ROLE OF INTESTINAL MICROBIOTA IN PROGRAMMING OF THE ENS

INFLUENCE OF INTESTINAL MICROBIOTA ON THE POSTNATAL DEVELOPMENT OF ENTEROCHROMAFFIN CELLS AND THE ENTERIC NERVOUS SYSTEM

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TITLE: Influence of Intestinal Microbiota on the Postnatal Development of Enterochromaffin Cells and the Enteric Nervous System

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ABSTRACT

At birth the gastrointestinal (GI) tract is rapidly colonized by microbial organisms which exhibit considerable fluctuations in composition across the first two years of life. During this period, the enteric nervous system (ENS) continues to undergo significant structural and functional changes. In the present study, we investigated whether exposure to intestinal microbiota influences the postnatal development of the ENS. We focused our investigations on dopaminergic neurons as they are among the latest populations of neurons to differentiate during enteric development. The myenteric plexus of specific pathogen-free (SPF) and germ-free (GF) mice were examined in whole-mount preparations of the small and large intestine at three time-points: postnatal day 1 (P1), P7, and P28. The density of dopaminergic neurons did not differ significantly between SPF and GF mice in any region of the intestine examined at P1. However, at P7, GF mice had significantly fewer myenteric dopaminergic neurons in the ileum than did SPF mice, and this difference was maintained at P28.

The proportion of enteric dopaminergic neurons has been shown to be dependent upon the availability of serotonin. In the GI tract, serotonin can be of neuronal or enterochromaffin (EC) cell origin. We therefore tested the hypothesis that reductions in myenteric dopaminergic neuron densities in the ileum of GF mice were secondary to changes in enteric serotonergic neuron densities or EC cell frequencies. Neither serotonergic neurons nor EC cell numbers were affected by GF status during the postnatal period. The reduction in dopaminergic neurons seen in GF mice must therefore be attributable to a mechanism that has yet to be determined.

These findings are consistent with the notion that enteric microbiota can influence the development of late-born neuronal populations. The reduced proportion of dopaminergic neurons in the ileum of GF mice at P7 and P28 may contribute to the previously described altered motility patterns in postnatal GF mice.

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LIST OF ABBREVIATIONS

ASF	Altered Shaedler flora
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
EC	Enterochromaffin
ENS	Enteric nervous system
ESD	Extreme studentized deviate
GABA	γ-aminobutryic acid
GF	Germ-free
GI	Gastrointestinal
HPA	Hypothalamic-pituitary-adrenal
HuC/D	Human neuronal protein HuC/HuD
IBD	Inflammatory bowel disease
IL-1β	Interleukin-1beta
MPTP	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
OCT	Optimal cutting temperature
P#	Postnatal day
PBS	Phosphate buffered saline
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
SPF	Specific pathogen-free
TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-alpha
5-HT	5-hydroxytryptamine

DECLARATION OF ACADEMIC ACHIEVEMENT

This project was initially conceived by Dr. Elyanne Ratcliffe. Experiments were designed by the combined efforts of Kal Mungovan, Dr. Ratcliffe, and Rajka Borojevic. All experiments were performed by Kal Mungovan and Rajka Borojevic. All whole-mounts were prepared by Rajka Borojevic. Data analysis and interpretation of results were conducted by Kal Mungovan under the guidance of Dr. Ratcliffe. Insightful direction was provided by Dr. Jane Foster, Dr. Waliul Khan, Dr. Kathy Murphy, and Dr. Alison Holloway, who were members of Kal Mungovan's advisory committee. Kal Mungovan wrote this dissertation with contributions from Dr. Ratcliffe.

1. INTRODUCTION

1.1 Background

Disorders of gastrointestinal (GI) motility such as gastroesophageal reflux disease, abdominal pain, and constipation are among the most common diagnoses for which children require medical attention (Chitkara et al., 2007). These conditions, moreover, are associated with significant co-morbidity, including increased risk of psychiatric disorders (Campo et al., 2001), respiratory disease, malnutrition, and reduced quality of life (Gariepy & Mousa, 2009). Despite the fact that they are so common, the pathophysiologies of many GI disorders remain largely unknown. Nevertheless, there is growing evidence in support of the notion that abnormal GI function may arise from developmental abnormalities of the enteric nervous system (ENS) (Krishnamurthy et al., 1993; Törnblom et al., 2002; Gershon & Ratcliffe, 2004; Burns et al., 2009).

1.2 The Enteric Nervous System

The ENS is the largest and most complex division of the autonomic nervous system. Its major functions include coordinating motility reflexes, controlling water and electrolyte exchange across the mucosal epithelium, regulating local blood flow, and modulating immune processes (Costa et al., 2000). It is estimated that the ENS contains a greater number of neurons than does the entire spinal cord (Gershon et al., 1993). Neurons of the ENS are arranged into two major plexuses that line the length of the GI tract. Neuronal cell bodies in each plexus are grouped into regularly spaced ganglia connected to one another by bundles of nerve fibers called internodal strands. The

myenteric plexus is nested between the longitudinal and circular muscle layers and is primarily responsible for controlling digestive motility. Internal to the circular muscle, embedded within the submucosal connective tissue, lies the submucosal plexus, which plays an important role in sensing the luminal environment and coordinating absorption and secretion reflexes. In addition to these intrinsic networks, the GI tract is also innervated by extrinsic fibers, receiving inputs from the sympathetic and parasympathetic branches (Trudrung et al., 1994) and sending afferent sensory information via the nodose and dorsal root ganglia (Powley, 2000). Although the ENS and the central nervous system (CNS) communicate, the ENS exhibits considerable autonomy from the CNS as evidenced by the overall functionality that persists following severance from the CNS (Furness et al., 1995). Such functional independence is unique among peripheral organs and arises from complex microcircuits of intrinsic primary afferent neurons, interneurons, and motoneurons found in the gut wall (Furness et al., 1998).

1.2.1 Development of the ENS

The ENS is primarily derived from cells of the vagal segment of the neural crest (Young et al., 2000). During early embryonic life, pluripotent precursor cells migrate out of the neural crest and into the oral end of the developing intestinal tract where they proceed to populate the entire length of the gut (Burns, 2005). The population of invading cells is heterogeneous, comprising both post-mitotic fully differentiated neurons and actively dividing neural precursors (Pham et al., 1991). These cells ultimately give rise to

the full range of cell types seen in the mature ENS, comparable in diversity to that of the brain (Costa et al., 1996).

The terminal phenotype of enteric neural crest-derived cells is determined in part by their enteric microenvironment (Gershon et al., 1993). The earliest evidence for this comes from studies of chicks that received heterotopic transplantations of sections of quail neural tube (Le Douarin & Teillet, 1974). In these chimeras, the fate of transplanted crest-derived cells depended more on the site of implantation in the host than on the level of the neural tube from which the cells originated in the donor. Thus, when the vagal crest of a chick was replaced by truncal crest from a quail, the truncal crest-derived cells migrated out of the region of the vagal crest to give rise to bowel-appropriate neuronal circuitry (Le Douarin & Teillet, 1974). The development of serotonergic neurons in the truncal crest-colonized bowel is especially revealing given that the truncal crest does not normally give rise to this cell type (Rothman et al., 1986).

In vitro experiments have further substantiated the hypothesis that the fate of enteric crest-derived cells is partly dependent upon their microenvironment. For example, Ziller and colleagues (1987) showed that different cell-types could be obtained from isolated neural crest cells simply by altering the medium in which they were grown. In another paradigm, primary crest cells gave rise to different proportions of serotonergic, γ-aminobutryic acid (GABA)-ergic, and catecholaminergic cells when cultured in the presence of gut extract (Coulter et al., 1988). More recent studies have focused on identifying the factors responsible for inducing specific cellular phenotypes. For example, growth factors of the bone morphogenetic protein family appear to increase the

proportion of late-arising neurons in the ENS including dopaminergic neurons (Chalazonitis et al., 2008). Li and colleagues (2011) recently found that loss of neuronal serotonin leads to a decrease in the proportion of dopaminergic and GABAergic neurons, suggesting that serotonin is also part of the microenvironment influencing the fate of late-born neurons.

