

## ABSTRACT

MEDLAND, JULIA ELISE. Development of the Enteric Nervous System in a Porcine Model of Early Life Stress: Implications for Long-Term Intestinal Disease Susceptibility. (Under the direction of Dr. Adam Moeser).

Early life stress is a major factor in the development of gastrointestinal diseases in adulthood, such as the inflammatory bowel diseases and irritable bowel syndrome. It is believed that these diseases involve dysfunction of the brain gut axis, with a specific emphasis on alterations in the nervous and immune systems. The studies outlined in this thesis seek to determine the impact of early life stress on the development of the enteric nervous system in a pig early weaning model. In this study, early weaning stress in piglets was used to investigate the effects of stress on the enteric nervous system. Specifically, we investigated whether early weaning stress impacted enteric nervous system by measuring veratridine-induced short circuit current ( $I_{sc}$ ) in intestinal mucosal tissue mounted on Ussing Chambers. Furthermore, we investigated whether there were phenotypic changes in the enteric nervous system by measuring expression of neural markers via immunofluorescence, ELISA and qRT-PCR. Intestine from early weaning stressed animals (weaned at age 16 days) exhibited increased veratridine-induced  $I_{sc}$  responses compared with late weaned control pigs (weaned at age 28 days). Female early-weaned pigs show greater functional changes than their male counterparts. Early weaned animals also failed to display the normal loss of neurons throughout the neonatal period. Furthermore, early-weaned intestine demonstrated increased cholinergic tone, as determined by response to atropine, increased expression of acetylcholinesterase (AChE), and choline acetyl transferase (ChAT), which may be contributing to the disease state. More studies will be required to determine the mechanisms

and pathways behind this nervous hypersensitivity and increased survival induced by early weaning.

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Development of the Enteric Nervous System in a Porcine Model of Early Life Stress:  
Implications for Long-Term Intestinal Disease Susceptibility

by  
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## **BIOGRAPHY**

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## ACKNOWLEDGMENTS

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**CHAPTER 1: EARLY LIFE ADVERSITY AND ENTERIC NERVOUS SYSTEM  
DEVELOPMENT IN GASTROINTESTINAL DISEASE:  
LESSONS FROM ANIMAL MODELS**

## **Introduction**

Early life adversity is recognized as a major risk factor for developing gastrointestinal conditions like irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) in adulthood. These conditions are chronic disorders with a significant cost to quality of life. Given the growing evidence supporting a role for the nervous system in gastrointestinal disease pathogenesis, and the vulnerability of the enteric nervous system during the postnatal period, the focus of this thesis will center on the developing enteric nervous system (ENS) in the neonate, and how it can be affected by stress. Given the complexity of diseases like IBS and IBD, and the interplay between brain and the gut, it is important to consider a large animal model, such as the pig, when investigating these diseases.

### **A link between early life adversity and gastrointestinal disease development**

#### *Early life adversity and gastrointestinal disease in humans*

Individuals who experience adverse events early in life are at far greater risk for functional gastrointestinal diseases in adulthood, like irritable bowel syndrome and inflammatory bowel disease, than the general populace (Bradford et al, 2012; Talley et al, 1994; Drossman et al, 1990; Agostini et al, 2010a; Agostini et al, 2010b; Ercolani et al, 2010). These stressors can vary widely from emotional abuse to sexual abuse and physical punishment, with adult disease manifesting most prominently in women (Bradford et al, 2012; Fumery et al, 2014). In addition to an increased risk of functional bowel disorders, children who experience these traumas also had an increased likelihood of experiencing abdominal pain (van Tilburg et al, 2010). Childhood trauma has also been linked with

elevated levels of cortisol during stressful or painful events in adulthood, which contribute to IBS severity (Vidlock et al, 2009). Furthermore, there is evidence to support that symptoms of functional bowel diseases in adult victims of child abuse are linked with symptoms of mood disorders like anxiety and depression (Jones et al, 2013).

*Neonatal maternal separation – a rodent model for early life adversity*

One of the best-studied models for early life stress-induced gastrointestinal disease is neonatal maternal separation stress (NMS) in rodents (Barreau et al, 2004; Garcia-Rodenas et al, 2006; O'Mahony et al, 2009). In this model, neonatal rat or mouse pups are separated from the dam during neonatal days 4-19 for 3 hours each day and then returned (Gareau et al, 2007). The model is used primarily to study functional gastrointestinal diseases such as IBS, but is also used to study depression and anxiety (Kuhn and Schanberg, 1998).

In addition to these similarities with human IBS, many changes within the enteric nervous system have been observed with this model, giving much needed insight in to the pathogenesis of IBS. The hypothalamic –pituitary-adrenal axis develops during the neonatal period, and as such is vulnerable to damage during this period, which may result in adult disease (O'Mahony et al, 2010). In rodents, postnatal days 4-14 are considered a period of stress hyporesponsiveness (Rosenfeld et al, 1992), which overlaps with a period of neuronal growth and myelination (Morgane et al, 2002). Low levels of corticosterone are thought to be required for normal development during this period (Meyer and Joy, 1985). Neonatal stress has long-lasting implications for the HPA axis, including an increased neuroendocrine response to stress, increased anxiety and higher levels of cortisol in adulthood (Aisa et al,

2009; Maccari et al, 2014). Maternal separation has also been shown to result in higher adulthood CRF transcription in brain tissues (Chen et al, 2012). Interestingly, this stress hyporesponsive period is also thought to exist in humans (Gunnar and Donzella, 2002), and babies born prematurely and exposed to neonatal stress or pain have been shown to have higher levels of cortisol later in life (Brummelte et al, 2015).

Rodents subjected to neonatal maternal separation stress exhibit many of the symptoms associated with human IBS. In this model, IBS like pathophysiology and symptoms including visceral hypersensitivity (Rosztoczy et al, 2003; Chung et al, 2007), disturbed motility (Zhang et al, 2010; Holmes et al, 2005), increased permeability (Barreau et al, 2004; Gareau et al, 2007; Lennon et al, 2013), and an altered intestinal microbiota (Garcia-Rodenas et al, 2006) have all been observed.

Many changes are known to occur in both the central and peripheral nervous systems with NMS. The primary change observed in the central nervous system is up-regulated 5-hydroxytryptamine (5-HT) signaling (Daniels et al, 2004). In the enteric nervous system, changes in serotonergic signaling are again observed, primarily through an up-regulation of SERT and an increased number of enterochromaffin cells (Bian et al, 2010), which corresponds with human literature (Dunlop et al, 2005). 5-HT is also known to have a role in nervous development, possibly through 5-HT<sub>4</sub> receptor (Liu et al, 2009). Increases in macromolecular permeability (a symptom of IBS) after NMS have been associated with changes in cholinergic signaling, and can be inhibited with muscarinic receptor antagonists (Gareau et al, 2007). The other arm of the autonomic nervous system, adrenergic tone, is from the sympathetic nervous system and is also thought to change with NMS (Schemann et

al, 2010). Changes in ENS plasticity have also been observed, and may be related to colonic mast cell signaling (Barreau et al, 2008).

Although this is a valuable model, and much of our understanding of IBS stems from NMS studies, this model requires genetically susceptible rats or mice (Julio-Pieper et al, 2012; Büchler et al, 2012; O'Malley et al, 2014) and even in this population, altered stool consistency (diarrhea, constipation, alternating) is not observed, which is one of the defining symptoms of IBS in humans.

There are other rodent models that display some symptoms of IBS, particularly visceral hypersensitivity, including neonatal colonic inflammation, and exposure to neonatal noxious stimuli. Neonatal colonic inflammation is induced with daily administration of bacterial components such as lipopolysaccharide or chemical irritants such as mustard oil or acetic acid during neonatal days 3-21 and induces HPA axis dysfunction and increased pain sensitization in adult animals (Al-Chaer et al, 2000; Shanks et al, 2000; Breivik et al, 2002; Boisse et al, 2005). Neonatal noxious stimuli is induced via pain sensitization, such as putting a needle in the paw, or colonic distension at hourly intervals from birth to age 21 days, which results in hypersensitivity and HPA axis dysfunction (Lidow et al, 2001; Rude et al, 2000; Anand et al, 1999; Anand et al, 2000; Lin and Al-Chaer, 2003). A Comparison of these rodent models is presented below in Table 2.

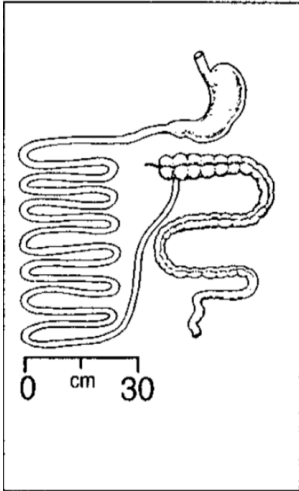
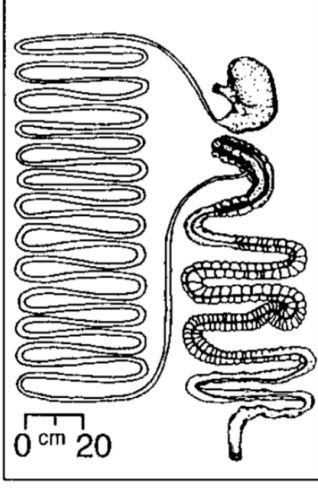
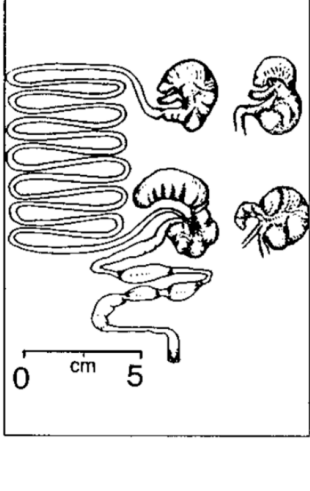
#### *Early weaning stress in piglets – a porcine model for early life adversity*

The shortcomings of the NMS model in rodents highlight the need for a large animal model that more closely represents the human condition. For studies of the gastrointestinal

tract, the pig is an excellent model for humans due to their anatomical similarity, especially when compared to existing rodent models (see Table 1 below). Specifically, pigs and humans share a more similar body size and gastrointestinal composition than humans and rodents. In addition to this anatomical similarity, pigs are also more similar to humans than rodents in their susceptibility to pathogens, and microbiota composition (Patterson et al, 2008; Pang et al, 2007; Gonzalez et al, 2015). They also share a more similar gastrointestinal transit time to humans than rodents (Graham and Aman, 1987). Pigs, like humans, are also true omnivores, and so share many digestive adaptations (Kararli, 1995; Swindle and Smith, 1998; Gonzalez et al, 2015).

Table 1. Comparison of the anatomy of human, porcine and rat gastrointestinal tracts

(Karalarli, 1995). The anatomical similarity between the gastrointestinal tracts of humans and pigs contributes to the validity of the pig as a model for human gastrointestinal disease.

	Human	Pig	Rat
Small intestine % of GI tract	78% (Ritschel, 1991)	78% (Stevens, 1977)	86% (Hebel and Stromberg, 1976)
Colon % of GI tract	17% (Ritschel, 1991)	21% (Stevens, 1977)	6.9% (Hebel and Stromberg, 1976)
Diagram of Gastrointestinal system (Stevens and Hume, 1998)	<p>Body length: 180 cm</p> 	<p>Body length: 125 cm</p> 	<p>Body length: 17 cm</p> 

For studies of the central nervous system, pigs have been highlighted as an important model with many similarities to humans. Specifically, pigs are more similar to humans in brain development and morphology than commonly used rodent models (Conrad and Johnson, 2015). Pigs reach puberty at approximately 24 weeks, and over this time, brain volume increases approximately 120% and this development has been shown to be similar to humans (Conrad and Johnson, 2015). Pig brains show similar sexual dimorphisms, displaying, for example, the same altered hippocampal growth rates in females observed in the human brain (Conrad and Johnson, 2015). The timeline of neurodevelopment of the pig is compared to that of humans in Figure 1 below.

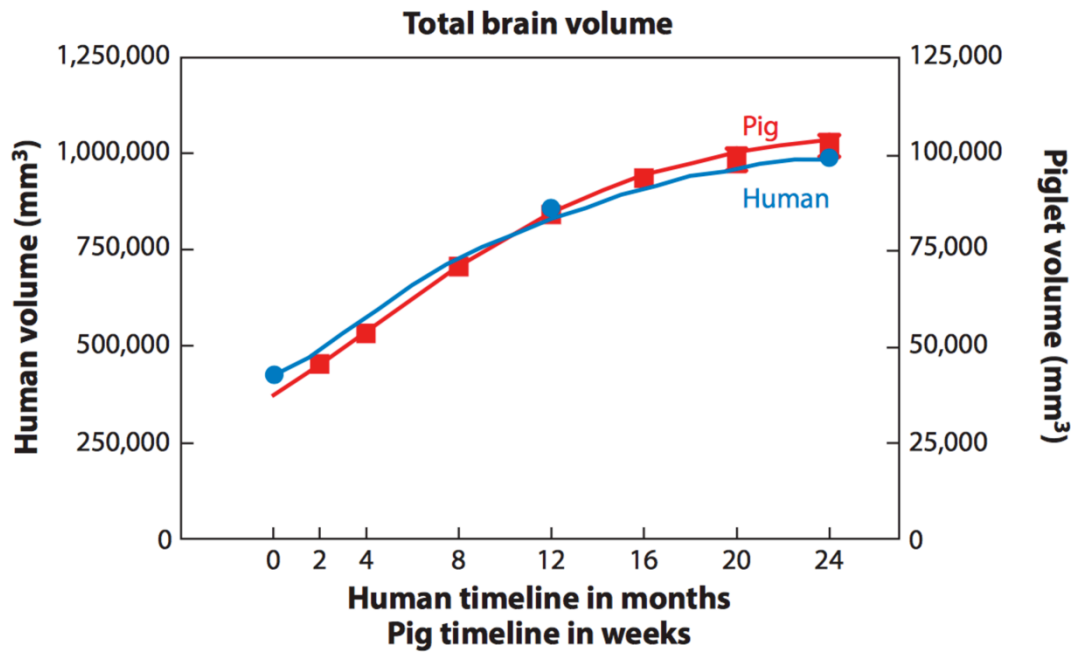


Figure 1. Comparison of human and pig neurodevelopment (from Conrad and Johnson, 2015). Brain growth over time in humans and pig, while occurring on a different timescale, is similar, and thus a desirable model for neurodevelopment studies.

In addition to brain structure, similarities in neurotransmitter function have also been highlighted. The distribution of serotonergic neurons is very similar between pig and human brains, particularly in infants (Niblock et al, 2005). This is relevant to GI disease as dysregulation of serotonin signaling has been implicated in IBS (Lee and Park, 2014). In addition to this, pigs, like humans, have most of their enteric primary afferent neurons located in the inner submucosal plexus (Brown and Timmermans, 2004). The intestinal contractile responses of pigs are also more similar to humans than that of common rodent models such as rats and mice (Brown and Timmermans, 2004). Morphologically, the intestinal mucosal projections of pigs appear to be more similar to that of humans than guinea pigs, which are commonly used in ENS research (Hens et al, 1999).

Because of these similarities in neurodevelopment and the gastrointestinal tract, the piglet is an excellent model animal to examine the effects of stress on the development of the enteric nervous system. Early weaning stress in piglets represents a valid and new model to study early life stress (Moeser et al, 2007; Smith et al, 2009; McLamb et al, 2013) to study the effects of stress on the gastrointestinal tract. These studies have shown the deleterious effects of weaning at a young age (16 days, compared to 28 days) on the gastrointestinal tract, including increased intestinal permeability (Smith et al, 2009) and impaired immune responses to subsequent pathogens (McLamb et al, 2013), and is directly comparable to NMS in rodents. HPA axis dysfunction has also been observed, particularly in CRF function and mast cell activity (Smith et al, 2009; Moeser et al, 2007).

While this model is similar to neonatal maternal separation in rats and mice, piglets undergoing this stress display more clinical signs of gastrointestinal disease, namely diarrhea,

than their rodent counterparts (Melin et al, 2004). Early weaning stress in piglets is a multifactorial stressor, which includes nutritional changes, changes in social milieu (mixing stress), changes in environment and stresses of transportation and handling (Campbell et al, 2013). This model represents an exciting opportunity for translational medicine with application to both human disease and animals in production, as piglets in agriculture are typically weaned as early as 14 days and this is linked with GI disorders. A summary table of models of early life adversity and the effects on gut health is presented below in Table 2.

Table 2. Models of neonatal stress. Commonly used rodent models of neonatal stress including neonatal maternal separation, neonatal colonic inflammation and neonatal noxious stimuli are compared to a large animal model, early weaning stress in pigs. Disadvantages and advantages and outcomes of each model are compared.

