Bi) Ileal mast cell numbers 20 week old Male-C and female pigs. Bii) LWC ileum mucosa

Figure 2.3. (cont'd)
and Biii) EWS ileum mucosa. Arrows indicate red staining mast cells. C) Ileal mast cell numbers determined by Toluidine blue staining in 7 week old pigs. Ci) Ileal mast cell numbers determined by Toluidine blue staining in 7 week old Male-C and female pigs. D) Colonic mast cell numbers determined in 7 week old pigs. Di) Colonic mast cell numbers in 7 week old Male-C and female pigs. Data presented are means ± standard error (n=6/experimental group) Two way ANOVA with Fisher's LSD post hoc test. **p<0.01,*p<0.05. LWC=Late Weaned Control. EWS=Early Weaning Stress, Male-C = male castrated pigs. All images 20x magnification. Scale bar = 50 µm. LWC=Late Weaned Controls. EWS=Early Weaning Stress.

Previously in juvenile (9 weeks of age) EWS pigs, we demonstrated that mast cell activity and protease expression were increased in EWS and contributed to increased intestinal permeability (57) To gain further insight into the chronicity of this response, we conducted *ex vivo* mast cell stimulation experiments with EWS and LWC ileum (20 week old pigs) and measured tryptase release in supernatants. Stimulation of ileal explants with the mast cell secretagogue, c48/80 induced a release of tryptase which tended to be higher in EWS pigs compared to later weaned controls *(p=0.09)* (**Figure 2.4A**). Comparison between Male-C and Female groups revealed that Female EWS ileum exhibited a greater (by ~ 1.6-Fold) release of tryptase compared with Female LWC and Male-C pigs (**Figure 2.4B**). Given that intestinal mast cell numbers were similar between female and Male-C EWS pigs, these data suggest that Female pigs exposed to EWS released more tryptase upon activation.

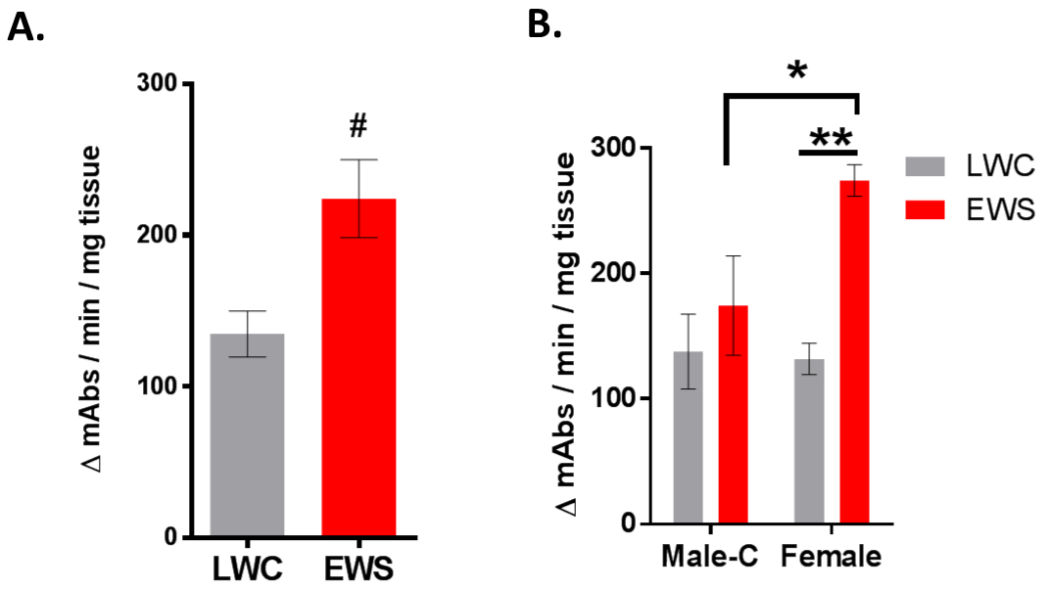

Figure 2.4. Influence of early weaning stress on ileal mast cell tryptase release. Ileal mucosal explants from 20 week old pigs were treated with secretagogue c48/80 and tryptase was measured in the supernatant via a substrate-based tryptase activity assay. A) EWS pigs tend to release more mast cell tryptase when treated with c48/80. B) EWS female ileum sections released more mast cell tryptase into supernatants compared LWC females and EWS Male-C pigs. Two Way ANOVA with Fisher's LSD post hoc test (*p<0.01); (**p<0.005). LWC=Late Weaned Controls. EWS=Early Weaning Stress, Male-C = male castrated pigs

Mast cells infiltrate enteric nerve ganglia in EWS pigs

Previous studies in IBS patients have reported mast cells to be in increased association with the peripheral enteric nervous system.(5, 19) Additionally, in other FGIDs such as functional dyspepsia, mast cells have been found in have increased associations with submucosal plexus.(16) Therefore, we next examined whether EWS in pigs increased the number of intestinal mast cells associated with the SMP and MP. At the 7 week time point, ileum from EWS pigs exhibited increased numbers of tryptase-positive mast cells (**Fig 2.5A-Aii.**) and T-blue mast cells (**Figure 2.5B-Bii).** Increased tryptase positive mast cell numbers in the MP were also found in EWS pigs compared

to LWC pigs (**Fig 2.5C-Cii).** The increased association of mast cells with enteric plexi was found in both Female and Male-C (data not shown).

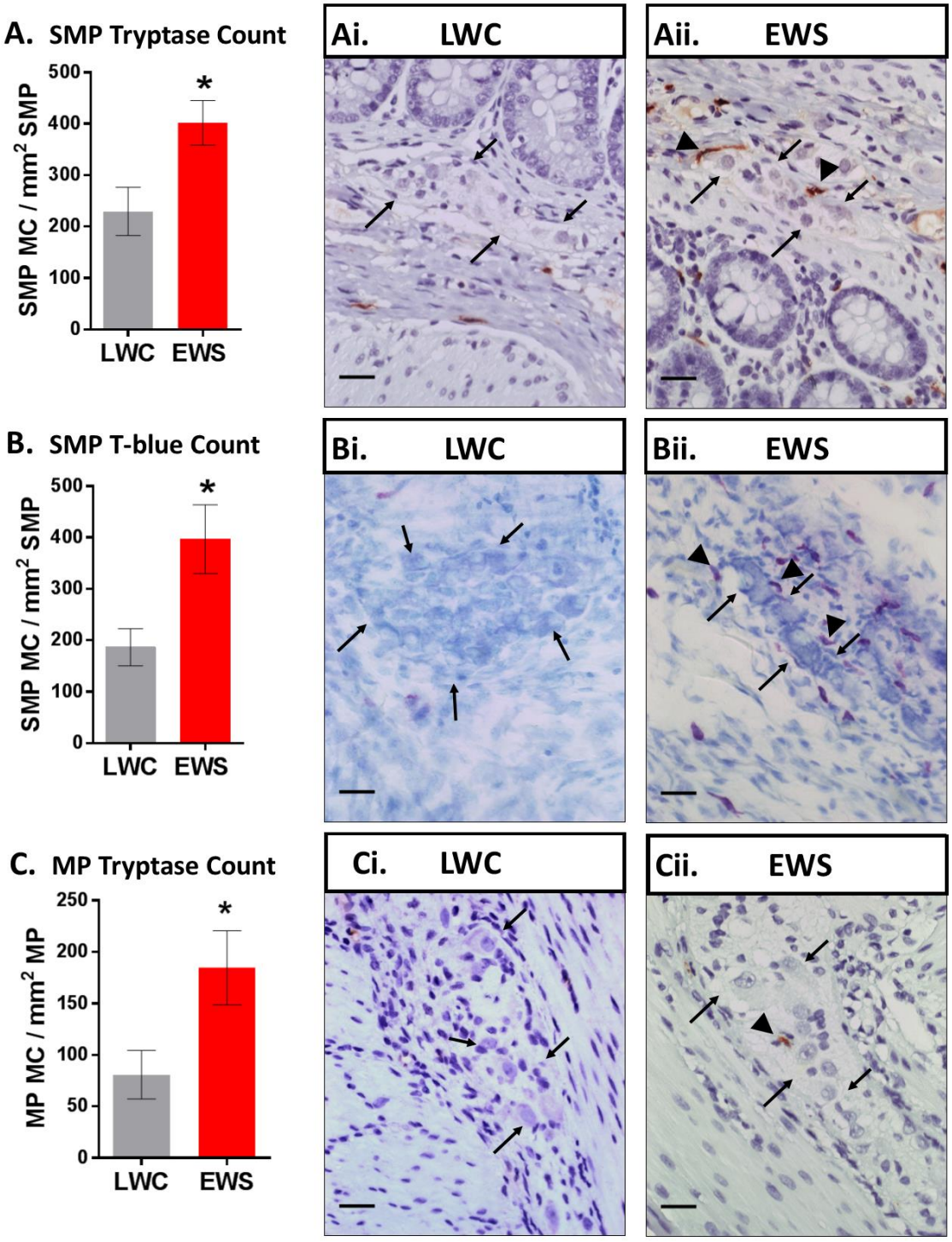

Figure 2.5. Influence of early weaning stress on the numbers of enteric ganglia-associated mast cells in pigs. Ileal mucosa/submucosa segments from 7 week old

Figure 2.5. (cont'd)
EWS and LWC pigs (Male-C and female pigs combined within each weaning experimental group) were stained for mast cell tryptase or T-blue and the number of mast cells (MC) co-localized with neuronal ganglia in the submucosal plexus (SMP) and myenteric plexus (MP) were quantified. A) SMP-associated MCs (tryptase-positive) in LWC and EWS pigs Ai) LWC ileum SMP tryptase stained, Aii) EWS ileum SMP tryptase stained. B) SMP-associated MCs (T.blue stained) in LWC and EWS pigs. Bi) LWC ileum SMP, T-blue stained. Bii) EWS ileum SMP, T-blue stained. C) MP-associated MCs (tryptase-positive) in LWC and EWS pigs Ci) LWC ileum MP tryptase stained, Cii) EWS ileum MP tryptase stained. B) SMP-associated MCs (T.blue stained) in LWC and EWS pigs. Images are 40x. Scale bar = 25 μm. Black arrows indicated periphery of ganglia. Black arrowheads identify mast cells. Data (A-C) are means ± standard error. Students T-test (* $p<0.05$) LWC=Late Weaned Controls. EWS=Early Weaning Stress.

Discussion

Early life adversity and environmental challenges during postnatal development represent a significant risk factor for chronic GI diseases in adult life (14, 15, 20); however, the mechanisms remain poorly understood. Here we show that, ELA induced by EWS in piglets, induces lasting disturbances in ileal barrier function, mast cell numbers, mast cell-nerve association, and functional diarrhea. This study also revealed that female pigs exposed to EWS exhibited a higher incidence and severity of diarrhea, heightened and persistent illeal barrier defects, and increased mast cell tryptase release, compared with Male-C pigs.

ELA in piglets induces chronic, relapsing functional diarrhea

In the present study, piglets exposed to ELA through EWS developed a chronic, relapsing functional diarrhea that persisted into adulthood. The frequency of diarrhea in the EWS pigs was high, as male and female pigs had chronic relapsing diarrhea approximately 40% of the time. Moreover, EWS female pigs presented more frequently with severe diarrhea, compared with Male-C pigs. The pathophysiologic mechanisms driving the diarrhea in EWS are not completely understood. In line with the present

findings, we have previously demonstrated that EWS pigs exhibited increased diarrheal disease in response to a live oral challenge with enterotoxigenic *E. coli*. (40) In addition, more recently we showed that EWS pigs exhibited a persistent, heightened sensitivity to neural-evoked secretomotor responses mediated largely by an upregulated cholinergic nervous system (41) that was more severe in EWS female pigs compared with Male-C pigs. Additional contributing factors contributing to diarrhea in EWS pigs could be the heightened excitability of intestinal mast cells. Mast cell activation and release of mediators including histamine and proteases can activate enteric secretomotor neurons (8) and (or) induces intestinal barrier defects (24, 47) as observed in EWS pigs leading to enhanced intestinal secretion and diarrhea. Together, findings in the present study and our previous studies suggest that EWS leads to developmental alterations in the ENS (e.g. upregulated cholinergic system) and immune system (e.g. mast cell activation) leading to a hypersensitivity to intestinal secretory stimuli which in turn could manifest as chronic functional diarrhea or heightened sensitivity to later life stress.

While the link between ELA and chronic diarrheal disease in humans is poorly defined, FGID's associated with ELA such as IBS exhibit chronic alterations in bowel habits. As stated above, both Male-C and female EWS pigs exhibited diarrhea but females exhibited more frequent bouts and severe diarrhea compared with Male-C pigs. In human IBS, men report more diarrhea predominant IBS (IBS-D), while females more commonly present with constipation-predominant (IBS-C) diarrhea. (1). However, women reporting childhood abuse were equally at risk for developing adult IBS-D or IBS-C.(39) Therefore the timing and nature of the stress could potentially dictate whether an individual will develop diarrhea vs constipation. Currently, the IBS literature

is ambiguous on how the age at which abuse occurs, type of abuse, severity of abuse, and duration or times of abuse influence the development of one bowel habit over the other (IBS-D vs IBS-C). It seems plausible that some combination of abuse events and environmental factors may influence development of one IBS subtype over another. Therefore, EWS female pigs could more closely resemble a subset of IBS patients who have experienced ELA event.

ELA in piglets induces lasting increases in ileal permeability in female pigs

Intestinal barrier defects marked by increased intestinal permeability is a central pathophysiologic mechanism of GI disease and results in increased translocation of novel food and bacteria antigens. (12, 22, 60) Subsequent exposure of antigens to the immune and enteric nervous systems can initiate mucosal inflammation, neuronal hypersensitization and clinical symptoms of abdominal pain and altered bowel habits as seen in IBS (9). In the present study, Male-C and female pigs in the LWC group exhibited a similar ileal permeability suggesting no inherent differences between Male-C and female pigs. Male-C and female pigs subjected to EWS exhibited increased ileal permeability relative to their LWC controls in the adolescent time point (7 weeks of age); however, the severity of the permeability defect in EWS pigs was greater in female pigs at 7 and 20 weeks. In comparison with the present study, human females tend to have increased intestinal permeability when exposed to acute stressors.(2, 3) In line with these findings, our previous studies in mice showed that, compared with adult intact males, female mice exhibited greater ileal permeability in response to acute restraint stress. (36) As the pigs aged and approached sexual maturity in the present study, EWS females continued to exhibit heightened ileal permeability while Male-C EWS pig

did not. These data suggest that Male-C pigs may have recovered from EWS-induced permeability disturbances while female EWS pigs did not. The reason for the persistence in ileal permeability defects in EWS females but not Male-C pigs is unclear but may be related to the increased mast cell activity (increased mast cell tryptase release) observed in female EWS pigs, compared with Male-C pigs. We and others have shown previously that mast cell proteases such as tryptase and chymase induce intestinal permeability (24, 47). Also in line with the current findings, our recent studies showed that despite having similar numbers of tissue mast cells, female mice released more mast cell mediators in response to psychological restraint stress and passive systemic anaphylaxis and exhibited increased intestinal permeability compared with intact male mice.(36) Another potential factor contributing to persistent ileal permeability in EWS females could be the increased cholinergic tone and acetylcholine release which was demonstrated in our previous study.(42) Given the female-specific response, potential estrogen effects are also a consideration as estrogen has been shown to both positively and negatively modulate intestinal barrier properties. (11, 18, 34) However, given there were no differences in intestinal permeability between LWC Male-C and LWC female pigs at the 7 (prepubertal) or 20 week (pubertal) time point, the role of postnatal estrogens, while possible, does not fit with our the present findings. Experiments with ovariectomized female pigs would provide a more definitive conclusion regarding its role in the heightened and persistent intestinal barrier defects in EWS females.

While functional bowel disorders such as IBS are associated with increased intestinal permeability, the focus has been predominantly on the large intestine (21, 23,

45, 51, 53, 56). However, through the use of oral sugar studies in IBS patients, the small intestines have been identified as source of increased intestinal permeability with the small intestine found to be the most significant source of permeability in IBS-D patients after controlling for other confounding factors.(45) Therefore, our observations in the ileum in the present study might be relevant to study the pathophysiology of human GI diseases associated with increased small intestinal permeability.

ELA in pigs induces an increase in intestinal mast cell numbers and localization to SMP and MP

In the present study, piglets that were exposed to EWS exhibited increased intestinal mast cell numbers in the ileum (at 7 and 20 weeks of age) and colon (at 7 weeks of age). In comparison, humans with IBS-D have been found to have increased mast cell numbers in the ileum.(50, 61) Similarly, rodent models have shown that NMS stress results in a persistently elevated increase in intestinal mast cell numbers (7). In addition, the present studies revealed that in EWS pigs, mast cells were more closely associated with enteric neuronal ganglia, suggesting an enhanced neuro-immune communication between mast cells and nerves as a result of EWS in pigs. An enhanced intestinal mast cell-nerve co-localization has been demonstrated in IBS patients, correlating with severity of abdominal pain.(5) In human IBS, female patients were shown to exhibit a higher number of mast cells compared with male IBS patients. (17) In the present study Male-C EWS pigs but not EWS females were statistically different from their respective LWC. While these data imply that EWS Male-C pigs exhibited increased mast cell numbers in response to EWS while EWS females did not, caution should be taken with this conclusion as other factors may have influenced the numbers

of mast cells. First, although the increased in mast cell numbers induced by EWS was statistically different Male-C pigs, EWS Male-C and female EWS pigs had similar numbers of tryptase-positive mast cells. The slightly higher number of mast cells in LWS female pigs might have influenced the ability to detect statistical differences (via a 2 Way ANOVA) between LWC and EWS females. Second, mast cell numbers were based upon tryptase and T-blue staining which are both intracellular mast cell granule stains. Therefore, any potential differences in the mediator composition or content between Male-C and female pigs could have influenced mast cell counts. For example, an enhanced released of tryptase could have resulted in less tryptase-stained cells thus leading to an interpretation of less mast cell numbers. In support of this explanation, Male-C and female EWS exhibited comparable numbers of tryptase and T-blue-positive mast cells, but EWS females released greater amounts of tryptase upon c48/80 stimulation. Therefore, an enhanced release of MC tryptase in EWS females pigs could have artificially reduced the number of mast cells in the intestinal tissues in female pigs. We have previously shown that mast cell tryptase and chymase expression can differ significantly in chronically stressed pigs (33) and that female mast cells obtained from rat and mice contain higher amounts of intracellular mediators at baseline (non-stressed conditions) compared with sexually mature intact males (36) suggesting the composition of mediators in female and male mast cells are different and can change in response to stress.

The underlying mechanism for enhanced ileal tryptase release in female EWS pigs remains to be elucidated. Given the present findings and our previous studies in mice and rats, female mast cells possess the ability to synthesize, store and release

more mast cell mediators compared with male MCs (36), the enhanced tryptase release could be a result of increased tryptase content in female EWS mast cells. In addition differences in adult sex hormones, such as increased estrogen in female pigs or reduced androgens in Male-C pigs could have contributed to these changes. Unfortunately we did not stage the estrous cycle in female pigs in the present study and therefore we cannot conclude the influence estrous or sex hormone levels on enhanced mast cell activity or numbers. While previous *in vitro* experiments showed that application of estrogens to cultured mast cells can induce mast cell degranulation(62), very little is known about the influence of estrogens on mast cell activity *in vivo*. If high estrogen levels during the estrous cycle were influencing the ileal mast cell tryptase response observed in the present study, we would have anticipated an elevated mast cell tryptase released in LWC females compared with LWC Male-C pigs, which was not observed in the present study. In mice, we showed that the elevated mast cell mediator levels and release observed in adult female mice were not influenced by different stages of the estrous cycle. (36)

Comparisons between female and Male-C pigs in their response to EWS: potential role of biological sex factors

In the present study we observed that diarrhea, intestinal permeability and mast cell activity were increased in both EWS Male-C and female pigs; however, EWS females exhibited a more severe EWS phenotype. It is known that biological sex is an important determinant in the susceptibility to stress-related GI disorders such as IBS with females at increased risk by up to 2-3 fold (1, 14, 20, 35, 54, 55, 59). Furthermore, recent studies indicated that females, but not males with IBS have a greater prevalence

of ELA suggesting a potential interaction between ELA and biological sex in IBS. (10, 28). The precise mechanisms underlying sex differences in human FGID's such as IBS remain poorly understood. In general, biological sex differences are determined in large part by sex hormones and (or) chromosomal (XX vs XY) factors. Furthermore, sex differences arise from organizational period events (e.g. perinatal gonadal androgen surge in males) which influence early sexual differentiation and prepare the system for later life activational effects of adult sex hormones upon puberty.(32) In the present study, we compared the responses of female pigs with gonectomized (castrated) male pigs (Male-C), which lack the influence of postnatal gonadal androgens. We understand that Male-C pigs are not representative of an intact male and should not be interpreted or compared with intact females as such; however, the dimorphic responses observed between Male-C pigs and females in response to EWS at both prepubertal and adult stages imply that sex factors are likely influencing the response to EWS in this porcine model. However, studies where intact male boars are compared with Male-C and female pigs are required to conclusively define the nature of the dimorphic response observed in the present work. Relevant to this proposed study is a working paradigm in sex biology that suggests that gonadal hormones and sex chromosomes (XX vs XY) can have compensatory, counteracting functions resulting in sexual monomorphism (both sexes having similar phenotype).(4) Therefore, it is possible that intact EWS Male-C pigs would exhibit a similar disease phenotype with EWS females. A monomorphic phenotype in this case however would not imply sexual equivalence because the present studies revealed that Male-C and females are inherently biologically different in their response to EWS. Instead a monomorphic response would suggest that the route

by which male and female EWS pigs achieved a similar EWS disease phenotype is via different mechanisms (e.g. gonadal androgens would be involved in creating the disease phenotype in male pigs which would not be the case in females). This could have potential implications for sex-specific therapeutic interventions (4). Furthermore, with comparison to a Male-C pig, a monomorphic response to EWS in intact male and female pigs would implicate testosterone as a detrimental component; however, in male IBS patients, there is evidence of a negative correlation between testosterone levels and visceral pain (26), suggesting that testosterone might be protective. Therefore, it is also possible that use of intact EWS males might have resulted in even larger differences compared to EWS females. In summary, the present study revealed dimorphic responses to EWS between Male-C and female pigs with female pigs exhibiting heightened sensitivity and GI disease. Future studies comparing intact males will be required to determine the nature and mechanisms of this difference.

In summary, the present study demonstrates that EWS in piglets induces lasting alterations in GI function that mimic some of the key features of human FGID's including chronic functional diarrhea, intestinal permeability, and enhanced mast cell activity and localization with enteric nerves. Potential sex differences were evident as females pigs exhibited a more severe clinical and pathophysiological EWS phenotype compared with Male-C pigs. Therefore the EWS porcine model holds promise as a pre-clinical translational model for studying the pathophysiology of FGIDs such as IBS-D that are associated with ELA.

APPENDIX

	B. hyodysenteriae	*B. Hampson ii*	*L. intracellularis*	*Salmonella spp.*
EWSF 1	CT >40	CT >40	CT >40	CT >40
EWSF 2	CT >40	CT >40	CT >40	CT >40
EWSF 3	CT >40	CT >40	CT >40	CT >40
LWC 1	CT >40	CT >40	CT >40	CT >40
LWC 2	CT >40	CT >40	CT >40	CT >40

Table S.2.1. PCR reactions on mucosal tissue of 20 week old EWS and LWC pigs for porcine diarrheal pathogens. CT values >40 indicate non-detectable genetic products of these pathogens. LWC=Late Weaned Controls. EWS=Early Weaning Stress

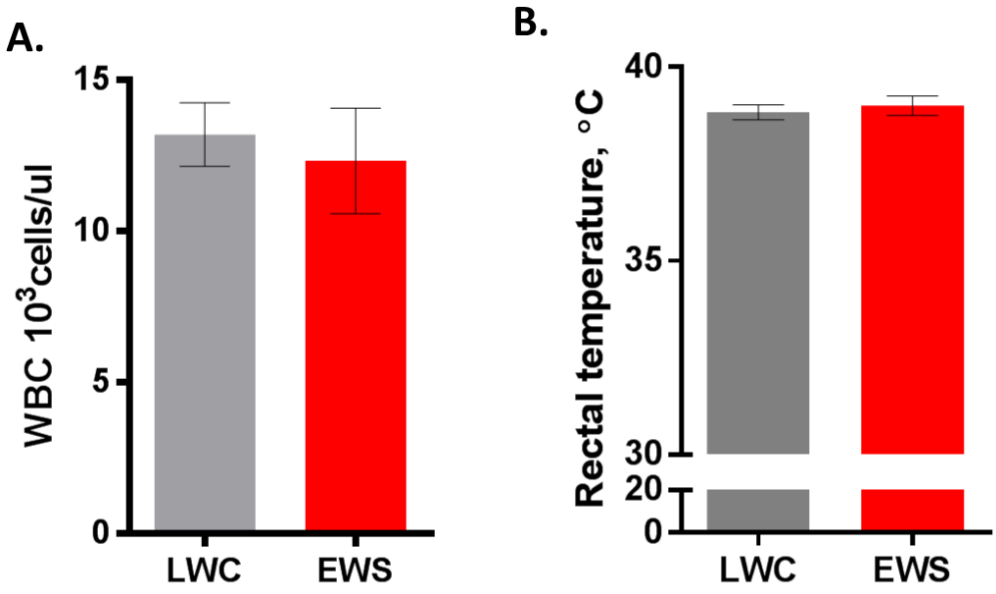

Figure S.2.1. WBC and rectal temperatures of EWS and LWC piglets.

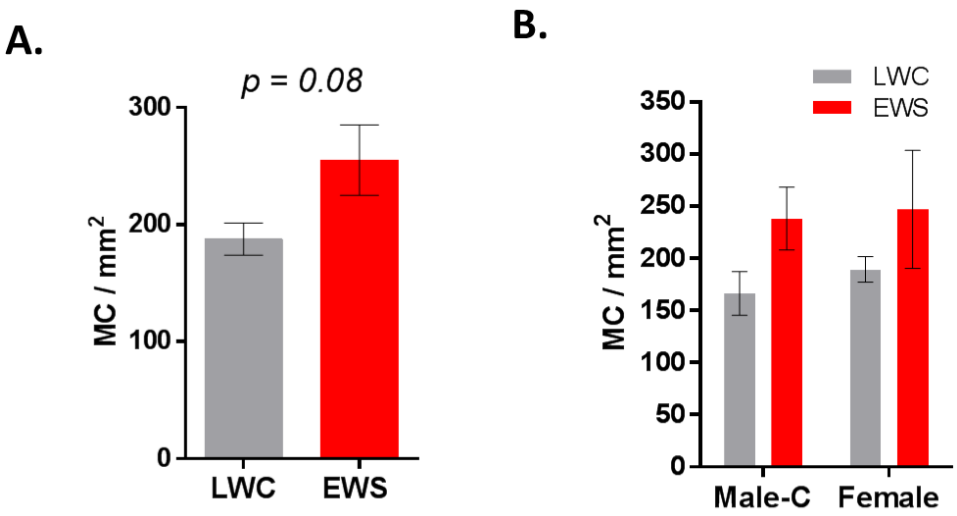

Figure S.2.2. Tryptase-positive mast cell numbers in the colon from 20 week old EWS and LWC female and Male-C pigs. Distal colon sections from 20 week old pigs were stained for mast cell (MC) tryptase and tryptase-stained cells were quantified to determine MC numbers. A) Colon MC numbers in LWC and EWS pigs, B) Colon MC numbers in Male-C and female LWC and EWS pigs. Data are means ± standard error. Student's T-test (A) and Two way ANOVA with Fisher's LSD post hoc test (B) were conducted. LWC = Late Weaned Control, EWS = Early Weaning Stress, Male-C = male castrated pigs.

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CHAPTER 3

Acute and long term impact of early life adversity on enteric cholinergic system

Abstract

Early life adversity is a major risk factor for manifestation of GI disease later in life; however the underlying mechanism linking early events to disease later in life remain elusive. Here we demonstrate that ELA, in a rodent model of neonatal maternal separation (NMS), increased an individual's sensitivity to stress induce GI dysfunction in adulthood. This underlying phenomena appeared to be regulated by a hyperactive enteric cholinergic nervous system. On Ussing Chambers, we observed NMS mice to have increased magnitude of electrogenic ion transport in response to the cholinergic agonist, physostigmine hemi-sulfate in the colon compared to controls. NMS mice also had elevated baseline electrogenic ion transport. NMS mice demonstrated dysfunction in colonic motility, with stronger and longer duration colonic migrating motor complexes under resting conditions. Though fecal pellet output and intestinal permeability were no different between NMS and control mice under resting conditions, a mild, 2 hour restraint stress induce significantly more fecal pellet output and intestinal permeability in NMS mice compared to controls. Additionally, NMS mice demonstrate a selective upregulation of colonic genes associated with metabolic dysfunction, inflammation, and cell death. Interestingly, stress induced motility, permeability, and gene expression all appeared to be under the function of the cholinergic system, as pretreatment with hemicholinium-3, the choline transporter important in acetylcholine synthesis, prevented these stress induced changes in NMS mice. Functional changes in the cholinergic enteric nervous system were not dependent on differential expression of the enteric cholinergic system, suggesting pathology is not due to differential expression of cholinergic signaling molecules. Though difference in expression of the cholinergic

system were not apparent in adults, we observed an increased expression of choline acetyltransferase (ChAT), in the mucosa of juvenile animals immediately following early life trauma. Together these findings support that ELA promotes risk of GI disease later in life through hyperfunction of the enteric cholinergic nervous system. Dysregulation of the cholinergic nervous system may begin at exposure of ELA, however, how the cholinergic system becomes permanently changed remains unknown.