The ENS continues to undergo significant changes throughout human fetal development and into the first 12 to 18 months of life (Bisset et al., 1999). Whereas prenatal enteric development has been characterized in great detail, few studies have examined the postnatal changes in the ENS. Among these limited studies are reports that neurons continue to be added to the enteric plexuses throughout the first two postnatal weeks in mice (Pham et al., 1991). Hagl and colleagues (2005) used two-dimensional gel electrophoresis to demonstrate significantly altered protein expression in the rat myenteric plexus across the first two weeks of life. Postnatal changes in the three-dimensional architecture of the myenteric plexus have also been reported in both the murine and human intestine (Schäfer et al., 1999; Wester et al., 1999).

These structural observations are complimented by functional studies, which have shown that postnatal mice have immature colonic motility patterns (Roberts et al., 2007). Likewise, in humans, intestinal motor patterns are not fully developed at birth (Berseth, 2006). De Vries and colleagues (2010) related changes in neurochemical coding of the myenteric plexus in the rat colon to altered neuromuscular transmission across the first three weeks of life. Most recently, Foong et al. (2012) found that the electrophysiological

properties of myenteric neurons in the mouse small intestine have not reached maturity by the second week of postnatal life.

The fact that the ENS is still developing during the postnatal period supports the notion that it is still plastic and therefore sensitive to changes in the enteric environment (Gershon, 2012). One important change that coincides with this timeframe is the colonization of the GI tract by a dense mixture of microbial species (Mackie et al., 1999).

1.3 Intestinal Microbiota

The fetus is thought to exist in a sterile environment in utero with time of birth representing the first exposure of the infant to bacteria (Fanaro et al., 2003). The intestinal flora develops rapidly thereafter and is subject to a number of influences, including gestational age, mode of delivery, type of infant feeding, and antibiotic use (Penders et al., 2006). A mature microflora comprises more than 400 species of bacteria (Eckburg et al., 2005), reaching concentrations of $10^{11} – 10^{12}$ microbes/ml of luminal content (Whitman et al., 1998). In healthy humans, the highest concentrations of bacteria are found in the ileum and colon (Blaut & Clavel, 2007). Commensal microbiota are critical for host physiology and have been shown to play a role in the developmental programming of host adaptive immunity (Hooper et al., 2012), epithelial barrier function (Hooper et al., 2003), and intestinal angiogenesis (Stappenbeck et al., 2002). Given that the ENS is still developing throughout the period of initial colonization, it is conceivable that the colonizing bacteria influences postnatal programming of enteric circuitry.

1.3.1 Intestinal microbiota and the ENS

Observations in neonatal germ-free (GF) mice by our lab have yielded preliminary support for the hypothesis that intestinal microbiota affect the development of the ENS. At postnatal day 3, the ENS of GF mice was structurally abnormal. Compared to the repeating lattice-like arrangement of the myenteric plexus in specific pathogen-free (SPF) control animals, the myenteric plexus in the jejunum, ileum and colon of GF mice appeared less ordered with thinner internodal fibres (Collins et al., 2013). The total nerve volume density and number of neurons per ganglia were also significantly reduced in these areas of the myenteric plexus in GF mice (Collins et al., 2013). Colonization of GF dams with Altered Shaedler Flora (ASF), a standardized flora comprised of only 8 bacterial strains, was sufficient to restore the normal patterning of the ENS in their offspring (Collins et al., 2013). These structural observations are strengthened by functional data showing impaired GI motility in corresponding regions of early postnatal GF mice. Specifically, the frequency and amplitude of intestinal contractions were significantly reduced in the jejunum and ileum of GF mice. Failure of the sodium channel blocker, lidocaine, to dampen contractions in GF tissues suggests that the reduced contractile activity seen in GF intestines is at least partly attributable to neurogenic abnormalities (Collins et al., 2013). Structural and functional abnormalities have also been described in the ENS of adult GF rodents. Dupont and colleagues (1965) reported enlarged neuronal cell bodies in the myenteric plexus of the GF rat cecum. Moreover, the excitability of intrinsic primary afferent neurons was found to be significantly reduced in adult GF mice, suggesting that commensal microbiota are

necessary for the development of normal electrophysiological profiles in enteric neurons (McVey Neufeld et al., 2013).

Which neuronal populations are affected by intestinal microbiota remains to be determined. Previous findings of altered proportions of nitrergic neurons in the ENS of postnatal GF mice provide precedent for a change in chemical coding in these animals (Collins et al., 2013). Di Giancamillo and colleagues (2010) reported increased density of galanin- and calcitonin gene-related peptide (CGRP)-immunoreactive neurons in the ileum of piglets treated with probiotics, suggesting that even subtle changes in the composition of the intestinal flora can affect the development of the ENS.

Enteric nerve terminals are separated from the lumen by the intestinal epithelium which acts as a barrier against bacterial invasion (Raybould, 2002). Although the integrity of the epithelial layer can become compromised during certain disease states (for example, Ma, 1997; Vogelsang et al., 1998), under physiological conditions, tight junctions between neighbouring epithelial cells effectively seal out encroaching bacteria. Thus, in order for the intestinal microbiota to exert their presumptive effects on the developing ENS, there must be some intermediary mechanism to relay signals from the microbes in the lumen to the sensory neurons in the lamina propria. One candidate cell type for this purpose is the enterochromaffin (EC) cell (Rhee et al., 2009).

1.4 Enterochromaffin Cells

EC cells comprise the single largest endocrine cell population of the GI tract (Sjölund et al., 1983). They are widely distributed throughout the gut and occur in the

stomach and all segments of the small and large intestines (Ahlman & Nilsson, 2001). Located within the epithelial layer, these cells are uniquely poised to convey information about the luminal environment to underlying enteric neurons. Microvilli on the apical end of EC cells project into the lumen of the gut where they monitor physical and chemical changes. The major secretory product of EC cells is serotonin (5-hydroxytryptamine, or 5-HT). 5-HT is released across the basolateral membrane in a calcium-dependent manner (Racké & Schwörer, 1993) in response to a variety of stimuli including changes in luminal pH (Kellum et al., 1983), nutrient detection (e.g. glucose) (Kim et al., 2001),), certain tastants and olfactants (Kidd et al., 2008), vagal stimulation (Grönstad et al., 1987), and distension of the gut wall (Tsukamoto et al., 2007). Secreted 5-HT acts on serotonin receptors on intrinsic and extrinsic afferent nerve terminals in the lamina propria to initiate peristaltic and secretory reflexes (Kirchgessner et al., 1992; Blackshaw & Grundy, 1993; Cooke et al., 1997; Tuladhar et al., 1997).

1.4.1 Enterochromaffin cells and intestinal microbiota

There are several lines of evidence to suggest that EC cells may also play a role in monitoring the microbial environment of the lumen (Rhee et al., 2009). Short-chain fatty acids (SCFA), a metabolic byproduct of bacterial fermentation, have been shown to elicit 5-HT release in the rat colon (Fukumoto et al., 2003). However, the absence of SCFA receptors on EC cells suggest that this response is more likely mediated either by 5-HT containing mast cells, which are also present in the lamina propria of the rat intestine, or by indirect activation of EC cells by other enteroendocrine transmitters (Karaki et al.,

2006; Tazoe et al., 2009). Researchers have also found that both human EC cells and a murine enteroendocrine cell line express Toll-like receptors (TLRs), which are pattern recognition receptors of the innate immune system that respond to microbial factors (Bogunovic et al., 2007). A number of bacterial toxins, including lipopolysaccharide (Kidd et al., 2009), cholera toxin (Bearcroft et al., 1996), and Staphylococcal enterotoxin (Hu et al., 2007) have been shown to stimulate EC cell secretion, and the presence of certain pathogenic strains of bacteria is associated with increased mucosal 5-HT release (Grøndahl et al., 1998). Interestingly, receptors for certain enterotoxins appear to be under developmental control such that the immature intestine is especially sensitive to their effects (Chu & Walker, 1993). Thus, the early postnatal period may constitute a critical window for microbial influences on the ENS not only because of the enduring plasticity of the ENS, but also on account of the heightened sensitivity of the developing gut to microbial signals.