	<b>Neonatal Maternal Separation (Mice, rats)</b>	<b>Neonatal colonic inflammation (Mice, rats)</b>	<b>Neonatal noxious stimulation (Mice, rats)</b>	<b>Early weaning stress (pigs)</b>
<b>Details of Model</b>	On neonatal days 4-19, animals are separated from the dam for 3 hours (Gareau et al, 2007)	Between neonatal days 3 and 21, the GI tract is exposed several times to inflammatory stimuli such as LPS, mustard oil, acetic acid etc (Shanks et al, 2000; Al-Chaer et al, 2000)	Between neonatal days 1 and 21, painful stimuli such as a needle in the paw or colorectal distension is administered hourly (Anand et al, 1999)	Piglets are subjected to early weaning stress are removed from the sow at approximately 16 days of age, compared to controls animals, weaned at approximately 28 days (Smith et al, 2009; McLamb et al, 2013)
<b>Symptoms of GI Disease</b>	Colonic barrier dysfunction (Gareau et al, 2007; Barreau et al, 2007; Lennon et al, 2013), HPA axis dysfunction (Aisa et al, 2009); Altered motility (Holmes et al, 2004), visceral hypersensitivity (Chung et al, 2007)	Hypersensitivity (Boisse et al, 2005; Al-Chaer et al, 2000), HPA Axis dysfunction (Shanks et al, 2000)	Hypersensitivity (Lidow et al, 2001; Rude et al, 2000), HPA axis dysfunction (Sternberg et al, 2005).	Decreased ability to fight infection (McLamb et al, 2013), gastrointestinal barrier dysfunction (Smith et al, 2009), HPA axis dysfunction (Smith et al, 2009), increased mast cell activity (Moeser et al, 2007)
<b>Advantages</b>	Well studied, model can be coupled with knock out mice (IL 10 -/- etc)	Good model for neonatal infection causing inflammation, can be coupled with genetically manipulated animals	Good model for long-term effects of neonatal pain, can be coupled with genetically manipulated animals	Closely models human disease state, shows full range of IBS symptoms, large animal model with similar gastrointestinal tract and peripheral nervous system to humans

Table 2 Continued

				Relevant psychosocial stressors with dual benefit to understanding production and companion animal diseases
<b>Disadvantages</b>	Does not show altered stool consistency	Invasive, must be induced by exogenous irritant, changes in permeability and motility not observed	Invasive, no changes observed in permeability or motility	Relatively new model, expensive, genetic manipulation difficult

## **The Enteric Nervous System: A potential link between early life stress and gastrointestinal disease**

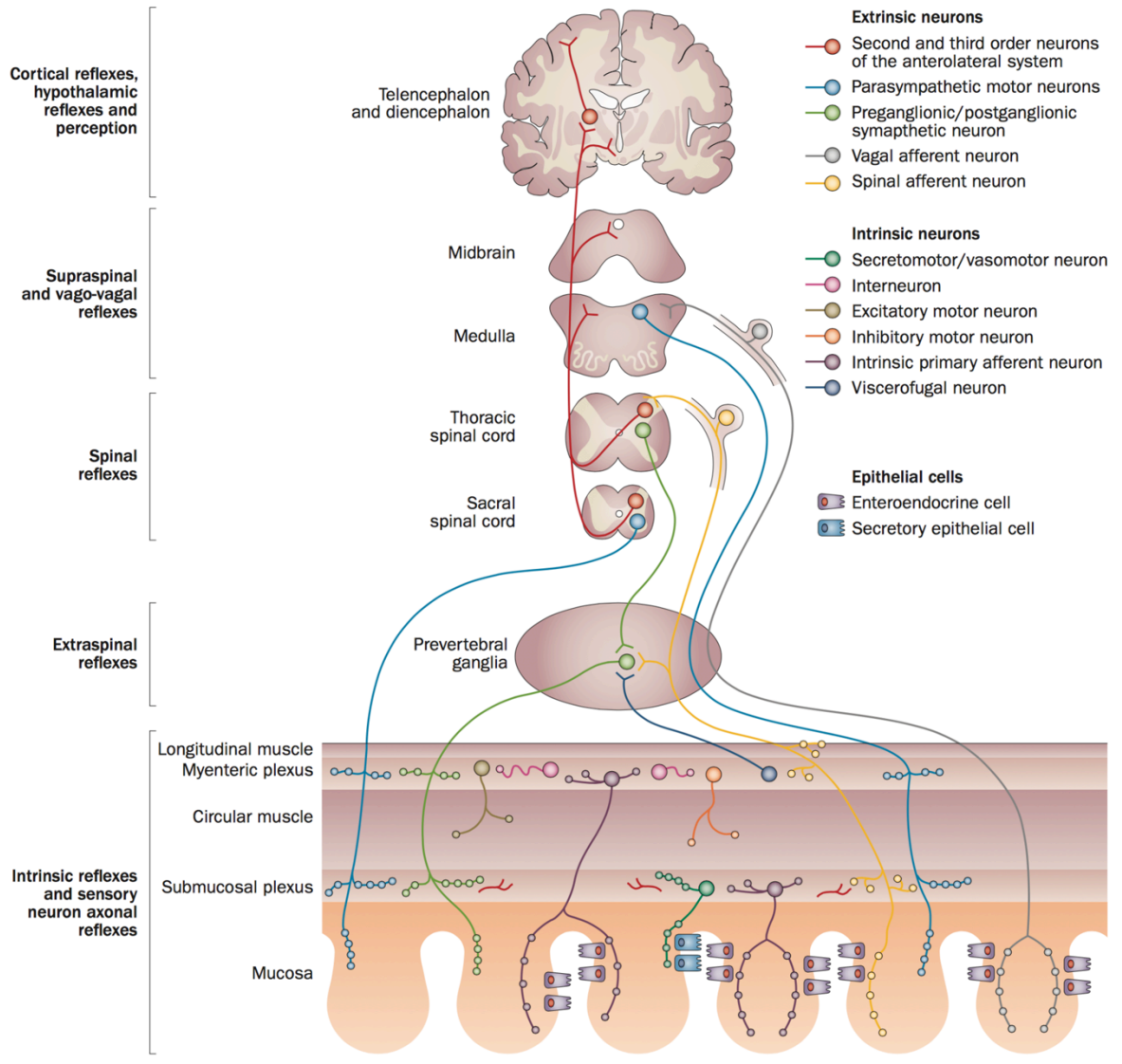
The enteric nervous system has been implicated as a major player in early life stress-induced gastrointestinal disease and is an area that deserves more research (Gareau et al, 2007; Bian et al, 2010; Dunlop et al, 2005). The enteric nervous system undergoes many developmental changes during the neonatal period and, as such, is particularly vulnerable to damage from neonatal stress. It is thought that the developing enteric nervous system may be altered with neonatal stress, and thus contribute to the symptoms of adult gastrointestinal disease such as visceral hypersensitivity and increased permeability (Gareau et al, 2007; Bian et al, 2010; Demir et al, 2013).

### *Anatomy of the Enteric Nervous System*

The gastrointestinal tract is controlled by the enteric nervous system, which is capable of functioning independently of the central nervous system and includes both intrinsic and extrinsic nerves. The GI tract contains about 100 million neurons, which is comparable to the number in the spinal cord (Furness and Bornstein, 1995). Intrinsic nerves in the gastrointestinal tract control motility, blood flow, endocrine and exocrine release, and secretion and absorption across the intestinal barrier (Zhang and Xu, 2003). Extrinsic innervation comes from the autonomic nervous system, which bridges the central nervous system and the gastrointestinal tract (forming the brain-gut axis), composed of both parasympathetic (primarily cholinergic, from the Vagus and Splanchnic nerves) and sympathetic (primarily noradrenergic from the Lumbar and Thoracic spinal cords)

innervation (Zhang and Xu, 2003). Extrinsic nerves terminate on intrinsic nerves, which send projections into the gastrointestinal mucosa (Zhang and Xu, 2003). Different types of neurons in the gut are shown below in Figure 2.

Figure 2. Innervation in the gastrointestinal tract (from Brierly and Linden, 2014). Extrinsic innervation to the gastrointestinal tract comes from parasympathetic neurons in the sacral spinal chord and the medulla, and from sympathetic neurons in the thoracic spinal chord. Afferent neurons from the gastrointestinal tract are spinal afferent neurons and vagal afferent neurons. Intrinsic enteric neurons, self-contained within the gastrointestinal tract, include secretomotor neurons, interneurons, primary afferent neurons, and excitatory and inhibitory neurons. All of these neurons connect with cells in the epithelium including enterochromaffin cells and secretory cells.



There are two major nerve plexi within the intestinal tract; the myenteric plexus, located between the longitudinal and circular muscle layers, and the submucosal plexus, located between the circular muscle layer and muscularis mucosae (Furness, 2012). The anatomy of the enteric nervous system is shown below in Figure 3. In both the myenteric and submucosal plexus, neuron cell bodies are grouped into small ganglia (Zhang and Xu, 2003). The myenteric plexus runs from the upper esophagus to the anal sphincter and primarily controls motility. The submucosal plexus, absent in the esophagus, and most prominent in the small and large intestines, primarily controls secretory activity in the gastrointestinal tract. Both plexuses are known to have projections into the muscle layers and the mucosa (Furness and Bornstein, 1995).

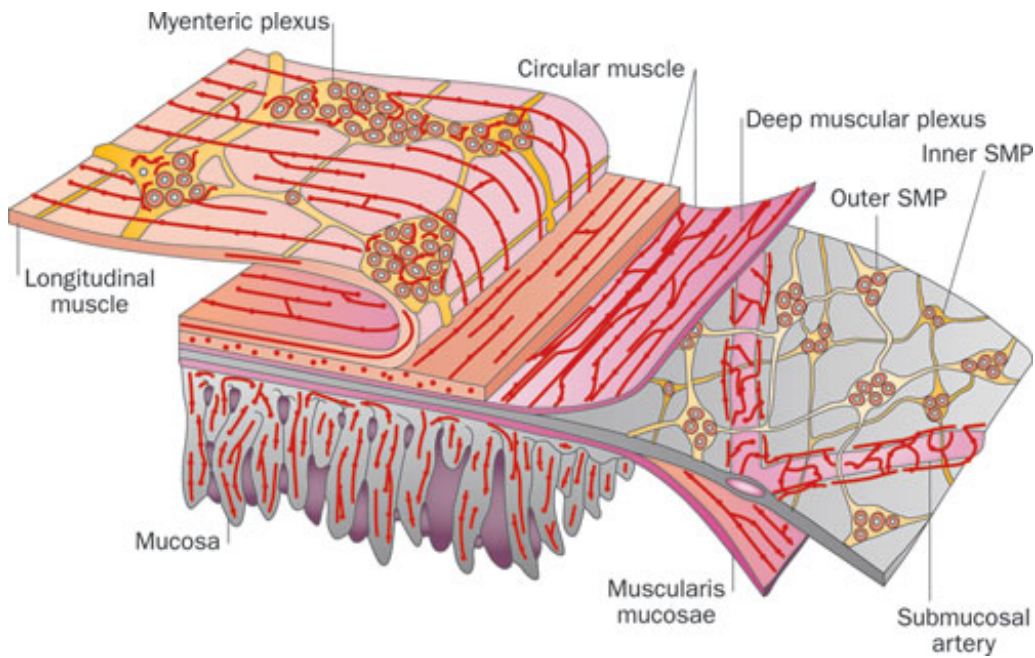


Figure 3. Anatomy of the enteric nervous system (from Furness, 2012).

The anatomy of the enteric nervous system is shown above with the myenteric plexus being the outermost group of neurons, located between the longitudinal muscle and the circular muscle layers. The submucosal plexus is located between the circular muscle and the muscularis mucosae. Both of these plexi house cell bodies that project into the mucosa and muscle, and also communicate with the central nervous system.

Enteric neurons can be broadly classified as motoneurons, sensory neurons or interneurons. Motoneurons can be further classified as excitatory, inhibitory, secretory (primarily in submucosal plexus) or vasodilatory (Zhang and Xu, 2003). There are different neurotransmitters associated with each type of motoneuron, with excitatory motoneurons being mediated by acetylcholine and tachykinins, inhibitory motoneurons being mediated by NO, ATP, VIP and NPY and secretory motoneurons being mediated by acetylcholine, VIP, CGRP and Substance P (SP) (Zhang and Xu, 2003). Extrinsic innervation in the gastrointestinal (GI) tract comes from the parasympathetic and sympathetic branches of the autonomic nervous system. Sympathetic innervation, mediated by norepinephrine, is generally inhibitory (Costa and Furness, 1984), while parasympathetic innervation, mediated primarily by acetylcholine, is generally excitatory in the gastrointestinal tract (Brookes et al, 1991; Keast et al, 1985).

#### *Development of the Enteric Nervous System*

In the developing embryo, the enteric nervous system is derived from enteric neural crest cells, which proliferate and migrate to populate the intestines (Lake and Heuckeroth, 2013). After migration, the cells respond to different stimuli, such as glial derived neurotrophic factor (GDNF), endothelin (ET)-3, and SOX10, and differentiate into glia and neuronal subtypes (Lake and Heuckeroth, 2013).

During the postnatal period, the major changes occurring in the enteric nervous system are (1) the formation of functional nervous circuits (e.g. those that regulate motility and secretion), (2) gangliogenesis, (3) the differentiation of mature neuronal phenotypes and

(4) cell death (Sasselli et al, 2012). In most other areas of the nervous system, approximately 50% of fetal neurons undergo apoptosis (Hamburger, 1992; Oppenheim, 1991), but this is not the case for enteric neurons. While there is apoptosis of migrating enteric neural crest cells, little apoptosis of enteric neurons occurs prior to birth, with most apoptosis occurring in the post-natal period (Aoki et al, 2007). This postnatal apoptosis lends confidence to the concept of “postnatal pruning” (Chalazonitis et al, 2012). Pruning becomes necessary in the developing nervous system as neurons generated in excess are not required and eliminated (Buss et al, 2006). For neurons to survive, it is thought that some signal is needed. In the developing, postnatal enteric nervous system, one of these signals could be serotonin, as mice lacking the 5-HT<sub>4</sub> receptor showed a significant loss of neurons between the ages of 1 and 4 months, and receptor agonists induced increased neuronal survival (Liu et al, 2009). GDNF is involved in the process of promoting survival of fetal enteric neurons (Heuckeroth et al, 1998), but its involvement in postnatal neuron survival is unknown. It is possible that during the postnatal period, with fewer survival factors available than during the fetal period, the enteric nervous system is more vulnerable to a disruption of this normal apoptotic process (Chalazonitis et al, 2012).

While no new postnatal neurons are observed in mice from postnatal day 10 onwards (Laranjeira et al, 2011), many other changes in the postnatal enteric nervous system are observed. Neurite projection length and morphology also change in the postnatal enteric nervous system, with ectopic expression of GDNF influencing nerve fiber distribution (Foong et al, 2012; Wang et al, 2010). Nerve growth factor (NGF), which is secreted by a number of cell types in the gastrointestinal tract, including glia and mast cells, enhances

neuron survival and neurite projections (Kobayashi et al, 1999; von Boyen et al, 2006). In the postnatal enteric nervous system, NGF expression can be altered through exposure to inflammatory cytokines, and can thus alter the enteric nervous system (Kobayashi et al, 1999; von Boyen et al, 2006). While neuron numbers in the enteric nervous system decrease during the postnatal period, the size of surviving neurons increases (Schafer et al, 1999; Gabella, 1970). In pigs, it has been noted that axonal thickness increases between birth and 12 weeks of age, and that mucosal innervation also increases during this time (Paran et al, 2008; Paran et al, 2006).

Different neuronal phenotypes appear at different times throughout development. It is generally accepted that serotonergic neurons are the first group to appear, followed by cholinergic neurons (marked by choline acetyl transferase) followed much later by VIP and CGRP neurons, though all populations still appear prior to birth (Pham et al, 1991). While adrenergic innervation is first observed in fetal stages, innervation continues to develop in the pig until 12 weeks of age (Paran et al, 2007). It is also known that the proportion (not total number) of ChAT immunoreactive neurons increases in the postnatal period (de Vries et al, 2010) and that appearance of these neurons is slightly delayed in the submucosal plexus compared to the myenteric plexus (Vannucchi and Faussone-Pellegrini, 1996).

The neurochemical makeup of neurons in the submucosal plexus is thought to be approximately 45% non-cholinergic secretomotor/vasodilator neurons, 44% cholinergic secretomotor neurons and 11% primary sensory neurons (Furness, 2000). It is important to keep in mind that these neurons express multiple different neurochemical markers, such as ChAT (choline acetyl transferase), VIP, NPY, calretinin etc (Furness, 2000). Interestingly,

the neurochemical composition of neurons underneath Peyer's patches is different than in the rest of the intestine, indicative of neuro-immune interactions between gut associated lymphoid tissue (GALT) and the ENS, which is an area of investigation in IBS research (Kulkarni-Narla et al, 1999).