Introduction

A growing body of evidence convincingly links early life adversity (ELA) with manifestation of persistent chronic inflammation (14), metabolic disorders such as obesity and diabetes (15, 27, 42), and chronic gastrointestinal disorders, including irritable bowel syndrome (IBS) (6, 9, 10, 18) and inflammatory bowel disease.(1, 2) The incidence of ELA in the United States is staggering, with 35 million individuals reporting at least one adverse life event, and 16 million people experience 2 or more adverse life events (23), making ELA a major concern for public health.

Understanding how ELA leads to chronic disease onset later in life remains to be fully understood; however, evidence suggests that risk of disease may be due to individual susceptibility to stress later in life. For example, individuals with prior experience of ELA have hyperactive hypothalamic-pituitary-adrenal (HPA) axis responses to stress later in life, which has been observed in humans (24, 25, 38) and corroborated in animal models (39, 43). Importantly, (HPA) axis dysfunction has been observed in patients suffering with obesity (34, 54) and IBS (8), leading to the possibility that adult life stress may increase susceptibility to these chronic conditions in individuals with prior experience of ELA. Indeed, in animal models of ELA, an adult psychological

stress was shown to increase motility (13), colonic visceral hypersensitivity (13, 19, 46, 49, 53), intestinal permeability (44, 48), and intestinal mucosal electrogenic ion transport (37, 48) compared to controls raised in a stress free environment. These studies highlight the predisposition of individuals stressed early in life to GI disease when exposed to mild stressors as adults.

Though a relationship between ELA, subsequent stress, and gastrointestinal (GI) disease activation is apparent, little is known about the factors linking these systems together. Previous work from other groups in a rodent model of ELA, called neonatal maternal separation (NMS), demonstrated that an early upregulation of the cholinergic system lead to increased intestinal electrogenic ion transport and intestinal barrier permeability immediately following ELA in weanling animals.(20) In adult animals, our group demonstrated that early life adversity in a porcine model induced by early weaning stress (EWS), induced persistent upregulation in function of the enteric cholinergic nervous system, leading to enhanced mucosal electrogenic ion transport into adulthood.(37) Combined, these data demonstrate an important role of the cholinergic system in contributing to GI dysfunction. These findings lead us to hypothesize that ELA mediates increased risk of GI dysfunction through a hyperactive enteric cholinergic system during adult life stress. We tested our hypothesis by measuring intestinal electrogenic ion transport, motility, permeability and gene expression under basal, non-stress conditions, and under conditions of mild, acute restraint stress (RS) in adult mice previous exposed to NMS. Furthermore, we assess the cholinergic contribution to stress induced GI disease by pretreated mice with an indirect anti-cholinergic drug, hemicholinium (HC-3). Finally, we sought to determine the

time course of intestinal cholinergic disturbances during early life in the porcine ELA model, EWS.

Here were demonstrate that ELA, via murine NMS, lead to adult GI dysfunction, characterized by heightened secretomotor function, hypermotility and intestinal permeability compared with NH mice. Adult NMS mice had increased body weight and secertomotor dysfunction compared to controls under a non-stress condition. When exposed to a mild stressor as adults, we found that NMS mice demonstrated exacerbated intestinal motility and intestinal permeability, which was largely mediated by cholinergic signaling. Finally, adult stress in NMS mice lead to a significant upregulation in colonic genes linked to inflammation and obesity compared to non-stressed mice. Upregulation in colonic gene expression in NMS mice was found to be under the control of cholinergic signaling. Finally, using the EWS porcine model, we observed that an early source of enteric cholinergic dysfunction appears to origin from lymphoid associated mucosa. Together, the findings reported here demonstrate a significant role of the enteric cholinergic system in mediating susceptibility to GI disease later in life, particularly when exposed to a subsequent stressor. The original source of cholinergic dysfunction appears to originate from a cellular source in lymphoid associated mucosa.

Methods

Animals

Mice: C57Bl/5 mice were utilized for all experiments. Experimental mice utilized were generated by sibling mating within the colony. Animals were house on a standard

12 hour light dark cycle with ad libitum access to chow and water. Mice were co-housed at 2-3 animal per cage in Optimouse racks.

Pigs: Pigs were obtained from Michigan State University swine farm. At weaning, pigs were transported from their original facility to crates and given ad libitum access for feed and water with a 12 hour light dark cycle.

Early life stress

Mice: Neonatal maternal separation (NMS) was performed as previously reported, with a few exceptions.(31) Briefly, NMS mice were separated from their parity one mothers every day between postnatal days 1-18 for 3 hours, with birth considered postnatal day 0. During separation, mothers were separated to a clean cage without nesting but with *ad libitum* access to food and water. Pups were isolated from each other in cups to prevent co-mingling. Normally handled (NH) mice served as controls and were reared per standard protocol. For all experiments, mice were weaned at 21 days of age, with the exception of animals used to determine fecal pellet output, permeability, and gene response to stress. In these studies, we added an additional stressor of early wean to the NMS mice in an attempt to exacerbate the phenotypic difference we were already observing. Early weaned NMS mice were weaned at 18 days of age. NH mice were weaned later at 28 days of age. Experiments and tissue collection was conducted on animals once they were between ages of 10-13 weeks.

Pigs: Early weaning stress (EWS) was induced by weaning pigs from their mother at 16 days of aged and transporting them to a new facility. Later weaned controls were similarly treated, however they were weaned at 26 days of age. Baseline

un-weaned pigs were taken directly from their mother and sacrificed for tissue collection.

Restraint Stress

RS was performed by inserting mice in to 50mL conical tubes, which had air holes drilled through the tip of the tube and 8 other holes drilled along the wall of the tube. Mice were pushed to the front of the tube with 2-3 pieces of cotton and inserted horizontally in a tube rack. Mice were not permitted to see each other during restraint stress. Mice were restraint for 2 hours unless noted otherwise after which time they were immediately scarified. Basal, unstressed control were taken directly from their cage and euthanized.

Body Weight

Mice: Weanling body weights were measured at 21 days of age NH: 6 males, 5 females; NMS 15 males, 9 females. Adult body weight was measured between age matched litters between 10-12 weeks of age NH: 22 males, 25 females; NMS: 31 males, 34 females.

Pigs: Pigs aged 20 weeks were weight, n=6 per group.

Statistical analysis by multiple t-tests for body weight

Ussing Chambers

Ussing chambers were utilized to measure electrogenic ion transport utilizing methods previously reported.(31, 37) Animals were sacrificed by cervical dislocation. Ileum and colon tissue were mounted on sliders after opening along mesenteric border and rinsing in mouse Ringer's solution with 1% gentamycin. Electrogenic ion transport responses were collected by voltage clamping (Physiological Instruments) such that a

net negative current to the musical surface was recorder as a positive change in current. Cholinergic sensitivity of the tissue was assess by application of 500uM physostigmine hemisulfate (Tocris, 0622) to the serosal surface of the tissue. The nature of the ion transport and neuronal and cholinerigc contribution in physostigmine responses was determine by pretreating tissue with 10uM bumetanide (Cayman Chemical, 14630), 100uM atropine (Sigma, A0257), 1uM tetrodotoxin (Abcam, ab120055) for 30 minutes. Sodium free Ringers solution was generated by substituting sodium chloride with 130mM d-glucamine and 24mM choline. Electrical field stimulation (EFS) was performed by attaching a Grass field stimulus isolation unit to sliders specially outfitted with silver electrodes (Physiological Instruments, San Diego). Tissues were stimulated at noted frequencies for 0.5ms and 10V for a 10 second duration. Tissues were repeatedly stimulated in ascending frequencies one initial responses had subsided (about 10 minutes). Basal and physostigmine response n= NH 12 male, 14 female; NMS 14 males 14 females. No effect of sex was observes, so all data was combined. Physostigmine characterization was performed using starndard reared, health control mice in a separate experiment. n= 4 per each treatment (2 males and 2 females). In physostigmine studies, no effect of sex was observed. Electrical field stimulation studies n= NH: 6 males 5 females; NMS 15 males and 8 females. Statistical analysis was performed by student's t-test.

Colonic migrating motor complexes

Experiments were performed as previously reported.(35) n= NH: 5 males and 4 females; NMS: 5 males and 6 females. No effect of sex was observed. Statistical analysis performed by one way ANOVA with multiple comparisons for sex,

HC-3 injections

Mice were injected with 10ug/mL HC-3 at 25ug/kg IP or with a PBS vehicle. This dose was chosen as in reveal minimal to mild clinical signs of respiratory suppression on a dose response curve (data not shown). For permeability and gene array studies, HC-3 was injected at time of oral gavage and 3 hours prior to restraint stress.

Stool pellet output

Basal stool pellet output was determine by singling housing individual mice in a clean cage for a 2 hour period. Fecal pellet output following restraint stress was quantified by measuring the number of fecal pellets immediately following restraint stress. No effect sex effect on fecal pellet output was observed, so all data were combined. Basal n= NH 4 males; 6 females; NMS 5 males, 4 females. 15 minute RS n= NH 2 male, 3 female; NMS 3 males; 4 females. 2hr RS n = NH 10 male, 13 female; 14 males, 15 females. Impact of HC-3, RS n= NH 4 males, 1 female; NMS 5 males, 3 females; HC-3 injection n= NH 4 males, 2 females; NMS 4 males and 4 females. Statistical analysis performed by student's t-test and One-way ANOVA with Sidka post hoc test for multiple comparisons.

Permeability

Intestinal permeability was determined by oral gavage of 125mg/mL FITC conjugated 4kDa Dextran (FD4) in PBS at 450mg/kg body weight with a 22 gauge straight gavage needle after a prior 3 hours fast from food. Blood was collected by submental bleeding with a 5mm lancet or after CO2 asphyxiation by cardiac puncture with a 25 gauge needle. To determine basal permeability, blood was collected 5 hours after oral gavage. To determine the influence of 2hrs restraint stress on intestinal

permeability, mice place in RS holders 3 hours after oral gavage with FD4. After two hours of RS, mice were sacrificed and blood was collected. Collected blood was mixed in a microvette EDTA treated tube and held on ice until spun at 10,000rpm for 10 minutes. Plasma was collected and flash frozen in liquid nitrogen. To quantify FD4 in plasma, samples were diluted to 1 part plasma:2 parts PBS. A standard curve of FD4 was generated with by diluting stock FD4 such that the final standards contained 1 part plasma from mice that were not gavaged with FD4 (we found that plasma itself is autofluorescent and must be included in the standard curve samples to account for background). Samples were read on a Synergy H1 plate reader (Biotek) with excitation at 485nm and emission at 528nm. Basal n= NH: 2 males, 2 females; NMS 2 males; 2 females; Restraint stress n= NH: 5 males, 1 female; NMS 5 males, 1 female. Impact of HC-3, RS n= NH 4 males, 1 female; NMS 5 males, 3 females; HC-3 injection n= NH 4 males, 2 females; NMS 4 males and 4 females. No effect of sex was observed, but may be due to insufficient powering of the study. Statistical analysis performed by One-way ANOVA with Sidka post hoc test for multiple comparisons.

RNA isolation

Mid-colon segments of tissue were collected and snap frozen in liquid nitrogen after being opened and rinsed free of fecal material. Tissue were stored at -70^{o}C until further used. Tissue was homogenized with Percelly's homogenizer and 2.3mm zirconia/silica beads (Biospec, 11079125z) at 63,000rpm for 30 seconds in RLT buffer from Qiagen and total RNA was isolated from colonic tissue utilizing RNeasy Mini Kit (Qiagen, 74106) utilizing manufacturer's instructions. DNase digestion and cDNA

synthesis of mRNA was performed with Maxima First Strand Kit (ThermoFisher, K1671).

Wafergen gene array

Multiple genes were screen for simultaneously utilizing the 384 well Wafergen PCR Chip method with Power SYBR Green Master mix with starting cDNA conenctration of 2.5ng/ul. Primers were obtained from PrimerBank https://pga.mgh.harvard.edu/primerbank/ added at concentration of 250nM. See supplemental table 2.1 for list of primers and gene screened.

Gene expression was normalized by the 2^{-ddCT} method by subtracting target gene expression from average housekeeping gene expression for each sample and normalizing again NH basal control dCT. No differences were detected, between basal NH and NMS, so the average dCT of these values were combined to create a pooled basal group. Effect of RS and impact of HC-3 on RS induced gene expression was normalize to the pooled baseline values. Significance of differential gene expression was determine performing a t-test for every gene screened. False discorery rate of 10% was set to determine true significance; however, genes with $p < 0.05$ were also inspected further inspected. Significance of effect by NMS v NH in RS or treatment with HC-3 was determined using One-way ANOVA with Sidka post hoc test for multiple comparisons. Basal n= NH: 2 males, 2 females; NMS 2 males; 2 females; RS n= NH 4 males, 1 female; NMS 5 males, 3 females; RS+HC-3 injection n= NH 4 males, 2 females; NMS 4 males and 4 females.

Taqman qPCR

Gene expression was performed utilized TaqMan primer probe sets provide by Therom Fisher, and specific genes and primer probe set catalog number appear in Supplementary Table 2.2. For each sample, gene expression was normalized to the house keeping gene HPRT. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method by normalizing samples the dCT of NH controls. Since no difference were observed between NH and NMS mice, pooled basal controls were utilized for dCT normalization in RS animals. Basal n= NH 9, NMS 12, equal numbers male and females. RS: NH 9, NMS 14, equal numbers males and females.

Western Blot

Pigs: Ileal mucosa and mesenteric lymph node protein samples were diluted to 1µg/µL in Laemmli Buffer (Bio-Rad, #161-0737) + 5% 2-mercaptoethanol and heat denatured at 70^{o}C for 10 minutes. 10 µg of protein sample was run on a TGX-Stain Free gel (Bio-Rad #5678095). Protocol for electrophoresis, wet to wet transfer, and stain free, lane total protein quantification was performed as published in Criterion^{TM} Precast Gels: Instruction Manual and Application Guide and Western Blot Normalization Using Image Lab^{TM} Software (Bio-Rad). The PVDF membrane was blocked in 5% BSA at RT for 1hr prior to incubation with monoclonal 1.B3.9B3 anti- porcine ChAT antibody (Millipore Sigma #MAB5270) at a concentration of 1:1000 in 1xTBS + 5% BSA + 0.1% Tween-20 overnight at 4^{o}C. The following morning, the blot was washed and an HRP linked anti-mouse antibody in 1xTBS + 5% BSA + 0.1% Tween-20 (Cell Signaling, #7076) at (1:1000) was incubated with the membrane for 1hr at RT. Chemiluminescence was performed using Clarity ECL (Bio-Rad, #1705060).

Densitometry was performed utilizing Bio-Rad Image Lab™ Software v5.2.1 and band density was normalized to lane total protein per Western Blot Normalization Using Image Lab™ Software (Bio-Rad) protocols.

Mice: colonic tissue was utilized, but performed similarly as described above. Immunodectection for CHAT was performed utilizing AB144p at 1:500 with a HRP conjugated bovine anti-goat secondary antibody for detection.

Immunofluorescence

Colonic tissue was opened along the mesenteric boarder and pinned out on Sylgard filled tissues for overnight fixation at 4°C with Zamboni's fixative (American MasterTech, FXZAMPT). Tissues were wased 3 x 10min in dimethyl sulfoxide and then 3 x 10min in phosphate buffered saline (PBS). Fixed tissues were stored in PBS + 0.4% sodium azide (Sigma, S8032) at 4°C until further used. Submucosa and longitudinal/myenteric plexi were dissected free under a stereoscopic microscope. ~5mm x 5mm pieces of mid colon were blocked in 4% normal donkey serum + 0.4% Triton X – 100 + 1% bovine serum albumin in PBS, then immuno-labeled for ChAT 1:100, (Millipore, AB144P) and HuC/D 1:200, (Invitrogen, 16A11) a marker of neuron cell bodies in 1% NDS + 0.4% Triton X – 100 in PBS for a 3 day period at 4°C. Tissues were washed 3 x 10min in PBS prior to application of secondary antibodys: AlexaFluor 488 Donkey anti-goat, 1:100; AlexaFluor 555 Strepavidin, 1:200, and nuclear stain To-Pro3 (1:1000) in 0.4% Titon X – 100 + 1% bovine serum albumin in PBS. Samples were wash 3 x 10min in PBS then mounted in ProLong Diamond Antifade Mountant (ThermoFisher, P36965). 0.5um Z-stacked images of plexi were captured on an Olympus FluoView 1000 Filter-based confocal laser scanning microscope with a 1.3

N/A, 40x oil objective at 3x zoom. Images were capture by exciting the specimen with an argon gas, green helium neon gas laser, and a red helium neon gas laser with excitation wavelengths/filters at 488/BA505-525nm, 543/BA560-620, and 633nm/650IF, respectively. *n* = 8 control and 13 CSDS mice for this section. Composite z-stacked images were analyze on Olympus FLUOVIEW Viewer Software v4.2. Ten plexi were images per animal and analyzed.

Acetylcholinesterase activity

Acetylcholinesterase activity was performed on protein isolated from colon using Amplex^{TM} Acetylcholine/Acetlycholinesterase Assay Kit (ThermoFisher Scientific cat#A12217) per manufacturer's instructions. n = 8 NH and 8 NMS.

Acetylcholine quantification

ACh was quantified utilizing liquid chromatography and mass spectroscopy (LC-MS) with the aid the Michigan State University LC-MS Core. ACh samples were standardized to a loading control of salbutamol and normalized to starting amounts of tissue. n= 8 NH and 8 NMS.

Results

Impact of NMS on physical condition

Throughout the experiment NH and NMS mice appear healthy without any obvious signs of clinical disease both at weaning and in adulthood. At weaning age, we did not detect any difference in body weight between NMS and NH control mice (**Sup Figure 3.1A**). However, in adult mice, we consistently observed that both NMS male and female mice were consistently heavier, by about 1 gram, compared to their sex matched NH controls (**Sup Figure 3.1B**). Similarly we observed this phenomena in

adult pigs exposed to ELA through early weaning stress (EWS). Though the significance was selectively found to occur EWS females compared to later weaned control females, male EWS pigs did have a larger mean body weight than their later weaned controls (**Sup Figure 3.1 C**)

Secretory dysfunction in NMS exposed mice

Utilizing Ussing chambers and the voltage clamp method, we assess colonic secretion/absorption by measuring electrogenic ion transport in NMS mice compared to NH controls at 10 weeks of age. In non-stimulated colon tissue, we found basal short circuit current (*Isc)* to be higher in NMS adult animals compared to controls, implying elevated electrolyte and fluid secretion (**Figure 3.1A**).

Previously, we have shown that pigs exposed to ELA, through early weaning stress (EWS), have increased secretory responses, mediated by hyperactive enteric cholinergic neurons.(37) Similarly, we hypothesized, that mice exposed to ELA through NMS, may have a hyperactive enteric cholinergic nervous system. Testing this hypothesis, we treated NMS colonic tissue with a cholinergic agonist, physostigmine hemisulfate, which blocks AChE function, and allows accumulation and persistent signaling of endogenously release ACh. Treating colonic tissues with physostigmine, we observed a bi-phasic electrogenic response (**Figure 3.1B**). The initial response to physostigmine resulted in a significant drop in Isc, we called *Phase I***,** with a subsequent rebound, we've called *Phase II*. Treating NMS and NH colonic tissue with physostigmine generated both a larger magnitude *phase I* (**Figure 3.1 B,C**) and *phase II* (**Figure 3.1 B, D**), indicating increased cholinergic sensitivity in the NMS mice compared to NH controls.

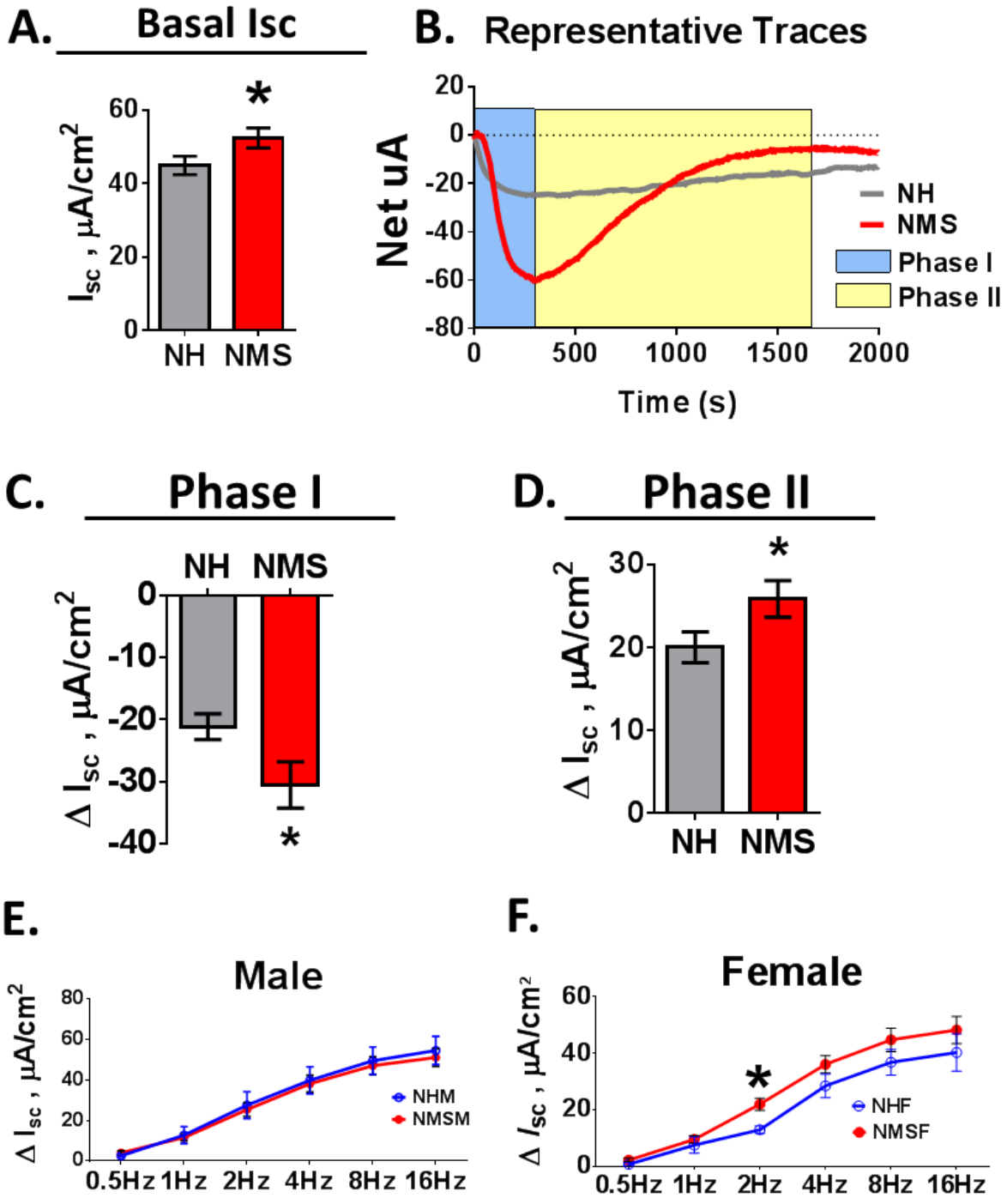

Figure 3.1. Influence of NMS on cholinergic intestinal electrogenic ion transport. A) Basal, resting short circuit current (Isc) of colonic tissue on Ussing Chambers between NH and NMS mice at 10 weeks of age. B) Representative change in current measure in unites of microamps and normalize to staring baseline Isc) in response to serosal application of 500uM physostigmine hemisulfate in both NH and NMS mice. Blue box highlight initial, phase I response, which was observed to be a strong reduction in current. Yellow box highlights the phase II response, which was found to be

Figure 3.1. (cont'd)
a rebound in current following phase I. Note that magnitude of both phase I and II were typically stronger in NMS animals compared to controls. C-D) Quantification of net current change (delta Isc) for phase I (C) and phase II (D). E-F) Net change in Isc in response to increasing electrical field stimulation frequencies for male (E) and females (F).

As physostigmine application to colonic tissue is not well described, we sought to determine the electrolyte and cholinergic signaling methods responsible for *Phase I* and *Phase II* responses on colonic tissue from controls*. Phase I* response could be blocked by pre-incubating colonic tissue with sodium free Ringer's solution (**Sup. Figure 3.2A**); atropine (ATR), a muscarinic receptor blocker (**Sup. Figure 3.2C**); and tetrotodoxin (TTX) a pan neuronal blocker (**Sup. Figure 3.2D**), but not bumetanide (Bumet), a basolateral $Na^{+}K^{+/}2Cl^{-}$ transporter blocker which is the predominant mechanism for basolateral Cl^{-} entry (**Sup. Figure 3.2B**). These observations confirmed the cholinergic neuronal nature of the physostigmine response, which mediate electrogenic sodium transport in *Phase I*. The *Phase II* response was bumetanide sensitive (**Sup Figure 3.2B**); however, neither ATR nor TTX inhibited *Phase II* responses. These observations indicated that *Phase II* represents physostigmine induced chloride secretion that occurs independent of enteric nerves or muscarinic receptor signaling. These observations confirm that physostigmine is acting, in part, via enteric nerves and cholinergic signaling. Furthermore, the observations in **Sup Figure 3.2**, indicate that there is elevated cholinergic and enteric nerve function in NMS mice, as observed by exacerbated *Phase I* and *Phase II* responses compared to NH controls (**Figure 3.1C-D**).

We also investigated whether the ileum had electrogenic ion transport dysfunction comparative to the colon. Utilizing EFS on Ussing chambers, we identified

that at lower frequencies, NMS females had stronger amplitude current responses compared to NH females (**Figure 3.1 F**). However, this observation was only observed in females and the difference was not apparent between males. (**Figure 3.1 E-F**).

Cholinergic mediated motility and motor defects in ELA

Previously, in Chapter 2, we demonstrated that adult pigs exposed to ELA, via EWS, experienced increased frequency of diarrhea.(44) We sought to determine if colonic tissue from adult mice demonstrated abnormal motility patterns compared to NH controls, since hypermotility is a function of diarrhea. Measuring colonic migrating motor complexes (CMMCs) via force transduction, we observed that NMS mice had increased contraction strength (**Figure 3.2A**). Furthermore, duration of strong contractions in NMS mice was longer compared to controls (**Figure 3.2B**). Stimulating colonic tissue with EFS generated stronger contractions in NMS mice compared to controls; however, there was no difference in relaxation response following EFS (**Figure 3.2C**). Together, these results support that NMS mice have stronger, longer CMMCs, which are also activated by the enteric nervous system, compared to controls.

We next investigated fecal pellet output in NMS mice compared to NH control, to determine if the CMMC patterns had any clinical significance. Housing NH and NMS mice for 3hours under standard (basal) conditions did not demonstrated any difference in fecal pellet output. However, application of a minimal, 15 minute RS revealed a trending increase in fecal pellet output from NMS mice compared to NH controls. Prolonging the RS to 2 hours revealed a significant, 20% increase in fecal pellet output from NMS mice compared to controls **(Figure 3.2D**).

Since enteric cholinergic function increases colon contraction and motility, we sought to determine if the elevation in fecal pellet output from NMS mice was under control from elevated cholinergic signaling. To assess cholinergic nerve function, we injected mice with a low dose of HC-3, which blocks choline uptake from nerve terminals, prevents re-synthesis of ACh, and leads to subsequent ACh depletion. Following HC-3, or PBS vehicle injection, mice were subjected to 2hrs of RS stress. Again, NMS, PBS control mice demonstrated an elevation in fecal pellet output compared to NH, PBS control mice (**Figure 3.2E**). Assessing the contribution of the cholinergic nervous system under these conditions, we observed that HC-3 significantly reduced fecal pellet output by 50% in NMS mice compared to NMS, PBS controls. Furthermore, fecal pellet output in NMS, HC-3 mice was no different from NH vehicle or HC-3 mice. No effect of HC-3 was observed in NH mice (**Figure 3.2E**).