Studies of GF animals suggest a role for intestinal microbiota in EC cell regulation, although the nature of this relationship is unclear. Nogueira and Barbosa (1994) reported enlarged, strongly immunostained serotonin-positive cells in the mucosa of some GF mice, but this was not observed consistently. Similarly, using stereological techniques, Uribe and colleagues (1994) found increased total volumes of serotonin-immunoreactive cells in the ileum and colon of GF rats. However, more recent data from the laboratory of Dr. Khan indicates a reduction in EC cell numbers and total 5-HT content in the GF colon (unpublished data). Mice infected with an enteric parasite had significantly greater numbers of EC cells in the colon, an effect which was shown to be

mediated in part by cytokines from activated immune cells (Wang et al., 2007). A similar mechanism may be responsible for the reported up-regulation of EC cell numbers in post-infectious irritable bowel syndrome (Spiller et al., 2000; Wheatcroft et al., 2005).

1.4.2 Enterochromaffin cells and the ENS

EC cells appear relatively early in fetal development, long before the circuitry in the ENS has matured (Singh, 1963; Branchek & Gershon, 1989). Thus, mucosal 5-HT could potentially play a role in ENS development. In keeping with its known neurotrophic actions in the CNS (Lauder & Krebs, 1978; Hamon et al., 1989), 5-HT appears to stimulate neurogenesis in the ENS (Fiorica-Howells et al., 2000; Liu et al., 2009). Two receptors have been implicated in serotonin's neurotrophic actions in the gut, namely the G-protein coupled receptors 5-HT_4 and 5-HT_{2B}. Activation of the 5-HT_4 receptor has been shown to promote proliferation and survival of enteric neurons both perinatally and into adulthood, and the total number of enteric neurons is significantly reduced in 5-HT_4 knock-out mice at 12 months of age (Liu et al., 2009). Similarly, activation of the 5-HT_{2B} receptor stimulated neuronal differentiation and neurite outgrowth in cultured enteric crest-derived cells (Fiorica-Howells et al., 2000). Expression of 5-HT_{2B} in primordial enteric neurons appears to be developmentally regulated, with heightened levels coinciding with periods of terminal differentiation in populations of late-arising neurons (Fiorica-Howells et al., 2000). These late-born neurons are characterized by their independence from the proneural gene *Mash-1*, which

is critical to the development of a subset of early-differentiating neuronal populations (Blaugrund et al., 1996).

Li and colleagues (2011) recently showed that development of enteric dopaminergic neurons, which arise perinatally of a *Mash-1*-independent lineage (Li et al., 2004), is regulated by 5-HT. Addition of 5-HT to cultured enteric crest-derived neurons increased the proportion of dopaminergic neurons. However, it is unclear whether this effect is mediated by mucosal 5-HT or 5-HT secreted from serotonergic neurons, which are among the earliest to differentiate during embryonic development (Pham et al., 1991). Studies of mice deficient in either neuronal or mucosal 5-HT suggest that survival of dopaminergic neurons depends more on neuronal than EC cell 5-HT (Li et al., 2011). However, the possibility of compensatory mechanisms in these knock-out mice cannot be discounted and further experiments are needed before the hypothesis that mucosal 5-HT influences late-born neuronal populations can be conclusively rejected.

1.5 Dopaminergic Neurons

Although it has long been known that the bowel contains dopamine (Eaker et al., 1988), the existence of intrinsic dopaminergic neurons was difficult to prove given that a major source of dopamine is from extrinsic sympathetic fibers in which dopamine serves as the precursor to norepinephrine. However, using reverse transcription polymerase chain reaction and immunohistochemistry for several markers of dopaminergic neurons, researchers have conclusively established dopamine as an intrinsic neurotransmitter in both the human (Anlauf et al., 2003) and mouse ENS (Li et al., 2004). The distribution of

dopaminergic neurons in the mouse GI tract appears to follow a similar pattern to that of the human, with the highest numbers occurring in the upper small intestine and progressively declining towards the colon (Anlauf et al., 2003; Li et al., 2004). The enteric circuitry in which dopaminergic neurons participate is largely unknown, but there are several lines of evidence supporting an inhibitory role in intestinal motility (Chevalier et al., 2008).

Administration of dopamine *in vitro* relaxes the rat jejunum (Lucchelli et al., 1986) and smooth muscle isolated from dog colon (Grivegnee et al., 1984). Moreover, combined blockade of dopamine receptors D_1 and D_2 increases both smooth muscle tone and the amplitude of spontaneous contractions in the mouse colon (Walker et al., 2000). Genetic deletion of the D_2 receptor resulted increased intestinal motility (Li et al., 2006), whereas deletion of the dopamine reuptake transported had the opposite effect (Walker et al., 2000).

Changes in dopaminergic neuron densities have also been reported in some GI pathologies. Specifically, an increase in myenteric dopaminergic neurons has been reported in the ileum of patients with Crohn's disease (Belai et al., 1997), whereas a loss in dopaminergic neurons is seen in the myenteric plexus of Parkinson's disease patients (Singaram et al., 1995). The MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) mouse model of Parkinson's disease also exhibited a reduction in enteric dopaminergic neurons, and was associated with a transient increase in colonic motility (Anderson et al., 2007). Dopamine receptor antagonists have long been used as prokinetic agents for patients suffering from upper GI motility disorders (Weihrauch & Ehl, 1981). Thus,

determining the factors that influence the development of dopaminergic neurons is of clear clinical relevance.

1.6 Critical Period

A critical period is a window of time during development during which an organism exhibits a heightened sensitivity to certain exogenous stimuli that are necessary for normal development (Knudsen, 2004). The postnatal period may represent a critical window during which disruptions of the intestinal flora, referred to as dysbiosis, can exert lasting effects on the ENS, and, in turn, on GI function. Whereas the core microbiota of the healthy adult remains essentially stable over time (Vanhoutte et al., 2004), that of the infant is more variable, undergoing dramatic restructuring during the first 2-4 years of life (Palmer et al., 2007; Koenig et al., 2011). Such instability leaves the infant's flora especially vulnerable to extrinsic influences such as dietary changes and antibiotic use (Fanaro et al., 2003). Moreover, establishment of the enteric circuitry is far from complete during these early years. Given the key role of the microenvironment in the programming of enteric neurons (Gershon et al., 1993), changes in the early intestinal flora are likely to carry important consequences for the development of the ENS.

Indirect support for this hypothesis has come from epidemiological studies. For example, antibiotic use early in life is associated with an increased incidence of recurrent abdominal pain (Uusijarvi et al., 2012) and an increased risk of childhood onset inflammatory bowel disease (IBD) disease (Shaw et al., 2010; Hviid et al., 2011). Moreover, breast-feeding, which is known to affect the composition of early intestinal

flora (Penders et al., 2006), is associated with a lower incidence of IBD later in life (Le Huërou-Luron et al., 2010). Finally, mode of delivery, also a known determinant of early intestinal flora makeup (Penders et al., 2006), has been linked to GI health later in life, with cesarean deliveries associated with a greater risk of developing celiac disease (Decker et al., 2010) and possibly IBD (Bager et al., 2012).

The notion of a critical period during which developmental processes can be influenced by intestinal microbiota is also substantiated by behavioural studies. Colonization of GF mice by exposure to SPF flora normalized behaviour when done preconception but failed to do so when done in adulthood (Heijtz et al., 2011; Neufeld et al., 2011). Moreover, conventionalization of GF mice with SPF flora reversed their heightened hypothalamic-pituitary-adrenal (HPA) response to stress when done at an early stage of development (6 weeks), but not when done later in life (14 weeks) (Sudo et al., 2004). Vagotomy studies in mice have verified that a number of behavioural effects of intestinal microbiota are mediated by neuronal pathways (Bravo et al., 2011; Bercik et al., 2011). Given the extensive communication between intrinsic and extrinsic nerve fibers in the GI tract, changes in the postnatal ENS may be responsible for the development of these bacterially induced behavioural effects. Thus, the existence of a critical window early in life during which the intestinal microbiota can exert lasting changes on host behaviour may be a result of the combined effects of the unstable flora and immature enteric circuitry that characterize this period.