Neurons are not the only source of neurotransmitters in the gastrointestinal tract. In fact, most of the body's serotonin is stored within enterochromaffin cells in the intestinal epithelium. Enterochromaffin cells have a large capacity to mediate secretory function in the GI tract through their connection with secretomotor neurons in the submucosal plexus (see Figure 4 below), which is mediated by primary afferents leading from enterochromaffin cells to submucosal ganglia (Cooke, 2000). Neurons from the submucosal ganglia then terminate on epithelial cells, which receive cholinergic signals primarily through muscarinic receptors, and adrenergic signals primarily through alpha receptors (Valet et al, 1993). Throughout the postnatal period, levels of serotonin in the GI tract change significantly, and are initially insensitive to the serotonin transporter SERT, which is expressed only at low levels in the neonatal period (Zhao et al, 2011). It has also been observed that these enterochromaffin cells have intricate basal processes, which likely convey electrochemical signals to neurons (Bohoroquez et al, 2014).

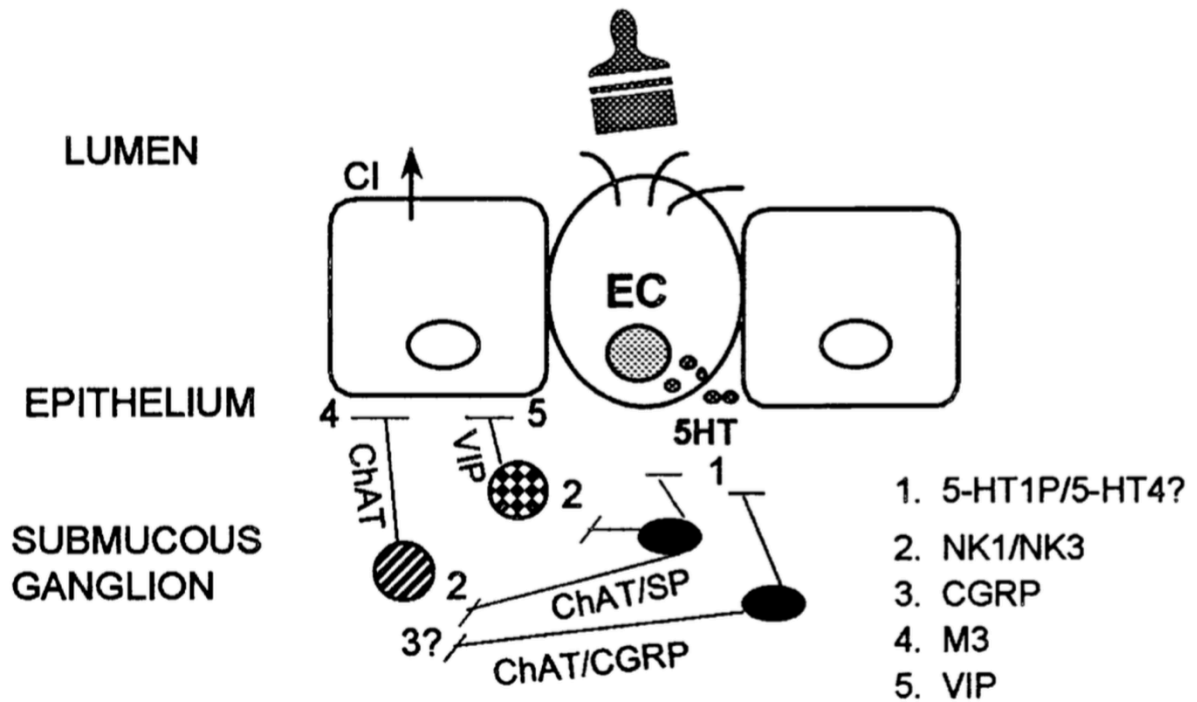


Figure 4. Interaction between enterochromaffin cells and submucosal neurons (from Cooke, 2000). Enterochromaffin cells in the epithelium of the gastrointestinal tract survey the mucosal environment and can signal to secretory neurons which terminate on epithelial cells, to cause changes in secretion.

The great degree of plasticity in the postnatal enteric nervous system leaves it open to damage from a host of environmental factors. For example, neuronal excitation has been demonstrated to change the patterns of neurochemical differentiation observed, leading to increased tyrosine hydroxylase expression (which marks dopaminergic and adrenergic neurons) in neurons (Chevalier et al, 2008). Additionally, the intestinal microbiota has been shown to influence neuron number, nerve density, neurochemical composition and nerve function in the postnatal period (Collins et al, 2014). Injuries sustained during this period also result in particularly deleterious effects on the enteric nervous system, indicating that this may be a critical period, when appropriate conditions are vital for normal development (Lowrie et al, 1986).

#### *Early life adversity and enteric nervous system plasticity*

Many functional bowel disorders, including irritable bowel syndrome, display structural and functional enteric neuroplasticity (Demir et al, 2013). This plasticity usually includes changes in tissue innervation, changes in neurotrophic factor expression (NGF, GDNF etc.), changes in neurochemical coding of neurons (an increase in nociceptive type neurons, such as CGRP, is common), and increased numbers of pain-related ion channels such as TRPV1 and TRPA1 (Demir et al, 2013). In addition to these morphological changes, altered neuronal activation results in changes in motility, secretory function and sensory thresholds (Demir et al, 2013).

Injury in early life, in the form of inflammation or stress, can cause long-lasting changes to the enteric nervous system that result in visceral hypersensitivity. Neonatal

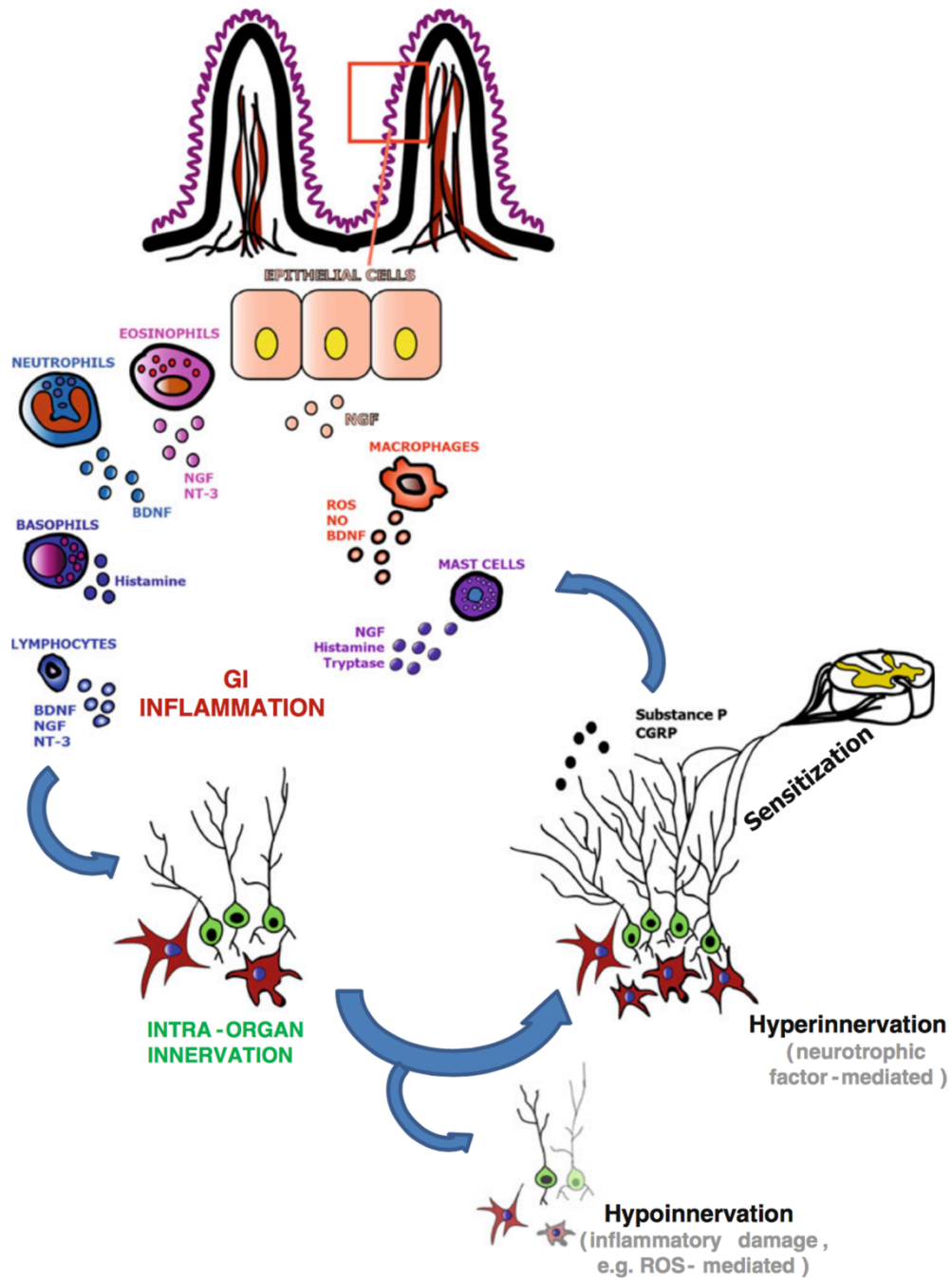
maternal separation, in conjunction with an acute stressor in adulthood (such as water avoidance stress), has been shown to increase visceral hypersensitivity, likely through serotonergic pathways, which are also changed (Bian et al, 2011). Rats subjected to neonatal maternal separation also showed increased levels of Fos immunoreactivity (a marker of neuron activation) in response to colorectal distension than their control counterparts (Chung et al, 2007). This hypersensitivity induced by NMS is thought to be mast cell mediated, with a definite role for ion channel TRPV1 (Van den Wijngaard et al, 2009). CRF receptor 1 has also been shown to have a role in NMS-induced hypersensitivity (Schwetz et al, 2005). Interestingly, NMS hypersensitivity is more pronounced in females, and is partially mediated by estradiol (Chaloner and Greenwood-Van Meerveld, 2012). This model is also known to induce changes in cholinergic tone in the GI tract (Gareau et al, 2007). A comparison of these changes in the enteric nervous system with changes observed in IBS is shown below in Table 3.

Neonatal colonic inflammation, induced by acetic acid, has been used to study plasticity in the enteric nervous system in response to injury (Winston et al, 2007; Xu et al, 2008; Qu et al, 2013). This model shows chronic visceral hypersensitivity mediated by primary afferent neuron components such as P2X receptors and voltage-gated sodium channels (Beyak and Vanner, 2005; Xu and Huang, 2002; Qu et al, 2013). As previously mentioned, the intestinal microbiota can influence the enteric nervous system, and *Lactobacillus rhamnosus GG* has been shown to prevent hypersensitivity resulting from neonatal inflammation, and alter the expression profiles of serotonin, norepinephrine and

dopamine in the central nervous system (Kannampalli et al, 2014). Some of the mechanisms by which ENS plasticity can result from inflammation are shown below in Figure 5.

Figure 5. Neuroplasticity and inflammation in the GI tract (from Demir et al, 2013).

Signals from immune cells in the gastrointestinal tract signal to neurons and can cause hyper innervation and hyper sensitization. These neurons in turn can affect the activity of immune cells in the mucosa.



### *Enteric nervous system dysfunction in human functional bowel diseases*

Irritable bowel syndrome is a functional bowel disorder with a prevalence of up to 15% in the US population (Lee and Park, 2014). IBS is diagnosed by the Rome III Criteria, which includes repeated episodes of abdominal pain with a change in bowel habit (Longstreth et al, 2006). Efforts to determine the pathophysiology of IBS have centered around dysmotility, visceral hypersensitivity and dysregulation of the brain-gut axis, but also include low-grade inflammation, post-infectious conditions, an altered microbiota, diet, the role of mast cells and the role of enterochromaffin cells (Lee and Park, 2014).

Visceral hypersensitivity is the defining hallmark of IBS, and is the result of a reduced pain threshold (Longstreth et al, 2006). Several ion channels and receptors on neurons have been associated with visceral hypersensitivity in IBS, particularly voltage-gated sodium channels, purinergic P2X receptors, and TRPV capsaicin-activated channels (Rocha et al, 2014).  $Na_v1.7$  and TRPV1 channels were observed in increased numbers in IBS patients compared to healthy control subjects (Yiangou et al, 2007; Chan et al, 2003). IBS patients have also been observed to have a greater skin conductance response to visceral distension than healthy subjects (Tillisch et al, 2005).

IBS is considered to be a disease of the brain gut axis, and many different components of this axis have been investigated in IBS patients. In a recently published paper, increased nerve fiber outgrowth and increased NGF were noted in the mucosa of IBS patients (Dothel et al, 2015). Serotonergic signaling is also known to be altered in IBS (Moses et al, 2003) and signaling in the autonomic nervous system has been shown to be altered in IBS and IBD patients (Aggarwal et al, 1994; Straub et al, 1997). Additionally, neuron

degeneration and infiltration of lymphocytes into ganglia have been observed in IBS patients (Tornblom et al, 2002). A comparison of changes in the enteric nervous system in IBS with changes observed in animal models is shown below in Table 3.

To further complicate the origin of these disorders, IBS is up to four times more common in women than men, a factor that is frequently overlooked in basic research (Chial and Camilleri, 2002). Sex hormones are thought to play a role in the skewed distribution of IBS patients, but the exact mechanism is undetermined (Meleine and Matricon, 2014). Women in the general population also display differences in nociceptive pathways, and a greater vulnerability to life stress (Berkley, 1997; Young and Korszun, 2010). This susceptibility to life stress highlights the need to further investigate the interaction between sex and stress hormones in IBS (Chaloner and Greenwood-Van Meerveld, 2013).

In both IBS occurring after infection (post infectious IBS) and IBD, inflammation is shown to cause persistent changes in the enteric nervous system. Post-infectious IBS arises after gastrointestinal infection when IBS symptoms and nervous plasticity are observed in up to 36% of patients after infection with *Campylobacter*, *Salmonella*, *Shigella*, *E.coli* or *Giardia* species (Barbara et al, 2009; Spiller and Garsed, 2009). In cases of post infectious IBS (PI-IBS), neuroplasticity resulting from the infection is maintained long after the infection is cleared, and fails to return to a healthy state.

Inflammatory bowel disease includes Crohn's disease and ulcerative colitis, both characterized by chronic, relapsing inflammation of the intestines (Brierly and Linden, 2014). These diseases are thought to develop through an over active immune response to antigens in the lumen, and are influenced by the GI microbiota and individual genetics (Baumgart and

Sandborn, 2012). Symptoms of IBD include abdominal pain, diarrhea, gastrointestinal bleeding and nutrient malabsorption.

In cases of IBD, reports about the exact changes to the enteric nervous system are conflicting, but clearly support an altered ENS (Collins et al, 1997; Geboes and Collins, 1998; Belai et al, 1997). One study showed increased serotonin and neurofilament (a neural marker) immunoreactivity in the myenteric plexus of Crohn's disease patients (Belai et al, 1997). Apoptosis of enteric neuroglial cells have also been shown to be dysregulated in IBD patients (Bassotti G, et al, 2009). Similar to IBS patients, IBD patients also experience reduced pain thresholds (Rao et al, 1987), and are shown to have elevated TRPV1 expression (De Fontgalland et al, 2014). Additionally, autonomic function is reported to be dysregulated in IBD, with sympathetic control increased and parasympathetic control diminished with respect to healthy controls (Furlan et al, 2006).

Many forms of stress, including infection, can affect the development of the enteric nervous system. For example, infection during childhood is a major risk factor for the development of IBS in adulthood (Cremon et al, 2014). NGF is also increased in the mucosa of children with D-IBS, suggesting a role for sustained neural survival (Willet et al, 2012). This, and the previously mentioned risk of early life adversity to the development of IBS (Talley et al, 1994), highlights the susceptibility of the enteric nervous system to stress and injury during development.

Table 3. Changes in the enteric nervous system observed in IBS and animal models of early life stress-induced gastrointestinal diseases. Many of the same changes observed in irritable bowel syndrome have also been observed in animal models of early life stress-induced gastrointestinal disease, which supports the importance of neonatal stress in ENS dysregulation and disease.

	<b>Models of early life adversity</b>	<b>Irritable bowel syndrome</b>
<b>Plasticity</b>	Increased NGF and innervation (Demir et al, 2013)	Increased NGF and mucosal innervation (Dothel et al, 2015)
<b>Visceral Hypersensitivity</b>	Increased visceral hypersensitivity (Demir et al, 2013; Chung et al, 2007)	Increased visceral hypersensitivity (Longstreth et al, 2006)
<b>Changes in pain-related receptors and ion channels</b>	Increased TRPV1 and TRPA1 (Demir et al, 2013; Van den Wijngaard et al, 2009)	Increased TRPV1, Na <sub>v</sub> 1.7, and P2X (Rocha et al, 2014; Yiangou et al, 2007; Chan et al, 2003)
<b>Changes in serotonin signaling</b>	Increased luminal serotonin and enterochromaffin cell density (Bian et al, 2011)	IBS-D patients show more serotonin than IBS-C patients and healthy controls (Moses et al, 2003; Kimar et al, 2012; Cremon et al, 2011; Faure et al, 2010)
<b>Changes in the autonomic nervous system</b>	Increased cholinergic tone (Gareau et al, 2007)	Increased sympathetic tone in diarrhea predominant IBS and lowered cholinergic activity in constipation predominant IBS (Aggarwal et al, 1994)

## **Conclusions**

Given the vulnerability of the enteric nervous system during the neonatal period, more work is needed to fully understand what happens during injury, so that GI disorders resulting from early life insults can be effectively treated and prevented. Rodent models have been invaluable, but without the full scope of clinical signs observed in humans, they cannot be the only models utilized. Using a large animal model to investigate the development of the enteric nervous system after injury will be an invaluable tool in understanding the pathogenesis and the risk factors involved in gastrointestinal diseases like IBS.