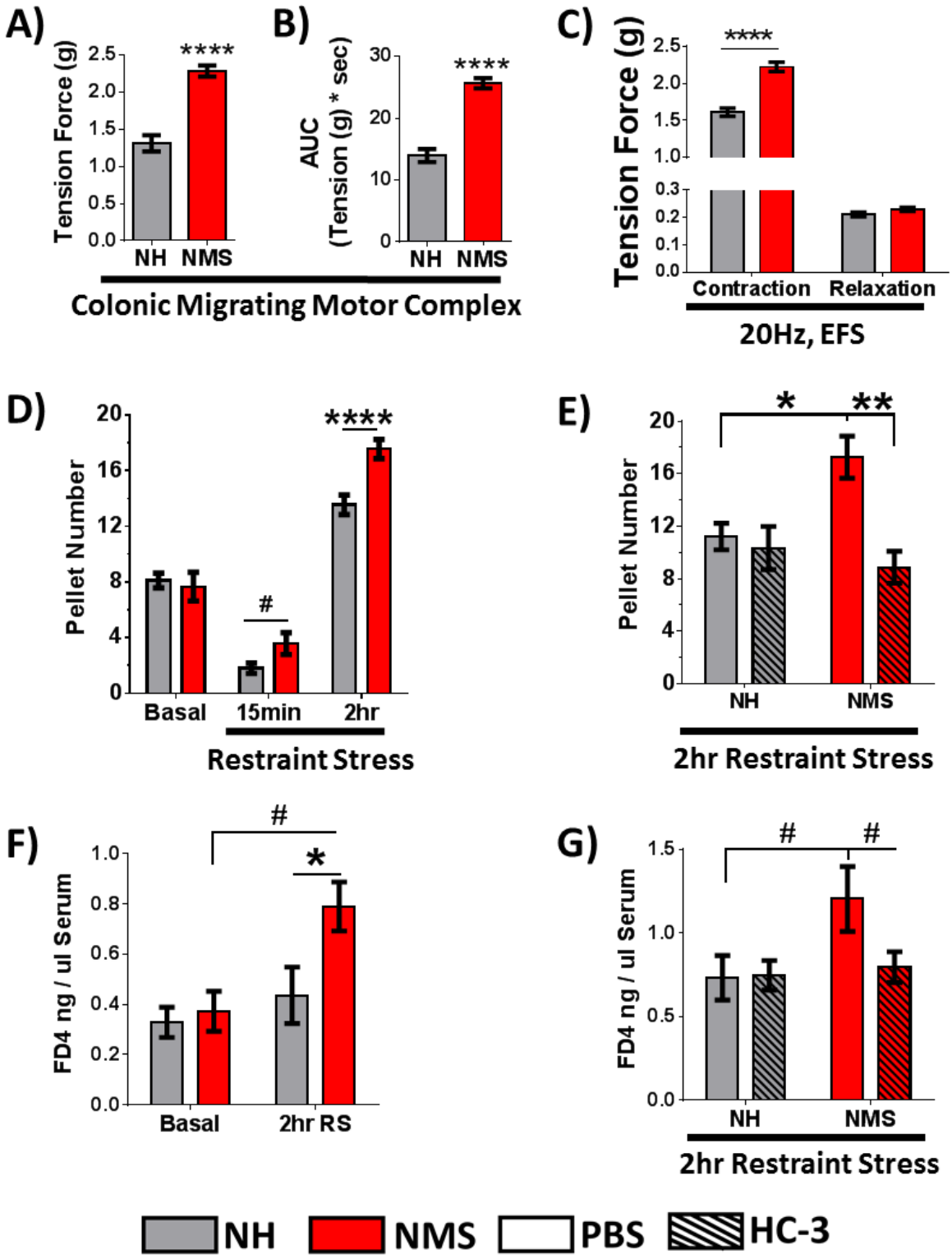

Figure 3.2. Elevated cholinergic signaling in NMS mice contributes to increased motility and intestinal permeability under mild stress. A-B) Endogenous colonic migrating motor complexes (CMMCs) from baseline, non-stressed NMS mice compared to control. A) Contraction strength B) Area under the curve as a product of contraction

Figure 3.2. (cont'd)
strength and duration of contraction. C) Colonic contractile and relaxation responses to 20 Hz, electrical field stimulation between non-stressed adult NH and NMS mice. D) Fecal pellet output from non-stress, basal NH and NMS mice after a 2 hour period and from NH and NMS mice after 15 minutes and 2 hours restraint stress. E) Influence of cholinergic signaling, assessed by HC-3 pretreatment (hashed bars), on fecal pellet output from NH and NMS mice exposed to 2hr restraint stress. F) Intestinal permeability assessed oral gavage of FD4 and subsequent serum quantity in non-stressed, basal and 2hr restraint stressed NH and NMS mice. G) Influence of cholinergic signaling, assessed by HC-3 pretreatment (hashed bars), intestinal permeability from NH and NMS mice exposed to 2hr restraint stress.

Cholinergic mediation of intestinal barrier dysfunction following ELA

Increased intestinal permeability is often found in patients suffering from functional bowel disorders, and we have previously demonstrated that adult pigs exposed to EWS have elevated intestinal permeability.(44) To assess intestinal permeability we performed oral gavage of mice with FD4 and measured accumulation of FD4 in plasma. Under standard housing, basal conditions, no difference in intestinal permeability was detected in NMS compared to NH controls (**Figure 3.2F**). However, application of a mild, 2hr RS exacerbated intestinal permeability, selectively in NMS mice compared to NMS baseline mice. Further intestinal permeability in NMS mice was significantly increased, by nearly two fold, in NMS RS mice compared to NH RS mice. Previous groups have demonstrated that intestinal permeability can be increased by cholinergic nerve signaling.(7) To test if the increase in intestinal permeability observed in NMS RS mice was a function of cholinerigic activity, we pre-treated mice with HC-3 or PBS prior to restraint stress. Increased intestinal permeability observed in NMS, PBS control mice was ameliorated with pre-pretreatment of HC-3 ($p<0.1$) (**Figure 3.2G**). No effect of HC-3 was observed on NH, RS mice compared to NH PBS treated RS mice.

Stress responsive genes in NMS mice are under cholinergic control

As demonstrated above, the GI tract is very response to acute stressors, and homeostatic adaptation to environmental changes is often mediated by complex gene interactions. Since the strongest, most consistent functional differences were observed in colonic tissue, we focused on the impact of stress and the cholinergic system in colon tissue. We developed a panel of genes (**Sup Table 3.1**) known to be associated with stress response to the GI tract or early life adversity and investigated if these genes are differentially regulated in the colon of adult NH control mice compared to mice exposed to NMS. Additionally, we asked if any of the genes differentially expression following acute 2hr RS were under the control of cholinergic signaling by pretreating mice with HC-3. In non-stressed, basal NH and NMS adults, we did not find any difference in colonic gene expression (*data not shown*), but this may be due to low power of the number of basal mice used. In order to identify genes most significantly impacted by acute, 2hr RS, we combined all NH and NMS mice in the basal and RS groups. Plotting the Log_{10}(*p* value) against Log_2(Fold Change), we generated a volcano plot to better visual change in colonic gene expression due to acute stress (**Figure 3.3 A**).

Significant difference in gene expression was determine by performing multiple t-tests for each gene screened, followed by accounting for a 10% false discovery rate. Significantly different genes were further analyzed to determine effect of NMS and the impact of the cholinergic system. Genes found to be significantly different after account for 10% false discovery rate included NR3c1, Fos, an Retnlb, and are color coded in red (**Figure 3.3A**)

In the event that the NMS group or NH group were selectively driving differences in gene expression following an acute stress, we also further analyzed genes which had

$p < 0.05$ after student's t-test, but were not considered significantly different after accounting for a 10% false discovery rate. These genes included Ptgs2, Rorc, Gpx2, and Fadd and are color coded in green (**Figure 3.3A**).

Down regulated genes included NR3c1, Ptgs2, and Rorc. Further investigation into these genes revealed that only NR3c1(the glucocorticoid receptor), was significantly down regulated in both NH and NMS RS mice compared to the pooled basal controls. There was no difference between NH and NMS stressed mice in NR3c1 expression (**Figure 3.3B**). Though NR3c1 expression was significantly reduced following acute RS in both NMS and NH mice, blockade of cholinergic signaling did not appear to prevent down regulation. (**Figure 3.3B**) Further investigation in Ptgs2 (also known as the gene encoding cyclooxygenase-2 (COX-2)) and Rorc expression revealed no effect of RS in either NH or NMS mice compared to non-stressed animal, nor any difference between expression of NH and NMS stressed mice (**Figure 3.3 C-D**, respectively).

Genes upregulated in response to 2hr RS include Fos (c-Fos), Retnlb (resistin like molecule beta or RELMbeta), Gpx2 (glutathione peroxidase 2), and Fadd (Fas associated via death domain). On further observation, Fos was found to be selectively upregulated in NMS RS mice compared to non-stress basal controls; however, change in Fos expression was no different from that of NH mice exposed to RS, and no effect of cholinergic blockade with HC-3 on Fos expression was observed (**Figure 3.3E**). Retnlb expression was significantly upregulated nearly 6 fold in NMS RS mice compared to unstressed, basal controls; however, there was no significant upregulation of Retnlb in NH RS mice compared to unstressed controls. Interestingly, the upregulation of Retnlb

in NMS RS mice was found to be controlled by cholinergic signaling, as pretreatment of NMS mice prior to RS with HC-3 inhibited upregulation of RetnlB (**Figure 3.3F**). Upregulation of Gpx2 in RS, similarly, appeared to be selectively driven by NMS RS mice, as upregulation of Gpx2 was significantly different from unstressed, basal mice and NH RS mice. NH RS mice were not significantly different from basal, unstressed controls. Additionally, we observed that upregulation of Gpx2 in NMS RS mice appeared to be under control of cholinergic signaling as HC-3 pretreatment prior RS blocked an increase in Gpx2 expression ($p < 0.1$) (**Figure 3.3 G**). Upregulation of Fadd was also selectively driven by NMS RS mice, and upregulation of Fadd in NMS RS mice was significantly different from NH RS mice. Fadd upregulation in RS NMS mice was also found to be impacted by cholinergic blockade as pretreatment with HC-3 prior to NMS prevented up regulation of this gene (**Figure 3.3 H**).

In summary, Retnlb, Gpx2, and Fadd all demonstrated selective upregulation in NMS mice exposed to RS compared to NH RS and unstressed basal controls (**Figure 3.3 A, F-H**). The most robust changes were observed in Retnlb and Fadd expression which increased 6 and 3 fold, respectively compared to unstressed controls. (**Figure 3.3 F and H**). Interestingly upregulation of Retnlb, Gpx2, and Fadd in NMS RS mice all appeared to be under the influence of cholinergic function as increased expression of these genes was significantly inhibited when mice were pretreated with HC-3 prior to 2hr RS (**Figure 3.3 F-H**).

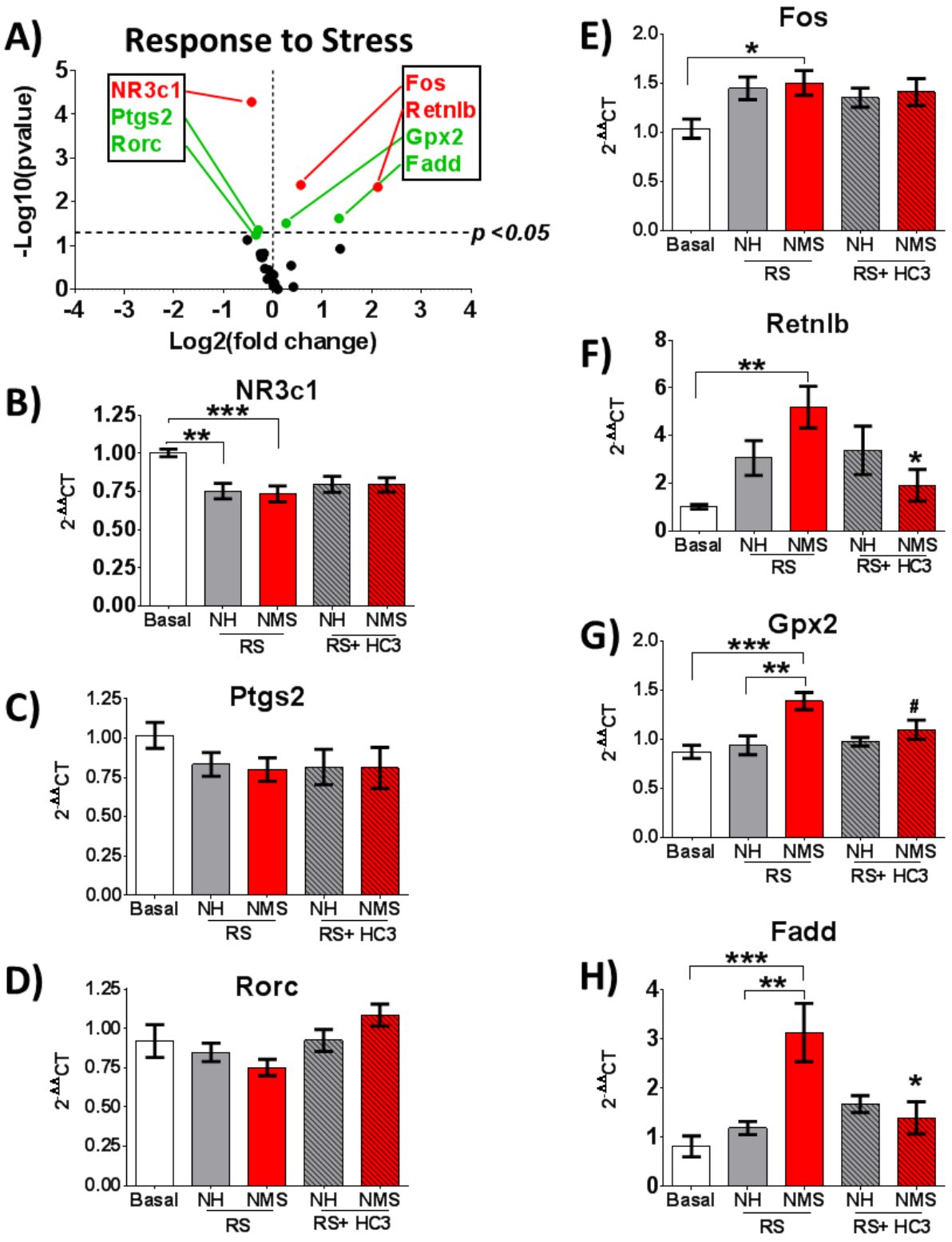

Figure 3.3. Cholinergic control of genes upregulated selectively in NMS mice exposed to acute stress. A) Volcano plot demonstrating significance vs fold change in colon gene expression from NH and NMS 2hr restraint stressed mice compared to

Figure 3.3. (cont'd)
unstressed, basal NH and NMS mice. NR3c1, Fos, and Retnlb were gene found to be significantly different following 2hr restraint stress after account for a 10% false discovery rate (highlighted in red). Ptgs2, Rorc, Gpx2, and Fadd are genes that had *p* – values <0.05, but not considered significant after accounting for 10% false discovery rate are highlighted in green. B-H) Genes upregulated with 2hrs restraint stress and listed above were further investigated for the influence of NMS and cholinergic function, assess by HC-3 injection. B-D) Includes all down regulated genes in response to 2hrs restraint stress. E-H) Includes all upregulated genes in response to restraint stress. Hashed bars indicated group pretreated with HC-3 prior to restraint stress in order to assess the role of cholinergic signaling on gene expression.

Origins of cholinergic dysfunction

It is apparent from the above results that there is cholinergic system dysfunction in adults exposed to early life adversity. To identify the origin of cholinergic dysfunction, we sought to determine how the enteric cholinergic system was differentially expressed early in life during early life adversity. To test this hypothesis, we utilized the porcine model of early life adversity, EWS.(37, 44, 45) Ileum tissue collected 24hours after weaning early, at 16 days, or weaning later at 26 day was collected an immunoblotted for ChAT expression. Generally, we found that weaning induced an upregulation in ileum ChAT expression at 24h hours (**Figure 3.4 A-B**). Further investigating the impact of early weaning compared to weaning later on 24hr expression of ChAT, we found that EWS pigs had a significant upregulation on ChAT expression compared to their age-matched un-weaned controls and LWCs 24 hours post weaning (**Figure 3.4 A and C**)**.**

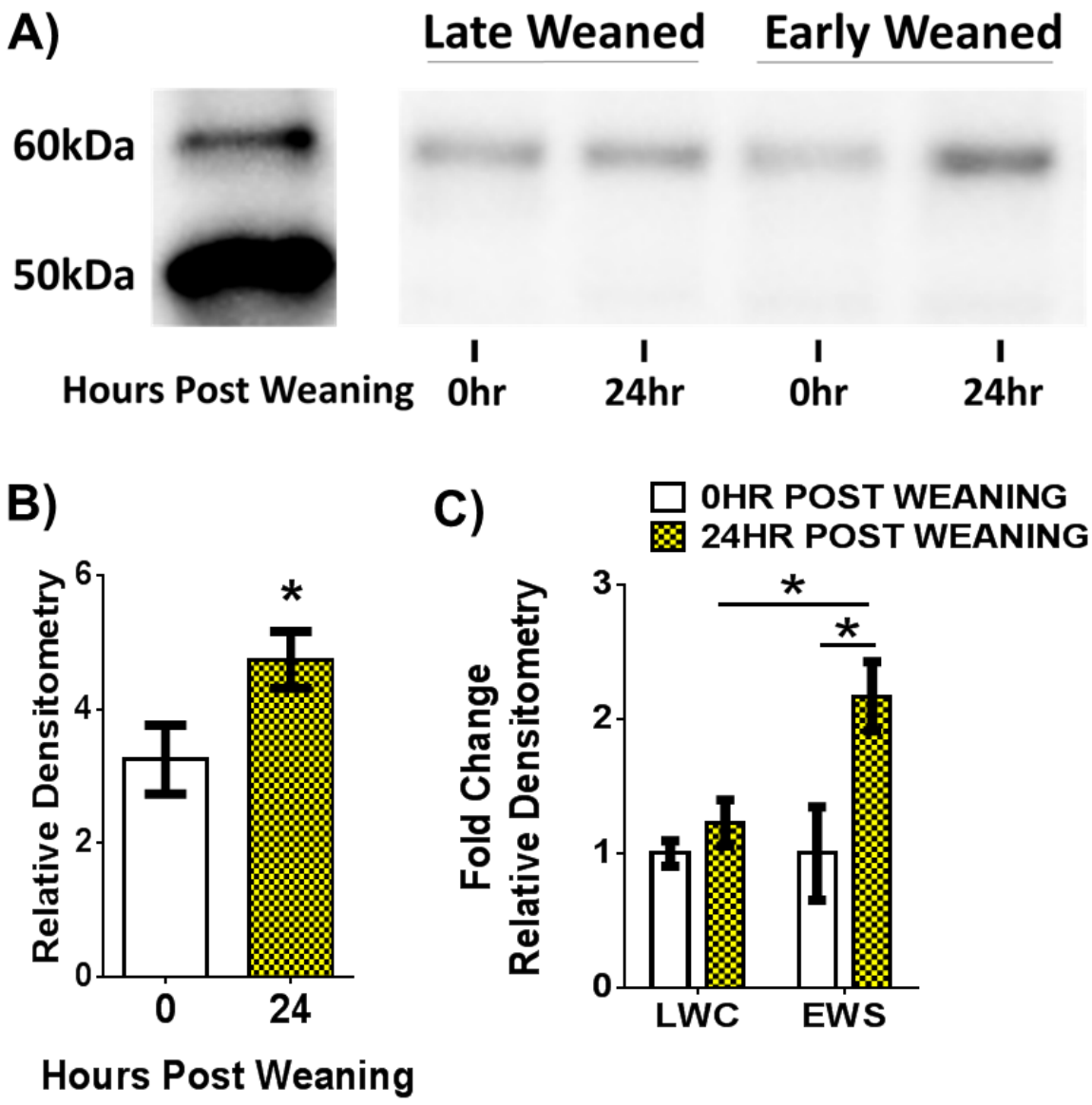

Figure 3.4. Acute upregulation of ChAT immediately following early weaning in pigs. A-B) Upregulation in expression of ileum ChAT in pigs within 24h hours of weaning. C) Upregulation of ChAT 24 hours post weaning is most significant in pigs weaned early (EWS), at 16 days of age, compared to pigs weaned later (LWC) at 26 days of age. Values were normalized to un-weaned, time zero time point within each group.

To further dissect the kinetics and origin of the changes to the cholinergic system during early life adversity, we compared change in ChAT expression between un weaned (0hr), and 3, 8, and 24 hour post early and late weaned pigs. Furthermore, we

bisected to the ileum into follicle free mucosa and follicle associated mucosa (mucosa over the Peyer's patch) to determine if there was particular site within the GI tract that might be driving differences in ChAT expression. Utilizing this approached we observed that ChAT expression from follicle free mucosa in EWS and LWC both mildly increased within 24hrs, and were not significantly different from each other (**Figure 2.5 A**). The increase in ChAT expression from follicle free mucosal was only about 1.5 fold compared to un-weaned pigs. A 10 and 5 fold increase in ChAT expression from EWS follicle associated mucosa was observed at 8 and 24 hours, respectively, and these strong upregulation in CHAT expression were significantly different from LWC at corresponding time points (**Figure 3.5 B**). Since we observed changes in lymphoid follicle associated mucosa, we asked if neighboring lymphoid tissue might also demonstrate an upregulation in ChAT expression. Investigating the mesenteric lymph nodes for ChAT expression, we found a trending, 2 fold, trending upregulation of ChAT expression from EWS pigs 24hrs post weaning compared to LWCs (**Figure 3.5 C**).

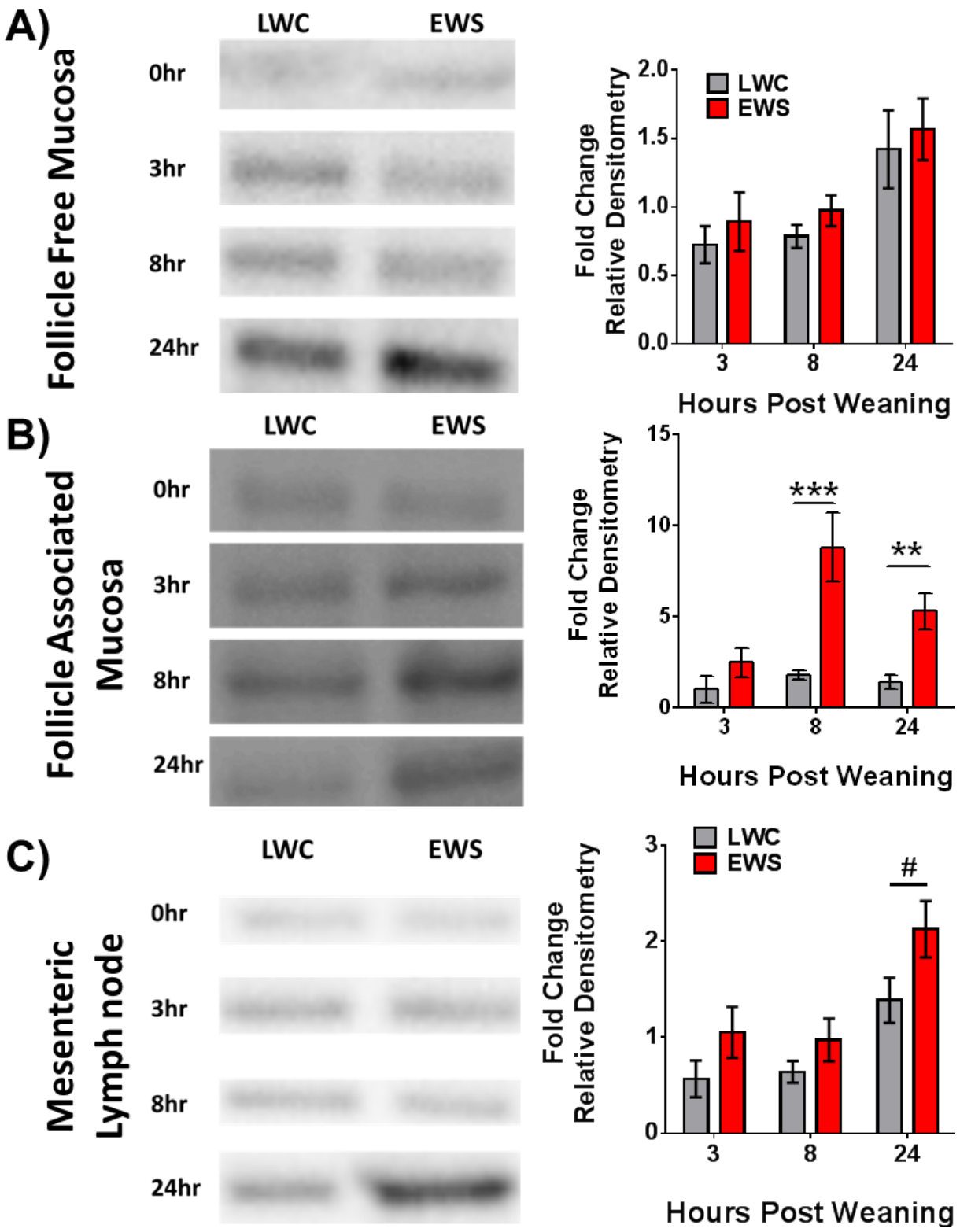

Figure 3.5. Immediate upregulation of ileum ChAT in EWS pigs is associated with mucosal lymphoid tissues. Ileum ChAT expression assessed immediately followingearly weaning (16 days) or later weaning (26 days) in un-weaned (0hr) and 3,

Figure 3.5. (cont'd)
8, and 24hr post weaning. A) Lymphoid follicle free mucosa, B) Lymphoid follicle associated mucosa, C) Mesenteric lymph node. Values were normalized to un-weaned, time zero time point within each group.

NMS does not impact colon cholinergic gene expression

Thus far, we observe that adult animals, with prior experience of ELA have increased enteric cholinergic function, which mediated increased secreto-motor responses, increased intestinal permeability, and increased gene expression in response to stress. Furthermore, early in life during periods of stress and adversity, aspects of the cholinergic system become upregulated in expression. Considering these changes, we next asked if the functional differences observed in adult animals previously exposed to early life adversity might be due to a persistent upregulation of the enteric cholinergic system. Measuring mRNA transcripts from colonic tissue of NH and NMS mice under basal non-stressed conditions, we did not observed any difference in expression of cholinerigic enzymes, ChAT or AChE; nor cholinergic transporters, VAChT or CHT-1 (**Figure 3.6 A**). Furthermore, we did not observe any difference in cholinergic muscarinic or nicotinic receptor expression NH or NMS mice (**Figure 3.6 F**). Since we did not observe cholinergic hyper functional changes in NMS mice until a mild stress was utilized, we assessed cholinergic gene expression under 2hr RS conditions. No difference in ChAT, AChE, VAChT, or CHT-1 gene expression was observed between non-stress basal (white bar) animals or between NH and NMS mice exposed to mild RS (**Figure 3.6 B-E**). Similarly, no difference in gene expression was observed between basal, non-stress controls or between NH and NMS mice in cholinergic muscarinic receptors 1 (CHRM1) and 3 (CHRM3) (**Figure 3.6 G, I**). In both NH and NMS mice, 2hrs RS induced a significant suppression in muscarinic receptor 2

(CHRM2), expression compared to non-stress basal controls, however there was no difference in CHRM2 receptor expression between stressed NH and NMS mice (**Figure 3.6 H**). A similar pattern was overserved in expression of CHRNA7 where stress resulted in a significant gene suppression in NH and NMS compared to non-stress basal mice; however, no difference in gene expression was observed between stress NH and NMS mice (**Figure 3.6 J**).