1.7 Clinical Relevance

Digestive disorders affect approximately 20 million Canadians and represent a significant burden to society, accounting for an estimated annual loss of $18 billion in health care costs and lost productivity (The Canadian Digestive Health Foundation, 2009). As mentioned above, intestinal dysbiosis has been implicated in a number of GI disorders including irritable bowel syndrome, IBD, gastric ulcers, and colon cancer (Nicholson et al., 2012). The mechanisms by which dysbiosis can lead to these chronic conditions, however, remain unknown. With the increased incidence of intestinal dysbiosis in Western populations over the past 60 years (Nicholson et al., 2012), and rising rates of many digestive diseases (The Canadian Digestive Health Foundation, 2009), uncovering these mechanisms is a growing priority. Serotonin and dopamine currently represent two of the most important targets of clinical pharmacology in the treatment of disordered GI motility (Tonini & Pace, 2006; Manabe et al., 2010; Ahmad et al., 2006). Thus, through investigations of the effects of microbiota on intestinal serotonin and dopamine, the present study will seek to shed light on one potential mechanism of action.

1.8 Hypothesis and Aims

The overall aim of this project is to understand how the intestinal microbiota and the ENS interact during early development. The hypothesis under investigation is that the presence of microbiota affects the developmental programming of the ENS. The experiments described herein seek to address the following specific aims:

1. Determine whether the presence of intestinal microbiota affects the density of dopaminergic neurons in the myenteric plexus of the small and large intestines across early development;
2. Determine whether the presence of intestinal microbiota affects the density of serotonergic neurons in the myenteric plexus of the small and large intestine across early development; and
3. Determine whether the presence of intestinal microbiota affects the frequency of EC cells in the small and large intestine across early development.

2. MATERIALS AND METHODS

2.1 Animals

Timed-pregnant SPF NIH Swiss (outbred strain) mice were purchased from Harlan Laboratories, Inc. (Indianapolis, IN, USA) and maintained in ultraclean conditions using ventilated racks. GF NIH Swiss mice were obtained from the Farncombe Family Axenic Gnotobiotic Unit at McMaster University (Hamilton, ON, Canada), which is strictly controlled to ensure a sterile environment. Cages were checked daily for offspring and the day the litters were found was considered postnatal day 0. SPF pups were weaned at three weeks of age while GF pups required an additional week with their mother before weaning. There have been no studies to our knowledge reporting sex differences in the programming of the ENS. Thus, in order to minimize the number of litters used, gender was not controlled for. GF and SPF mice (n = 5) were harvested at three key time-points: 1 day after birth (P1), 1 week after birth (P7) and four weeks after birth (P28). These time-points were selected because they represent important milestones in mouse development. Since we did not expect 1 day of exposure to intestinal microbiota to have significant effects on the programming of the ENS or the number of EC cells, the P1 time-point served as a baseline for comparisons between GF and SPF mice and provided insight into the potential contribution of the prenatal environment to postnatal differences between groups. The vast majority of enteric neurons, including dopaminergic neurons, have differentiated by P7 (Pham et al., 1991) such that differences in enteric programming between groups were expected to emerge by this time-point. Finally, the P28 time-point provided an indication as to whether these differences persist into the

post-weaning period. Mice were euthanized by exposure to carbon dioxide gas followed by cervical dislocation. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

2.2 Tissue Preparation

The entire lengths of the small and large intestines were removed immediately following sacrifice and immediately placed in phosphate-buffered saline (PBS; pH 7.4). The small intestine was divided into three portions according to the anatomical map published by Lefrançois and Vezys (2001) (Fig. 1). Tissue from P1 animals was collected for a single experiment whereas tissue from P7 and P28 animals was divided in two, with the proximal portions of each intestinal segment used for cross-sections and the distal portions used for whole-mounts. To avoid potential confounds from litter effects, animals within each group were taken from different litters.

2.2.1 Whole-mount preparation

Whole-mounts were prepared according to a protocol modified from Li et al. (2006). Briefly, the specimens were cut open along the mesenteric border and the contents flushed away with PBS. The tissue was stretched flat and pinned to a Sylgard silicone-coated plate (Dow Corning, Midland, MI, USA) with the mucosa facing up. Once all segments were pinned, the tissue was fixed for 2 hours in 4% formaldehyde

(freshly prepared from paraformaldehyde; pH 7.4), washed three times (5 minutes each) in PBS to remove residual fixative, and stored at 4 °C.

To visualize the myenteric plexus, the mucosa, submucosa, and submucosal plexus were carefully peeled away from the underlying muscle layers using fine forceps under a stereomicroscope. Although typically removed in whole-mount preparations, the circular muscle layer was left intact in these samples as preliminary experiments showed it was not yet thick enough to significantly interfere with staining at these early time-points. Once stripped of the mucosa and submucosa, the tissues were stored at 4 °C in PBS containing 0.01% sodium azide to prevent bacterial growth.

2.2.2 Cross-section preparation

Tissues for cross-sections from P1 and P7 animals were left as closed tubes to preserve the integrity of the mucosa, whereas P28 tissues were opened along the mesenteric border for better morphology. The tissues were fixed in 4% formaldehyde according to the procedure outlined above and stored in PBS at 4 °C. Samples were cryoprotected by overnight incubation in a 30% sucrose solution in PBS before embedding in Optimal Cutting Temperature (OCT) compound (Tissue-Tek, Miles Laboratories, Elkhart, IN, USA). Samples were frozen in liquid nitrogen and stored at -80 °C. Cryostat sections were cut at 10 µm and thaw-mounted onto Superfrost Plus microscope slides (Thermo Scientific, Waltham, MA, USA). Slides were stored at -20 °C until processed for immunohistochemistry.

2.3 Immunohistochemistry

2.3.1 Whole-mount staining

Whole-mount preparations were transferred to a 48 well plate and permeabilized and blocked by incubation in 4% normal horse serum and 0.4% Triton X-100 in PBS for 1 hour at room temperature. Primary and secondary antibodies were diluted to the desired concentration in blocking serum. To visualize neuronal cell bodies, all whole-mounts were incubated overnight at room temperature with antibodies to human neuronal protein HuC/HuD (biotinylated monoclonal mouse anti-HuC/D; dilution 1:50; Molecular Probes, Invitrogen Canada Inc., Burlington, ON, Canada). A subset of these tissues was further processed for visualization of dopaminergic neurons by overnight incubation at room temperature with antibodies to tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis (polyclonal sheep anti-TH; dilution 1:1000; Millipore, Cedarlane, Burlington, ON, Canada). Although TH is also present in noradrenergic and adrenergic neurons, the mouse bowel does not contain intrinsic neurons of those types (Keast et al., 1984; Qu et al., 2008). Thus, any TH-positive cell bodies could be conclusively considered to be dopaminergic neurons (Li et al., 2004). Serotonergic neurons were labeled by overnight incubation with antibodies to serotonin (polyclonal rabbit anti-5-HT; dilution 1:2000; ImmunoStar, Cedarlane). Sites of antibody binding were detected by incubation with streptavidin conjugated to Alexa Fluor 594 (1:500; Molecular probes) and either donkey anti-sheep (1:200; Molecular Probes) or donkey anti-rabbit antibodies (1:200; Molecular Probes) conjugated to Alexa Fluor 488 for 2.5 hours. Primary antibodies were applied separately whereas secondary antibodies were

applied together. Tissues were mounted on glass slides with Vectashield medium (Vector Laboratories Canada Inc., Burlington, ON) to minimize photobleaching.