**CHAPTER 2: EARLY WEANING STRESS IN PIGLETS CAUSES LIFELONG  
ENTERIC NERVOUS SYSTEM DYSFUNCTION AND  
PERMANENT MORPHOLOGICAL CHANGES**

## **Introduction**

Early life adversity has been shown to predispose individuals to gastrointestinal disease in adulthood, such irritable bowel syndrome (Bradford et al, 2012; Talley et al, 1994; Dorssman et al, 1990). Irritable bowel syndrome significantly reduces quality of life for sufferers and is a significant economic burden (Wang et al, 2012; Nellesen et al, 2013; Nalifboff et al, 2012). Sufferers of irritable bowel syndrome experience symptoms, listed by the Rome III Criteria, including episodic altered bowel habit, and abdominal pain.

Interestingly, approximately twice as many women suffer from IBS as men, so sex appears to play a role in disease pathophysiology (Bradford et al, 2012). Although a link between early life adversity and the subsequent development of diseases such as IBS exists, the biological mechanisms by which early life adversity leads to long-term GI disease susceptibility remain poorly understood and this impedes development of effective treatments.

It is thought, however, that IBS is a disease of the brain-gut axis, and that the enteric nervous system is dysregulated. A recent study in humans revealed increased innervation in the mucosa of IBS patients (Dothel et al, 2015), and several rodent studies have pointed towards cholinergic dysfunction, in particular (Gareau et al, 2007). In addition to this, changes in ion channels associated with nociception, such as TRPV1 that contribute to visceral hypersensitivity associated with IBS, have been observed (Yiangou et al, 2007; Chan et al, 2003). Altered serotonergic signaling has also been proposed as a contributing factor in enteric nervous dysregulation in IBS and in rodent models (Aggarwal et al, 1994; Bian et al, 2010). Together, these findings support a prominent role for dysregulation of the ENS in stress-induced GI disorders.

Currently there are several widely used rodent models of early life adversity-induced gastrointestinal disease, including neonatal colonic inflammation and neonatal maternal separation stress (Barreau et al, 2004; Garcia-Rodenas et al, 2006; Winston et al, 2007). While these rodent models have provided valuable insights into the pathogenesis of early life stress and GI disease, they have limitations. For example, rodent stress models are unnatural stressors (neonatal maternal separation, restraint stress), do not show the full clinical scope of human IBS symptoms, such as diarrhea and require specific rodent strains or chemicals to induce disease. Furthermore, it has become increasingly evident that rodent model findings alone are difficult to translate in to effective human therapies; therefore, there is a critical need for large animal translational model systems displaying the full range of symptoms observed in human disease. To fill the gap in animal models, our laboratory has developed a large animal model, early weaning stress in piglets, that more accurately represents the disease state in humans (Moeser et al, 2007; Smith et al, 2009; McLamb et al, 2013). In this model, early weaning is a natural stressor, which involves multiple sources of stress including being removed from the sow, being handled by humans, being exposed to a new diet and being mixed with unfamiliar littermates. In this model, piglets are weaned at  $\leq 18$  days of age (compared to late weaned control animals, weaned at  $\geq 24$  days). Early weaning stress in piglets results in lasting hallmarks of human GI disease including persistent increases in intestinal permeability, stress-hyper reactivity, impaired mucosal defense against pathogens, and mast cell activation (Moeser et al, 2007; Smith et al, 2009; McLamb et al, 2013).

Given our previous findings in the early weaning stress model and the suggested involvement of ENS dysfunction in human diseases associated with early life stress, we hypothesized that early life stress leads to long-term developmental alterations in the ENS, which in turn, contribute to the lasting intestinal dysfunction observed in this model. The pig represents an unique model to study early life stress and development as ENS in pigs is more complex than rodents and more similar to humans in anatomy and ontogeny.

In this study, we demonstrate that early life stress (early weaning stress) induces long-term functional and phenotypic changes in the porcine ENS which may provide valuable clues to the pathogenesis of stress-related GI diseases

## **Materials and Methods**

### *Animals*

The North Carolina State University Institutional Animal Care and Use Committee (IACUC) approved all studies. Yorkshire-duroc cross, female and castrated male piglets were purchased from Looper Farms (Granite Falls, NC) and weaned at 16 or 28 days of age, depending on the assigned weaning group. Weaned piglets were offered ad libitum access to water and feed. Animals were fed three diets depending on their age: from age 16 or 28 days to age 7 weeks, pigs were fed diet 1 (see table 1 below), from age 7 weeks to age 11 weeks, pigs were fed diet 2 (see table 1 below) and from age 11 weeks to 20 weeks, pigs were fed diet 3 (see table 1 below). Animals were housed in IACUC approved, environmentally controlled, 12 ft x 6 ft pens, with six animals per pen. All animals were euthanized via captive bolt. Segments of ileum were harvested immediately after euthanasia.

Table 1. Composition of diets for piglets

<b>Ingredient</b>	<b>Diet 1 (%)</b>	<b>Diet 2 (%)</b>	<b>Diet 3 (%)</b>
<b>Plasma, spray dried</b>	2.5	0	0
<b>Corn</b>	59.5	66	67.35
<b>Soybean meal</b>	32	30	24
<b>Wheat Bran</b>	0	0	6
<b>Dicalcium phosphate</b>	1.450	1.1	0.85
<b>Limestone</b>	0.9	0.8	0.8
<b>Corn Oil</b>	0	1	0
<b>Soweena 4-80</b>	2.50	0	0
<b>Hystidine-HCl</b>	0.150	0.1	0
<b>Methionine</b>	0.02	0	0
<b>Vitamins/Minerals</b>	0.5	0.5	0.5
<b>Salt</b>	0.5	0.5	0.5
<b>Total</b>	100	100	100

*Early weaning stress model*

In the early weaning stress model, piglets were separated into two groups: the early weaned group and the late weaned group. Early weaning occurred at 16 days and late weaning occurred at 28 days. To monitor development, samples were collected at four different time points. Samples were collected from 7 week old piglets (12 early weaned, 12 late weaned animals, equal numbers of castrated males and females in each group), 12 week old piglets (6 early weaned, 6 late weaned, all castrated males), 16 week old pigs (6 early weaned, 6 late weaned, all castrated males) and 20 week old pigs (12 early weaned, 12 late weaned animals, equal numbers of castrated males and females in each group).

### *Ussing Chambers*

Ileum was harvested from each animal immediately following euthanasia and opened lengthwise along the anti-mesenteric border. In oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) ringer solution (154 Na<sup>+</sup> mM, 6.3 K<sup>+</sup> mM, 137 Cl<sup>-</sup> mM, 0.3 H<sub>2</sub>PO<sub>3</sub> mM, 1.2 Ca<sup>2+</sup> mM, 0.7 Mg<sup>2+</sup> mM, 24 HCO<sub>3</sub><sup>-</sup> at pH 7.4) at 37°C, the seromuscular layer was removed from the tissue. Tissue free of Peyer's patches was then mounted in a 0.3cm<sup>2</sup> -aperture on Ussing chambers (Physiologic Instruments, Inc., Sand Diego, CA) as described in previous studies (Moeser et al, 2007). The tissue was bathed in ringer 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<sub>2</sub>, 5% CO<sub>2</sub>) and maintained at 37°C. Short circuit current ( $I_{sc}$ ) and trans epithelial resistance (TER) were measured at 30 second intervals for 90 minutes. Tissues were treated with atropine (Med-Pharmex, Pomona, CA) (1uM, added to mucosal and serosal chambers), phentolamine (Sigma Aldrich, St Louis, MO) (1 uM, added to mucosal and serosal chambers), or tetrodotoxin (Abcam, Cambridge, MA) (0.5 uM, Added to the serosal chamber) and allowed to equilibrate for 30 minutes. Veratridine (Abcam, Cambridge, MA) (5 uM, 30 uM or 100 uM) in DMSO was then added to the serosal chamber and  $I_{sc}$  and TER were monitored for 60 minutes.

### *Immunofluorescence.*

Ileum free from Peyer's patches was removed from the animals immediately after euthanasia, washed with PBS, pinned flat and fixed in 4% Paraformaldehyde solution (Thermo Fischer Scientific, Waltham, MA) for 3 hours at room temperature. The tissue was

then washed with PBS and stored in a solution of 10% sucrose and 0.5% sodium azide in PBS at 4°C for 24 hours. Tissue was then embedded in Tissue-TEK OCT (Sakura, Torrance, CA), frozen (stored at -80°C) and sectioned transversely at 5 µm on a cryostat on to Superfrost+ slides (Thermo Fischer Scientific, Waltham, MA) and stored at -80°C.

Slides were washed in PBS (2x 5 minutes), and then treated with 0.1% triton-X (Sigma Aldrich, St Louis, MO) in PBS for 10 minutes at room temperature. After washing again with PBS (2x 5 minutes), slides were treated with 20% normal donkey serum (Sigma Aldrich, St Louis, MO) and 0.5% bovine serum albumin (Sigma Aldrich, St Louis, MO) in PBS for 45 minutes at room temperature. Slides were then washed with 0.5% bovine serum albumin in PBS (5x 5 minutes) before being incubated for 24 hours at 4°C with primary antibodies (see Table 2 below) in PBS with 0.5% bovine serum albumin. Slides were washed with 0.5% bovine serum albumin in PBS (5x 5 minutes) and then incubated with the secondary antibodies (see Table 3 below) and a nuclear stain, ToPro (Invitrogen, Carlsbad, CA) at 1:1000 for 60 minutes at room temperature with 0.5% bovine serum albumin in PBS. The slides were then washed with PBS (5x 5 minutes) and cover-slipped with Faramount mounting media (Dako, Carpinteria, CA). Confocal images of stained sections were captured using a Nikon Eclipse C1 confocal microscope.

Table 2. Primary antibodies used in immunofluorescence

<b>Antigen</b>	<b>Immunogen</b>	<b>Manufacturer</b>	<b>Species</b>	<b>Dilution</b>
<b>PGP9.5</b>	Sequence: ASSEDTLKDAAKVCR	Neuromics	Rabbit polyclonal	1:500
<b>Choline acetyl transferase</b>	Synthetic peptide Sequence: GLFSSYRLPGHTQDTLVAQKSS	Abcam	Sheep polyclonal	1:1000
<b>Dopamine beta hydroxylase</b>	Purified bovine dopamine beta hydroxylase	Millipore	Mouse monoclonal	1:500
<b>5-HT</b>		ImmunoStar	Goat polyclonal	1:700

Table 3. Secondary antibodies used in immunofluorescence

<b>Name</b>	<b>Fluorescent conjugate</b>	<b>Manufacturer</b>	<b>Dilution</b>
<b>Donkey anti Rabbit</b>	Cy3	Jackson ImmunoResearch	1:1000
<b>Donkey anti Mouse</b>	FITC	Jackson ImmunoResearch	1:1000
<b>Donkey anti Sheep</b>	FITC	Jackson ImmunoResearch	1:1000
<b>Donkey anti Goat</b>	FITC	Jackson ImmunoResearch	1:1000

### *Image analysis*

Neuron cell bodies were counted if a ToPro stained nucleus was visible within a PGP9.5 positive cell body. Tissue area was measured using ImageJ (NIH) and measurements were expressed as percentage of neurotransmitter positive neurons, or neurons/mm<sup>2</sup>.

### *Acetylcholinesterase Assay*

Protein was extracted in MPER buffer (Thermo Fischer Scientific, Waltham, MA) and homogenized. Protein concentration was determined using Pierce BCA protein assay kit (Thermo Fischer Scientific, Waltham, MA). Acetylcholine esterase content of tissue was determined used Amplex<sup>®</sup> Red Acetylcholine/Acetylcholinesterase Assay Kit (Invitrogen, Carlsbad, CA).

### *RNA extraction and RT-qPCR analysis*

Total RNA was isolated from tissue using the RNeasy<sup>®</sup> Mini Kit (Qiagen, Valencia, CA). cDNA was derived from 3.5 ug total RNA using the Maxima First Strand cDNA synthesis kit for RT-qPCR with dsDNAse (Thermo Fischer Scientific, Waltham, MA). Quantitative real-time PCR, using LightCycler<sup>®</sup> 480SYBR Green I Master Mix (Roche, Nutley, NJ) and 30ng of cDNA, was performed using a 20 uL reaction volume with a LightCycler<sup>®</sup> 480 Instrument according to Roche protocols. All targets genes were normalized to RPL4. All primer sequences are listed below in Table 4.

Table 4. Primer sequences used for qRT-pCR

<b>Target gene</b>	<b>Forward</b>	<b>Reverse</b>
<b>RPL4</b>	AGCGAATGAGAGCTGGTAAAG	TTACGCCAAGTGCCATAGAG
<b>ENO2</b>	CTGCCGATCCTTCCCGATAC	GCTTCACTGACTGAGCCGAT
<b>CHAT</b>	GCTTTCACGGCTGCAAAGAA	TCAAGGTTGGTGTACCTGGC
<b>CHRM1</b>	GGCACACTCCAGAATGGTCA	CCTTGGGACTTGACGTGTGA
<b>CHRM2</b>	GACCAAGCAGCCTGCAAAAA	TCCACACTGTGTTGGGGATG
<b>CHRM3</b>	CCACAGGTAGTTCTCGGAGC	GAAGTGGCAGCGTCCATACT
<b>SLC6A4</b>	TCATCGCCTTCTACATCGCC	TCCTGGAGTCCCTTAGACCG
<b>TPH1</b>	GTCACTCTCTGGGAGAGAAGTTT	CTCTGTTGGCACAGTGGTCT
<b>ADRA1A</b>	AAGAAAGCGGCCAAGACACT	TCACTGAGGGAGATCGTGTG
<b>ADRA2A</b>	GGCGCGTCTATGTAGTCCTT	CTAGTGGGAGCAGCCCTAAA

#### *Statistical Analysis*

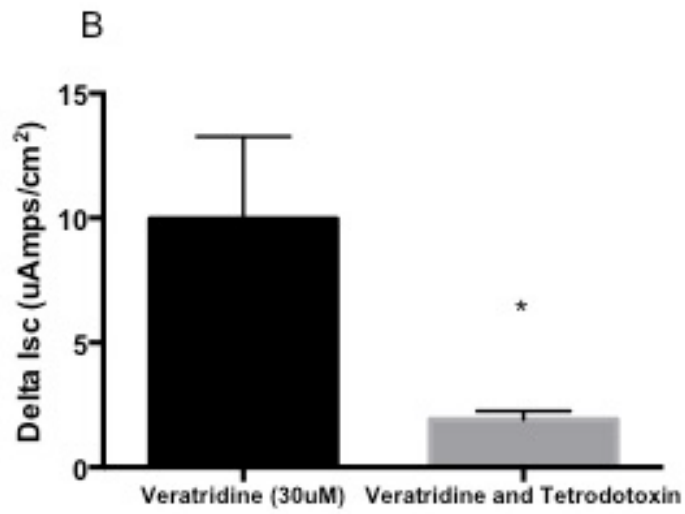
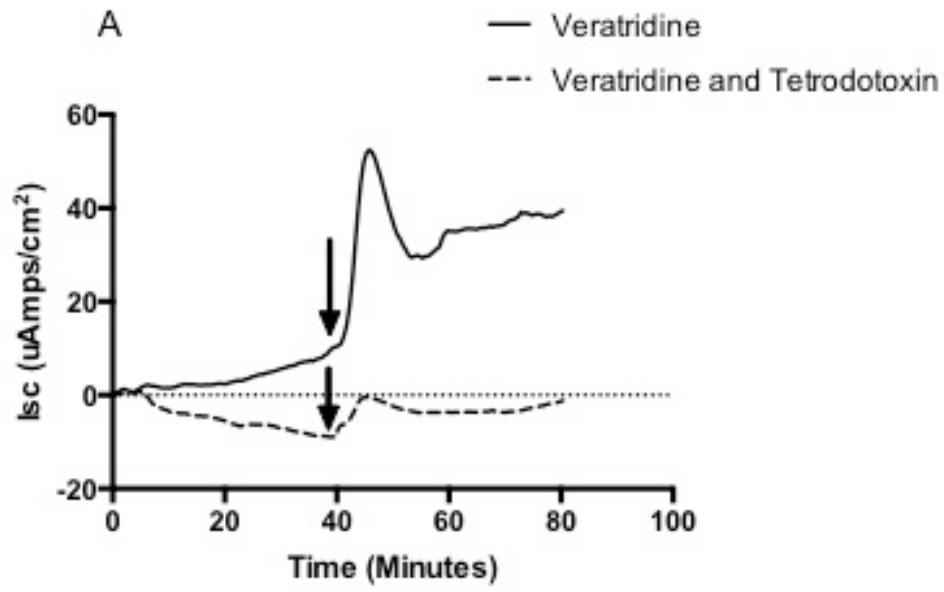
Data are reported as mean  $\pm$  SE based on experimental number ( $n$ ). Data were analyzed using a Student's T Test or a standard 2-way ANOVA using GraphPad Prism (GraphPad Software, San Diego CA) with weaning stress and age as the main effects. A post-hoc Tukey's test was used to determine differences between treatment groups following ANOVA.  $p < 0.05$  was considered significant and  $p$  values  $\geq 0.05$  and  $\leq 0.1$  were reported as trends.