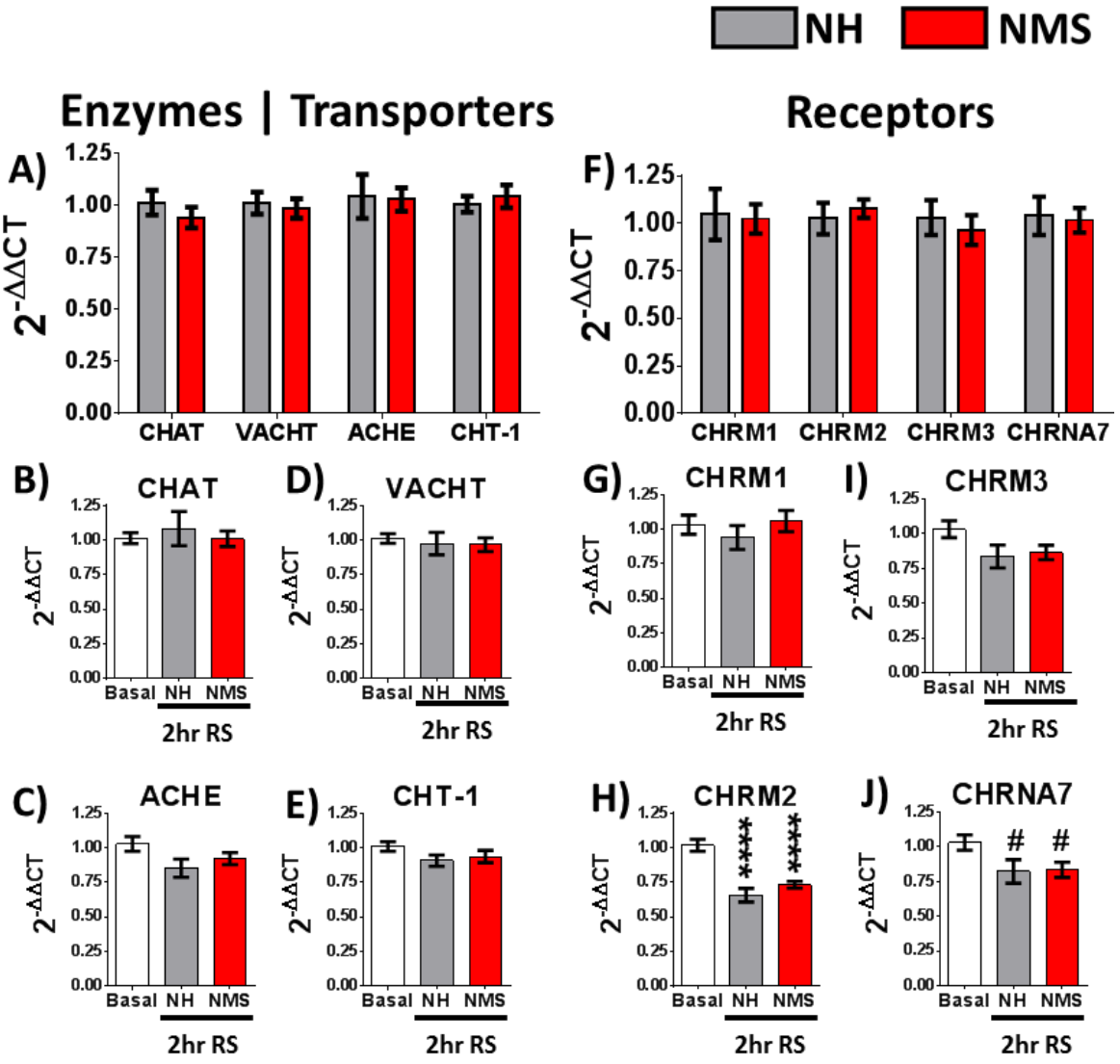

Figure 3.6. Colonic gene expression of cholinergic enzymes, transporters, and receptors do not differ between NH and NMS adult mice. A-D) Gene expression of cholinergic enzymes (ChAT and AChE, A-C) and transporters (VAChT and CHT-1 A, D-E) in non-stressed basal (A), and in response to 2 hours restraint stress (B-E). F-J)

Figure 3.6. (cont'd)
Gene expression of cholinergic muscarinic receptors (CHRM1-3, F-I) and cholinergic nicotinic receptor (CHRNA7, J) in non-stressed basal (F), and in response to 2 hours restraint stress (G-J).

NMS does not impact colon cholinergic nerve number of protein expression

Considering the possibility that gene expression may not truly represent the phenotypic expression of the cholinergic system within the colon, we sought to determine if there is a difference in cholinergic neuron number or a difference in protein expression of cholinergic enzymes. Immunolabeling SMP and MP for ChAT and the pan-neuron body and fiber marker PGP9.5, we counted the percentage of cholinergic positive nerves. No difference in percentage of cholinergic neurons was observes between NMS or NH submucosal plexus or myenteric plexus (**Figure 3.7 A-B**). No difference in total neuron number was observed either (data not shown).

We also assessed total choline ChAT protein expression by western blot, but did not observe any difference in expression between NH or NMS mice (**Figure 3.7 C**). Though there was no difference in ChAT neuron number or expression of CHAT protein, we did observe an upregulation of PGP9.5, a protein selective to neurons and nerve fibers, in colon tissue of NMS mice compared to controls (**Supplemental Fig 3.3**). We did not observe any difference in AChE enzymatic activity, between NH and NMS mice (**Figure 3.7 D**); nor did we find any difference in colon ACh concentration when measured by liquid chromatography – mass spectroscopy following 2 hours restraint stress (**Figure 3.7 E**).

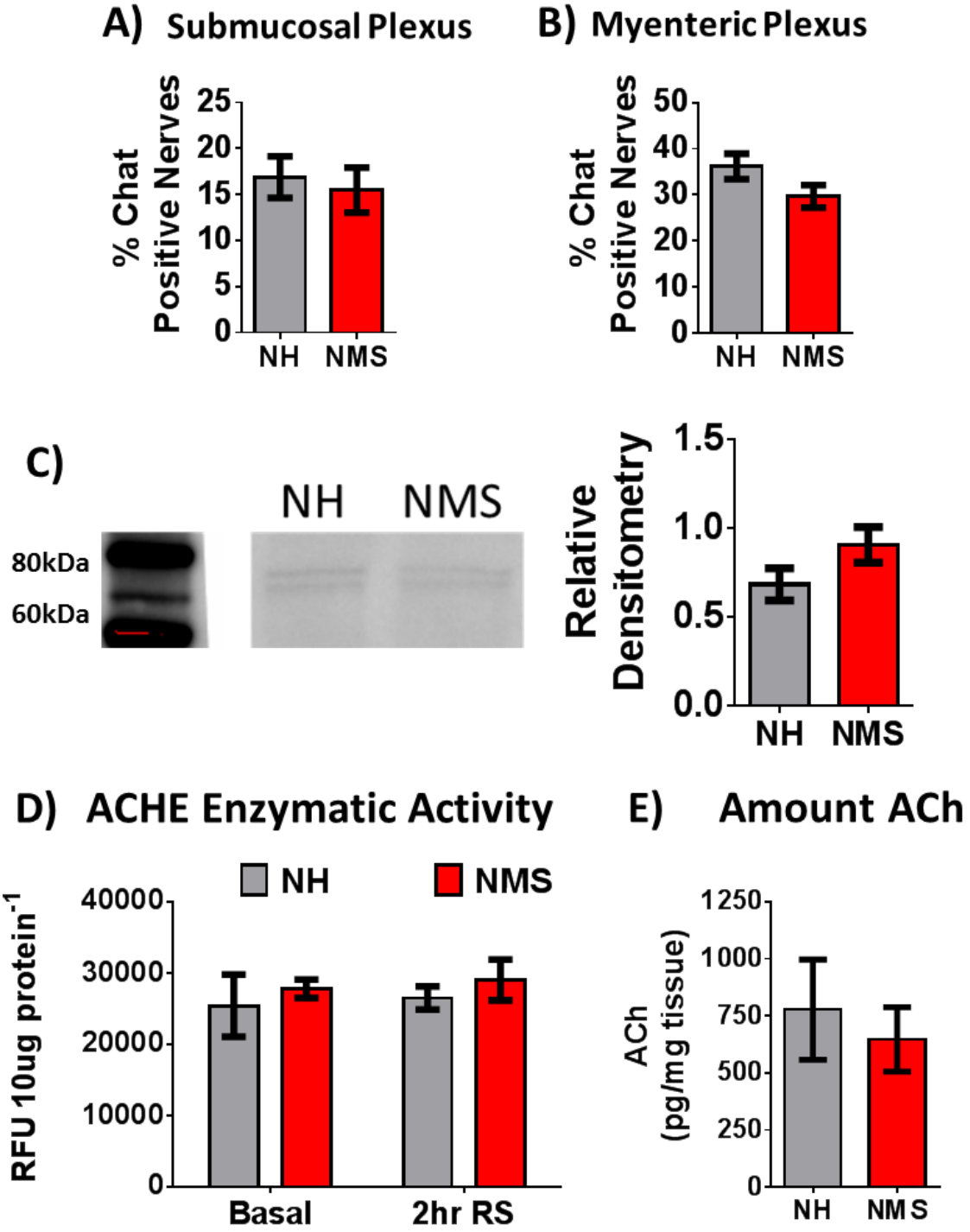

Figure 3.7. Colonic cholinergic enzymes and total acetylcholine non different between adult NH and NMS mice. A-B) quantification of cholinergic neurons from A) submucosal and B) myenteric plexus. C) Western blot for whole colonic ChAT expression, blot (left), histogram (right). D) Enzymatic activity of AChE in colon tissue

Figure 3.7 (cont'd)
from NH and NMS mice in non-stressed basal and 2 hour restraint stress mice. E) Quantification of whole colon ACh by LC-MS following 2hrs restraint stress.

Discussion

Here we demonstrate that NMS resulted in underlying secretomotor abnormalities in adults, which manifested clinically under exposure of acute stress. Resting, non-stressed NMS mice demonstrated increased intestinal electrogenic ion transport, increased endogenous colonic contractility the both of which could be exacerbated with neuronal stimulation and the former exacerbated with facilitation of cholinergic signaling through physostigmine hemisulfate. Exposure to a mild stress exacerbated underlying mucosal and motility dysfunction leading to increased intestinal permeability and fecal pellet output in NMS mice compared to baseline and compared to stressed NH mice. Importantly, NMS increased GI dysfunction following an acute stressor was blocked by HC-3 pre-treatment, indicating that the NMS vulnerability to a secondary stressor was mediated by cholinergic hyperfunction. Upregulated gene expression following mild stress almost selectively occurred in NMS mice compared to controls. These elevated gene expression in NMS mice following a secondary stress appeared to be under the control of cholinergic function, as HC-3 prevented their upregulation. Interestingly, we observed that RS appeared to upregulate intestinal permeability and gene expression selectively in NMS mice compared to NH controls, highlighting the sensitivity of the NMS mice to mild stress as adults. Finally, the increased cholinergic function in NMS mice did not appear to be due to any differential expression of canonical cholinergic enzymes, transporters, receptors, or concentration of ACh ligand. However, since gene expression was assess from full thickness colon

pieces, a measurement of neuron specific expression of these cholinergic mediators may reveal different answers. Nor was there any change in cholinergic neuron number, suggesting that the sensitivity of NMS mice to secondary stress is purely due to hyperfunction rather than over expression of any part of this system. Abnormalities in the cholinergic system first appear, immediately during the early life period, demonstrated by an upregulation of mucosal CHAT.

ELA induced disturbances in secretomotor function were noted by an increased basal electrogenic ion transport in NMS mice compared to NH mice, a finding that has similarly been shown in a rat NMS model (48) and in porcine EWS.(37) NMS mice also demonstrated elevated nerve mediated electrogenic ion transport in the ileum, at a low frequency of electrical stimulation, indicating that the enteric neurons of the NMS mice were hypersensitive as they could be more robustly activated at lower frequencies. Additionally, we demonstrated an elevated colonic mucosal electrogenic activity from NMS mice compared to NH mice in response to physostigmine application. Classically, magnitude of electrogenic ion currents is indicative of fluid transport, as water tends to follow along an osmotic gradient. Previous reports demonstrated that the increased current response in EFS are due to electrogenic transport of chloride ions, which is associated with a net movement of water from the tissue into the lumen.(12) Interpreting the electrogenic response of the colonic tissue is less clear; however the reduction in current response during *phase I* may be attributed to cholinergic nerve blockade of sodium absorption, resulting in a net reduction in current. This is supported by our supplementary data demonstrating that *phase I* responses were ablated in the absence of sodium and in the presence of TTX. Supporting our conclusion, others have

demonstrated that EFS and cholinergic stimulation of colon tissue resulted in a reduced net sodium absorption.(22, 30) Finally, the elevated *phase II* response in NMS mice appeared to be chloride dependent. Together, these data suggest that cholinergic stimulation results in increased luminal sodium and chloride, generating an osmotic gradient supporting increased fluid transport into the colon lumen. That the magnitude of these responses was most strongly observed in NMS mice suggest, that they are predisposed to increase water transport into the colonic lumen during cholinergic nerve stimulation, which would translate to increased risk for diarrhea. In summary, NMS adult mice appear to have an underlying hyperactive enteric cholinergic nervous system in both the ileum and the colon, similar to our findings in EWS pigs, where elevated enteric nerve function was found to be mostly mediated by cholinergic signaling through muscarinic receptors.(37)

Though we had previously demonstrated a persistent elevation in enteric cholinergic nerve secretion function following ELA in the porcine EWS model, we extended those finds here to demonstrate that the cholinergic system of adult NMS animals mediated increased gastrointestinal dysfunction following an acute stressor. Similar to others, we demonstrated that adult stress provoked an increase in fecal pellet output and intestinal permeability in NMS mice compared to controls.(13, 48) The role of the cholinergic system in mediating these responses was observed by HC-3 blocking the elevation in fecal pellet output and permeability following 2 hours of restraint stress. Additionally, we found significant upregulation of three different genes in NMS RS mice compared to control, and upregulation of these gene appeared to be controlled by cholinergic signaling, as HC-3 prevented upregulation in NMS RS mice pre-treated with

HC-3. However, since HC-3 prevents cholinergic signaling by depleting pre-synaptic acetylcholine stores, it is possible the both pre-synaptic (vagus (5) or sympathetic (28)) and post-synpatic (enteric (17)) cholinergic nerves were impacted by HC-3 treatment. Nevertheless, the body of evidence presented here, including exacerbated response to physostigmine and intrinstic colon nerve hyperactivity, with evidence demonstrating a hyperactive enteric cholinergic system in porcine EWS (37), support an effect of HC-3 on the enteric cholinergic nervous system.

The genes differentially expressed in NMS restraint stressed mice were Retnlb, Gpx2, and Fadd. Retnlb is a resistin like molecule found primarily in intestinal epithelium, particularly in goblet cells.(51) Classically, resistins are hormones secreted from adipocytes, which induce insulin resistance (50); however the role of Retnlb in the intestine appears to be diverse. From an immunological standpoint, increased Retnlb expression promotes inflammatory cytokine production during infection (41), and enhances chemical colitis severity.(3, 26, 36) Interestingly, expression of Retnlb in the colon correlated positively with metabolic dysfunction, where higher colonic Retnlb expression was observed in obese mice.(47) That Retnlb could be selectively upregulated in NMS mice during an acute, mild stress highlights the likelihood that this gene may contribute to stress susceptible induction of GI inflammation and metabolic disorders in individuals with prior ELA. Significantly increased induction of Retnlb and its link to obesity may explain why we observed heavier body weights in both adult NMS and EWS pigs. Even more interesting was that expression of this gene appears to be under control of cholinergic signaling, demonstrating a nascent role of the cholinergic enteric nervous system in metabolic diseases.

Gpx2 is a glutathione peroxidase, which breaks down hydrogen peroxide, and its upregulation potentially indicates high oxidative stress.(11) Selective upregulation of Gpx2 in NMS mice following stress further highlights the susceptibility of ELA individuals to developing GI disease as Gpx2 is a reliable marker of GI inflammation in chemical colitis and human inflammatory bowel diseases.(52) Fadd is the fas associated via death domain gene, which is known to be upregulated in cells undergoing apoptosis. Interestingly, a protective role for Fadd in limiting ileitis and colitis has been detailed, were Fadd expression in epithelium maintains intestinal barrier function by promoting expression of anti-microbial peptides.(55) Therefore, the upregulated Fadd expression in NMS stressed mice may be in response to increased intestinal permeability. Interestingly, the expression of both Gpx2 and Fadd was under the control of cholinergic signaling, as upregulated expression of these genes in NMS stressed mice was blocked by HC-3 application.

In these studies we reported an increase in intestinal cholinergic function, which contributed to intestinal permeability, motility, and mucosal electrogenic ion transport. To further understand the underlying mechanism of cholinergic dysfunction in adult animals with prior exposure to ELA, we sought to determine the expression level of canonical cholinergic enzymes, transporters, and receptors. Previously, we demonstrated the EWS resulted in a persistent upregulation of cholinergic enteric neurons, with dysregulated expression of muscarinic receptors and ACHE activity.(37) Conversely, in adult NMS mice, we did not observe any difference in cholinergic neuron numbers or enzyme, transporter, or receptor expression compared to NH controls. Further, no difference in ACh ligand expression was detected between NMS and NH. In

fact, ACH concentrations were numerically lower in NMS mice compared to controls, a phenomena previously observed by an other group.(21) Since NMS mice do not express any differences in the cholinergic system compared to NH mice, the observed role of the cholinergic system in mediating these different GI dysfunctional responses may be due to anatomical or functional difference of the enteric nervous system rather than difference in neurochemical expression. For example, membrane properties of enteric nerves were altered during and following resolution of chemical colitis, making the enteric nervous system hypersensitive to subsequent stimulation.(29, 32, 33) Since ELA can induce GI injury early in life (40), it is possible that the underlying enteric neurons develop abnormal membrane properties, making them hypersensitive to stimulation later in life, without necessarily changing expression of neurotransmitters. Supporting this hypothesis, other groups have demonstrated the electrical field stimulation of NMS colonic tissue resulted in an increased liberation of ACh.(16) Development of additional neuroanatomical structures or aberrant neuro-circuits following ELA may also explain functional difference in cholinergic signaling, as we observed increased neuron fiber density in NMS mice compared to controls on western blot, a phenomena observe by others in adult NMS rats.(4)

Finally, we observed that cholinergic dysregulation begins immediately during early life adversity with upregulation of mucosal ChAT expression in ileum of EWS. Increased epithelial ChAT expression was similarly reported immediately following NMS in rat pups, with cholinergic signaling contributing to excessive electrogenic ion transport and increased intestinal permeability.(20) However, we identify here that the changes in ChAT expression appear mostly over lymphoid follicle associate tissue. The

upregulation in cholinergic signaling over the lymphoid tissue could be to facilitate immune activation or development, as cholinerigc signaling has previously been shown to enhance antigen uptake over the Peyer's patch.(7)

Together these findings demonstrate a functional role of the cholinergic system which mediates stress induced susceptibility to GI dysfunction in mice previously exposed to early life adversity. Though there was no difference in expression, the function of the cholinergic system mediated increased secretomotor responses, elevated intestinal motility, increased intestinal permeability, and increased expression of genes which may enhance intestinal inflammation and predispose individuals to insulin resistance and weight gain. Therefore, the cholinergic system appears to be central in understand the risk factors associated ELA with subsequent disease later in life. Future therapies may focus on modulating nerve function rather than target expression of certain neurotransmitters to alleviate chronic disease associated with early life adversity.

APPENDIX

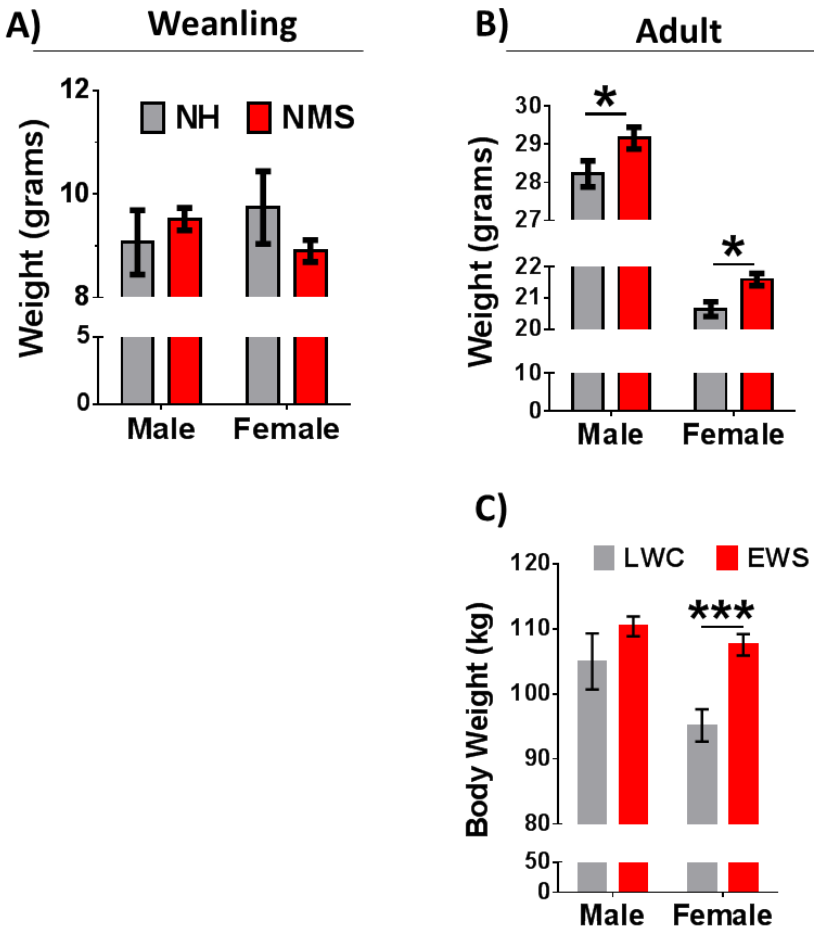

Figure S.3.1. ELA adversity induces heavier body weights in adult animals. A-B) Mice exposed to NMS or ELA. A) Weights at weaning of 3 weeks of age. B) Body weight of mice at 10 weeks of age. C) Body weight of adult pig 20 weeks of age.

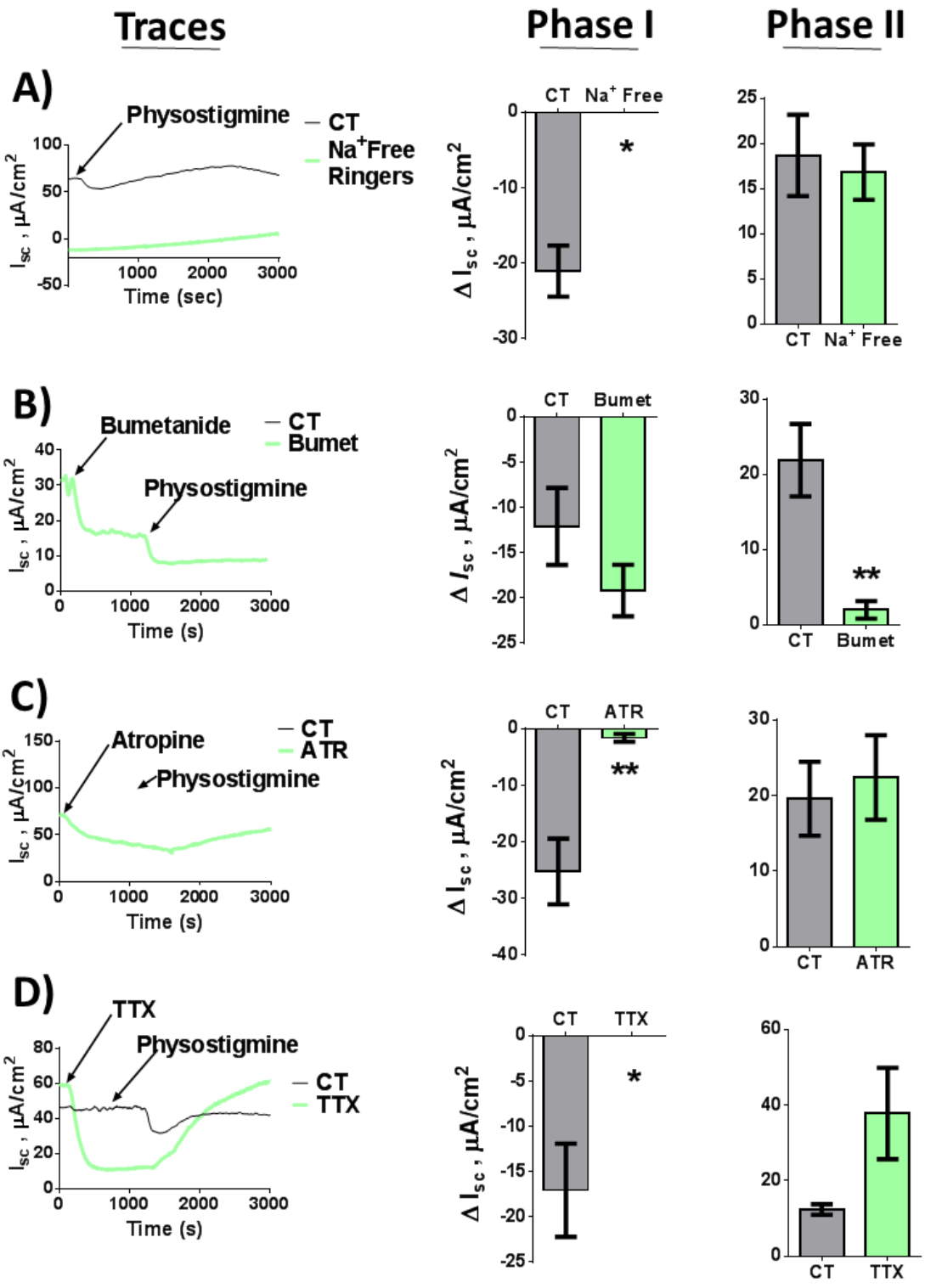

Figure S.3.2. Mechanisms of physostigmine response in murine colonic tissue. Colonic tissue from healthy, adult control mice was mounted on Ussing

Figure S.3.2. (cont'd)
Chambers. Mechanisms of phase I and phase II responses to serosal application of 500uM physostigmine hemisulfate were determine under conditions of A) sodium free Ringer's, B) chloride channel blocker bumetanide (Bumet), C) muscarinic receptor blocker 100uM atropine (ATR), and D) neuronal sodium voltage gate channel blocker (1uM) tetrototoxin (TTX).

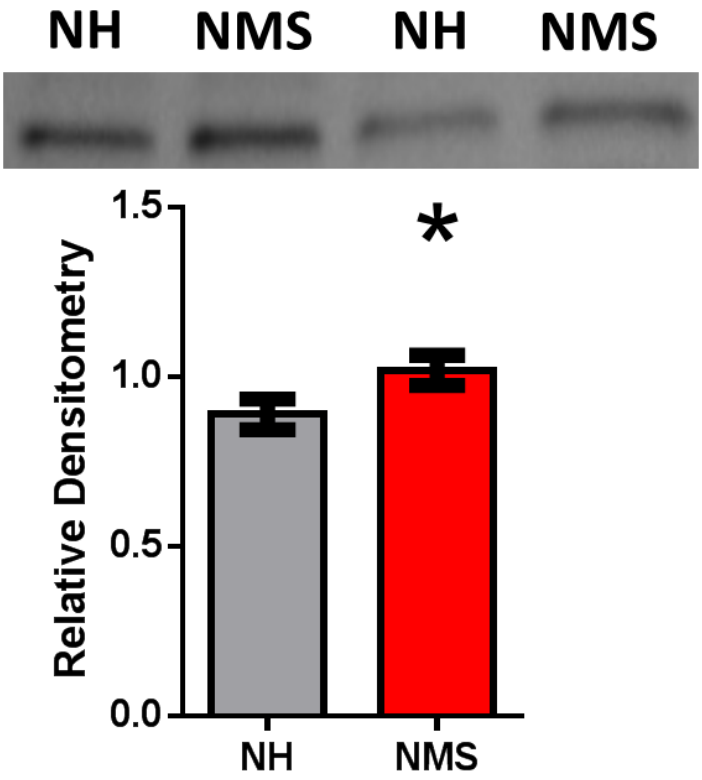

Figure S.3.3. Upregulation of colonic nerve fiber protein in NMS mice

Gene and PrimerBank Code	Sequence	
TNF	F	CCCTCACACTCAGATCATCTTCT
7305585a1	R	GCTACGACGTGGGCTACAG
MUC2	F	ctgaccaagagcgaacacaa
23956200a1	R	catgactggaagcaactgga
Muc3	F	cgtggtcaactgcgagaatgg
	R	cggctctatctctacgctctcc
Reg3g	F	aacagaggtggatgggagtg
	R	ggccttgaatttgcagacat
Retnlb	F	agctctcagtcgtcaagagcctaa
	R	cacaagcacatccagtgacaacca
GC-R (NR3c1)	F	AGCTCCCCCTGGTAGAGAC
6680103a1	R	GGTGAAGACGCAGAAACCTTG
Bcl2	F	GTCGCTACCGTCGTGACTTC
28916685a1	R	CAGACATGCACCTACCCAGC
Bax	F	TGAAGACAGGGGCCTTTTTG
6680770a1	R	AATTCGCCGGAGACACTCG
Hmgcs2	F	GAAGAGAGCGATGCAGGAAAC
31560689a1	R	GTCCACATATTGGGCTGGAAA
Ptgs2	F	TGAGCAACTATTCCAAACCAGC
31981525a1	R	GCACGTAGTCTTCGATCACTATC
Fos	F	CGGGTTTCAACGCCGACTA
6753894a1	R	TTGGCACTAGAGACGGACAGA
Ppp3r1	F	GAAGGAGTGTCTCAGTTCAGTG
13277370a1	R	ACGAAAAGCAAACCTCAACTTCT
Gpx2	F	GCCTCAAGTATGTCCGACCTG
17432429a1	R	GGAGAACGGGTCATCATAAGGG
Ccl2	F	TTAAAAACCTGGATCGGAACCAA
6755430a1	R	GCATTAGCTTCAGATTTACGGGT
Cxcl1	F	CTGGGATTCACCTCAAGAACATC
6680109a1	R	CAGGGTCAAGGCAAGCCTC
Il1b	F	GCAACTGTTCCTGAACTCAACT
6680415a1	R	ATCTTTTGGGGTCCGTCAACT
Il33	F	TCCAACTCCAAGATTTCCCCG
19527000a1	R	CATGCAGTAGACATGGCAGAA
Ccl11	F	GAATCACCAACAACAGATGCAC
6755418a1	R	ATCCTGGACCCACTTCTTCTT
Ptger2	F	GGAGGACTGCAAGAGTCGTC
26331428a1	R	GCGATGAGATTCCCCAGAACC
Ltc4s	F	ATGAAGGACGAAGTGGCTCTT

Table S.3.1 Primer sequences for Wafergen PCR Array

Table S.3.1. (cont'd)

20380551a1	R	CCTGTAGGGAGAAGTAGGCTTG
Fadd	F	GCGCCGACACGATCTACTG
6753812a1	R	TTACCCGCTCACTCAGACTTC
Rorc	F	GACCCACACCTCACAAATTGA
6755344a1	R	AGTAGGCCACATTACACTGCT
Gapdh	F	AGGTCGGTGTGAACGGATTTG
6679937a1	R	TGTAGACCATGTAGTTGAGGTCA
Hprt	F	TCAGTCAACGGGGGACATAAA
7305155a1	R	GGGGCTGTACTGCTTAACCAG
Actb	F	GGCTGTATTCCCCTCCATCG
6671509a1	R	CCAGTTGGTAACAATGCCATGT

Gene	**Taqman ID**
HPRT	Mm00446968_m1
CHRM1	Mm00432509_s1
CHRM2	Mm01701855_s1
CHRM3	Mm00446300_s1
Chrna7	Mm01317884_m1
CHAT	Mm01221880_m1
ACHE	Mm00477274_g1
VACHT	Mm00491465_s1
CHT-1	Mm00452075_m1

Table S.3.2 Taqman Primer Probe Catalog Numbers

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CHAPTER 4

S. Typhimurium challenge in juvenile pigs modulates the expression and localization of enteric cholinergic proteins and correlates with mucosal injury and inflammation

The text presented in this chapter is currently in re-submission as Pohl CS, Lennon EM, Yihang L, DeWilde MP, and Moeser AJ. *S.* Typhimurium challenge in juvenile pigs modulates the expression and localization of enteric cholinergic proteins and correlates with mucosal injury and inflammation. *Autonomic Neuroscience: Basic and Clinical*, 2018.