2.3.2 Cross-section staining

EC cells were visualized with antibodies to 5-HT. Briefly, slides were air-dried for 30-60 minutes at room temperature before being washed for 10 minutes in PBS to remove OCT. Sections were circled using a GnomePen hydrophobic marker (Frogga Bio, Toronto, ON, Canada) and blocked in PBS containing 4% normal goat serum and 0.4% Triton-X-100 for 1 hour to prevent non-specific binding. Slides were drained and the primary antibody solution applied (polyclonal rabbit anti-5-HT; dilution 1:2000; ImmunoStar). Following overnight incubation at room temperature in a humidified chamber, primary antibodies were washed away in PBS (three 5-minute washes) and drained. Sites of antibody binding were visualized by incubation with goat anti-rabbit IgG conjugated to Alexa Fluor 594 (1:200; Molecular Probes) for 2.5 hours at room temperature, and again washed three times in PBS. Both primary and secondary antibodies were diluted in blocking buffer. Nuclei were labeled with the fluorescent DNA stain, bisbenzimide (1 μg/ml in PBS for 4 minutes; Sigma-Aldrich, Oakville, ON, Canada). Slides were coverslipped with Vectashield mounting medium and stored at 4 °C. No immunostaining was seen when primary antibodies were omitted (data not shown).

2.4 Image Analysis

All slides were coded prior to analysis such that the investigator was blind to the experimental condition. Immunostained tissues were viewed and photographed with a Retiga QImaging digital camera mounted on a Leica DMRXA2 microscope (Nussloch, Germany) operated by a Macintosh computer (Apple Computers, Markham, ON, Canada). Images were viewed and analyzed using Volocity Imaging software (Improvision Inc., Montreal, QC, Canada).

To assess the relative density of myenteric neurons in GF versus SPF animals, HuC/D-stained tissues were photographed with a 40X objective. The high magnification was required to distinguish overlapping cells within a ganglion. Five fields of view were randomly selected for imaging, and the total number of HuC/D-positive neurons in each photograph was counted manually and expressed per area.

Dopaminergic neurons are quite rare, occurring at a frequency of less than 0.5% of all myenteric neurons in the murine small intestine (Qu et al., 2008). A large sample was therefore required to obtain a representative value for their frequency. Whereas in the smaller samples obtained from P1 animals, dopaminergic neurons were counted in the entire lengths of tissues, in the larger samples from P7 and P28 animals, a defined area was delineated for counting based on the size of the specimen and the quality of the tissue and staining. Dopaminergic neurons were counted by carefully scanning the region of interest at under a 40X objective and photographing all TH-positive cells to ensure none were counted in duplicate. Areas of the regions counted were calculated by photographing the tissues alongside a ruler for calibration with a PowerShot ELPH 110

HS digital camera (Canon Canada Inc., Mississauga, ON, Canada) and analyzing the photographs using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Serotonergic neurons are more abundant than dopaminergic neurons, representing approximately 1% of all neurons in the myenteric plexus of the mouse small and large intestines (Sang & Young, 1996; Qu et al., 2008). A smaller sample was therefore sufficient to obtain a reliable estimate of their frequency. 5-HT-immunopositive neurons were manually counted in ten randomly selected photographs taken with a 20X objective. To ensure that the entire length of the whole-mounts was sampled, the tissue was divided into three approximately equal areas and 3-4 images were taken from each.

Given reports of altered myenteric neuron density in the ENS of GF mice, some groups choose to normalize densities of neurons of interest to total neuronal density (Collins et al., 2013), while others opt not to (Anitha et al., 2012). Normalization is necessary for understanding the functional consequences of observed changes in neuron densities. For example, a reduced number of inhibitory neurons is functionally irrelevant if the number of excitatory neurons is also reduced. However, unnormalized values are useful for understanding which neuronal populations are specifically affected by the presence of intestinal microbiota and are driving changes in the total neuronal density. Since the primary focus of the present research was to understand the developmental processes underlying changes in the ENS of GF mice, dopaminergic and serotonergic neuron numbers per area were not normalized to total neuron numbers per area for comparisons between groups within each time-point. However, since there was a significant difference in the number of HuC/D-positive cells per area across the three

time-points, all cell counts were normalized to 1000 HuC/D-cells for comparisons between different time-points.

Lastly, EC cell frequencies were quantified in cross-sections of ileum and colon from mice harvested at all three time-points. EC cells were identified based upon positive 5-HT immunostaining, morphology, and localization to the epithelial layer. EC cells were quantified by counting the number of 5-HT-positive cells per villus-crypt unit in the small intestine, and per crypt in the large intestine, as previously published (Montomura et al., 2008). Three sections were quantified per animal. Villi and crypts were selected for counting based on morphology. Crypts were considered when sectioned along at least two-thirds of the length of the crypt lumen. The number of villi and crypts counted varied depending upon the quality of the sections, but a minimum of 10 was required for inclusion in the results. Results were expressed as the number of EC cells per 10 villus-crypt units or 10 crypts.

2.5 Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism Version 4 software (GraphPad Inc., La Jolla, CA, USA). Within each time-point, group differences were compared by unpaired Student's *t* tests. Welch's correction was adopted when group variances were significantly different. When outliers were suspected, the Kolmogorov-Smirnov test for normality was performed first, followed by the extreme studentized deviate (ESD) test if normality was confirmed. Statistically significant outliers were discarded (n = 2). Two-

way analysis of variance was used to compare group differences across time-points followed by Bonferroni tests where appropriate. A two-tailed significance criterion of $p < 0.05$ was applied to all statistical tests.

3. RESULTS

3.1 Total Neuron Densities

Myenteric ganglia were visualized by immunolabeling neuronal cell bodies with antibodies to HuC/D (Fig. 2A,B). At both P1 and P7, there was no significant difference between GF and SPF mice in the number of HuC/D-positive cell bodies per square millimeter in any region of the small and large intestine examined (Fig. 3A,B). At P28, however, GF animals have a significantly greater number of neurons per area in the jejunum and colon than do SPF mice ($p < 0.05$). No significant difference was apparent in the duodenum or ileum at this time-point (Fig. 3C).

3.2 Dopaminergic Neuron Densities

TH-immunoreactive cell bodies were counted in the duodenum, jejunum, ileum, and colon of P1 and P7 animals, but only in the ileum and colon of P28 animals (Fig 2A). At P1, the number of dopaminergic neurons per area in GF mice did not differ significantly from that of SPF mice in any region of the small or large intestine (Fig. 4A). At P7, no significant difference was found in the duodenum, jejunum, and colon of GF and SPF mice (Fig. 4B). However, GF ileums had significantly fewer dopaminergic neurons per area than did those of SPF mice at this time-point ($p < 0.05$; Fig. 4B). This effect persisted into P28 ($p < 0.05$; Fig. 4D). No significant difference was found in the colon of P28 animals (Fig. 4C). The proportion of dopaminergic neurons per 1000 HuC/D-positive neurons did not change significantly in the duodenum and jejunum of either GF or SPF mice between P1 and P7 (Fig. 5A,B,C,D). However, in the ileum, a

significant spike in dopaminergic neuron density was seen at P7 in SPF animals, followed by a drop at P28 (Bonferroni, $p < 0.001$; Fig. 5E). Neither of these effects was apparent in the ileums of GF animals, which showed no significant difference in dopaminergic neuron densities over time (Fig. 5F). Both SPF and GF mice exhibited a decrease in the proportion of dopaminergic neurons between P1 and P7, although this only reached statistical significance in GF mice (Bonferroni, $p < 0.05$; Fig. 5G,H). This difference persisted in GF animals into P28 (Bonferroni, $p < 0.05$; Fig. 5H).

3.3 Serotonergic Neuron Densities

Serotonergic neurons were visualized immunohistochemically with antibodies to 5-HT (Fig. 2B). The number of serotonergic neurons per square millimeter of ileum or colon tissue in GF mice did not differ significantly from that of SPF mice at P1, P7, and P28 (Fig. 6).