## Results

### *Early weaned pigs display elevated enteric neural sensitivity*

To determine whether early weaning stress influenced ENS function in the porcine intestine, we measured the neurosecretory responses of ileum mounted on Ussing chambers in response to the neuronal agonist, veratridine. Veratridine, which causes voltage gated sodium channels to remain active (Ulbricht, 1998), was added to the serosal side of pig ileal mucosa (with the serosal layer removed, leaving mucosa and submucosa), and mounted on Ussing chambers where short circuit current ( $I_{sc}$ ), an index of electrogenic ion transport induced by secretomotor neurons, was recorded over a 30 minute period. Initial experiments with porcine intestine demonstrated that veratridine addition induced an increase in  $I_{sc}$  (Figure 1A). The neuronal specificity of this response was confirmed, by the ability of tetrodotoxin, which binds to voltage-gated sodium channels on neurons and blocks the passage of ions (Figure 1B), to ablate the veratridine-induced  $I_{sc}$  response. Overall, these results confirm that veratridine elicits a temporary, neurally mediated increase in secretory function.

Figure 1. Veratridine excites neurons and causes an increase in short circuit current that is ablated by tetrodotoxin. Functional response of of veratridine on ileum was recorded in terms of short circuit current using Ussing chambers. A representative trace of the effects of veratridine (100uM) is and Veratridine when tissue is pretreated with tetrodotoxin, arrows denote when veratridine was added (A). The average increase in short circuit current with addition of veratridine is shown along with the average increase in  $I_{sc}$  when tissue is pretreated with tetrodotoxin (B). \*P<0.05 vs. veratridine alone. One-tailed student's T Test. N=12/treatment.



Baseline short circuit current (without veratridine) was measured to determine if differences between early and late weaned piglets in electrogenic ion transport activity were observed (Figure 2). No statistical differences in baseline  $I_{sc}$  were found between early weaned stressed piglets and their late weaned control counterparts at age 2 ( $p=0.266$ ) or 5 months ( $p=0.930$ ). This data indicates that baseline secretory activity, without neural stimulation, was unchanged with stress.

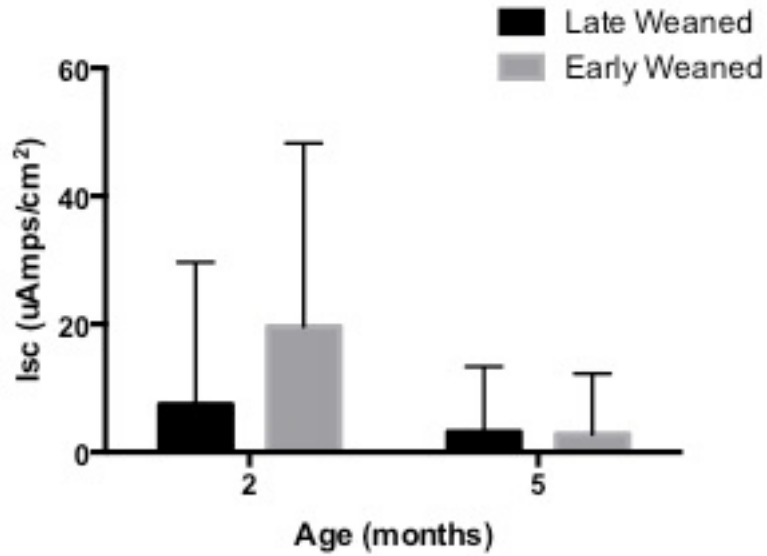
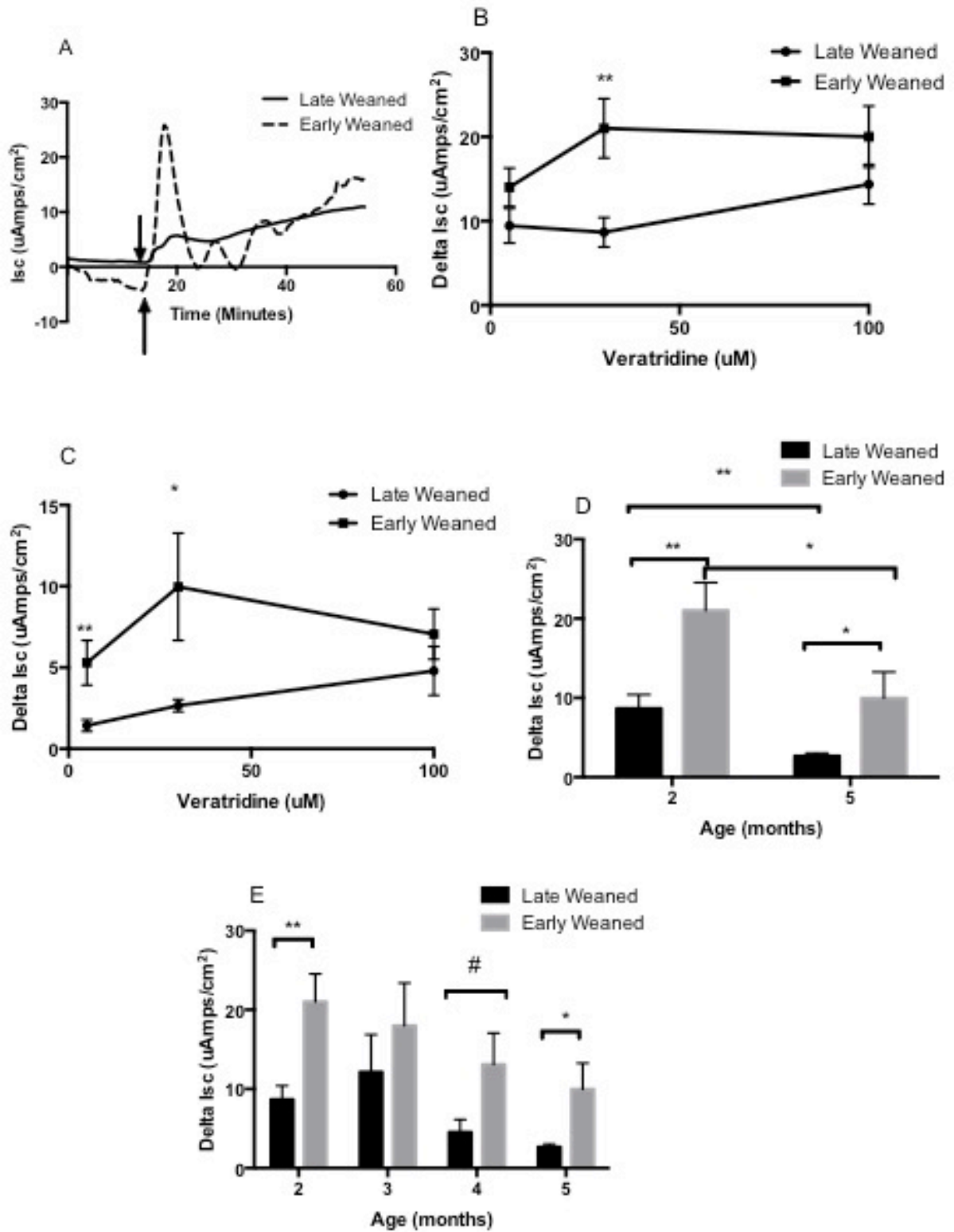


Figure 2. Average baseline short circuit current is not different between early and late weaned animals at either 2 or 5 months of age. The average short circuit current was recorded for the first 10 minutes of tissues being on Ussing chambers and not found to be different between groups. Two Way ANOVA. n=12/ treatment

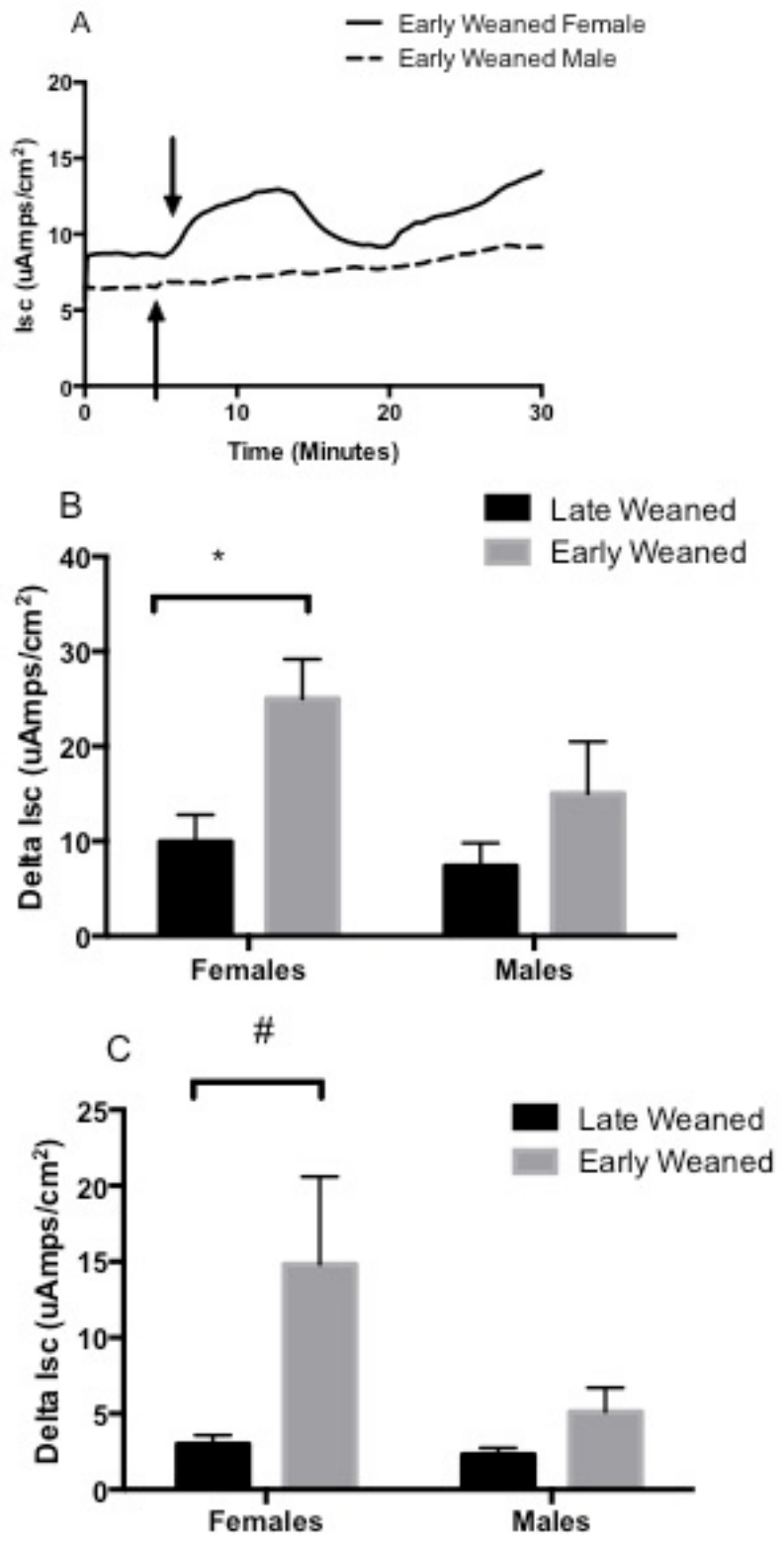
We next determined whether pigs subjected to an early weaning stress had increased enteric nervous system sensitivity to veratridine compared to late-weaned control counterparts, and whether this hypersensitivity would be long lasting. Ileum from 2 month old pigs subjected to early weaning stress displayed increased nervous sensitivity to low doses of veratridine when compared to tissue from late weaned control pigs as indicated by heightened  $I_{sc}$  response to lower doses of veratridine (Figure 3A, Figure 3B). This elevated sensitivity at low doses of veratridine was also observed in 5 month old pigs (Figure 3C). Although sensitivity to veratridine decreased with age, pigs subjected to early weaning stress were more sensitive than their late weaned control counterparts at age 5 months (Figure 3D). In fact, this elevated sensitivity to veratridine in early-weaned animals was noted at ages 2, 3, 4, and 5 months (Figure 3E). Interestingly, the only measurements in Figure 3E where significant differences between early and late weaned animals are observed is in 2 and 5 month old animals, where both males and females were included in the measurements. This may indicate that females are driving the difference in response to veratridine observed between early and late weaned animals. Together, these data demonstrate that early weaning stress induces hypersensitivity in enteric nervous function that persists long after the initial stressor.

Figure 3. Early weaned animals are more sensitive to veratridine than late weaned animals. (A) Showing a representative trace of veratridine (30uM) induced short circuit current in early and late weaned animals, arrows denote when veratridine was added. B and C show response of early and late weaned animals to different doses of veratridine at ages 2 months (B) and 5 months (C). N=12/treatment group, Two-way ANOVA. (D) Change in short circuit current to a 30 uM dose of a veratridine at ages 2 and 5 months, n =12/treatment. Two Way ANOVA. E Short circuit current response to veratridine of early and late weaned pigs at ages 2, 3, 4, and 5 months. n=12/treatment (males and females) at ages 2 and 5 months, n=6/treatment (males only) at ages 3 and 4 months Two Way ANOVA. \*\*= P<0.01, \*= P<0.05, #= P<0.10



Because both human and animal models have shown that the effects of stress on the GI tract are often sex specific (Bradford et al, 2012; Chaloner and Greenwood-Van Meerveld, 2012), and the lack of statistical significance observed in Figure 3E when only male pigs were included, we next considered whether sex played a role in the nervous response to veratridine described in Figures 1 and 3. In both 5 month old and 2 month old animals, female pigs subjected to early weaning stress displayed greater nervous sensitivity than their male, early weaned counterparts (Figure 4A, Figure 4B and Figure 4C). It is interesting to note this differential response to veratridine, as 2-month old pigs are not sexually mature, and this raises the possibility of sex differences resulting from both hormonal and chromosomal differences. Overall, these data show that the enteric nerves of female pigs subjected to early life stress are more sensitive to neuronal stimulation, which could contribute to the increased susceptibility to stress-induced hypersensitivity in females compared to males.