Abstract

In the present study we characterized the changes in expression of the enteric mucosal cholinergic system in pigs acutely challenged with *S.* Typhimurium and how these changes are correlated with tissue damage and mucosal inflammatory cytokine production. At 2 d post-challenge, a three-fold reduction in ileal acetylcholine (ACh) levels was observed in challenged animals, compared with controls. Ileal acetylcholinesterase (AChE) activity was decreased (by four-fold) while choline acetyltransferase (ChAT) expression was increased in both the ileum and mesenteric lymph nodes. Elevated ChAT found found to localize preferentially to mucosa overlying lymphoid follicles of the Peyers patch in challenged pigs, with more intense labeling for ChAT in *S.* Typhimurium challenged pigs compared to controls. Ileal mRNA gene expression of muscarinic receptor 1 and 3 was also increased in challenged pigs, while muscarinic receptor 2 and the nicotinic receptor alpha 7 subunit gene expression were unaffected. A positive correlation was observed between ChAT protein expression in the ileum, rectal temperature, and histopathological severity in challenged animals. These data show that inflammation from *S.* Typhimurium challenge alters enteric cholinergic expression by down-regulating acetylcholine concentration and acetylcholine degrading enzymes while increasing acetylcholine synthesis proteins and receptors. Given the known anti-inflammatory role of the cholinergic system, the divergent expression of cholinergic genes may represent an attempt to limit tissue damage by preserving cholinergic signaling in the face of low ligand availability.

Introduction

To survive infectious challenges, the host must balance pathogen inducing immunity and inflammation with responses that limit tissue damage. Therapeutic interest focuses on pathways that simultaneously limit tissue damage without comprising sterilizing immunity.(42, 44) Due to the intimate juxtaposition of microbes and host tissue, the gastrointestinal tract is an area requiring precise balance between immunity and host tissue survival, and the cholinergic system is considered to be an important factor mediating this balancing act during enteritis.(16)

Cholinergic signaling via the vagus nerve is a well-established mediator of inflammation in the gastrointestinal tract.(29) Several groups have shown that cholinergic function, mostly through action of the vagus nerve and nicotinic receptors, reduces gastrointestinal inflammation and prevents mucosal tissue damage in rodent models of chemical colitis (19, 20, 31, 36), endotoxemia (12), and postoperative ileus (30, 46). Further anti-inflammatory influence of the cholinergic vagal system is known by its role in promoting oral tolerance to foreign antigens (14).

The functional role of the cholinergic system in infectious enteritis is less well described; however, initial reports suggest that in contrast to the chemical colitis and postoperative ileus models, cholinergic signaling may enhance immunity by promoting inflammation. For example, pretreating mice with an acetylcholinesterase inhibitor prior to oral *Salmonella* infection increased serum inflammatory cytokine production, bacterial clearance, and host survival.(15) Another report demonstrated that cholinergic muscarinic receptors enhance T-cell pro-inflammatory activity and contribute to rapid convalescence and generation of adaptive immunity against both bacterial and parasitic

infection (10). However, certain bacterial and parasitic, infectious enteritis models were also shown to result in suppressed cholinergic enteric nerve activity and attenuated release of ACh(3, 9, 17). Together, previous studies in rodents indicate that the cholinergic system has significant, yet divergent actions during mucosal inflammation which may depend upon the inciting stimuli.

The cholinergic system has several components that regulate the synthesis, degradation and signaling of Ach. Acetylcholine (ACh), the primary endogenous ligand inducing cholinergic signaling can be synthesized in both neuronal and non-neuronal cells. Choline acetyltransferase (ChAT), is the primary enzyme that generates acetylcholine, and it can be found in epithelia (18), immune (13, 24, 40), and neuronal cells (49, 51). Once acetylcholine is liberated into the extracellular space, it acts on two classes of cholinergic receptors, known as muscarinic and nicotinic receptors. Muscarinic receptors are g-coupled protein receptors that have up to 5 different sub-classes.(28) Nicotinic receptors are pentameric, ionotropic receptors that are formed by heterogeneous or homogenous assembly of one of several different nicotinic subunits.(2) Both types of receptors can be found on several different cells, mediating vastly different homeostatic functions. Finally, cholinergic signaling is terminated primarily by acetylcholinesterase (AChE), an enzyme the breaks down ACh at high efficiency and is expressed in many different cell types. While the functions of each component of the cholinergic system is well-established, how each component is dynamically during infectious challenge remain poorly defined.

In the present study we characterized the changes in expression of the enteric mucosal cholinergic system components in pigs acutely challenged with *S.* Typhimurium and

how these changes are correlated with tissue damage and mucosal inflammatory cytokine production. Considering the significant contribution of acetylcholine to gut homeostasis, understanding the expression of the cholinergic system during inflammatory challenges will provide a foundational understanding for future research and therapies.

Materials and Methods

Animals and experimental design

Data was generated from tissues which were collected in a previously reported experiment (4), which was under an approved Institutional Animal care and Use Committee at North Carolina State University (protocol no. 12-051-A). As reported previously, animals used were Yorkshire-Large White piglets weaned at 16-17 days of age and housed at 8 pigs per pen with *ad libitum* access to water and feed. Sex was distributed equally across weaning groups. At 34 days post weaning, all piglets were transferred to isolation rooms and housed by treatment groups with continued *ab libitum* access to food and water. *S.* Typhimurium challenged pigs were orally inoculated with $3x10^9$ colony forming units in 4mls of culture media; while uninfected controls were feed 4mls of sterile culture media. Inoculated pigs were house separately from unchallenged controls; however, both were housed under the same facility in identical rooms. *S.* Typhimurium DT104 strain culture were grown overnight at 37^{o}C in Luria broth agar and added to 0.7% sterile saline for form final concentrations of $7.5x10^8$ colony forming unites per mL (4). Animal were euthanized 2 days post pathogen challenge, and collection of ileum mucosal scrapes and lymph nodes were performed and stored as previously reported (4).

Ileum and mesenteric lymph node protein isolation

For SDS-PAGE and Western blot, 0.5cm^3 pieces of ileal mucosa scrapes and mesenteric lymph node were collected over dry ice and homogenized in RIPA buffer (Thermo Scientific, #89900) in the presence of 1x protease inhibitor cocktail (Sigma Aldrich, #P8340) and 1x Halt Phosphatase Inhibitor (Thermo Scientific, #78420). Samples were spun at 13,300rpm at 4^{o}C for 15min. Supernatant was collected, aliquoted and frozen at -70^{o}C. Protein concentration was determine with Pierce BCA kit (Thermo Scientific, #23225), and samples were diluted to working a concentration of 2µg/µl.

For TNF and IL-8 ELISA and myeloperoxidase assay, samples were isolated as previously reported.(4)

SDS PAGE | *Western Blot*

Ileal mucosa and mesenteric lymph node protein samples were diluted to 1µg/µL in Laemmli Buffer (Bio-Rad, #161-0737) + 5% 2-mercaptoethanol and heat denatured at 70^{o}C for 10 minutes. 10 µg of protein sample was run on a TGX-Stain Free gel (Bio-Rad #5678095). Protocol for electrophoresis, wet to wet transfer, and stain free, lane total protein quantification was performed as published in Criterion^{TM} Precast Gels: Instruction Manual and Application Guide and Western Blot Normalization Using Image Lab^{TM} Software (Bio-Rad). The PVDF membrane was blocked in 5% BSA at RT for 1hr prior to incubation with monoclonal 1.B3.9B3 anti- porcine ChAT antibody (Millipore Sigma #MAB5270) at a concentration of 1:1000 in 1xTBS + 5% BSA + 0.1% Tween-20 overnight at 4^{o}C. The following morning, the blot was washed and an HRP linked anti-mouse antibody in 1xTBS + 5% BSA + 0.1% Tween-20 (Cell Signaling, #7076) at

(1:1000) was incubated with the membrane for 1hr at RT. Chemiluminescence was performed using Clarity ECL (Bio-Rad, #1705060). Densitometry was performed utilizing Bio-Rad Image Lab™ Software v5.2.1 and band density was normalized to lane total protein per Western Blot Normalization Using Image Lab™ Software (Bio-Rad) protocols.

Acetylcholine quantification and acetylcholinesterase activity

Acetylcholine and acetylcholinesterase activity was performed on protein isolated from ileum mucosal scrapes using Amplex™ Acetylcholine/Acetlycholinesterase Assay Kit (ThermoFisher Scientific cat#A12217) per manufacturer's instructions.

Gene Expression Analysis

Total RNA samples were isolated from frozen intestinal mucosal scrapings using the Qiagen RNeasy Mini kit. First-strand cDNA was synthesized from 4 µg RNA using Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific, K1641) according to the manufacturer's instructions. Semi-quantitative, real-time PCR was used to determine the relative quantities of transcripts of the genes of interest. Beta-actin (*ACTB*) was selected and validated as suitable internal reference genes. The relative gene expressions of cholinergic receptor muscarinic 1 (*CHRM1*), 2 (*CHRM2*), and 3 (*CHRM3*), cholinergic receptor nicotinic alpha 7 subunit (*CHRNA7*) were determined. Primer sequences for all genes are provided in **Supplemental Table 4.1.** All PCR reactions were subjected to a melt curve analysis to validate the absence of nonspecific products. The data are presented as 2-ΔΔCT in gene expression relative to control group, normalized to the *ACTB* before

Correlation Analysis

Correlations were performed between mucosal acetylcholine concentrations, acetylcholinesterase activity, and the ChAT band identified on Western blot and rectal temperature, ileum histopathological scores, or ileum mucosal cytokine levels reported in a previous publication.(4) For methods on histopathology and mucosal cytokine analysis, please refer to our previous manuscript.(4) Comparisons between ChAT and cytokines of interest was performed per each animal, and two tailed Pearson correlations were run on each group to identify any positive or negative association between ChAT expression and cytokine protein.

Immunohistochemistry and Image analysis

Sections of ileum were fixed in 10% neutral buffered formalin and paraffin embedded. Sections were prepped and immunohistochemically labeled for ChAT with B3.9B3 anti-porcine ChAT antibody (Millipore Sigma #MAB5270) at 1:100. Detection of ChAT in section was performed by using secondary anti-mouse-on-Farma HRP polymer for 30min at RT and treatment with Romulin AEC. Slides were counter stained with hematoxylin at 1:10. All sample preparation and labeling was performed by Michigan State University's Investigative Histopathology Laboratory. Total mucosal area of ChAT positive labelling and integrated density of ChAT labelling were determine to generally assess the number of cells positive to ChAT and the intensity with which ChAT was expressed, respectively. Using ImageJ (U.S. NIH, Bethesda, MD, USA), total area and integrated density of ChAT expression was determined by using the threshold function on the blue stack for each RGB image. Using the threshold function, we were able to assess the area and integrated density of the ChAT labeling alone, independent of the

hematoxylin counter stain. The threshold was set from 0-61 and was consistently used on each image assess. The percentage area of ChAT positive staining was determined by dividing the area of ChAT positive labeling by the total area of the mucosa in the field. 10 images were captured for each individual tissue slide in order to make the calculations.

Statistics

Two-tailed student's t-test were performed on most data comparing unchallenged controls to *S.* Typhimurium challenged animals. Unless reported otherwise, all values reported are means and standard error of means. Two tailed student t-tests and Pearson correlations and figures were generated with GraphPad Prism 6, v6.04 (GraphPad Prism Software)

Results

Summary of S. Typhimurium *clinical and histopathological findings as previously reported*

In **Table 4.1**, we summarize clinical and ileal histopathological features between uninfected controls and *S.* Typhimurium challenged animals as previously reported.(4) *S.* Typhimurium challenge induced significant diarrhea and pyrexia. Histopathology scores demonstrate that *S.* Typhimurium induced intestinal mucosal injury with moderate to severe villus blunting, mild villus fusion, and moderate lymphoid depletion in the ileum. **Table 4.1** summarizes the clinical and histopathological effects of the *S.* Typhimurium challenge in this porcine model. For representative photos of histopathological findings, see **Supplemental Figure 4.1** in the Appendix.

		Control	*S. typhimurium* challenged	p-value	sig
Fecal Score		1.20	3.33	0.0012	y
Rectal Temperature (^{o}C)		39.70	40.64	0.0238	y
Histopath Scores	Villus Blunting	0.00	2.20	0.0172	y
	Villus Fusion	0.00	1.40	0.0479	y
	Lymphoid Depletion	0.20	2.00	0.0063	y

Table 4.1. Previously reported clinical and histopathology effects of *S.* Typhimurium
Data in this table are summarized from a previously reported study.(4) Fecal consistency was scored on a 4 point scale by an individual blinded to the study design (1= no diarrhea; 4= severe, profuse diarrhea). Histopathology scoring was performed as previously reported. Villus blunting was scored on a 5 point scale with 0 = 1:4 crypt to villus height and 4 = no villus present. Villus fusion and lymphoid depletion were scored on a 4 point scale with 0 = normal and 3 = severe. n=5-6 per group. *p-values* generated by two tailed students t-test. For significance, y=yes; n=no.

S. Typhimurium *challenge reduced ileal acetylcholine levels*

The immune-modulatory role of the cholinergic system is mediated primarily through the ligand acetylcholine, so we asked whether bacterial enteritis would affect ileal mucosal acetylcholine concentration. Compared with controls, *S.* Typhimurium-challenged animals exhibited a 3-fold reduction (*p=0.0142*) in ileal mucosal acetylcholine concentration (**Figure 4.1A**).

S. Typhimurium *challenge down-regulates ileal mucosal acetylcholinesterase activity*

Since challenged animals demonstrated reduced concentrations of acetylcholine in ileal mucosa, we reasoned that AChE, the enzyme that degrades acetylcholine, might be upregulated during enteritis. In contrast to what we expected, measurements for AChE enzymatic activity in ileal mucosa demonstrated over a 2-fold reduction in challenged animals compared with controls (**Figure 4.1B**).

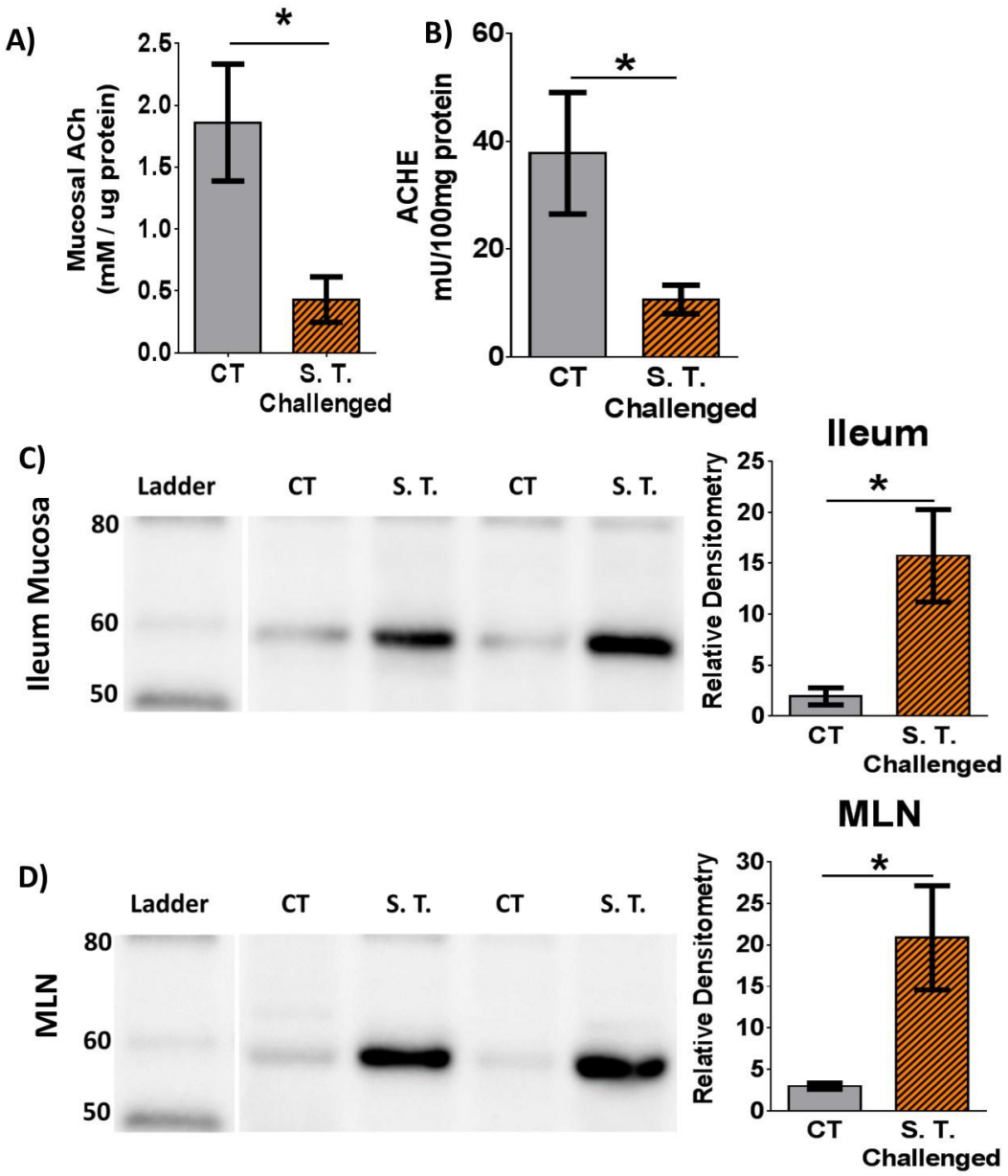

Figure 4.1. Impact of *S.* Typhimurium challenge on acetylcholine and cholinergic enzymes in ileum mucosa

A) Acetylcholine concentration in ileal mucosa. B) Acetylcholinesterase activity ileal mucosa. C) Western blot of ileal mucosa for porcine ChAT. Prominent band is between 60 and 50kDa. Histogram quantifying relative density directly to the right. D) Western blot of mesenteric lymph node (MLN) for porcine ChAT. Respective histograms represent relative density normalized to lane total protein. CT = Control, S.T. = *S.* Typhimurium. Bars and SEM represented. *n* = 5 controls and 6 challenged per bar. Student's t-test compared controls vs *S.* Typhimurium challenge. * = $p<0.05$.

S. Typhimurium *induced enhanced ChAT protein expression*

Since alterations in AChE did not explain the reduction in acetylcholine concentrations, we next asked if the enzyme that produces acetylcholine, ChAT, was down-regulated in *S.* Typhimurium-challenged pigs compared to controls. Since there are several ChAT isoforms, we utilized Western blot to determine if there was a particular isoform that was increased following infectious enteritis. Again in contrast to our expected results, in ileal mucosa, we identified a ~60 KDa band that was increased in *S.* Typhimurium-challenged animals compared with controls (**Figure 4.1C**). This band corresponds to a ChAT isoform known as peripheral ChAT, which has been previously reported in porcine, non-human primate, rat, and guinea pig peripheral neurons, including the enteric nervous system.(5, 8, 27, 35, 48) No difference was found in the conical 80kDa ChAT isoform, commonly associated with the central nervous system, between challenged and control pigs (*data not shown*).

We next screened the mesenteric lymph nodes to determine if the change in cholinergic regulation extended beyond the gastrointestinal mucosa. In the mesenteric lymph nodes, a band similar in size and intensity to the ileum was found in *S.* Typhimurium challenged animals compared to controls (**Figure 4.1D**). In both the ileum and mesenteric lymph nodes, the expression of ChAT was significantly increased in challenged animals compared with controls (**Figure 4.1C-D**, respectively).

Cellular source of ChAT protein upregulation in S. Typhimurium challenged ileum

To gain further insight into the mechanism contributing to upregulated ChAT expression, we investigated the localization patterns of ChAT expression in ileal mucosa of control and challenged animals via immunohistochemistry. In agreement with

Western blot analysis, ileum from *S.* Typhimurium-challenged pigs exhibited a larger area of ChAT expression (% Mucosa ChAT, Fig 3.2A-D, p=0.06). ChAT positive cells included villus and crypt epithelia (**Figure 4.2E, F**), lamina propria mononuclear cells (F) and submucosal and myenteric ganglia (not shown). Further, the % area of ChAT-positive staining and intensity of staining was most pronounced within the epithelium of the Peyer's patch follicle associated mucosa (FAM) compared to follicle free mucosa (FFM) (**Figure 4.2G-J**).

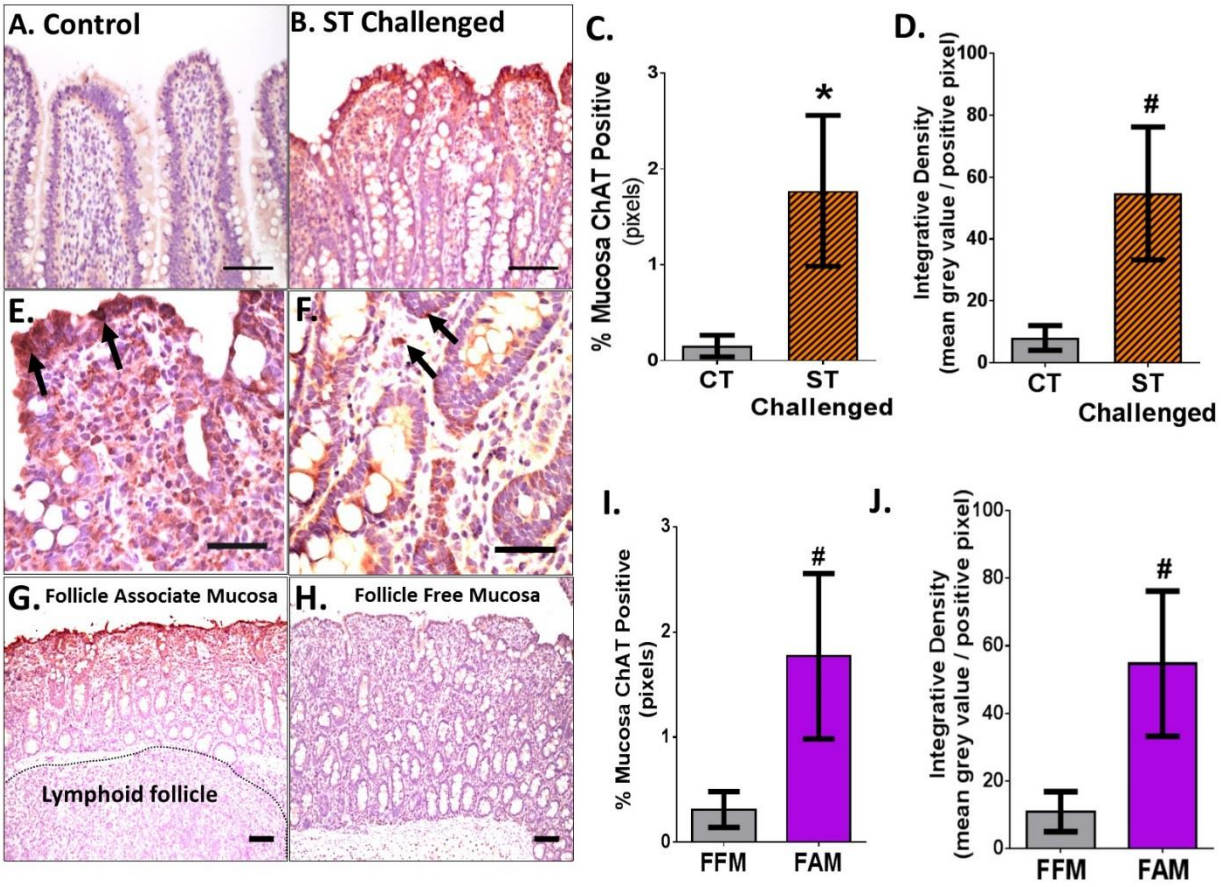

Figure 4.2. ChAT is elevated in epithelium and round cells of lamina propria over the Peyer's patch following *S.* Typhimurium challenge
A-B: 20x images of control (A) and ST challenged (B), scale bar = 100uM. **C-D:** % of area of ChAT positive mucosa (C) and integrated pixel density analysis (D) between control and ST challenged pigs. **E,F:** 40x representative images from ileum from an *S.* Typhimurium challenged animal demonstrating the enhanced epithelial expression (arrows) and staining within lamina propria monocytes (E,F; arrows). **G-J:** 10x image of

Figure 4.2 (cont'd)
(G) follicle free mucosa (FAM) and (H) follicle associated mucosa (FAM) from ST challenged pig. In (G), note the Peyer's patch lymphoid follicle tissue directly under the mucosa. **I,J:** % area ChAT positive and integrated density between (I) FFM and (J) FAM.Scale bar – 100um. CT = Control, ST = *S*. Typhimurium. Mann-Whitney t-test was used in (C) and (G). Students T-test used in (D) and (H). # *p<0.1, * p<0.05*.

Changes in cholinergic receptor gene expression following S. Typhimurium *challenge*

Considering that *S.* Typhimurium-challenged pigs had reduced acetylcholine concentrations in ileal mucosa, we sought to determine if cholinergic receptor gene expression was altered. Cholinergic muscarinic receptors 1, 2, and 3 (CHRM1, CHRM2, and CHRM3, respectively) were selected due to their known prevalence in mediating inflammation.(22, 47) Utilizing real time - reverse transcription PCR with quantitation relative to control values, we determined that mRNA transcripts for CHRM1 and CHRM3 were significantly upregulated in ileal mucosa of pigs challenged with *S.* Typhimurium (**Figure 4.3**). Specifically, CHRM1 expression was increased by ~ 4 fold compared with controls and CHRM3 expression was increased by ~2 fold relative to controls. There was no effect of *S.* Typhimurium challenge on CHRM2 expression (**Figure 4.3**). We next measured mRNA expression of the cholinergic nicotinic alpha 7 receptor subunit (CHRNA7), which contributes to formation of the homopentameric receptor known to influence inflammatory responses.(30, 50) We found no effect of *S.* Typhimurium challenge on CHRNA7 expression in pig ileal mucosa (**Figure 4.3**).

Correlation of mucosal ileal ChAT expression with clinical symptoms and histopathology

Since *S.* Typhimurium challenge influences several enzymes and receptors of the cholinergic system, we asked whether ileal mucosal ChAT expression was correlated with symptom severity and histopathological findings. Correlation analysis of

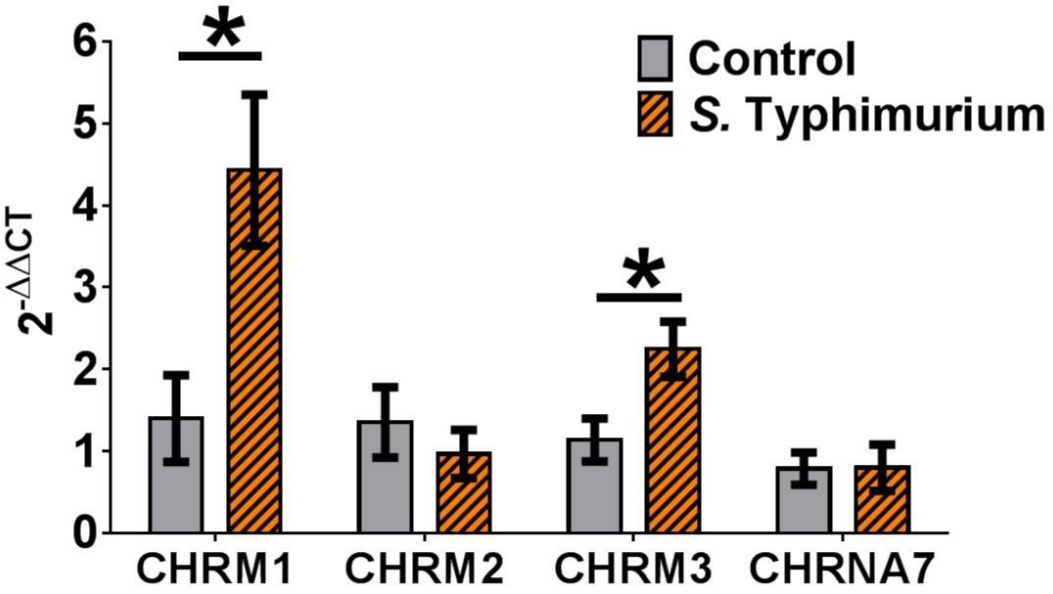

Figure 4.3. Impact of *S.* Typhimurium challenge on ileum mucosa cholinergic receptor gene expression
Salmonella Typhimurium enhances muscarinic receptor mRNA gene expression. mRNA gene expression was quantified utilizing two step reverse transcriptase quantitative PCR (RT-qPCR) for cholinergic muscarinic receptors 1, 2, 3 and cholinergic nicotinic receptor alpha 7 subunit (CHRM1, CHRM2, CHRM3, and CHRNA7 respectively). The data are presented as $2^{-\Delta\Delta CT}$ in gene expression relative to control group, normalized *to beta-actin* (*ACTB)* before statistical analysis. *n* = 6 controls and 6 challenged per bar. Student's t-test compared controls vs *S.* Typhimurium challenge of each cholinergic receptor. * = $p<0.05$.