3.4 EC Cell Frequencies

We sought to establish a timeline for EC cell development in GF and SPF mice across the first four weeks of life by immunohistochemistry (Fig. 2C). The ileum and colon were chosen for examination of EC cell frequency since these are the most densely colonized regions of the GI tract. Moreover, these are the regions in which differences in EC cell frequencies have been reported between GF and SPF mice (Uribe et al., 1994; Khan, unpublished data). We found no significant difference in EC cell numbers in either the ileum or colon of GF and SPF mice at any time-point examined (Fig. 7). The

developmental trajectory of EC cells in the ileum of GF mice followed closely that of SPF mice, except that the increase in EC cells between P7 and P28 reached statistical significance in GF mice only (Bonferroni, $p < 0.01$; Fig. 8A,B). However, there was no significant difference in either group between P1 and P28. The number of EC cells in the colon remained stable across the three time-points examined in both rearing conditions.

4. DISCUSSION

In the present study, we investigated the contribution of intestinal microbiota to early programming of the ENS. We found that the density of myenteric dopaminergic neurons was significantly reduced in the small intestine of GF mice. This change was independent of both the number of EC cells and the number of serotonergic neurons, which were unchanged in these animals.

4.1 Effects of Intestinal Microbiota on Dopaminergic Neuron Density

Our values for the proportions of dopaminergic neurons in the small intestine of SPF mice were comparable to some reports (Qu et al., 2008; less than 0.5% of myenteric neurons), but lower than others (Li et al., 2004; approximately 9% of myenteric neurons). This discrepancy may be attributable to strain differences.

The finding of reduced enteric dopaminergic neurons in the ileums of GF mice at P7 and P28 lends support to the hypothesis that the early intestinal microbial environment can program lasting changes in enteric circuitry. The functional importance of this difference remains to be determined. While dopaminergic neurons make up only a small percentage of all myenteric neurons, they play an important role in modulating intestinal motility. However, they do not appear to be critical to the enteric circuitry underlying the peristalsis reflex, which remains intact in D_2 knockout mice (Li et al., 2006). Rather, the reduced intestinal transit time (increased motility) seen in these D_2 knockout mice suggests that dopaminergic neurons are capable of modulating the intensity of peristaltic waves (Li et al., 2006). The physiological relevance of the observed reduction in

dopaminergic neurons in GF mice is uncertain given that these animals have delayed intestinal motility relative to SPF mice (Anitha et al., 2013; Collins et al., 2013), contrary to what would be predicted based on the studies in D_2 knockout mice. However, given our long-standing mutualistic relationship with intestinal microbiota, the GF state can be expected to lead to several compounding defects which may have masked the functional importance of a reduction in dopaminergic neuron density. The effects of changes in the microbiota on the density of dopaminergic neurons and on intestinal motility should therefore be examined under more physiological conditions, such as following probiotic or antibiotic administration in an already colonized host.

4.2 Mechanisms of Action

4.2.1 Serotonergic neurons

Development of enteric dopaminergic neurons has been shown to be dependent upon the availability of 5-HT (Li et al., 2011). 5-HT in the gut may be of neuronal, mucosal, or microbial origin, although there is evidence to suggest that neuronal 5-HT plays a greater role in the developmental programming of dopaminergic neurons (Li et al., 2011). We therefore examined the number of serotonergic neurons in the GI tract of GF mice.

Immunolabeling of serotonergic neurons proved more difficult than anticipated. Neuronal fibers and cell bodies were only faintly visible even after optimizing the staining protocol, such that many samples had to be discarded. The low sample size in several groups must be taken into consideration when interpreting these results. Future

studies may benefit from pre-loading samples with serotonin and pre-treatment with a monoamine oxidase inhibitor to amplify the signal, as previously published (Wardell et al., 1994; Young & Furness, 1995; Sang & Young, 1996). Despite our limited sample size, the densities of serotonergic neurons in P28 animals were consistent with previously reported values in adult mice (Sang & Young, 1996; Furness, 2006).

We found no significant difference between SPF and GF mice in the number of serotonergic neurons in the ileum at any time-point examined. Thus, the changes in dopaminergic neuron density reported here cannot be attributed to changes in serotonergic neuron numbers. This was consistent with our expectations given that enteric serotonergic neurons differentiate early in fetal development (Pham et al., 1991), and are thus unlikely to be affected by the postnatal environment. Nevertheless, the fact that differentiation of serotonergic neurons is unaffected in GF mice does not eliminate the possibility of altered neuronal activity in these cells, which could, in turn, contribute to the programming of dopaminergic neurons. The ability of enteric microbiota to affect neuronal activity was demonstrated by McVey Neufeld and colleagues (2013) who found reduced excitability in intrinsic primary afferent neurons of GF mice. Electrical and chemical stimulation of enteric neurons has been shown to affect the proportion of dopaminergic neurons (Chevalier et al., 2008). Altered neuronal activity in GF mice may thus account for the reduced numbers of dopaminergic neurons in these animals.

4.2.2 Enterochromaffin cells

The EC cell frequencies reported here were consistent with previously published values in adult rodents after making the necessary conversions (Estienne et al., 2010; Wang et al., 2007). We found no significant difference between GF and SPF mice in the number of EC cells in the ileum and colon across early development. The observed reduction in dopaminergic neurons in the GF ileum at P7 and P28 cannot, therefore, be attributed to previously reported changes in EC cell regulation in GF mice (Khan, unpublished data; Nogueira & Barbosa, 1994; Uribe et al., 1994), which, according to our observations, must not occur until later in development. In both groups of mice, EC cell numbers remained largely stable across early development, with the only significant difference occurring in the ileum of GF mice between P7 and P28. However, they did not differ significantly in the ileum of GF mice between P1 and P28, suggesting that despite some minor fluctuations, the number of EC cells remains largely stable in the ileum across early development regardless of microbial status.

As with serotonergic neurons, there remains the possibility that EC cell activity may be altered in GF mice. Indeed, bacterial products have been shown to stimulate 5-HT release from EC cells (Kidd et al., 2009; Hu et al., 2007). This possibility could be tested by *in vitro* amperometric monitoring of spontaneous 5-HT secretions from the mucosa of SPF and GF ileal whole-mounts by microelectrode (Patel et al., 2007).

4.2.3 Direct interactions with the ENS

Several other potential mechanisms exist that could account for, or contribute to, the observed reduction in dopaminergic neurons in the GF ileum. One possibility is the direct interaction between intestinal microbiota and enteric nerve fibers. The presence of TLRs has been demonstrated in the mouse (TLR4) (Rumio et al., 2006; Anitha et al., 2012) and human (TLR3, 4 and 7) (Barajon et al., 2009) ENS. TLR4 knockout mice have reduced numbers of nitrergic neurons in the ENS and delayed intestinal transit, suggesting that activation of these receptors plays a role in programming of enteric neurochemical phenotypes (Anitha et al., 2012). Intestinal motility was preserved in wild-type mice expressing TLR4 knockout bone marrow, implying that this effect was not a consequence of interactions with TLR4-expressing immune cells of hematopoietic origin (Anitha et al., 2012). Activation of TLRs on enteric neurons would require entry of intestinal microbes into the lamina propria, which is normally protected from bacterial invasion by a mucus layer and epithelial cell barrier (Camilleri et al., 2012). However, direct interaction with enteric neurons may contribute to changes in the neurochemical coding of the ENS in disease states characterized by compromised barrier function, such as irritable bowel syndrome and IBD (Martínez et al., 2012; Belai et al., 1997).

Alternatively, changes in neurochemical coding of the ENS may be mediated by neuroactive substances secreted by intestinal microbes. A number of neurotransmitters, including serotonin, dopamine, GABA and acetylcholine, have been isolated from various microbial species that inhabit the intestinal tract (Lyte, 2011). Bacteria-derived neurotransmitters secreted into the intestinal lumen may diffuse across the intestinal

epithelial layer to stimulate receptors on enteric nerve fibers in the lamina propria and alter neuronal development. Penetration of the mucosal barrier by neuroactive molecules is far more likely to occur under physiological conditions than passage of whole bacteria. Delivery of neurochemicals to intrinsic and extrinsic nerve fibers in the gut has been proposed as a mechanism of action for the effects of probiotics on CNS activity (Lyte, 2011).