Figure 4. Early weaned females are more sensitive to veratridine than early weaned male pigs. (A) A representative trace of short circuit current of early weaned male and female pigs (aged 5 months) in response to 30 uM veratridine, arrows denote when veratridine was added. Average change in short circuit current displayed for male and female early and late weaned 2 (B) and 5 (C) month old pigs in response to 30 uM of veratridine. Two way ANOVA, n=6/treatment \*= P<0.05, #= P<0.10



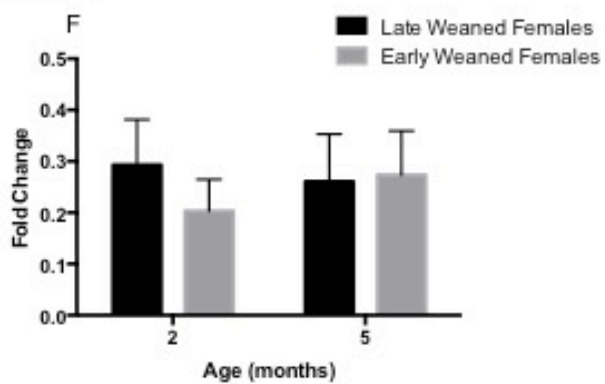
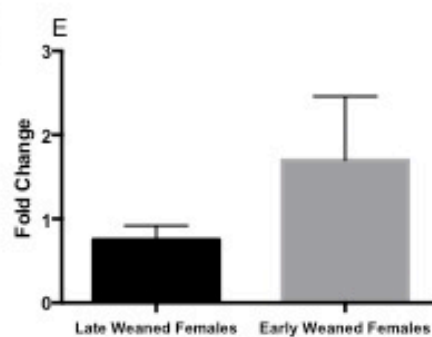
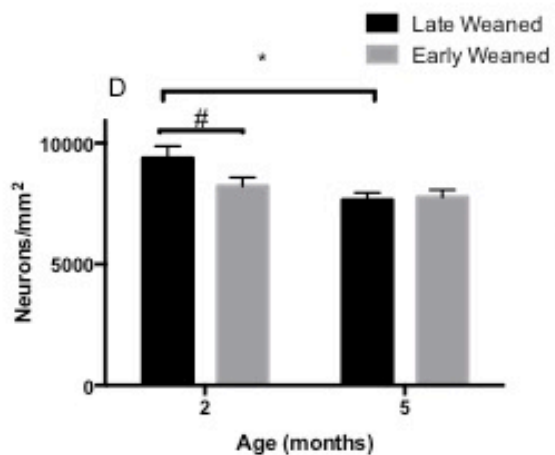
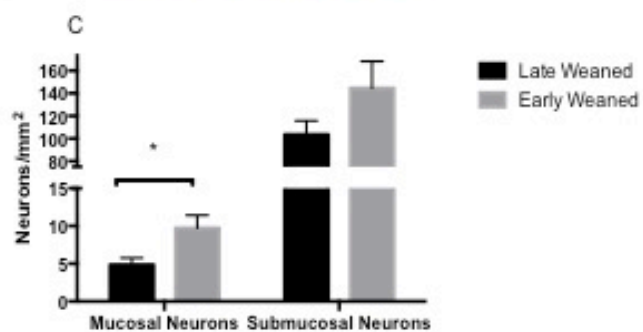
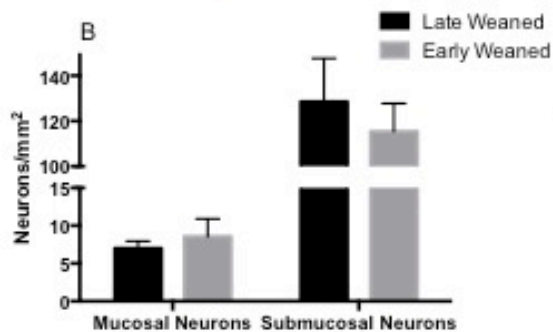
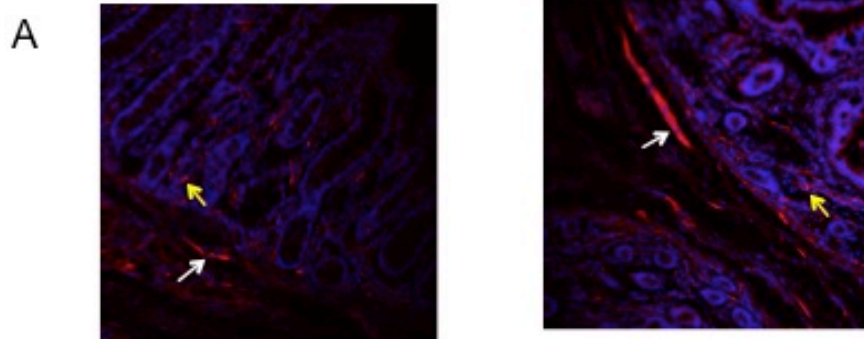
*Innervation is increased in early weaned animals*

Given the functional changes observed with early weaning stress (Figures 3 and 4), we sought to determine whether changes in innervation were also observed in early-weaned animals. Using immunofluorescence, we quantified the number of neurons, determined by the number PGP9.5-positive stained cells, in both the ileal mucosa and submucosa. In general, there were more neurons in the submucosal plexus compared with the mucosal plexus, which has been demonstrated previously in multiple species and validated our neuronal staining techniques. In late weaned control pigs, enteric neuronal numbers in both the submucosa and mucosa declined with age (Schaefer et al, 1999; Gabella, 1970). This normal, developmental decline in neuronal numbers is in line with previous investigations (Aoki et al, 2007; Chalazonitis et al, 2012). In contrast, enteric submucosal and mucosal neurons persisted in early weaning stressed pigs and mucosal neurons were significantly greater in number at 5 months of age in early-weaned compared to late-weaned pigs (Figures 5A-C).

Additionally, it is known that over time, as neuron numbers decrease during the neonatal period, neuron size increases (Schaefer et al, 1999; Gabella, 1970). We used ganglion density, or the numbers of neuron cell bodies per ganglionic area, to measure neuron size. We observed that in late weaned control animals, neuron density in ganglia decreases with age, indicative of increasing neuron cell body size, but this same decrease is not evident in the early weaned pigs (Figure 5D). In addition to these data from immunofluorescence, PCR of neuron marker ENO2 (encoding the gene for neuron-specific enolase) indicates that in the submucosa of 5 month old piglets, a slight (not significant,  $p=0.2964$ , likely due to low

numbers of sample) increase in the ENO2 transcription is seen (Figure 5E). However, differences in ENO2 transcription in 2 and 5 month old piglets were not observed in the mucosa, as there are relatively few neuron cell bodies located in this tissue (Figure 5F). These data together indicate that early weaning stress induces morphologic dysregulation of normal development of the enteric nervous system, possibly including a fault in normal cell death or “pruning” of the enteric nervous system during the neonatal period.

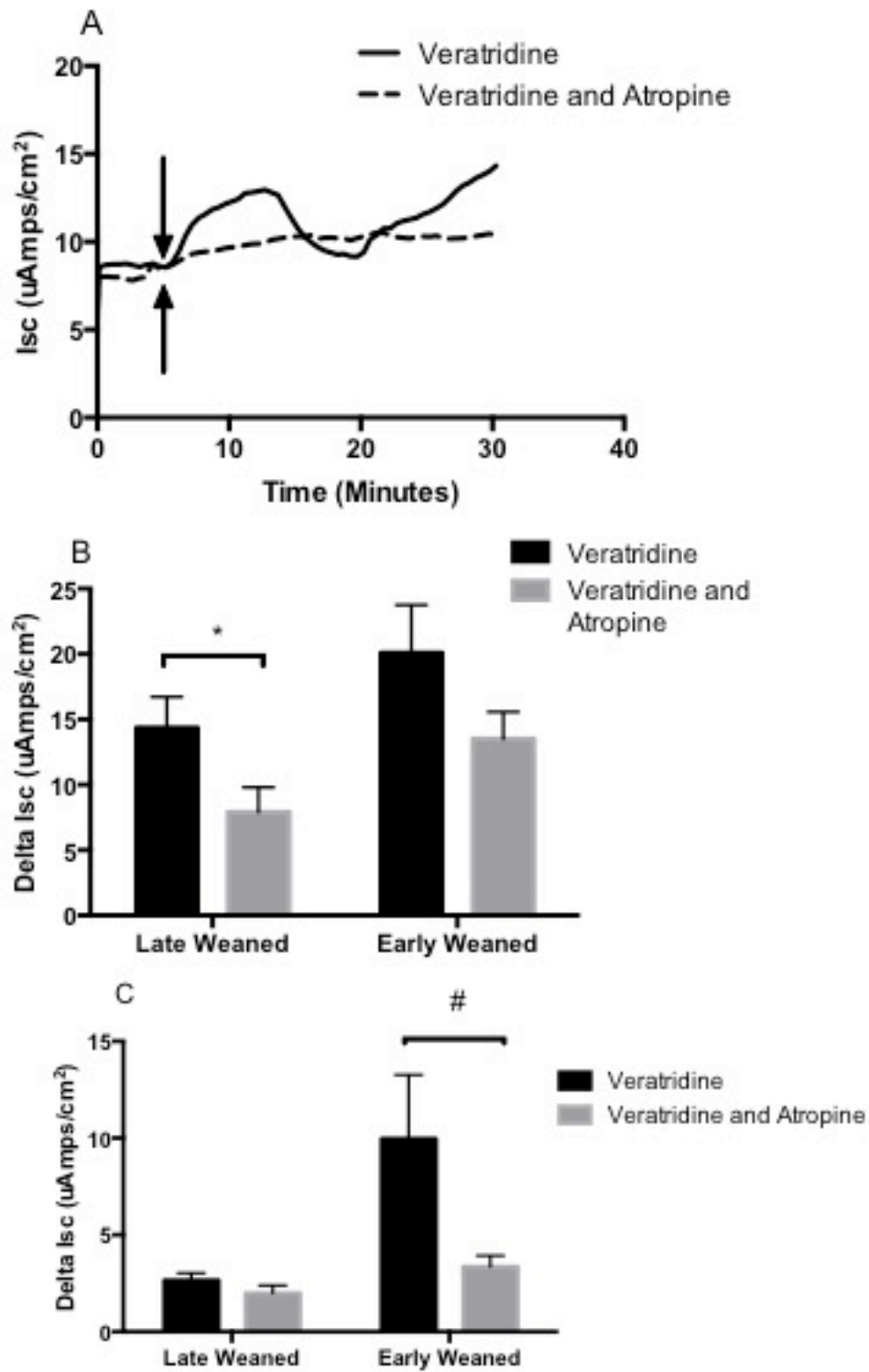
Figure 5. At age 5 months, the mucosa and submucosa of early weaned animals is more innervated than their late weaned counterparts. (A) Representative images of early and late weaned ileum at 5 months with PGP9.5 in red and ToPro (a nuclear stain) in blue. White arrows indicate submucosal ganglia and yellow arrows indicate mucosal neurons. Mucosal and submucosal neurons were counted in immunofluorescence images in 2 month old (B) and 5 month old (C) early and late weaned animals. (D) Ganglion density was measured as the number of visible nuclei with PGP9.5 positive cytoplasm per square millimeter of submucosal ganglion. Two Way ANOVA, n=6/treatment. (E) Neuron marker ENO2 was measured using qRT-PCR in the ileal submucosa and normalized to RPL4. Student's T Test n=5/treatment. (F) Neuron marker ENO2 was measured in the ileal mucosa and normalized to RPL4. Two-way ANOVA. N=9/treatment. \*=P<0.05, #=P<0.10.



*Early weaned pigs show elevated cholinergic tone and innervation*

As shown in rodent models, neonatal stress can induce changes in the development of the enteric cholinergic system (Gareau et al, 2007). We first sought to evaluate cholinergic function of enteric nerves in early-weaned animals. Using atropine, a non-specific muscarinic acetylcholine receptor antagonist, on Ussing chambers, the  $I_{sc}$  response to veratridine was blunted (Figure 6A), however, differential responses were observed between early and late weaned pigs. In 2-month old piglets, atropine significantly blunted the  $I_{sc}$  response to veratridine in late weaned animals, but not significantly ( $p=0.134$ ) in early weaned animals (Figure 6B). In 5-month old pigs however, atropine only significantly inhibited the response to veratridine in early-weaned animals (Figure 6C). These data demonstrate that 4 months after the initial weaning stress, animals subjected to early weaning display significantly increased cholinergic function than their late weaned control counterparts and that this cholinergic function can be blocked using atropine.

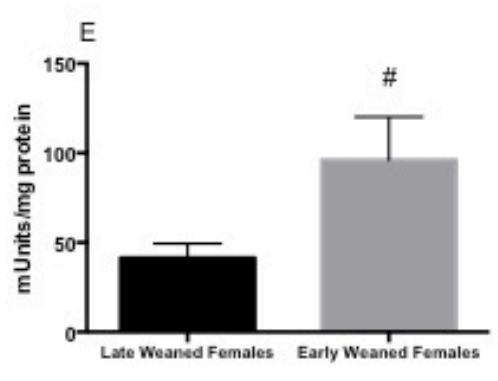
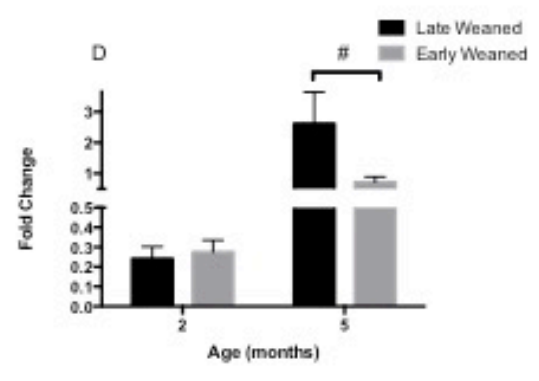
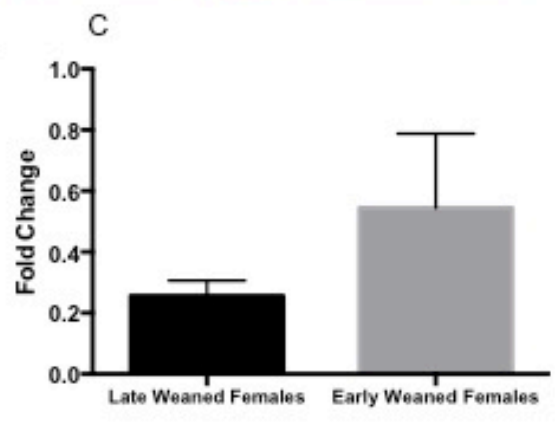
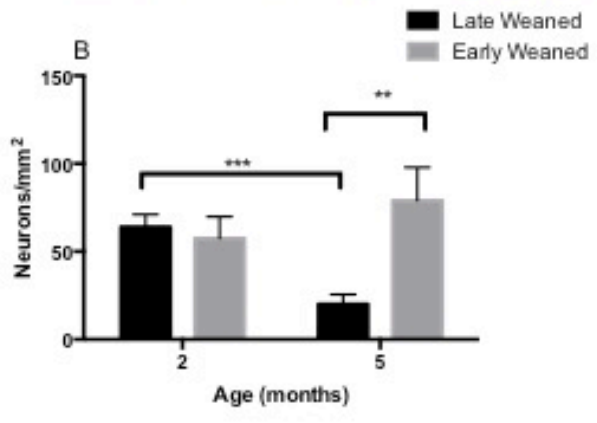
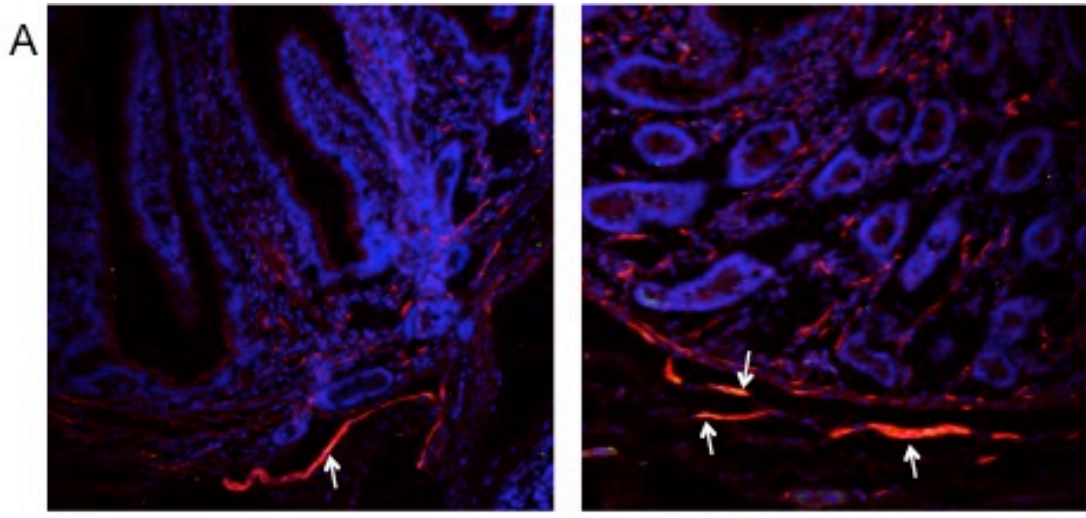
Figure 6. Atropine inhibits cholinergic responses to veratridine. (A) Representative trace of short circuit current in response to 30  $\mu$ M veratridine in 5 month old early weaned animals with and without atropine pretreatment, arrows denote addition of veratridine. Short circuit current response to 100  $\mu$ M veratridine in 2 month old early and late weaned animals (B) with and without atropine treatment and to 30  $\mu$ M veratridine in 5 month old early and late weaned animals (C) with and without atropine treatment. Two way ANOVA, n=12 / treatment. \*=P<0.05, #=P<0.10.