ChAT expression intensity between rectal temperatures were performed separately for both controls and *S.* Typhimurium challenged animals. In controls, there was no correlation between ChAT expression and rectal temperature (*r=0.1363, p=0.8270*), (**Figure 4.4A**). However, in *S.* Typhimurium challenged pigs, a significant, positive correlation was found between ileum ChAT expression and rectal temperatures (*r=0.8985, p=0.0149*), (**Figure 4.4B**).

Correlations between ileal mucosal ChAT expression and ileum histopathological scores for villus blunting, villus fusion, and lymphoid depletion were also performed separately for both controls and *S.* Typhimurium challenged animals. There was no villus blunting or villus fusion found in control pigs; therefore, no correlation could be performed (**Figure 4.4C and 4.4E**). While one control pig was found to have mild lymphoid depletion, overall there was no correlation between ileum ChAT expression and lymphoid depletion in controls was found (*r=0.1289, p= 0.5528*), (**Figure 4.4G**). However, in *S.* Typhimurium challenged pigs, mucosal ChAT expression positively correlated with villus blunting (*r=0.9374, p=0.0185*) and villus fusion (*r=0.9366, p-0.0190*), (**Figure 4.4D and 3.4F**, respectively). Also, ileal mucosal ChAT expression had a trending positive correlation with lymphoid depletion; however, the relationship was not significant (*r=0.6317, p=0.1081*), (**Figure 4.4H**). A negative correlation was found between AChE and lymphoid depletion in *S.* Typhimurium-challenged animals (*r = -0.9296, p<0.0222, data not shown)*.

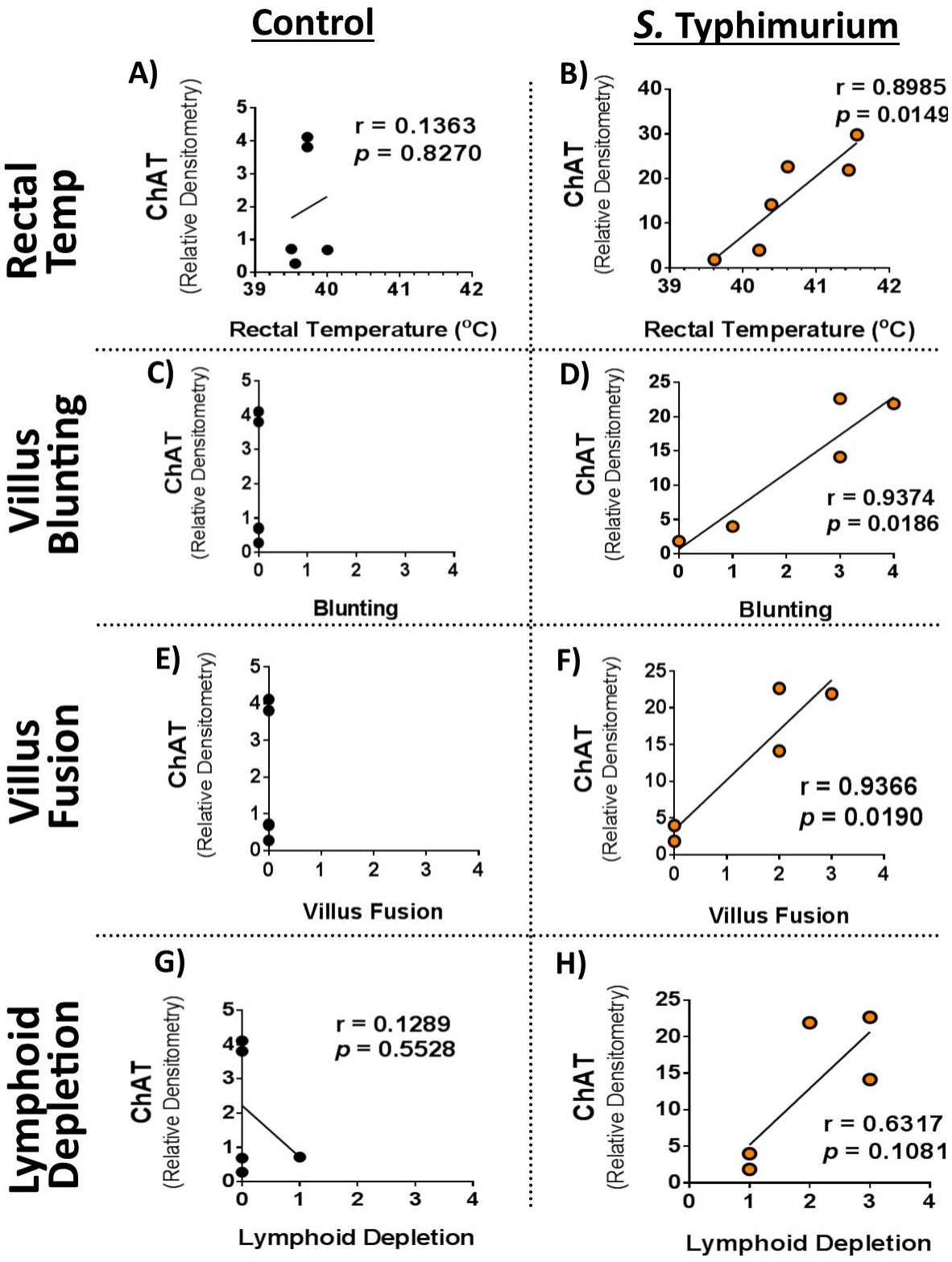

Figure 4.4. Correlation of ChAT with body temperature and ileum histopathology
Rectal temperatures and histopathological scores were correlated with ileum mucosal ChAT expression from **Figure 4.1**. Correlations were performed independently on

Figure 4.4. (cont'd)
controls A, C, E, G) and *S.* Typhimurium challenge B, D, F, H) pigs. Pearson correlations were performed between mucosal ChAT and rectal temperature A, B); Villus Blunting C, D); Villus Fusion E, F); and lymphoid depletion G, H). Pearson correlation, r values and p values reported on figures.

Correlation of mucosal ileum ChAT expression with mucosal ileum cytokine expression

Given the positive correlation between ileal mucosal ChAT expression and rectal temperature and histopathology in *S.* Typhimurium challenged animals, we next asked if mucosal ChAT expression correlated with inflammatory cytokine production. No correlation between mucosal ChAT expression and TNF, IL-8, or myeloperoxidase (MPO) expression was found in controls (**Figure 4.5A, 4.5C, 4.5E**). However, in *S.* Typhimurium challenged pigs, a trend towards a positive correlation was found between ileum mucosal ChAT expression and ileum mucosal TNF expression, (*r=0.7354, p=0.0955)*, (**Figure 4.5B**). No significant correlation was found between ChAT and IL-8 or MPO in *S.* Typhimurium challenged animals (**Figure 4.5D and 4.5F**). We also performed correlation analysis between acetylcholine concentrations or AChE activity with ileum mucosal cytokine levels which showed no significant correlations (*data not shown)*.

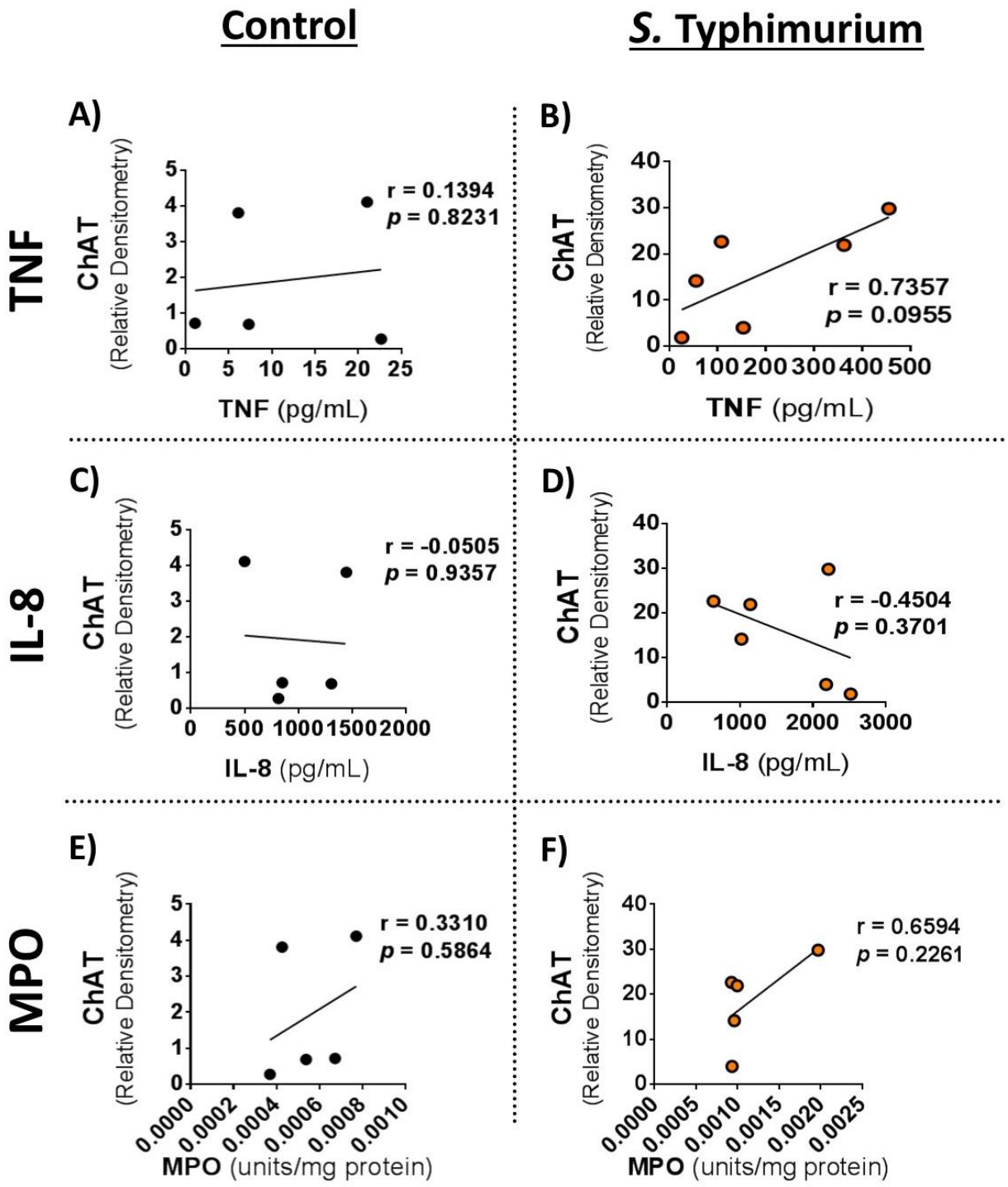

Figure 4.5. Correlation of ChAT with ileal mucosal cytokines
Mucosal cytokine protein expressions were correlated with ileal mucosal ChAT expression. Ileal mucosal ChAT and cytokine correlations from A, C, E) controls and B, D, F) *Salmonella* Typhimurium challenge. ChAT correlation with mucosal TNF A, B); IL8 C,D); and MPO E,F). Cytokine protein levels were determine previously by ELISA and protein expression of ChAT, determined in Figure 1, from Western blot. Pearson

Figure 4.5. (cont'd)
correlation, r values and p values reported on figures. Each point represents an individual animal. R and *p* values for two tailed Pearson correlation were analyzed and are reported on each figure.

Discussion

S. Typhimurium *challenge in pigs alters the expression of cholinergic proteins involved in ACh synthesis, receptor signaling and degradation*

Results from the present study showed that acute oral *S.* Typhimurium challenge in pigs resulted in significant alterations in the expression of cholinergic system markers characterized by reduction in ileal mucosal ACh concentrations, down-regulation of acetylcholine degrading enzyme activity, AChE, and upregulation of acetylcholine synthesis enzyme, ChAT. Cholinergic muscarinic receptor 1 and 3 expression was also upregulated in *S.* Typhimurium challenged pigs.

To date, nematode-induced enteritis in rodents has been the most commonly utilized animal model to study pathogen-mediated modulation of the enteric cholinergic system, and many of these studies focused on the cholinergic myenteric plexus and motility. Commonly demonstrated across these studies, and very similar to our data, are that parasitic enteritis acutely induces reduced production and release of acetylcholine (9, 11), elevated ChAT activity and expression (11, 37), and diminished AChE activity (11, 37) in the small intestine. Only a few studies exist reporting the impact of bacterial and viral infection on the enteric cholinergic system following chronic infections. In one study, *Helicobacter pylori* and Herpes Simplex Virus-1 infection were shown to induce chronic down-regulation of acetylcholine release, suggesting that like acute infection as observed in the present study, chronic infections may also act to suppress acetylcholine

availability.(3, 7). Similar to the acute infectious enteritis models, an acute chemical ileitis model of 2,4,6-trinitrobenzenesulfonic acid (TNBS) resulted in impaired ACh release with paradoxical increased ChAT expression when compared to controls. (49) Chemical colitis models utilizing TNBS and dextran sodium sulfate (DSS) have been shown to impact ChAT expression, though the effects were dichotomous, with TNBS reducing ChAT expression and DSS enhancing ChAT expression. (51) In the present study, our results demonstrated similar paradoxical expression of impaired ACh production, with increased ChAT expression and reduced AChE function following acute bacterial enteritis. Therefore, previous reports and our data here suggest a highly conserved cholinergic response to acute intestinal injury across animal species and etiological agents.

Potential benefits and consequences of dynamic, enteric cholinergic expression changes during pathogen challenge

As mentioned above, the present study and previous work in rodent models support evidence for a conserved cholinergic response to intestinal injury characterized by a reduction in ACh levels and alterations in cholinergic system components involved in Ach synthesis and muscarinic receptor expression, and decreased ACh degredation. Together, this response may represent a compensatory mechanism by the host to preserve and recover cholinergic signaling in the face of diminished ACh levels. The functional significance of this response is unclear at this time but might represent a beneficial response to limit intestinal mucosal injury. In support of this, cholinergic signaling through muscarinic receptor subtype 3 was shown to limit intestinal tissue damage in response to acute chemical colitis.(23) In the presence of inflammatory

cytokines, TNF and INF-γ, muscarinic signaling preserved barrier function and limited release of neutrophil chemotactic cytokine IL-8, (26) demonstrating a protective role of cholinergic-mediated muscarinic signaling on the intestinal barrier potentially via control of the inflammatory milieu. Additionally, endotoxin induced upregulation of B lymphocyte ChAT expression was shown to negatively regulate neutrophil influx through action of acetylcholine on endothelium muscarinic receptors.(40) Likewise, down-regulation of mucosal AChE expression in response to endotoxin challenge was also shown to be necessary to limit acute cytokine production. (43). Increased cholinergic, muscarinic signaling, via muscarinic receptors may also play a role in adaptive immune responses. When challenged with enteric nematode infection, muscarinic receptor 3 knock out mice demonstrated an attenuated ability to generate humoral cytokines necessary to upregulate mucus production or alter motility, and this attenuated response resulted in increased worm and egg burden compared to wild type infected controls.(33) Further, muscarinic receptor 3 deficient mice infected with the enteric pathogen *Citrobacter rodentium* also demonstrated an impaired pathogen clearance (32) potentially linked with impaired adaptive immunity. Featuring a more direct role of muscarinic receptor signaling in adaptive immunity, muscarinic receptor 3 deficient lymphocytes demonstrated reduced cytokine production to cholinergic agonists with impaired memory immune responses and reduced activation and cytokine production in response to both humoral and cell mediated immunity inducing pathogens.(10).

ACh can also bind and signal via α 7 nicotinic receptors, which have been more intensively studied in rodent chemical colitis models as major anti-inflammatory mechanism. In murine pathogen challenge models, α 7 nicotinic receptor genetic

deficiency resulted in enhanced neutrophil recruitment which coincided with improved bacterial clearance compared with control animals. (21) Pharmacological blockade of nicotinic receptors was shown to reduce intestinal villus damage induced by invasive *Shigella* infection.(45). In the present study, unlike muscarinic receptors, we did not observe significant changes in the expression of ileal α 7 nicotinic receptors in response to *S.* Typhimurium challenge. However, lack of changes in expression of α 7 nicotinic receptors following S. Typhimurium challenge does not necessarily imply an insignificant role for this receptor. For example, based on the preponderance of literature showing an inhibitory role of α 7 nicotinic receptors in immune activation, an unchanged expression level during pathogen challenge may facilitate bacterial clearance. Related to this, the reduced concentration of ACh in *S.* Typhimurium challenged pigs in the present may be an effort by the host to selectively activate muscarinic receptors over nicotinic receptors, since muscarinic receptors are known to have a much higher affinity for ACh compared with nicotinic receptors.(1, 25, 38)

Together, results from our study with *S.* Typhimurium challenge in pigs and the literature support a critical role of the cholinergic response in balancing a robust immune responses required for pathogen clearance and development of adaptive immunity while at the same time limiting excessive inflammation and loss of intestinal barrier integrity. Therefore, conditions that result in suppression or hyper-active enteric cholinergic tone could have significant consequences for host immunity and survival.

Increased ChAT expression and localization to the Peyer's patch epithelium in S. Typhimurium *challenged pigs*

Choline acetyltransferase (ChAT) has been shown to be expressed in neuronal and non-neuronal cell types. To gain more insight into the potential role of upregulated ileal ChAT expression in *S.* Typhimurium-challenged, investigated the localization of ChAT expression via IHC. As expected, we identified ChAT-positive cells within submucosal and myenteric neuronal ganglia and lamina propria which was increased in challenged pigs. Unexpectedly, we found the greatest intensity of ChAT-positive cells within the epithelium covering the Peyer's patch lymphoid mucosa. The mechanism for upregulation of ChAT, particularly in the Peyer's patch epithelium in challenged pigs remains to be determined. *S.* Typhimurium is an intracellular bacterial pathogen which establishes infection by transportation through microfold cells (M cells) over the Peyer's patches in the ileum or through invasion of absorptive ileum enterocytes.(6, 34) After crossing the intestinal epithelial barrier, *S.* Typhimurium enters the lamina propria where the pathogen is phagocytosed by macrophage and dendritic cells of the immune system. The pathogen resides and replicates within these leukocytes, permitting dissemination to other organs such as the mesenteric lymph nodes.(6, 41). Therefore, focal pattern of ChAT localization observed in challenged pigs in present study follows the pathway of *S.* Typhimurium invasion. Others have shown that bacterial products such as LPS upregulate ChAT expression in mucosal associated lymphoid tissue, peritoneal B cells, macrophage, and dendritic cells. (24, 40). Specifically in line with the current study, *S.* Typhimurium infection was shown to elicit induction of intestinal $CD4^+$ Th17 lymphocytes, (39) a group of cells that has recently been shown to by ChAT

positive.(13) Therefore we hypothesize that specific localization patterns ChAT expression reveled in the present study is critical in modulating bacterial invasion and subsequent modulation of innate and adaptive immune response.

Summary

In summary, while the vagal cholinergic input has been shown to be an important key modulator of gut inflammatory responses, results from the present study demonstrate that the intrinsic cholinergic system, which includes neuronal and non-neuronal cell types, is dynamically regulated in response to *S.* Typhimurium challenge. (**Figure 4.6**.) The precise cause, cellular source, and significance of these cholinergic responses to overall host defense and immunity remains to be elucidated; however, our data establishes a foundation for future mechanistic research aiming to better understand the role of the enteric cholinergic system in mediating a balance between immune activation required for pathogen clearance and host immunity and the prevention of excessive inflammation and epithelial barrier damage.

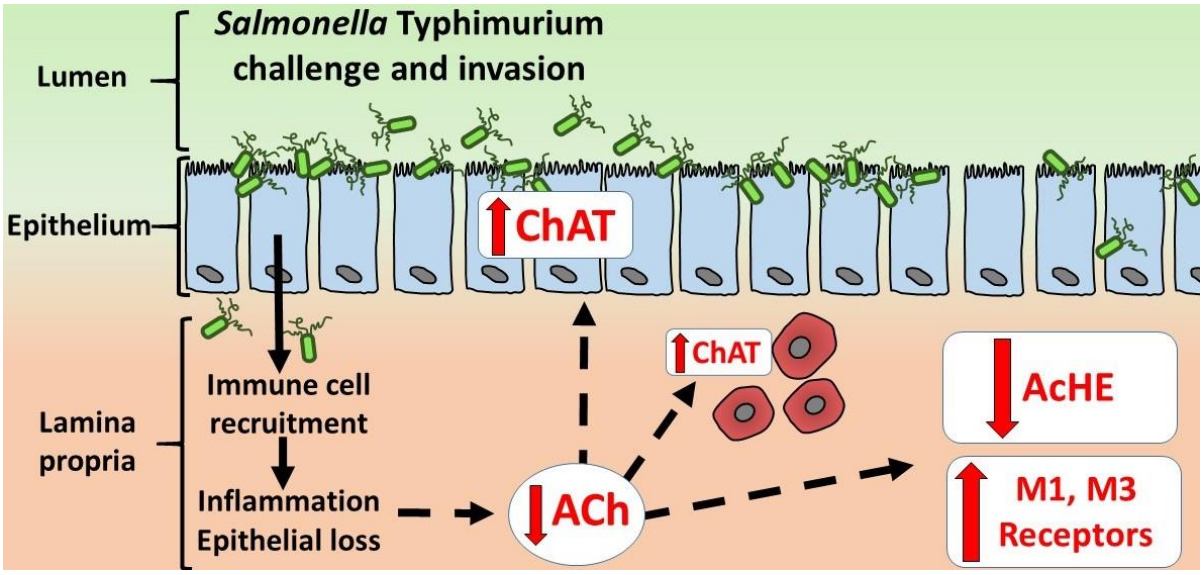

Figure 4.6. Alterations in the enteric cholinergic system induced by *Salmonella* Typhimurium challenge in pigs.

Figure 4.6. (cont'd)
Salmonella Typhimurium challenge and invasion triggers immune cell recruitment, inflammation and mucosal injury including epithelial cell loss. Subsequent reduction in acetylcholine (ACh) concentrations in the mucosa may induce a compensatory response to preserve and (or) amplify cholinergic signaling by **(1)** upregulating ACh synthesis via choline acetyltransferase (ChAT) in epithelial cells, lamina propria monocytes and enteric neurons (not shown) **(2)** down-regulating ACh degradation via suppression of acetylcholinesterase (AChE), and **(3)** enhancing of ACh signaling via increased expression of muscarinic M1 and M3 cholinergic receptors. Dotted lines indicate hypothesized mechanisms by which mucosal damage and inflammation may drive altered cholinergic expression.

APPENDIX

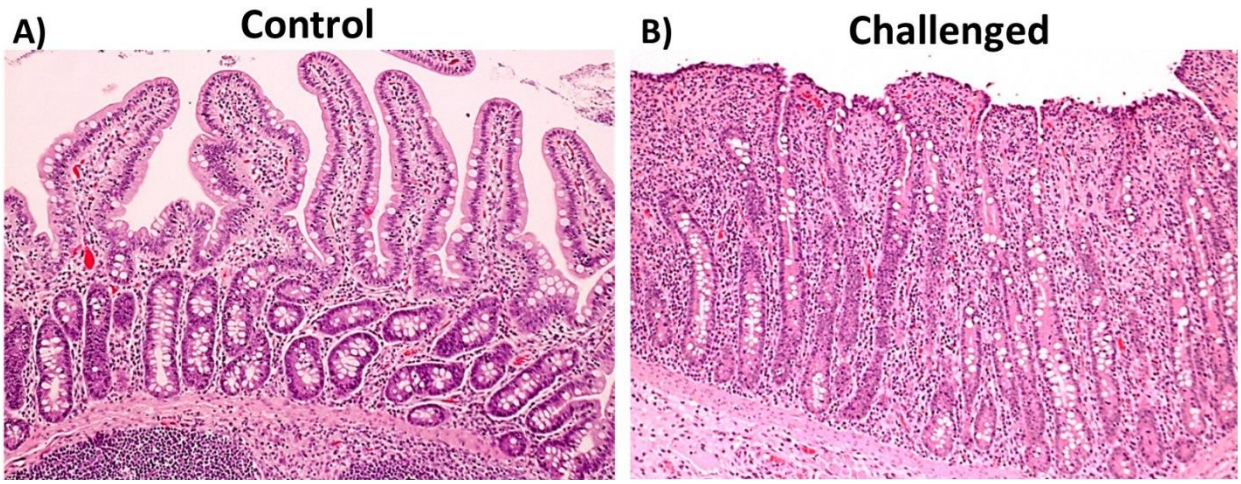

Figure S.4.1. H&E stained sections of porcine control and *S.*Typhimurium challenged pigs as published previously.(4) Section highlight differences displayed in Table 1 of previously published data. Not the villus blunting and fusion in the *S.* Typhimurium challenged pigs (B).

Target gene	Forward	Reverse
ACTB	GAAGCTCAGTCGGGCTTCTC	ATGTCGACGTCGCACTTCAT
CHRM1	*GGCACACTCCAGAATGGTCA*	*CCTTGGGACTTGACGTGTGA*
CHRM2	*GACCAAGCAGCCTGCAAAAA*	*TCCACACTGTGTTGGGGATG*
CHRM3	*CCACAGGTAGTTCTCGGAGC*	*GAAGTGGCAGCGTCCATACT*
CHRNA7	*CAGCGCCATTTCTACCAAGC*	*CCCCCTGACTTGACTGGTTC*

Table S.4.1. PCR forward and reverse primers

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CHAPTER 5

Chronic Social Defeat Stress Acutely Reduces Expression and Function of the Enteric Cholinergic Nervous System

Abstract

Stress and depression are well known factors that promote chronic inflammatory GI diseases, like inflammatory bowel disease (IBD); however the mechanism linking both conditions is not well understood. Here we demonstrated that in a murine model of chronic stress and depression, chronic social defeat stress (CSDS), leads to a strong suppression of the enteric cholinergic nervous system in the colon. Immediately following chronic stress, CSDS mice demonstrated reduce colonic cholinergic tone resulting in reduced secretomotor responses. Underlying the reduced cholinergic tone was a significant reduction in percentage of enteric cholinergic nerves of the colonic submucosal plexus (SMP) (about 25% reduction). Gene expression of choline acetyltransferase (ChAT), an enzyme that synthesizes acetylcholine (ACh), and acetylcholinesterase (AChE), an enzyme that degrades ACh, were both found to be reduced by 40% and 30% compared to controls. Colonic AChE enzyme activity was also significantly downregulated by nearly 50% in defeated. Gene expression of vesicular acetylcholine transporter (VAChT), a transport protein which loads ACh into neuro-vesicles and high efficiency choline transporter 1 (CHT-1), a transport protein the recycles ACh byproducts, were both down regulated by 50% in defeated mice compared to controls. Gene expression of cholinergic muscarinic and nicotinic receptors were also significantly down regulated in defeated mice compared to controls, with the largest down regulation in expression in cholinergic muscarinic receptor 2 and 3. Accompanying the reduction in cholinergic tone and expression, we observed colonic mucosal lymphoid follicle hyperplasia. These findings indicate that social defeat results

in abnormal gastrointestinal physiology, which is, in part, mediated by enteric cholinergic dysfunction.

Introduction

Inflammatory bowel disease (IBD) collectively represents a combination of chronic intestinal inflammatory conditions with etiologies including a combination of genetics and several environmental factors.(40) Of the environmental factors thought to contribute to IBD, stress is thought to be a leading cause. Prospective studies on IBD patients highlight psychological stress as a leading environmental risk factor leading to IBD flares or relapse compared to other possible risk factors like non-steroidal anti-inflammatory drug use or exposure to infection.(5, 20) Importantly, stress was associated with an increase in mucosal disease in IBD patients, liking psychological health with mucosal activation.(28) Further investigation reveals anxiety and depression induced by life stress, underpin stress induced IBD flares.(2, 10, 27) Though there is a clear association between psychological disease and manifestation and severity of IBD (9, 17, 23), the underlying mechanisms linking these co-morbidities is poorly understood and requires further investigation.