4.2.4 Indirect interactions with the ENS via the immune system

The observed differences in dopaminergic neuron numbers between GF and SPF mice may also be a result of indirect interactions between activated immune cells and the ENS. The immune system plays an integral role in the development of the nervous system. Cytokines affect the proliferation, survival, and phenotypic differentiation of neurons in the CNS during development (Zhao & Schwartz, 1998) and microglia, the resident macrophages of the CNS, are critically involved in shaping neuronal circuits in the postnatal brain (Schafer et al., 2012). Recent studies have shown that the immune system plays an equally important role in the development of the ENS. For example, Gougeon and colleagues (2013) found that the pro-inflammatory cytokines tumor necrosis factor-alpha (TNFα) and interleukin-1beta (IL-1β) promote neurite outgrowth in dissociated cocultures of enteric neurons, glia, and smooth muscle cells. Moreover, cytokines were suggested to contribute to the neurotrophic effects of breast milk on isolated rat myenteric neurons (Fletcher et al., 2011).

GF mice have many immunological defects, including underdeveloped Peyer's patches (lymphoid tissue of the intestinal tract) (Rhee et al., 2004), reduced numbers of regulatory and helper T cells (Round & Mazmanian, 2009), a dampened humoral response to antigens (Ohwaki et al., 1977), and altered cytokine profiles (Nicaise et al., 1993; Ikeda et al., 1999). Such immunological defects may contribute to the abnormal programming of the ENS in these mice reported here and elsewhere (Collins et al., 2013).

4.3 Limitations

Although the study of GF animals can yield valuable insight into the nature of our relationship with our bacterial symbionts, it carries with it several limitations that must be considered when interpreting the results. Chief among them is the inherent confound of the different prenatal environments in GF and SPF mice. In addition to a defective immune system, GF animals exhibit many abnormalities relative to their SPF counterparts, including altered neurological activity (Heijtz et al., 2011), reduced cardiac output (Wostmann et al., 1968), abnormal digestive functions (Husebye et al., 2001), and altered energy homeostasis (Musso et al., 2010). Such differences inevitably affect the prenatal environment of these animals. Indeed, GF mice have reduced litter sizes (Shinoda et al., 1980), which may indicate a less hospitable uterine environment in these animals. The prenatal environment is critical to ENS development, as evidenced by the ability of maternal diet to alter the neurochemical coding in pig offspring (De Quelen et al., 2011). Thus, the possibility that in utero differences contributed to the reduction in dopaminergic neuron densities in GF mice cannot be discounted. However, the fact that

dopaminergic neuron numbers in GF and SPF mice were comparable at P1 suggests that the differences seen at P7 and P28 are more likely attributable to differences in the postnatal environment. It also indicates that one day of expose to bacteria may not be sufficient to elicit major changes in enteric circuitry.

Another confounding variable in the study of GF mice is the delay in development that occurs in these animals. At one week of age, GF mice are less than two-thirds the weight of conventionally raised SPF mice, and this difference is maintained until four weeks of age (data not shown). In fact, GF mice require an extra week with their mother before they are big enough to be weaned. The difference in size between GF and SPF animals may account for the differences in total neuronal density seen at P28. As the animal grows, the enteric ganglia become increasingly spread out such that the number of neurons per area declines across development. Thus, the observation of increased neuronal density in the jejunum and colon of P28 GF mice relative to SPF mice may simply be a consequence of the growth retardation experienced by GF animals. Conversely, Anitha and colleagues (2012) found reduced numbers of neurons per field in the colon of GF mice at the same time-point. This discrepancy may be attributable to technical differences between protocols for preparing whole-mounts. Whereas Anitha et al. (2012) removed the circular muscle from the tissues to expose the underlying myenteric plexus, we left it intact. The presence of the circular muscle in our preparations may have occluded some neurons from view. This may have occurred to a greater extent in the larger SPF animals, which presumably have a thicker circular muscle layer, thus

accounting for the greater total neuron density observed in GF mice relative to SPF mice at P28. Further experiments should be conducted to resolve this discordance.

4.4 Future Directions

Despite the confounds associated with studies of GF animals, they represent an indispensible tool in dissecting the complex relationship between host and microbiota, and provide a useful starting point for more mechanistic and clinically relevant studies. Such experiments could involve examining the effects of probiotic or antibiotic use early in life on the development of the ENS and its associated functions in pre-colonized hosts. The use of gnotobiotic animals, which are colonized by a limited number of known microbial species, could help to determine which bacterial species are driving changes in intestinal physiology.

4.5 Conclusions

The results of the present study are consistent with the notion that enteric microbiota can influence the development of late-born neuronal populations. This observation can be added to the mounting evidence that the early microbial environment is critical to the development of the GI tract (Stappenbeck et al., 2002; Gaskins et al., 2008; Anitha et al., 2012; Collins et al., 2013). These findings carry important clinical implications, especially in light of the rising rates of digestive diseases linked to early intestinal dysbiosis (The Canadian Digestive Health Foundation, 2009). Further investigations are needed to uncover the means by which intestinal microbiota shape the

ENS. Ultimately, such studies may lead to the development of new preventative or therapeutic strategies to reduce the burden of GI disorders among children and adults.

APPENDIX

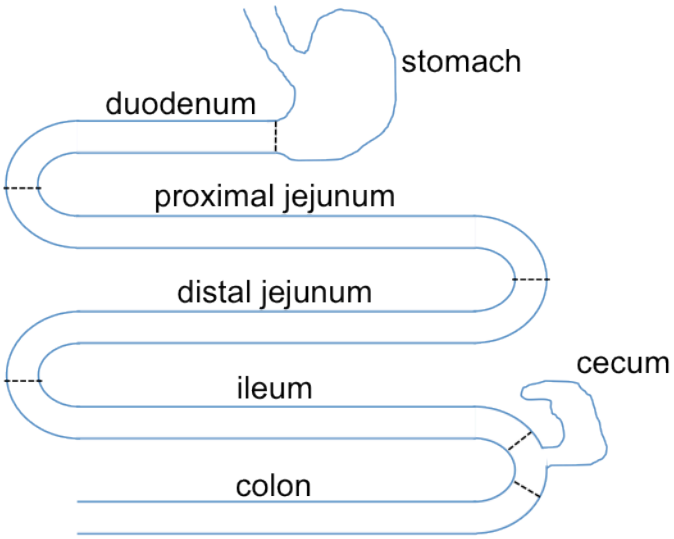

Figure 1. Diagram of the anatomical divisions of the GI tract as used in this paper, based upon Lefrançois and Vezys (2001).

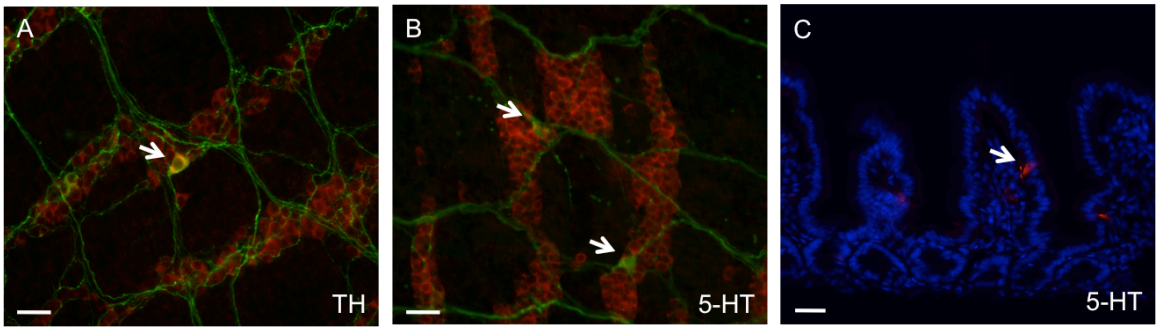

Figure 2. Representative photographs of immunofluorescence staining of intestinal whole-mounts and cross-sections from SPF animals. ***A,*** The myenteric plexus labeled with antibodies to HuC/D (red) and tyrosine hydroxylase (TH) (green). The arrow indicates a TH-positive neuronal cell body that was considered to be a dopaminergic neuron. ***B,*** The myenteric plexus labeled with antibodies to HuC/D (red) and 5-HT (green). Arrows indicate 5-HT-positive neuronal cell bodies. ***B,*** Cross-section of the ileum labeled with antibodies to 5-HT (red) and stained with the nuclear stain, bisbenzimide (blue). Arrow indicates an immunoreactive EC cell. *Scale bars,* 32 μm.