This increased cholinergic function may have been the result of increased levels of acetylcholine in the intestine of early weaned pigs, or of increased levels of acetylcholine receptors. We first sought to determine whether an increase in cholinergic innervation occurred in the enteric nervous system of early weaning stressed animals. We counted enteric neurons by counting PGP9.5-positive cells that were co-localized with ChAT (choline acetyltransferase, the rate limiting enzyme involved in the production of acetylcholine). Immunofluorescence staining revealed high numbers of ChAT positive neurons in the submucosal plexus (Figure 7A). At 2 months of age, the numbers of ChAT positive submucosal neurons were similar between early and late weaned pigs, however, by age 5 months, the number of ChAT positive neurons had significantly decreased in the late-weaned control intestine, but remained persistently elevated in in early-weaned pig intestine (Figure 7B). ChAT positive neurons in the mucosa were not observed in any images, and so were not quantified. PCR results showed that transcripts of CHAT were increased (not significant) in the submucosa of early-weaned animals at 5 months of age, compared to controls (Figure 7C). In the mucosal layer, however, where there are far fewer neurons, no differences between early and late weaned animals were observed, although CHAT transcripts did increase significantly by age 5 months in both populations (Figure 7D). Another measure of cholinergic activity is the amount of acetylcholine esterase activity (an enzyme that breaks down acetylcholine and correlates well with acetylcholine levels). Using an assay for acetylcholine esterase activity, we observed that early-weaned females had higher submucosal levels of acetylcholine esterase activity than late weaned females at age 5

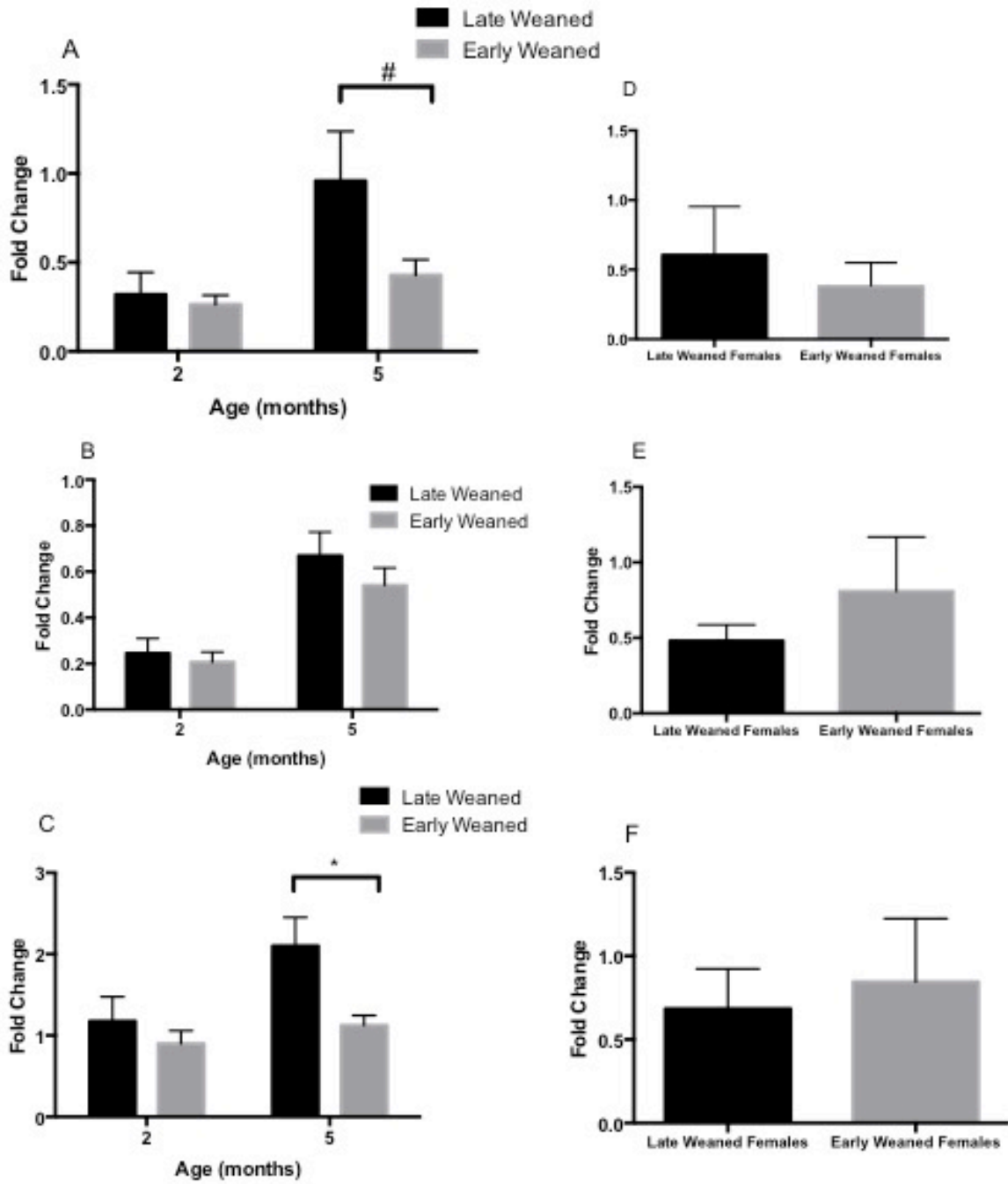
months (Figure 7E). These data show that cholinergic innervation and cholinergic activity is increased in the early weaned stressed animals.

Figure 7. Cholinergic neurons are present in increased numbers in 5 month old early weaned animals compared to controls. (A) Representative images of 5 month old late weaned (left) and early weaned (right) ileum, with PGP9.5 (neuron marker) in red, ChAT in green, and ToPro in blue. White arrows mark ChAT positive neurons (B) Submucosal cholinergic neurons at 2 and 5 months in early and late weaned animals counted in immunofluorescence images. Two Way ANOVA, N=6/ treatment. (C) CHAT transcripts measured using qRT-PCR in the submucosa of 5 month old female early and late weaned pigs (n=5/treatment) and normalized to RPL4. Student's T test. (D) CHAT transcripts in the mucosa of 2 and 5 month old early and late weaned pigs and normalized to RPL4. Two Way ANOVA, n=9/treatment. (E) Acetylcholine esterase activity per mg of submucosal protein in 5 month old early and late weaned females. Student's T test n=5/ treatment. # = P<0.10, \*\* = P<0.01, \*\*\*=P<0.001



To determine if muscarinic receptor expression changed with age or early life stress, along with cholinergic function and innervation, we performed PCR on mucosal and submucosal tissue from the ileum of early weaned and late weaned animals. In the mucosa of 2 month old pigs, there was no difference in levels of muscarinic receptor 1 expression between early and late-weaned animals, but in 5 month old animals, muscarinic receptor 1 expression was significantly down regulated in early-weaned animals compared to late-weaned controls (Figure 8A). In the submucosa of 5 month old animals, no difference between early and late-weaned animals was noted in muscarinic receptor 1 expression (Figure 8D). Conversely, although an age-dependent increase in transcription of muscarinic receptor 2 was noted, there was no difference in expression in mucosa or submucosa in the ileum of 2 month old or 5 month old early and late-weaned pigs (Figure 8B, Figure 8E). Similarly to muscarinic receptor 1 expression, no difference in muscarinic receptor 3 expression was noted between early and late-weaned animals at age 2 months, but at age 5 months in the mucosa, but not submucosa, a significant decrease in muscarinic receptor 3 expression in early-weaned animals was observed (Figure 8C, Figure 8F). These data indicate that age and early weaning stress influence the expression of mucosal muscarinic receptors in a subtype specific manner. The most pronounced differences in muscarinic receptors expression were observed at 5 months, when most differences in cholinergic function and innervation were observed.

Figure 8. Muscarinic receptors 1 and 3, but not 2 are down regulated in the mucosa of 5 month old early-weaned animals as compared to late-weaned controls. qRT-PCR was used to measure transcript levels of muscarinic receptors 1 (A), 2 (B) and 3 (C) in the mucosa of 2 and 5 month old pigs and normalized to RPL4. Two way ANOVA, n=9/treatment. Transcript levels of muscarinic receptors 1 (D), 2 (E) and 3 (F) in the submucosa of 5 month old female pigs were measured and normalized to RPL4 using qRT-PCR. Student's T Test n=5/treatment. \*=P<0.05, #=P<0.10



### *Early weaned pigs have less free serotonin*

The role of serotonin in irritable bowel syndrome and enteric nervous system dysfunction is unclear. Conflicting reports have shown serotonin in increased, decreased and unchanged levels in IBS patients (Cremon et al, 2011; Lee et al, 2008; Kerchoffs et al, 2012). Serotonin has also been pointed to as a survival factor for neurons in the neonatal period (Lin et al, 2009). There are two major serotonergic cell types in the GI tract; enterochromaffin cells in the intestinal epithelium and serotonergic neurons.

First we sought to investigate the population of serotonergic neurons in the ileum of early and late weaned animals via immunofluorescence looking specifically for colocalization of PGP9.5 and serotonin positive cell bodies (Figure 9A). In 2 month old pigs, early-weaned stressed animals have a lower percentage of serotonin positive neurons than their late-weaned counterparts (Figure 9B). However, by 5 months of age, both early and late weaned animals have significantly reduced percentages of serotonin positive neurons, and there is no difference between the two groups (Figure 9B). These data show early changes in serotonergic neurons in early-weaned animals that appear to be resolved by age 5 months. The exact role of these early differences between early weaning stress and late-weaned control is unclear; but changes may be influencing the development and survival of other populations of nerves as demonstrated previously (Lin et al, 2009).

The other source of serotonin in the GI tract is enterochromaffin cells. Enterochromaffin cell numbers in IBS patients is not agreed upon, with some studies showing increased numbers in IBS patients (primarily in post-infectious IBS) (Cremon et al, 2011; Lee et al, 2008) and others showing no differences between IBS patients and healthy

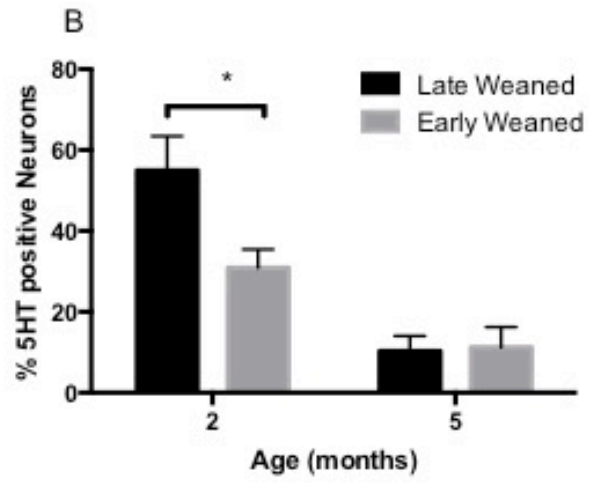
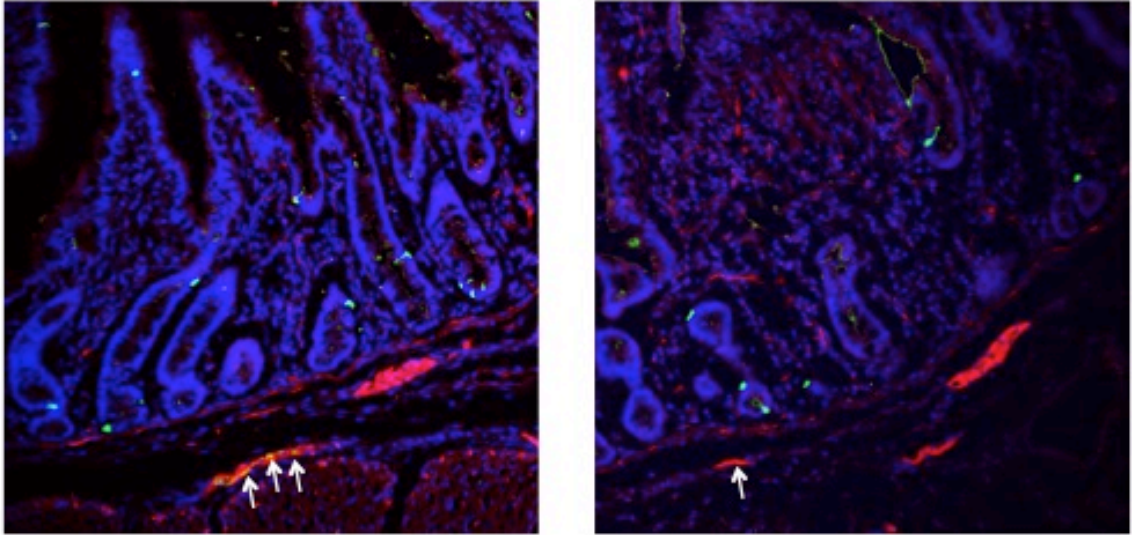
controls (Kerckhoffs et al, 2012). Studies have also shown that both SERT and TPH1 (which manufactures serotonin in non-neuronal cell types) expression are reduced in IBS patients (Kerckhoffs et al, 2012). Using immunofluorescence, we identified enterochromaffin cells in the GI tract as serotonin positive, triangular cells in the epithelium of the intestine (Figure 9C). We found that in 2 month old piglets, early weaned animals had significantly fewer enterochromaffin cells in the ileal epithelium than control animals (Figure 9D). At age 5 months, there was no difference in enterochromaffin cell numbers between early-weaned stressed animals and controls (Figure 9D). Tryptophan hydroxylase 1 (TPH1) was expressed at similar levels in the mucosa of 2 month old early-weaned and late-weaned animals, but by age 5 months, was expressed at considerably lower levels in early weaned animals compared to controls (Figure 9E). No differences in TPH1 expression were detected in the submucosa of 5 month of old early and late weaned animals (Figure 9F), which follows with our current understanding of TPH1 distribution, as primarily from enterochromaffin cells located in the intestinal mucosa. Together, these data indicate a lower amount of serotonin and serotonin-producing enterochromaffin cells in the mucosa of early-weaned animals compared to controls.

Another factor which may influence the amount of serotonin available to cells is the amount of the serotonin transporter (SERT), which terminates the activity of serotonin and recycles it. We examined SERT expression in the mucosa of 2 and 5 month old animals, and found that in 2 month old animals, SERT expression was considerably higher in early-weaned animals compared to controls (Figure 9G). Although overall expression levels increased, by age 5 months there were no differences in expression between early and late

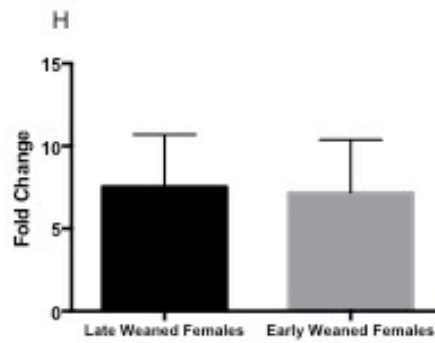
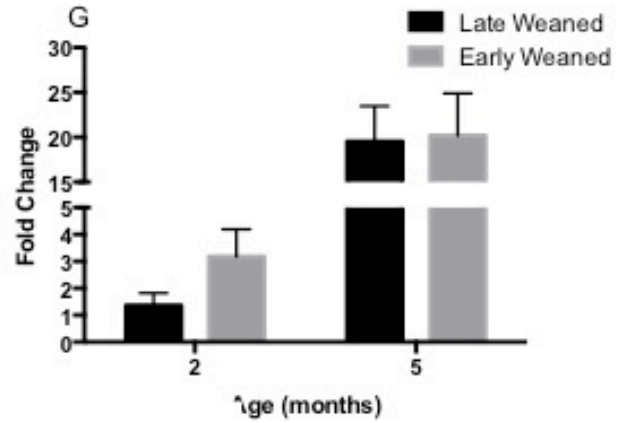
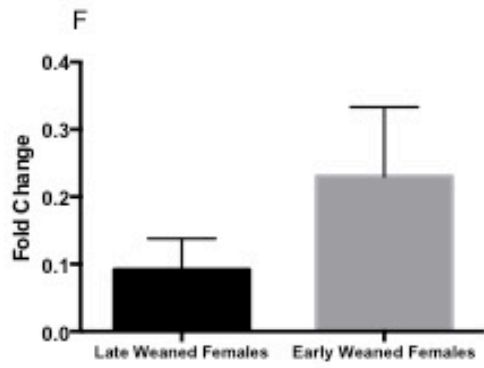
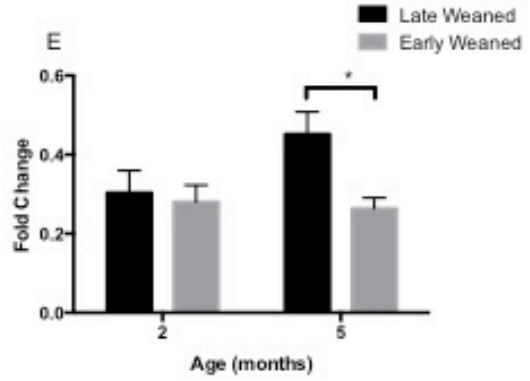
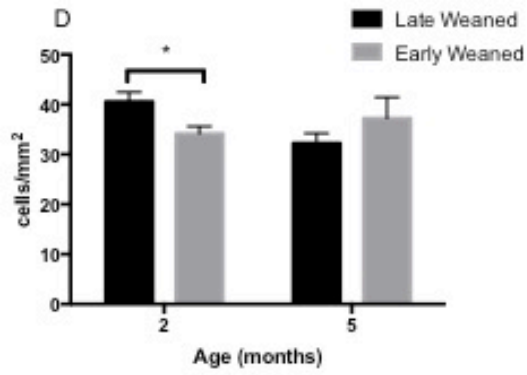
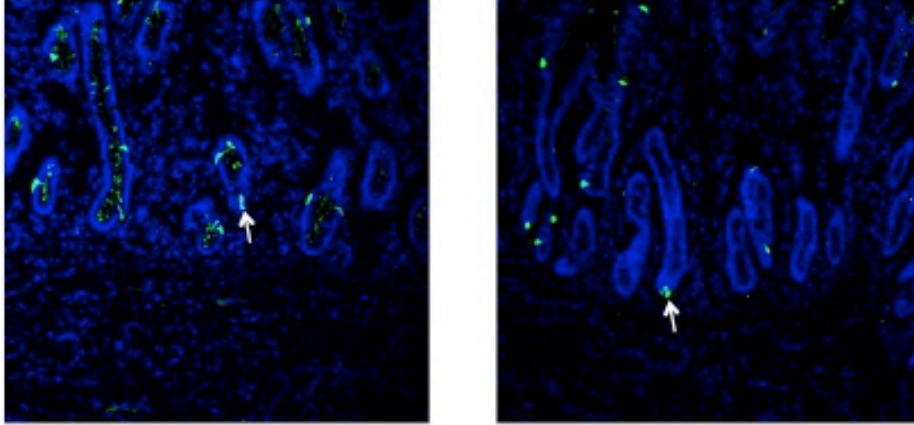
weaned animals (Figure 9G) and no observed differences between males and females. In the submucosa of 5 month old animals, there was also no detectable difference in SERT expression (Figure 9H). These data indicate that there is less free serotonin in the mucosa of 2 month old early-weaned stressed animals compared to late-weaned controls.