The cholinergic anti-inflammatory pathway is a leading candidate for the explanation of simultaneous brain and gut disease due the known anti-inflammatory influence of the central nervous system’s cholinergic vagus nerve on peripheral immunity.(8, 21, 29, 37) Indeed, murine depression models demonstrated that reduced activity of the cholinergic vagus nerve and reduced activation of nicotinic receptors on colonic macrophage, play a major role in facilitating chemically induced colitis.(15, 16) To put another way, vagal release of acetylcholine onto nicotinic receptors of colonic

macrophage prevents chemically induced colitis; however, depression blocks this activity by reducing vagal tone. Furthermore, preclinical trials studying vagal stimulation in IBD patients increased rates of remission, indicating the cholinergic signaling may be a valid therapeutic approach for IBD patients.(7) In depression induced susceptibility to gastrointestinal disease, these examples demonstrate a potential link between the central nervous system (cholinergic vagus nerve) and the gut (macrophage). However, speculation remains as to how the sparse innervation of the distal colon by the vagus nerve may independently mediate such a strong regional anti-inflammatory role, especially when the vagus nerve is found to primarily synapse on myenteric plexi.(6, 38)

In the work presented here, we asked if cholinergic expression and function, intrinsic to the colon was dysregulated in a stress induced depression model called chronic social defeat stress (CSDS). Furthermore, we sought to determine if stress induced depression models result in any kind of stimulation of the colonic mucosal immune system. Our findings suggest that CSDS results in depressive like behavior with simultaneous down regulation of the cholinergic system in the colon. The source of reduced cholinergic tone appears to originate from submucosal enteric neurons. Finally, depressive behavior and reduced colonic cholinergic expression and function in CSDS mice was associated with an increase in the size of mucosal lymphoid follicles.

Methods

Animals

All experimental data reported was collected from mice which were 10-12 week old, male, C57BL6 strain and housed as previously reported.(12) Briefly experimental 8-9 week old C57BL6 mice were purchased from Jackson laboratory and CD1 retired

breeders were acquired from Charles River and habituated in the animal facility for at least one week on new chow and a 12 hour light – dark cycle. All experiments were approved by Michigan State University Institutional Animal Care and Use Committee.

Chronic Social Defeat Stress

Social defeat was performed as previously reported.(12) Briefly, a retired CD1 breeders were tested and verified to be aggressors during home cage invasion. Chronic social defeat was performed by introducing an experimental 10 week old C57BL6 mice into the home cage of a CD1 aggressor mouse for a period of 8-10 minutes. Following physical stress exposure to the aggressor CD1 mouse, experimental BL6 mice were removed and co-housed with a different CD1 aggressor, but kept physically separate from the aggressor via a perforated Plexiglas divider for the remaining 24 hours in order to induce sensory stress. This protocol was repeated for 10 consecutive days between the hours of 8-11am. Control, non-stress C57BL6 mice were co-house with each other in standard cages by physically separated by Plexiglas dividers.

Collection

To assess the acute impacts of CSDS, controls and physically stressed mice were sacrificed 24 hours after the final day of social defeat. To assess the chronic impact of CSDS, controls and physically stressed mice were sacrificed 14 days after the final day of social defeat. Mice were sacrificed by cervical dislocation.

Ussing Chambers

Mid colonic tissue was collected and held in iced DMEM/F12 1:1 (ThermoFisher, 11039021) with 1% penicillin/streptomycin for under 1 hour. Pieces of colon were opened along the mesenteric boarder, mounted onto 0.3cm^2 sliders, and placed into

Ussing Chambers (Physiological Instruments) as reported previously.(26, 30) After a 15-30 minute equilibration period, basal short circuit current (Isc) readings were taken by voltage clamping the tissue. Functional cholinergic activity of tissue was assessed by serosal application of 500uM physostigmine hemisulfate (Tocris, 0622), an acetylcholinesterase inhibitor, which allows accumulation and persistent signaling of endogenous acetylcholine. Subsequent Isc responses were acquired every 15 seconds by continuously voltage clamping the tissue. *n* = 12 controls and 12 CSDS mice for the acute timepoint. *n* = 8 controls and 8 CSDS for the 14 day time point

Immunofluorescence

Colonic tissue was opened along the mesenteric boarder and pinned out on Sylgard filled tissues for overnight fixation at 4^{o}C with Zamboni's fixative (American MasterTech, FXZAMPT). Tissues were washed 3 x 10min in dimethyl sulfoxide and then 3 x 10min in phosphate buffered saline (PBS). Fixed tissues were stored in PBS + 0.4% sodium azide (Sigma, S8032) at 4^{o}C until further used. Submucosa and longitudinal/myenteric plexi were dissected free under a stereoscopic microscope. ~5mm x 5mm pieces of mid colon were blocked in 4% normal donkey serum + 0.4% Triton X – 100 + 1% bovine serum albumin in PBS, then immuno-labeled for ChAT 1:100, (Millipore, AB144P) and HuC/D 1:200, (Invitrogen, 16A11) a marker of neuron cell bodies in 1% NDS + 0.4% Triton X – 100 in PBS for a 3 day period at 4^{o}C. Tissues were washed 3 x 10min in PBS prior to application of secondary antibodys: AlexaFluor 488 Donkey ant-goat, 1:100; AlexaFluor 555 Strepavidin, 1:200, and nuclear stain To-Pro3 (1:1000) in 0.4% Titon X – 100 + 1% bovine serum albumin in PBS. Samples were wash 3 x 10min in PBS then mounted in ProLong Diamond Antifade Mountant

(ThermoFisher, P36965). 0.5um Z-stacked images of plexi were captured on an Olympus FluoView 1000 Filter-based confocal laser scanning microscope with a 1.3 N/A, 40x oil objective at 3x zoom. Images were capture by exciting the specimen with an argon gas, green helium neon gas laser, and a red helium neon gas laser with excitation wavelengths/filters at 488/BA505-525nm, 543/BA560-620, and 633nm/650IF, respectively. *n* = 8 control and 13 CSDS mice for this section. Composite z-stacked images were analyze on Olympus FLUOVIEW Viewer Software v4.2. Ten plexi were images per animal and analyzed.

Gene Expression

Mid-colon segments of tissue were collected and snap frozen in liquid nitrogen after being opened and rinsed free of fecal material. Tissue were stored at -70^{o}C until further used. Tissue was homogenized with Percelly's homogenizer and 2.3mm zirconia/silica beads (Biospec, 11079125z) at 63,000rpm for 30 seconds in RLT buffer from Qiagen and total RNA was isolated from colonic tissue utilizing RNeasy Mini Kit (Qiagen, 74106) utilizing manufacturer's instructions. DNase digestion and cDNA synthesis of mRNA was performed with Maxima First Strand Kit (ThermoFisher, K1671). Gene expression was performed utilized TaqMan primer probe sets provide by TheromFisher, and specific genes and primer probe set catalog number appear in **Supplementary Table 3.2**. For each sample, gene expression was normalized to the house keeping gene HPRT. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. *n* = 12 control and 12 CSDS mice for this assay for the acute time point, and *n* = 8 controls and 8 CSDS for the 14 day time point.

Acetylcholinesterase assay

Colonic tissue was collected as in the gene expression segment. Tissues were homogenized in RIPA buffer (ThermoFisher, 89901) in the presence of protease (Sigma, P8340) and phosphatase inhibitor (ThermoFisher, 78420). Acetylcholinesterase activity was detected utilizing the AmplexRed Acetylcholine/Acetylcholinesterase Assay Kit (ThermoFisher, A12217) per manufacturer's instructions.

Statistics

All histograms represent mean and standard error. All statistical analysis was performed by student's t-test, except for there percent cholinergic neurons, which was analyzed by Mann-Whitney t-test. Histograms and statistical analysis was performed with GraphPad Prism.

Results

Chronic social defeat stress results in reduced social interaction

Mice subjected to 10 consecutive days of CSDS were found to have reduced social score, a well reported finding and a hallmark of depressive like symptoms (**Figure 5.1A**).

Chronic social defeat induces cholinergic mediated secretomotor dysfunction

To test if chronic stress adversely impacts GI function, we utilized Ussing Chambers to assess electrolyte movement in colon tissue of controls or animals exposed to CSDS. Initially, we found that basal Isc, a measure of electrolyte transport across the epithelium, was significantly reduced in CSDS mice compared to controls (**Figure 5.1B**). Cholinergic signaling plays a significant role in mediating basal electrolyte transport in the gastrointestinal tract, so we asked whether cholinergic

signaling was intact in CSDS animals compared to controls. Applying physostigmine hemisulftate, an acetylcholinesterase inhibitor which allowed endogenous accumulation of ACh, to colonic tissue we observed a bi-phasic response, as noted in **Chapter 3**, **Supplemental Figure 3.2.** CSDS mice demonstrated a substantially weaker response to physostigmine initially in *phase I* (**Figure 5.1C**), but a similar *phase II* response compared to controls (**Figure 5.1D**). As *phase I*, responses were previously shown to be mediated by neuronal and muscarinic signaling **Chapter 3**, **Supplemental Figure 3.2**, CSDS mice appeared to have a reduction in enteric cholinergic neuronal function.

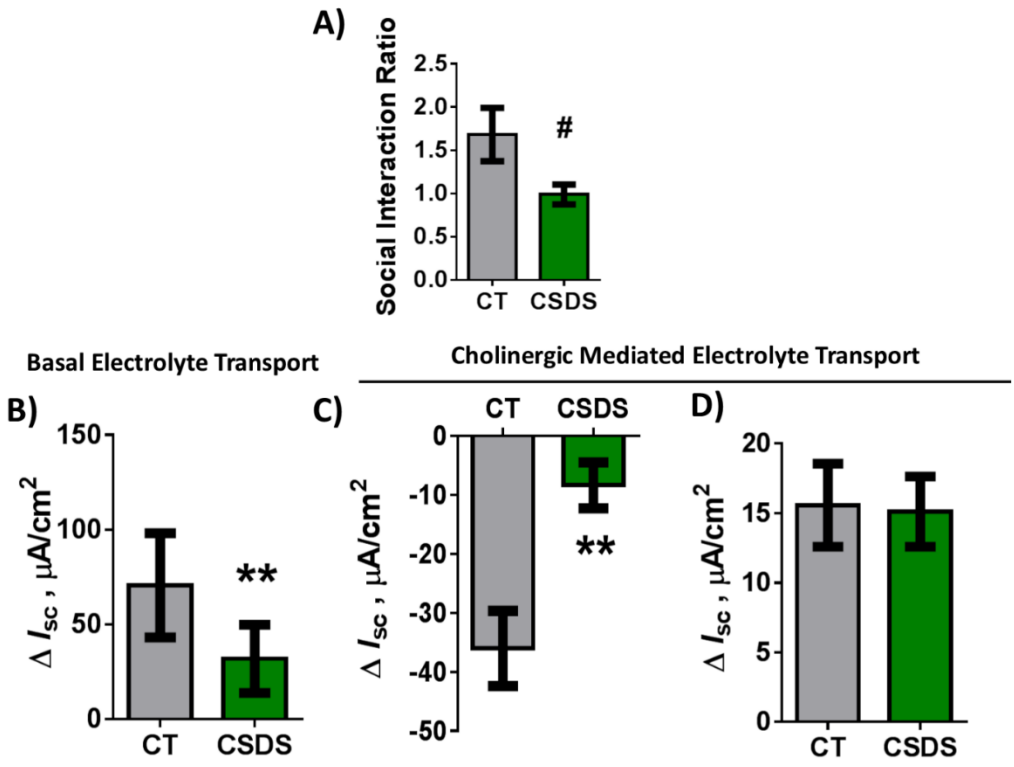

Figure 5.1. CSDS impacts behavior and GI function
A) Social interaction ratio of mice in controls or mice after 10 days of chronic social defeat. B) Basal electrogenic activity of colonic tissue on Ussing chamber. C-D) Change in short circuit current (Isc) of colonic tissue treated with 500uM physostigmine hemi-sulfate. C) Phase I response. D) Phase II response. ** - p< 0.01. # - p< 0.1. Student's t-test.

Secretory dysfunction in chronic social defeat stressed mice is not associated with dysregulation of electrogenic sodium transporters

In **Chapter 3**, **Supplemental Figure 3.2**, we previously demonstrated the *phase I* response to physostigmine application on colonic tissue was dependent on the electrogenic sodium channel, ENAC, as sodium free ringers ablated these response. To determine if the electrogenic abnormalities in CSDS colonic tissue might be due to dysregulated expression of ENAC, we measured gene expression the three subunits making up the electrogenic sodium channel. We found no difference in expression of any of the three ENAC subunits in CSDS mice compared to control. (**Supplemental Figure 5.1A-C**). This suggests that the electrogenic dysfunction we observed was due to cholinergic neuronal signaling, rather than electrolyte channel dysregulation.

Chronic social defeat reduces the percentage of enteric cholinergic neurons

Electrogenic data from Ussing chambers suggested that the enteric cholinergic nervous system may be dysfunctional in CSDS mice; therefore, we investigated the expression of the primary acetylcholine synthesis enzyme, ChAT, in the ENS. Since the majority of secretory responses in the gut are mediated by the submucosal plexus (SMP), we focused on ChAT expression in these neurons. While the number of enteric neurons per ganglia did not change, (**Figure 5.2A-C**) we found a significant reduction in the percentage of cholinergic neurons in CSDS mice compared to controls **(Figure 5.2A-B, D**). Control mice demonstrated about 20% ChAT positive nerves in the distal colon, a percentage observed previously by other groups.(14) There was nearly a 25% reduction in ChAT positive nerves from CSDS mice, with only 15% of the nerves in the

SMP of these animals positive for ChAT. No differences in ChAT expression within the myenteric plexus was identified (*data not shown*.)

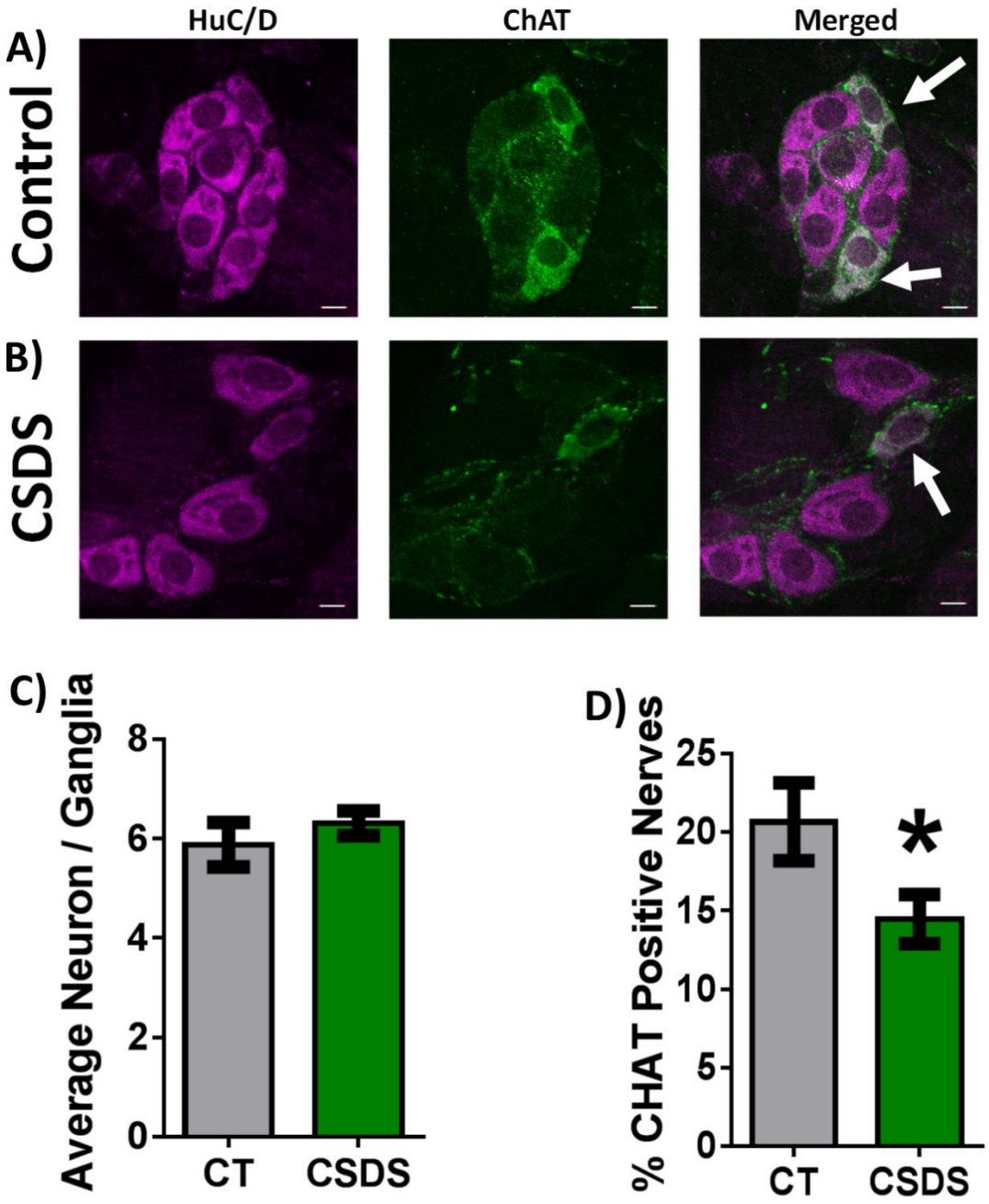

Figure 5.2. Impact of chronic social defeat on enteric nervous system
A-B) Representative micrographs of confocal z-stack images of the submucosal plexus of control (A) and CSDS mice (B) 24 hours after 10 days of CSDS. Green indicates

Figure 5.2. (cont'd)
ChAT, Magenta indicates HuC/D, a pan neuronal marker, and grey are areas of co-localization. White arrows indicate cholinergic neuronal cell bodies. Scale bar = 10um C) Histogram representing total neurons per plexus. D) Percentage of cholinerigc neurons per 10 randomly scored plexi per animal. * - $p<0.05$. Student's t-test.

Chronic social defeat induces a pan suppression of cholinergic enzymes and transporters

Given that CSDS mice had a reduction in cholinergic enteric neurons, we wanted to determine if other aspects of the cholinergic system might also be dysregulated. Similar to the reduced percentage of cholinergic neurons, we found a significant down regulation in gene expression of ChAT **(Figure 5.3A**). In addition, we found that transporters vital to acetylcholine neurotransmission, VAChT, and acetylcholine precursor recycling, CHT-1, we also significantly reduced in CSDS mice compared to controls (**Figure 5.3B-C**). Finally, we found a significant down regulation in both gene expression and enzymatic activity of the ACh degradation enzyme, AChE (**Figure 5.3D**). Together, these results demonstrate a substantial down regulation of all canonical genes involved in ACh synthesis and degradation.

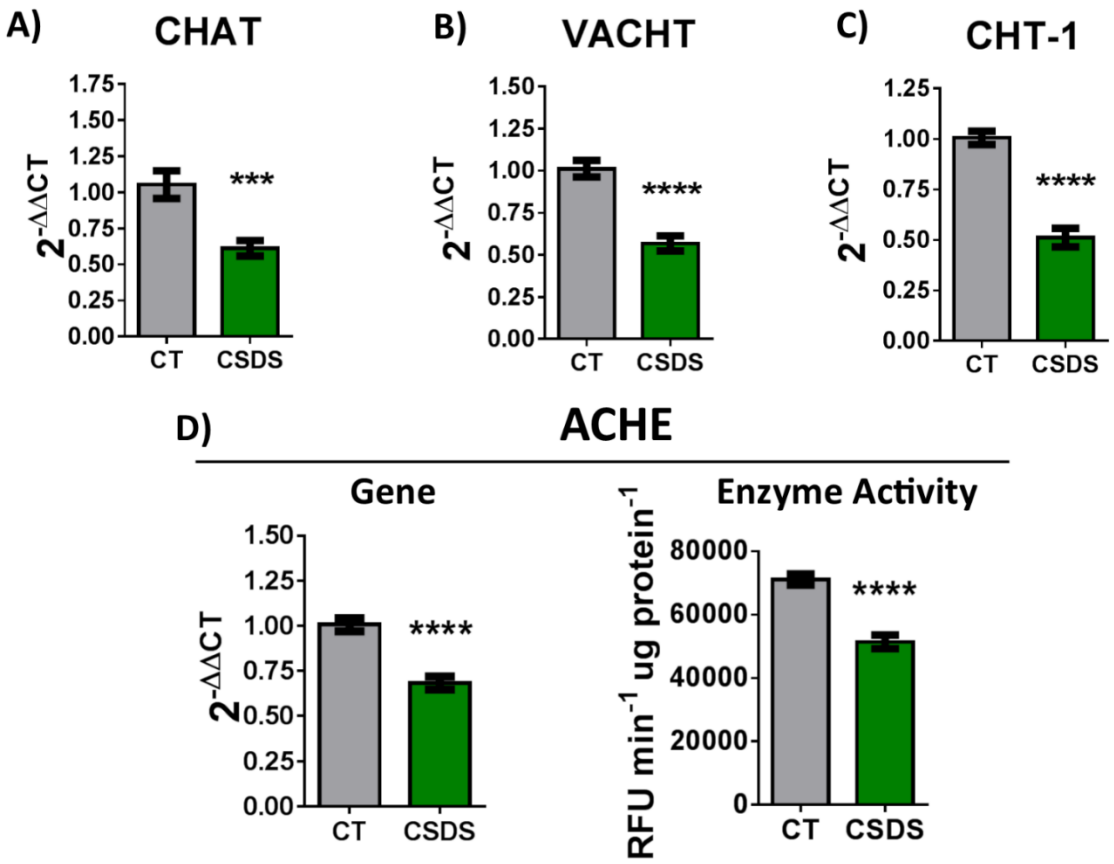

Figure 5.3. Influence of CSDS on colonic cholinergic gene expression
A-D) Gene expression of cholinergic enzymes and transporters reported by the 2^-ddCT method. D) *Right figure* is enzymatic activity of ACHE, verifying similar gene expression (*left*). ***-p<0.001, ****p<0.0001. Student's t-test.

Down regulation of colonic cholinergic muscarinic receptors in chronic social defeat mice

We next asked if the receptors which bind ACh and relay signals may be disrupted. No difference in expression of muscarinic receptor 1 (CHRM1), was found between CSDS and controls mice (**Figure 5.4A**). We observed a moderate down regulation of muscarinic receptor 2 (CHRM2) (**Figure 5.4B**) and a substantial down regulation in muscarinic receptor 3 (CHRM3) (**Figure 5.4C**) in CSDS mice compared to

controls. Together, these data demonstrate a reduction in expression of muscarinic receptors known to mediate GI secretomotor function.

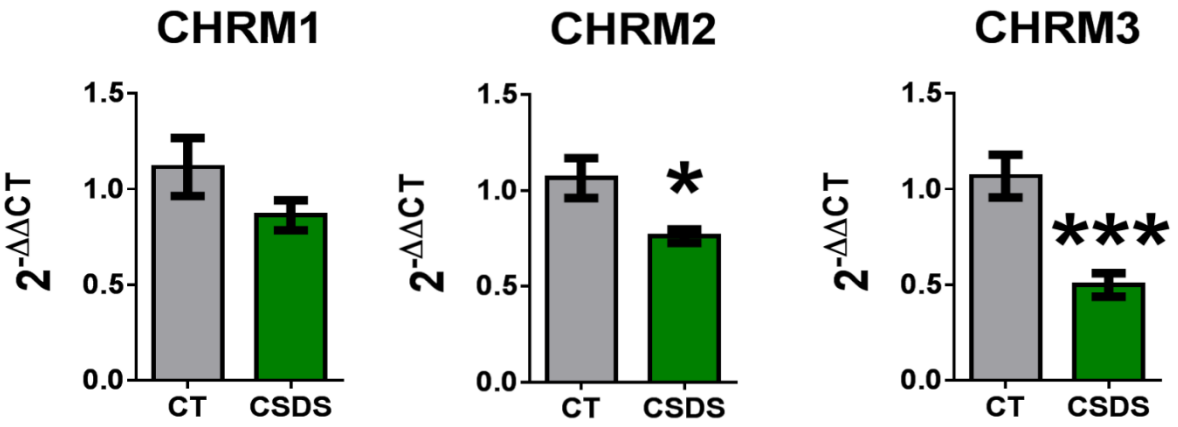

Figure 5.4. Impact of CSDS on cholinergic receptor expression
All histograms represent gene expression of cholinergic muscarinic receptors by the 2^-ddCT method. *-p<0.05, ***-p<0.001, Student's t-test.

Repeat 2 hours restraint stress induces similar down regulation of cholinergic transporters and cholinergic receptors

To determine if the suppression in colonic cholinergic gene express was conserved across types of chronic stress, we exposed mice to 2 hours restraint stress for 3 and 7 consecutive days. Similar to CSDS, we found a down regulation in CHRM2 and CHT-1 expression with repeated stress. Interestingly the down regulation of CHRM2 appeared to occur immediately at 1 day, 2 hours restraint stress (**Chapter 3**, **Figure 3.6 H**) with continues suppression at 3 and 7 days of consecutive restraint stress (**Supplemental Figure 5.2 A**). We also observed a down regulation on CHT-1 expression; however, this only occurred after 7 days of consecutive 2 hours restraint stress (**Supplemental Figure 5.2B**). Other cholinergic enzymes and transporters where screened at these time points; however, there were not found to be differentially expressed (data not shown).

CSDS induces colonic lymphoid tissue expansion

Given the known interactions between the cholinergic system and mucosal immunity, we asked if CSDS mice showed any indication of mucosal immune activation. Histological examination of CSDS tissue did not reveal any colitis or epithelial barrier defects; however, a significant increase in musical lymphoid follicle size was noted in CSDS mice compared to controls (**Figure 5.5A-C**, arrows). Accompanying the increase in lymphoid follicle size observed in CSDS mice, we also found a significant down regulation in the cholinergic nicotinic alpha 7 subunit, which is known to mediate suppress immune activation (**Figure 5.5D**). Collectively, these data indicate that immune activation was occurring, in conjunction with down regulation of a cholinergic receptor, which is known suppress immune activation.

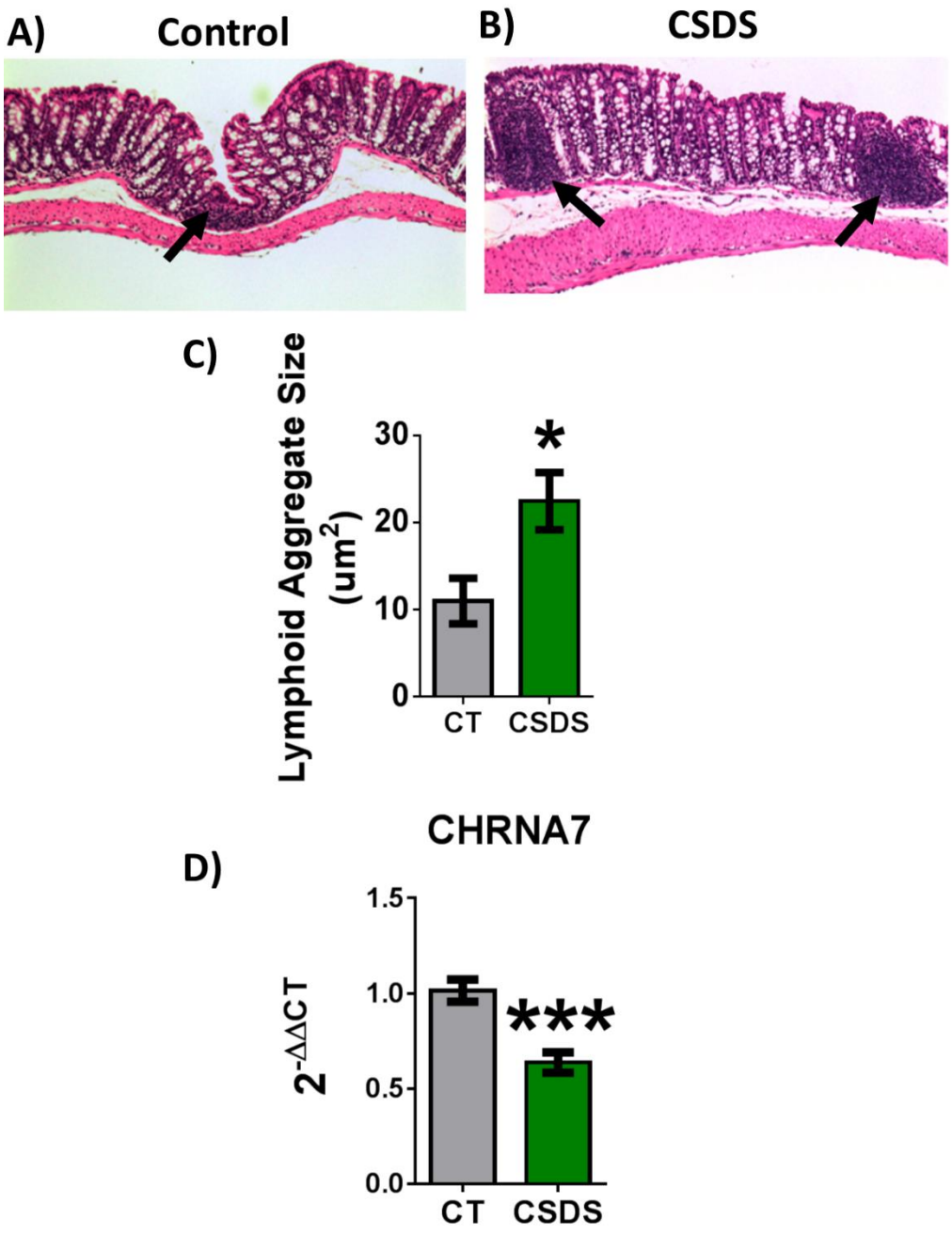

Figure 5.5. Immune activation in CSDS animals
A-B) H&E stained micrographs of control (A) and CSDS (B) 24 hours after chronic social defeat. Black arrow indicate mucosal lymphoid aggregates. C) represents average square area of lymphoid aggregates in controls and CSDS. D) Represent gene expression of the alpha-7 cholinergic nicotinic receptor subunit by the 2^-ddCT method. *-p<0.05; ***-p<0.001. Student's t-test.

Reduced colon cholinergic function and expression in CSDS mice resolves over time

We next asked if the cholinergic dysfunction observed immediately following 10 days of social defeat persisted chronically. Mice exposed to CSDS for 10 days, then left in standard housing for 14 days showed persistent reduction in social interaction, indicating a persistence in depressive like symptoms. (**Figure 5.6A**) Measuring electrogenic activity and responsiveness to physostigmine in mice 14 days following CSDS, we did not observe any difference between controls and CSDS mice (**Figure 5.6B-D**). Additionally, no difference in cholinergic enzyme, transporter, or receptor gene expression was observed between controls and CSDS at a 14 day time-point following chronic social defeat (**Figure 5.6 E-F**), except for muscarinic receptor 2, which was significantly upregulated in CSDS mice compared to controls. These results indicate that cholinergic dysfunction observed acutely following CSDS resolved with time.

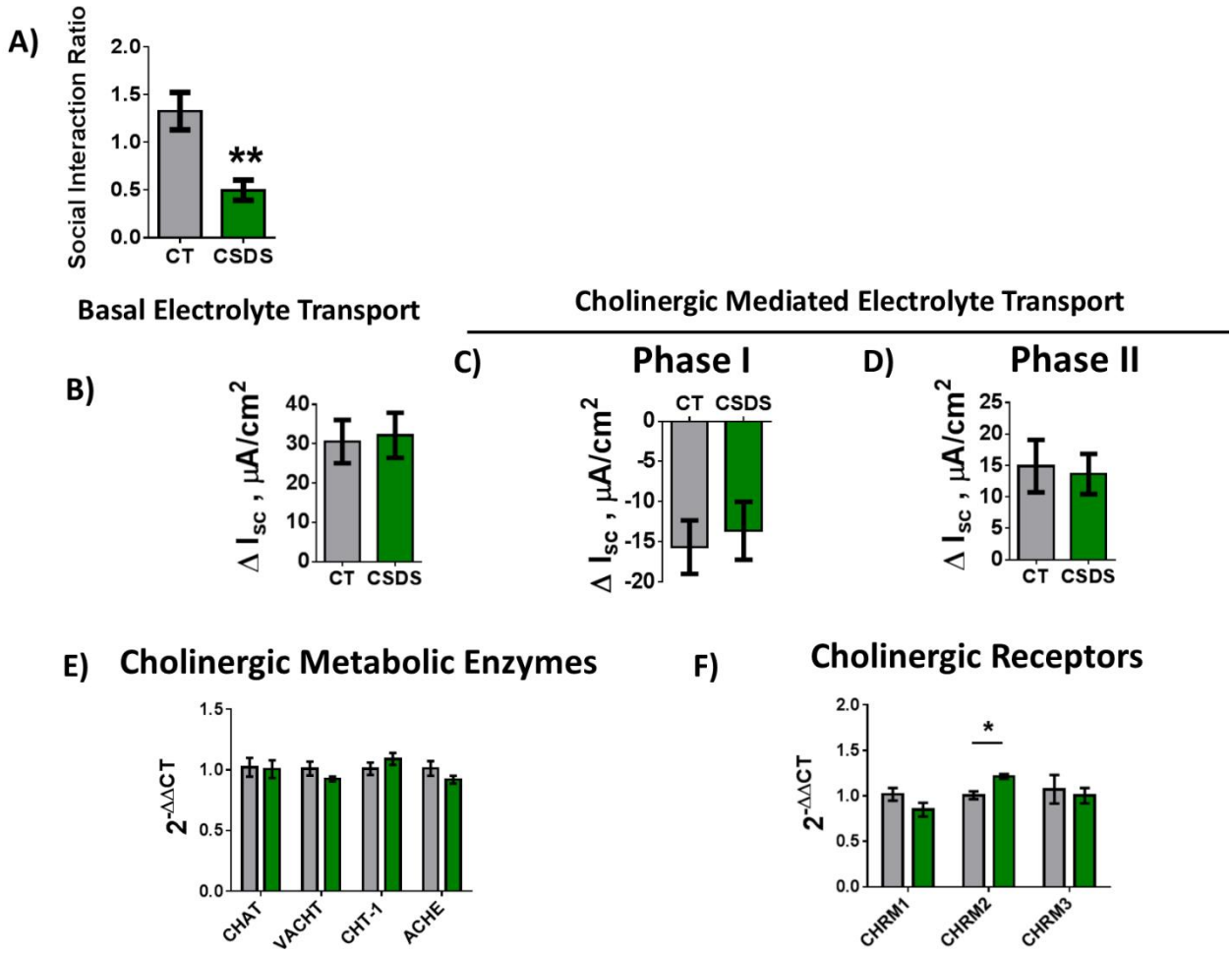

Figure 5.6. Long term impact of CSDS on behavior and colonic cholinergic function and expression.
All figure in this panel are from mice collected from standard handled controls and mice 14 days after a 10 day CSDS exposure. A) Social interaction B-D) Electrogenic activity of colonic tissue on Ussing Chambers. B) represents basal electrogenic activity. C-D) separately represent the biphasic changes in short circuit current following serosal application of 500uM physostigmine. C) Phase I and D) Phase II. E-F) Represent gene expression of all cholinergic genes previously measured by the 2^-ddCT method. *-p<0.05, **p<0.01. Student's t-test.

Discussion

The results presented here demonstrated that stress induced depression is associated with an acute, strong down regulation of many genes associated with normal cholinergic function. Furthermore, these changes appear, in part, to originate from reduced expression of cholinergic submucosal enteric neurons. Reduced social

interaction and reduced cholinergic tone was associated with an increase in lymphoid follicle size. Finally, though there was a robust, acute down regulation of the colonic, cholinergic system, most cholinergic differences self-resolved in the face of continued depressive phenotype.

Our findings fit well within previous reports investigating the connection between depression and susceptibility to colitis. Ghia et al utilized a chemically induced model of depression in combination with dextran sulfate sodium salt (DSS) to demonstrate that depressed mice experience more severe inflammation when exposed to DSS. Importantly, the authors demonstrate that reversal of depressive behavior with tricyclic antidepressants alleviated colitis only if there was an intact vagus nerve. This indicated that depressive susceptibility to colitis was mediated by vagal signaling. The authors go on to demonstrate that depression resulted in a significant reduction in colonic acetylcholine concentrations, and subsequently that depression must down-regulated cholinergic tone through inactivation of the vagus nerve. Finally, the authors conclude that reduce acetylcholine concentration removed the anti-inflammatory impact acetylcholine plays by acting on nicotinic receptors of macrophage.(15, 16) These results compellingly argue for a central role of the vagus nerve in mediating colitis susceptibility by down regulating colon acetylcholine availability in depression. However, they do not explain how the vagus nerve, which sparingly innervates the distal colon and only synapses superficially on the myenteric plexus, could lead to such robust decreases in colonic acetylcholine.(6)

Sources of cholinergic dysfunction

Though the colon receives cholinergic extrinsic cholinergic innervation, the enteric nervous system is comprised of a substantial amount of cholinergic neurons, which mediated many homeostatic functions. Our findings revealed that CSDS lead to a significant down regulation in the percentage of submucosal plexus cholinergic enteric neurons, which may more accurately explain the intrinsic loss of cholinergic tone in depressive phenotypes. Submucosal enteric neurons representing the major source of lost cholinergic tone in CSDS is substantiated by deficient colonic secretomotor function in CSDS mice compared to controls. Namely, strips of colon demonstrated reduced amplitude of phase I currents in CSDS mice compared to controls, and we previously demonstrated **Chapter 3**, **Supplemental Figure 3.2** that this response was TTX and muscarinic receptor sensitive. Since submucosal nerves are well known to mediate electrolyte transport, the reduction in percentage of cholinergic nerve likely explains the transport deficits observed in CSDS animals, and demonstrates a functional loss due to reduced percentages of cholinergic enteric neurons.

In addition to reduced percentage of cholinergic enter nerves, gene expression of cholinergic components required for canonical cholinergic synthesis, degradation, and signaling were also found to be reduced in CSDS mice. In the present study, the purpose and source of these changes is unclear. It is conceivable that the reduction in CHAT, VACHT, and CHT-1 can be attributed to the reduction in cholinergic neuron number, as these genes are required for neuronal acetylcholine synthesis.(13) Though, ChAT,(11) VAChT,(3) and CHT-1(18, 19) are known to be expressed in the enteric nervous system, there is some evidence that these systems exist, though without

function, in non-neuronal cells.(4) Therefore, it is possible that some of the down regulation in cholinerigic gene expression is occurring outside of the enteric nervous system, though we suspect this is unlikely as observed difference in phase I response to physostigmine are dependent on neuronal function.

Speculation on cholinergic response

Presumably, reductions in AChE may be a compensatory response by the host to preserve the limited cholinergic signaling observed acutely following CSDS. Indeed, other groups have shown that acute stress resulted in a down regulation in intestinal mucosal AChE activity, which promoted cholinergic signaling.(33) Similarly, the observed down regulation of muscarinic receptor expression in CSDS mice may have been a compensatory response to chronic stress.

Muscarinic signaling is known to contribute to elevated electrogenic intestinal secretion (32) and motility (22) under acute stress or acute exposure to stress peptides. Therefore, it is conceivable that a homeostatic response to chronic stress may result in down regulation of cholinergic signaling through down regulation of muscarinic receptors involved in both motility and secretion. Supporting this argument, we observed down regulation in muscarinic receptors 2 and 3, which both are known to play the major role in motility(24), while muscarinic receptor 3 is chiefly responsible for epithelial electrolyte transport.(1, 25)

Cholinergic dysfunction as a risk factor for colitis

Though much focus has been paid on alpha-7 nicotinic receptor function in preventing acute inflammation, little is known about how this receptor's expression may change in disease. Previous studies investigating the interaction of depression and

susceptibility to colitis demonstrate that colitis susceptibility in depressed mice could be mitigated with increased alpha-7 nicotinic receptor activation. These findings demonstrate a central role of the alpha-7 nicotinic receptor in mediating colitis susceptibility in depressed individuals; however these studies never focus on how expression of this receptor may change with disease. The current understanding of depression increased risk of colitis focused on a loss of vagal cholinergic signaling onto alpha-7 nicotinic receptors (15, 16); however, our findings suggest that a down regulation in alpha-7 nicotinic receptor expression, in combination with reduced colon intrinsic cholinergic tone, may also contribute to colitis risk. Though we did not observe colitis in the CSDS mice, we did find an increase in lymphoid aggregate size, indicating some form of immune induction. Expansion of this tissue may be linked to the reduction in alpha-7 receptor expression, but further studies would be required to validate this argument.

Though we did not observe outright significant colitis, such as large inflammatory cell infiltrate, edema, or epithelial barrier loss, we did observe an increase in lymphoid follicle size. This is an interesting finding and may indicate an initiation point in the mucosal immune system conferring increased risk of colitis from stress induce depression. Supporting this argument, in human patients, increase in colonic lymphoid aggregate size correlates with severity of colitis (31, 35, 36, 39); therefore, the expansion of lymphoid follicle size in CSDS mice may serve as a marker for pending risk to colitis, particularly in individuals with genetic susceptibility to IBD.

Though we observed robust down regulation of the cholinergic system immediately following 10 days of CSDS, at a two week follow up time point, during

which time the animals were under standard housing conditions, we observed that the changes to the cholinergic system had subsided, despite a continued deficit in social interaction. These results would suggest the changes to the colonic cholinergic system are transient and self-resolving once a stressor is removed. However, these findings do not mean that the intial down regulation of the cholinergic system does not have significance on either inflammatory predisposition or initiation of behavioral deficits. It is possible that chronic stress induced cholinergic deficits in the gut serve as a trigger to initiate colitis; however, once the stress is removed inflammation may persist despite a recovery in the cholinergic system.

Proposed future work

Future studies may focus on supplementing the cholinerigc system during chronic stress to determine if they prevent development of increased follicle size. Furthermore, testing this hypothesis on a colitic prone animal model may be provide more robust, reliable results.

Finally, understanding the temporal sequence of psychological or colonic cholinergic deficits may be a reward avenue to pursue. Though we observed behavioral and gastrointestinal dysfunction simultaneously in the CSDS mice, it is unclear which system becomes defective first. Interestingly, a recent study in IBD patients attempting to identify whether depression precedes colitis, or whether colitis precedes depression, found bidirectional effects. For example, depressed patients in IBD remission were more likely to have and IBD flare and psychologically healthy patients experiencing an IBD flare were more likely to become depressed.(17) Therefore, it is possible that

colonic cholinergic dysfunction precedes psychological disease; therefore, therapeutically targeting the cholinergic ENS may be beneficial adjuvant therapy.

Summary

The work from this project describes the results on chronic social stress on psychological and gastrointestinal health. Behavioral deficits were observed simultaneously with down regulation of enteric cholinergic nerve expression and function. The results provide, for the first time, how chronic stress impacts the colonic enteric nervous system, establishing a foundational evidence for of future investigation into how psychological and gastrointestinal health may interact.

APPENDIX

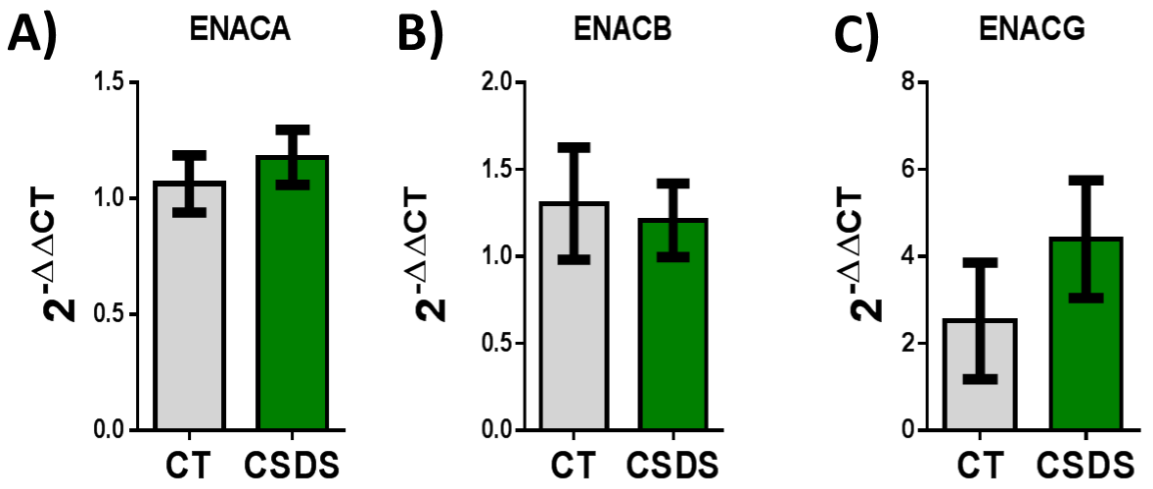

Figure S.5.1. Gene expression of electrogenic sodium transporter subunits in colonic tissue.

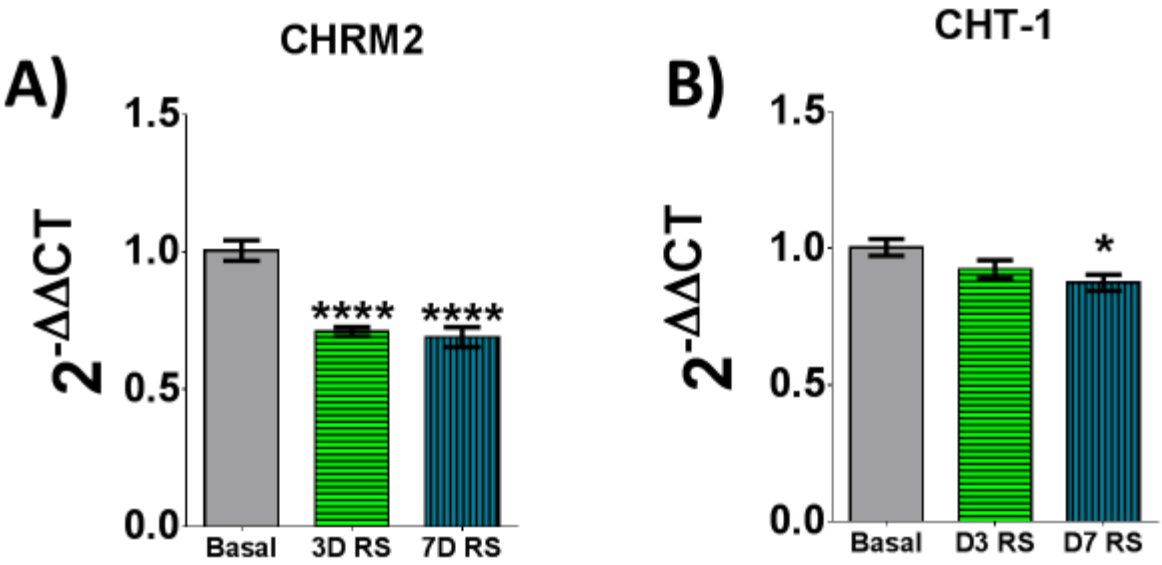

Figure S.5.2. Impact of repeated 2 hour restraint stress on colonic cholinergic gene expression. A) Cholinergic muscarinic receptor 2 (CHRM2) B) Choline high affinity transporter (CHT-1) expression in whole colonic tissue of mice subjected to 2 hours restraint stress for the specified number of consecutive days. (34)

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CHAPTER 6

Summary

Overview

Stress is a leading environmental factor contributing to GI disease such as IBS and IBD. Diverse modes of stress all appear to impact GI health, whether, they are ELA, acute stress, chronic stress, or infectious enteritis; however, the work presented here demonstrates that different types of stress may impact GI health through different mechanisms. Considering the central importance of the enteric cholinergic system in GI homeostasis, we focused on how different types of stress at different ages impact short and long term GI function.

In Chapter 2, we demonstrate with porcine EWS, that ELA adversity induces persistent GI dysfunction into adulthood. GI dysfunction was noted by persistent diarrhea, persistent intestinal barrier defects with an underlying increase in mast cell numbers and increased mast cell association with enteric neurons. Interestingly, these findings appeared to persist into adulthood only in female individuals rather than males, highlighting an important effect of biological sex on ELA and GI disease. These findings were in agreement with the sister study to this project, demonstrating that ELA resulted in a persistent upregulation of the enteric cholinergic nervous system.

In Chapter 3, we sought extrapolate the findings of ELA in the porcine model to the murine early life stress model, NMS. In this model, we found a persistent upregulation of colonic ion transport, which was exacerbated by cholinergic stimulation. Converse to the porcine model, we did not observe resting, baseline differences in fecal output, or permeability, though we did observe that NMS mice had increased colonic contraction strength and duration. Only upon stimulation with a mild 2 hour restraint stress did we observe NMS mice to present with GI dysfunction, reveal as increased

fecal pellet output and increased intestinal permeability. We also demonstrated that the function increase in fecal pellet output and permeability were mediated by increased by HC-3. Finally, we found NMS mice to demonstrate an increased expression of gene for inflammation, metabolic hormones, and inflammatory mediators following a mild stressor, which were also under the control of cholinergic signaling. Though we did not observe expression differences of the enteric cholinergic system in NMS mice, we did find that ELA induced immediate changes to mucosal cholinergic expression in the EWS pig model.

Surprisingly, many of the functional differences observed in the pig (such as fecal output and permeability) occurred at baseline, resting conditions, whereas a mild stressor was required to reveal these functional difference in the mice. Additionally, sex differences between male and female NMS mice was not as apparent as it was in the EWS model. Several factors may contribute to these differences. First, as highlight in Chapter 1, pigs have a much more complex central and peripheral nervous system, potentially making pigs more sensitive to environmental stress factors than mice. Housing conditions are also considerably cleaner and less stressful for mice than for pigs, potentially leading to a reduced total environmental stress factor. Second, the method of ELA is significantly different between pigs and mice. In pigs, EWS results in an abrupt loss of maternal care, change in diet, change in environment, and comingling with unfamiliar peers. In NMS, mice are only separated for 3 hours daily, for an 18 day period and, when mice are wean, they remained co-house with their littermates in a similar environment. Mice do not undergo the stressful transportation stress the pigs undergo when weaned (our EWS pigs were shipped from nearly 2 hours away to a new

location). Therefore, the NMS model has a much less severe impact on maternal loss and likely has little impact on diet, as pups continue to have exposure to breast milk throughout the duration of the ELA period. Third, in the EWS model, we focused on the influence of ELA on the ileum; however, in the NMS model, our studies focused on the colon. Though we did find some functional difference in the ileum of the NMS mice, through electrical field stimulation, the strongest differences from controls were observed in the colon. The difference in site specific pathology may indicate an effect of the microbiome as microbial populations and there metabolites are different between gut regions. In spite of these difference, we still found similarly cholinergic mediated GI dysfunction in both models, highlighting the comparative pathophysiology of ELA on GI disease and elevating the translational value of this research.

Additionally, in Chapter 3, we explore the acute impacts of weaning on the enteric cholinergic system. Though acute stress in adults did not appear to change expression of the cholinergic system, we observed a significant upregulation in mucosal ChAT expression in EWS pigs compared to later weaned controls. These findings illustrate that age at which stress occurs may results in different enteric cholinergic responses.

In Chapter 4, we explored the impact of non-psychological stressors on cholinergic expression. Similar to the effects of EWS, we observed that *Salmonella typhimurium* challenges generated an upregulation in ChAT expression in mucosa associated with lymphoid follicles. In addition, increase in ChAT expression correlated positively with the inflammatory cytokine TNF and histopathology scores, establishing a potential role for non-neuronal acetylcholine in mediated GI dysfunction. An

upregulation in ChAT was observed over the follicle associated mucosa in both EWS and infectious enteritis, suggesting that upregulation of non-neuronal CHAT expression is a conserved response, particularly in acute stressor of juveniles.

In Chapter 5, we explored the impact of chronic stress and depression, through murine chronic social defeat, on the enteric cholinergic system. Though ELA and acute stress in juvenile pigs appears to manifest as an upregulation of the cholinergic system function and expression, respectively, we observed that CSDS resulted in significant down regulation of the enteric cholinergic nervous system. Interestingly, in adults, even acute stress appears to initiate a down regulation in cholinergic receptors functioning as we found significant suppression of muscarinic receptor 2 immediately following 2 hours of restraint stress. Interestingly, after cessation of stress, expression and function of the enteric cholinergic system returns to control levels. Recovery of the enteric cholinergic system to control levels is opposite to the impact of ELA, which appears to result in a persistent hyperactivity of the enteric cholinergic system. These findings support the hypothesis that stress early in live impacts post natal development of the ENS, resulting in permanent changes. Understanding how adults can recover back to baseline levels of cholinergic expression and function following chronic stress may provide therapeutic insights for guarding or repairing ENS damage induced by ELA.

Together, these studies demonstrate a dynamic function and expression of the enteric cholinergic system with different modes of stress, and highlight the complexity of cholinergic signaling in stress induced GI disease.

Highlights of novel findings

A hyperactive enteric cholinergic nervous system contributes to long-term GI disease following ELA. This work identifies that the link between early life adversity and susceptibility to GI disease later in life is due to increased function of the enteric cholinergic nervous system. In the porcine EWS model we had previously demonstrated that there is persistent upregulation of enteric cholinergic nerve secretomotor function in *ex vivo* setting on Ussing chambers, however the work here demonstrates that 1) this dysfunction is translatable to a different animal model and raises the translational value of these findings 2) the upregulated cholinergic system also mediated increased fecal pellet output and intestinal permeability 3) increased gene expression in ELA individuals is under control of the cholinergic system. These findings highlight the significant of the enteric cholinergic system in patients suffering with ELA and may serve as a valuable therapeutic targets.

Psychological and infectious challenge in juvenile individuals upregulated an epithelial cholinergic system, which correlates with inflammation and tissue damage. Though the role of cholinergic nerve function in mediated stress induced GI disease is clear, our studies demonstrate that a non-neuronal cholinergic system is upregulated in stressed juveniles. Upregulation of ChAT over follicle associated mucosa in psychological and infectious challenge demonstrates a conserved response, increasing the significance of these findings. The role of this non-neuronal cholinerigc system is not clear; however, its upregulation is positively correlated with inflammation, suggesting that is may play a role in either limited or promoting the immune system. It remains

unclear if the epithelial cholinergic system is important in mediating adults stress responses, but should be address with future studies.

Acute and chronic stress in adults results in down regulation of the enteric cholinergic expression, and ultimately function. Despite the function role of the cholinergic system in promoting stress induced GI dysfunction, little was known about the underlying regulation of the cholinergic system. The data presented here demonstrates that stress in adults result in a significant down regulation in expression of the enteric cholinergic nervous system, with a loss in percentage of cholinergic nerves, as well as a reduction in total number of cholinergic receptors. Importantly, the down regulation in the cholinergic system was accompanied by a suppression in cholinergic function. Importantly, these findings are the first report that depression impacts expression of the cholinergic enteric nervous system. While acute stress promotes GI responses that would promote a diarrhea, phenotype (increased secretion, motility, and permeability), the chronic stress (CSDS) model may indicate factors leading to stress induced constipation.

Limitations

Use of pharmacological agents. Physostigmine hemi-sulfate and HC-3 were both used to promote and inhibit the cholinergic nervous system, respectively. A common problem with pharmacological studies is that they may have action on off target site or on cell types un-intended to study. However, physostigmine induced rapid changes, which were TTX sensitive, indicating that this drug was elevating nerve function. HC-3 treatments were administered systemically, which means they may have had an impact on other cholinergic neurons outside of the gastrointestinal tract;

however, our findings in the Ussing chambers from EFS and physostigmine application support that enteric cholinergic nerves were truly dysfunctional, and it is likely that the drugs was having its inhibitory impact at the level of the enteric neurons. Technical improvement of these applications could be achieved with transgenic approaches allowing specific activation or inhibition of enteric cholinergic nerves.

Future Directions

The work presented here provides a foundational understand about enteric cholinergic involvement and regulation during different types of stress, and serves as a starting point to look at both up- and down-stream mechanisms.

Determination if electrical physiology of nerves is dysfunctional. Knowing the importance of the cholinergic system in regulating long term risk to GI disease later it life, it will be important to understand how the cholinergic system becomes dysfunction. The data presented here suggest that the role of the cholinergic system may not be mediated by enhanced expression, but rather alterations in neuron excitability. Assessing membrane properties of the enteric neurons, either through patch clamping or *ex vivo* imaging with voltage sensitive dyes may help better reveal the underlying neurophysiology of the enteric nervous system.

Inciting factors of ENS dysfunction in ELA. The inciting events promoting neuron dysfunction remain elusive. Identifying mediators which induced changes to the postnatal enteric cholinergic nervous system would represent a significant leap in understanding the link between ELA and risk of GI disease later in life. Identification of mediators altering development of the postnatal ENS, could be achieved by specifically isolating mRNA and investigate changes in receptors expression specifically on enteric

nerves during and immediately following ELA. Identification of receptors pathways activated in the ENS during ELA could be used to help develop interventional therapies in children during traumatic events.

Functional role of the epithelial enteric cholinergic system. Though we demonstrated that the intestinal epithelial upregulate ChAT expression in two different forms of stress, a functional role of this system remains elusive. Additionally, it remains unclear if epithelial ChAT is also upregulated in adults experiencing stress or infectious enteritis, and should be address with future work. Studying the impact of stress on disease severity, immune function, and survival in a model with epithelial specific knock out of CHAT may help reveal the significance of this system.

Impact of suppressed cholinergic tone due to chronic stress. Observation of lymphoid hypertrophy with simultaneous down regulation of cholinergic nerve expression supports link between stress and increased risk of inflammation. Though we did not observe outright colitis, utilizing the CSDS model on a colitis prone background, such as IL-10-/-, may better reveal the relationship between suppressed enteric cholinergic nerve function and increased risk of inflammation. Future work may investigate if the reduction in cholinergic tone directly promotes immune activation, and could be tested by supplementing chronically stressed individuals with cholinergic agonists.

Work here highlights a significant role of the enteric cholinergic system in GI dysfunction. Future work should focus on factors impacting cholinergic nerve development, function, and expression. Furthermore, targeting epithelial cholinergic systems may also prove to be a fruitful. Both direct and indirect modulation of this

system may help alleviate the major public health issue of stress induced GI dysfunction.