Figure 9. Levels of serotonin in neurons and enterochromaffin cells are decreased in early weaned animals. (A) Representative images of serotonergic neurons (marked by colocalization of PGP9.5 in red and serotonin in green) in early (right) and late (left) weaned animals aged 2 months. Serotonergic neurons are indicated by white arrows. (B) These images were quantified and measurements were expressed as serotonin positive neurons as percentage of total neurons observed in the submucosa. Two way ANOVA, n=6/treatment. (C) Representative images of enterochromaffin cells (marked by serotonin positive cells in green with triangular shape in the epithelium) in the epithelium of late (left) and early (right) weaned animals at 2 months old, with arrows denoting enterochromaffin cells. (D) These images were quantified by counting serotonin cells in the epithelium of both 2 and 5 month old animals. Two way ANOVA, n=6/treatment. (E) TPH1 transcripts were measured via qRT-PCR in the mucosa of 2 and 5 month old animals, and data was normalized to RPL4. Two way ANOVA, n=9/treatment. (F) TPH1 transcripts were measured via qRT-PCR in the submucosa of 5 month old female early and late weaned pigs. Student's T Test, n=5/treatment. (G) SERT transcripts were measured via qRT-PCR in the mucosa of 2 and 5 month old animals, and data was normalized to RPL4. Two way ANOVA, n=9/treatment. (H) SERT transcripts were measured via qRT-PCR in the submucosa of 5 month old female early and late weaned pigs. Student's T Test, n=5/treatment. \* =  $P < 0.05$

A



C



### *Influence of adrenergic innervation*

As sympathetic function has been shown to have significant impact on stress-related disease (Schemann et al, 2010), we sought to determine the role of sympathetic innervation in the form of norepinephrine in the ileum of early and late-weaned animals.

To assess the functional role of adrenergic innervation in the ileum of piglets, phentolamine (a non-selective alpha adrenergic antagonist) was added to Ussing chambers prior to the administration of veratridine. A representative trace of the effects of phentolamine are shown in Figure 10A. In both 2 month old piglets (Figure 10B) and 5 month old pigs (Figure 10C), no difference in the effects of phentolamine is shown between early and late weaned animals, and no significant change in response to veratridine was noted with phentolamine administration.

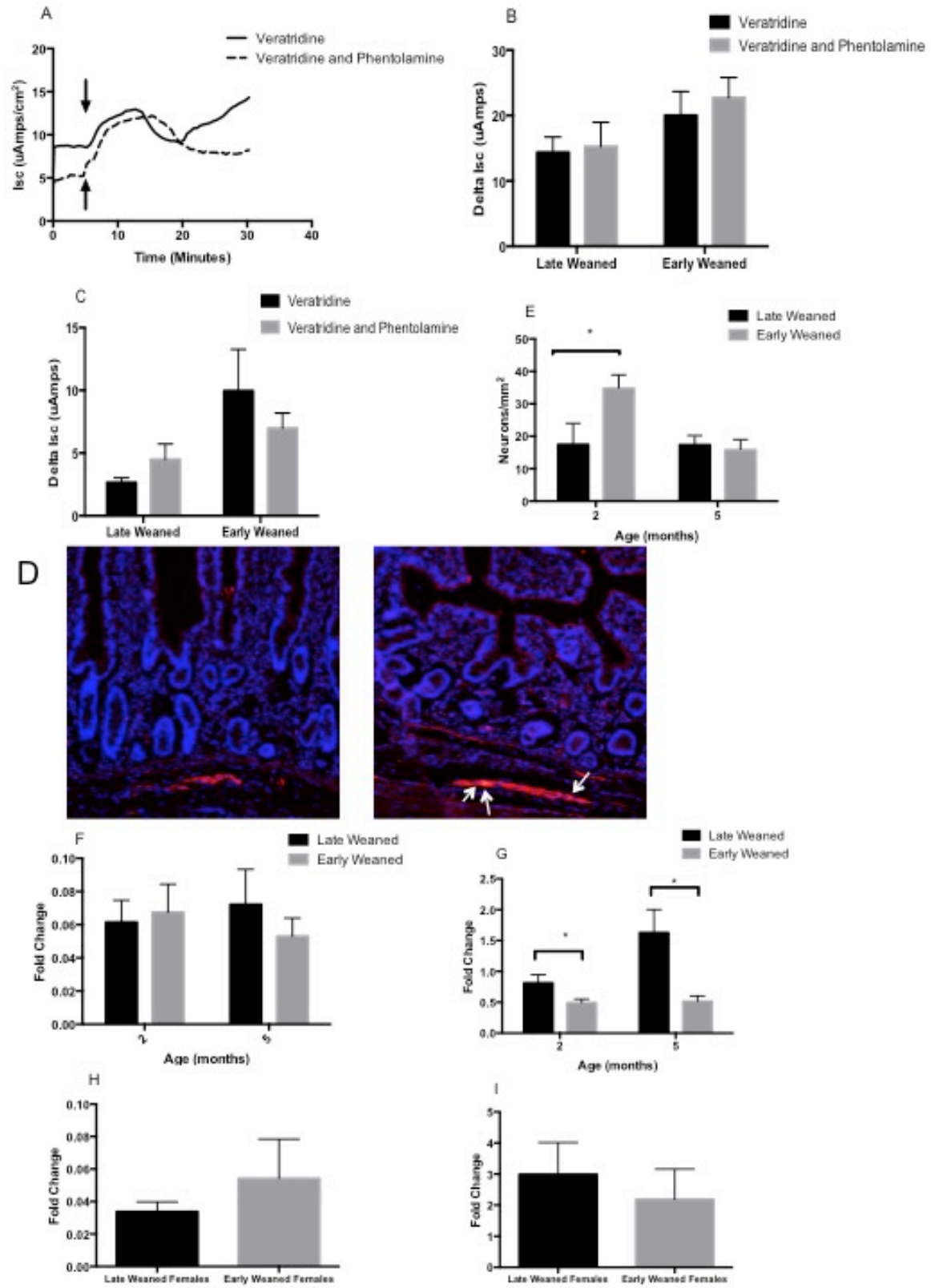
Although no functional differences were detected using phentolamine, using immunofluorescence to assess the numbers of noradrenergic neurons (marked by the enzyme which manufactures norepinephrine, dopamine beta hydroxylase, and PGP9.5 double positive cells). Representative images are shown in Figure 10D. In 2 month old animals, early-weaned piglets displayed a significantly higher percentage of noradrenergic neurons than controls (Figure 10E). However, at age 5 months, these differences were not observed (Figure 10E). These data indicate that, although functional changes were not detected, an increased number of noradrenergic neurons were observed in 2 month old early-weaned stressed pigs compared to controls.

We also investigated whether age or early weaning stressed influence the expression of adrenergic receptors in the porcine intestine. We focused alpha adrenergic receptors as

they are more prominent than beta adrenergic receptors in the intestinal tract. Alpha 1 receptor expression was not significantly different in the mucosa and submucosa of early and late weaned animals regardless of age (Figure 10F, Figure 10H). In contrast, Alpha 2 receptor expression, was significantly reduced in the mucosa of early weaned animals at both 2 and 5 months of age (Figure 10G). No difference in alpha 2 expression was noted in the submucosa. Mucosal-specific changes in alpha 2 receptors may indicate that primary changes in alpha 2 receptor expression occur on effector cells in the mucosa (Figure 10I).

While these data indicate changes in adrenergic innervation in the ileum of early weaned animals, at this early stage of investigation, it is difficult to determine exactly what changes are occurring and what functional role these changes play in the disease state.

Figure 10. Sympathetic innervation is altered in early weaned animals. (A) Representative trace of short circuit current in response to 30  $\mu$ M veratridine in 5 month old early weaned animals with and without phentolamine pretreatment, arrow denotes addition of veratridine. Short circuit current response to 100  $\mu$ M veratridine in 2 month old early and late weaned animals (B) with and without phentolamine treatment and to 30  $\mu$ M veratridine in 5 month old early and late weaned animals (C) with and without phentolamine treatment. Two way ANOVA. n=12/treatment. (D) Representative images of noradrenergic neurons (marked by colocalization of PGP9.5 in red and DBH in green) in early (right) and late (left) weaned animals aged 2 months. White arrows denote noradrenergic neurons. (E) These images were quantified measurements were expressed as serotonin neurons as percentage of total neurons observed in the submucosa. Two way ANOVA, n=6/treatment. (F-G) qRT-PCR was used to measure transcript levels of alpha adrenergic receptors 1 (F), and 2 (G) in the mucosa of 2 and 5 month old pigs and normalized to RPL4. Two way ANOVA, n=9/treatment. Transcript levels of alpha adrenergic receptors 1 (H), and 2 (I) in the submucosa of 5 month old female pigs were measured and normalized to RPL4 using 1 qRT-PCR. Student's T Test n=5/treatment. \*=P<0.05



## **Discussion**

The role that early life adversity plays in the development of gastrointestinal disease is well documented (Bradford et al, 2012; Talley et al, 1994; Drossman et al, 1990), but the mechanism by which this occurs is poorly understood, and likely to be multifactorial. The enteric nervous system, and its vulnerability during the neonatal period, has been the subject of recent studies investigating the pathogenesis of IBS (Gareau et al, 2007; Barreau et al, 2004; Bian et al, 2010). To our knowledge, this is the first study investigating the role of neonatal stress and the developing enteric nervous system in gastrointestinal dysfunction in a large animal model.

In this study, we have shown that early weaning stress in piglets causes nervous hypersensitivity, particularly in females. Additionally, we have shown that there is increased innervation of the ileum in 5 month old pigs subjected to early weaning, which may be related to the increased sensitivity observed. In particular, cholinergic innervation was determined to play a major role in both the hypersensitivity and hyper innervation observed in early-weaned pigs. Serotonin, from both neurons and enterochromaffin cells, was determined to be present at lower levels in the 2-month-old early-weaned animals.

The greater innervation, in both submucosal and mucosal layers of the ileum, in early weaned animals is likely to be the result of aberrant apoptosis of neurons in the neonatal period. Many studies have shown that, unlike the central nervous system, the enteric nervous system goes through a stage of “pruning” during the neonatal period (Chalazonitis et al, 2012; Aoki et al, 2007). Pruning occurs when excess neurons are generated during development, but are not required and subsequently eliminated (Buss et al, 2006). The fact

that pruning of neurons does not seem to occur to the same extent in early-weaned animals as in late weaned controls seems to suggest that these neurons may be receiving excessive stimulation, and thus not undergoing appropriate apoptosis. Enteric neuron hyperplasia has been shown to cause increased nervous activity (Taketomi et al, 2005), and increased neurite outgrowth has been demonstrated in IBS patients (Dothel et al, 2015).

One proposed survival factor for neurons in the enteric nervous system in serotonergic signaling via the 5-HT<sub>4</sub> receptor (Liu et al, 2009). Other growth factors such as GDNF, from cells in the submucosa and mucosa, may also be involved in this altered pattern of neuron survival (Heukeroth et al, 1998). Cholinergic tone, specifically acetylcholine and acetylcholinesterase, has also been implicated in controlling neurite outgrowth (Owen and Birdm 1995; Sternfeld et al, 1998). Given the large proportion of cholinergic neurons in the intestinal tract (Furness, 2000), it is of particular importance that the cholinergic system appears heightened in early-weaned animals and that increased neurite outgrowth is reported in IBS (Dothel et al, 2015).

The cholinergic component of the response to veratridine is mediated largely by muscarinic receptors on epithelial cells, which control secretion. In addition to increased numbers of cholinergic neurons, and a larger cholinergic contribution to the response to veratridine observed in early weaned animals, a decrease in muscarinic receptors 1 and 3, but not 2 is also observed. This decrease in receptor mRNA is likely part of a documented response of G proteins to excess ligand (in this case, acetylcholine), where the receptor is down regulated and internalized (Haga, 2013). Because differences in receptor expression

were observed in the mucosa and not the submucosa, it appears as though receptor expression is changing on effector cells in the mucosa.

The role of the other arm of the autonomic nervous system, the sympathetic nervous system, and its main neurotransmitter in the GI tract, norepinephrine, are also likely to be key in deciphering the changes occurring the enteric nervous system with early weaning stress. Figure 10 presents some preliminary findings on norepinephrine and its receptors in the early-weaned ileum, but the functional role of the sympathetic nervous system in early life stress-induced intestinal dysfunction remains poorly understood. Previous studies have shown that adrenergic tone is altered with NMS in rats (Schemann et al, 2010). In pigs, the adrenergic nervous system in the gut is still undergoing development until the age of 12 weeks (Paran et al, 2008) and stress during early life may result in plastic ENS changes influencing GI function later in life. While higher levels of catecholamines have been previously observed in males as compared to females (Crowley et al, 1978), no differences were observed in this study, possibly because male pigs were castrated.

Although functional differences between early-weaned males and females were observed, morphological differences between early-weaned female and male animals were difficult to discern. The functional differences observed between males and females were consistent with IBS as a disease that predominantly affects women (Bradford et al, 2012), but clear mechanisms behind this are not known. It is known, however, that women are generally more susceptible to stress and have different nociceptive pathways than men (Berkley, 1997; Young and Korszun 2010).

The profound differences in enteric nervous system function and morphology described in this study resulting from early weaning (16 days) compared to control weaning age (28 days) highlight the vulnerability of the system during this short window of time. This period is thought to be a critical window in development for several reasons, including stress hypo responsiveness, and a sharp decline in nervous growth factors. The stress hypo responsive period has been observed in rats aged between 4 and 14 days, and is marked by a subdued hypothalamic-pituitary-adrenal axis (Rosenfed et al, 1992). Interestingly, this period corresponds with a period of neuron growth and myelination (Morgane et al, 2002). It is possible that stress during this period is poorly tolerated, and disrupts the concurrent period of neuron growth and myelination and results in lifelong enteric nervous system dysfunction, which we observe in early-weaned piglets, and human victims of child abuse.

Additionally, during the postnatal period as compared to the prenatal period, there are far fewer growth factors stimulating neurons, and so an up or down regulation of any single growth factor, such as GDNF, or even serotonin, could result in functional and morphological abnormalities of the ENS during the postnatal period that would likely not occur during prenatal ENS development (Chalazonitis et al, 2012).

A dysregulated enteric nervous system may also be interacting with other cell types to cause intestinal dysfunction in early-weaned pigs and humans with IBS, such as mast cells. In IBS in particular, there is evidence for increased cross-talk between neurons and mast cells in colonic mucosa as a contributing factor to disease (Barbara et al, 2004). While mast cells have historically been considered effector cells, there is increasing evidence to support their influence over neurons. There is a growing body of information supporting that mast cells

have the ability to release serotonin, which could be contributing to the ENS dysregulation we observe in early weaned pigs based on its role as a growth factor (Nautiyal et al, 2012; Ji et al, 2011; Liu et al, 2009). Mast cells may also play a role in regulating other growth factors, such as NGF, through cleavage to its active form (Spinnler et al, 2011).

This study supports enteric nervous system dysregulation as a contributing factor in early life stress-induced gastrointestinal disease and begins to elucidate specific populations that are particularly sensitive to stress. While specific changes occurring in the enteric nervous system with neonatal stress have been documented in this study, more studies are required to determine what specific mechanisms allow for these functional and morphological changes, and to determine the interplay between enteric neurons and other cell types in gastrointestinal disease.

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