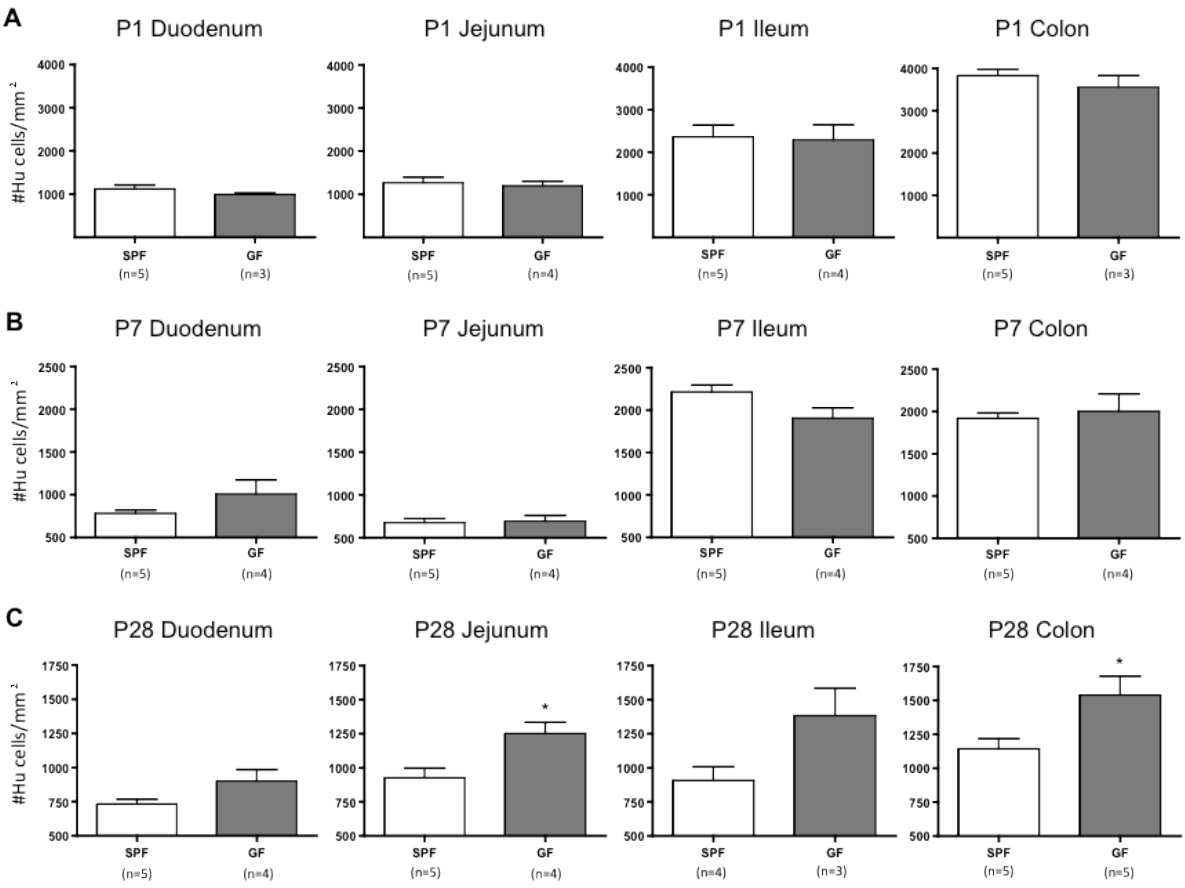

Figure 3. Total neuron density, expressed as the number of HuC/D-positive cell bodies per square millimeter of tissue, in SPF and GF mice at P1, P7, and P28. ***A,B,*** At P1 and P7, there was no significant difference in the number of HuC/D-immunoreactive cells per area between SPF and GF mice in any region of the intestine examined. ***C,*** At P28, however, the number of HuC/D-positive cells per area was significantly higher in both the jejunum and colon of GF mice relative to SPF mice (*$p < 0.05$).

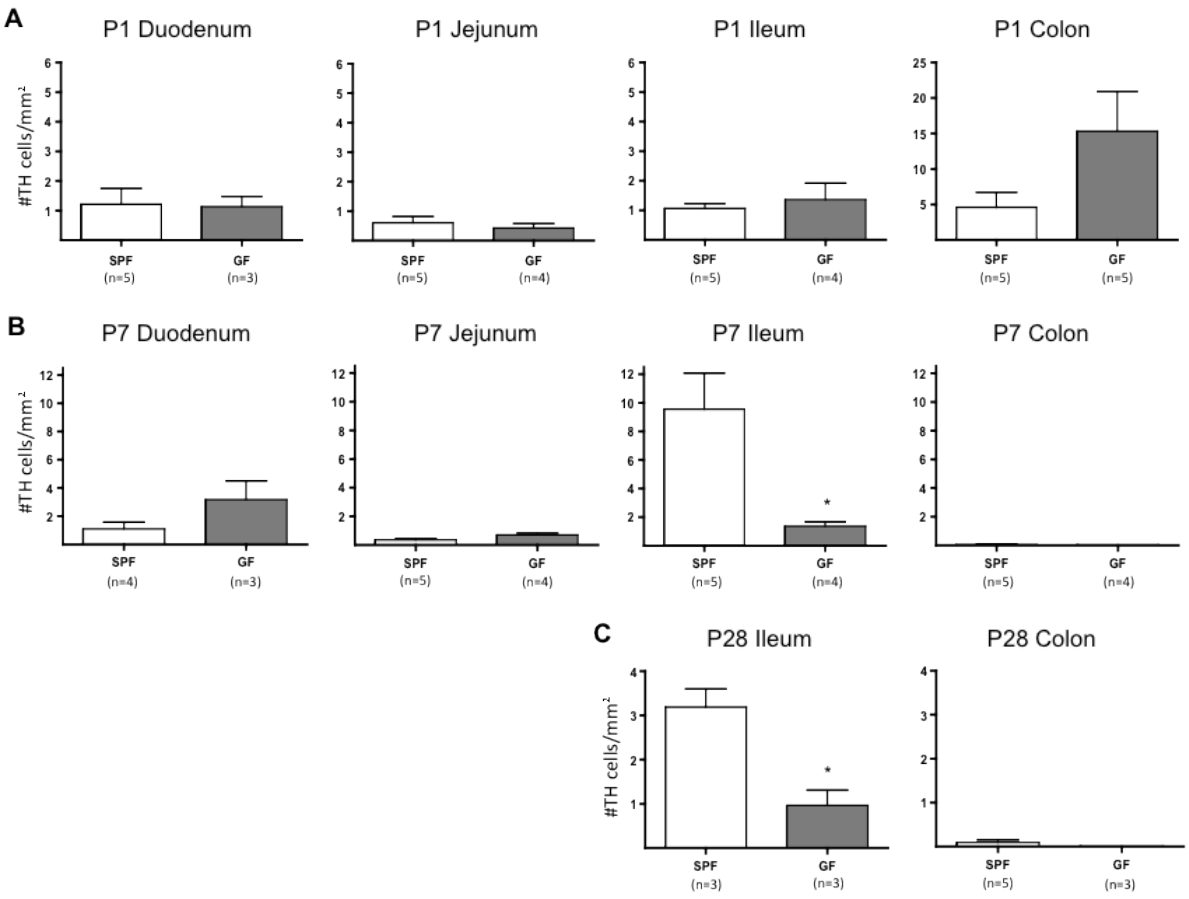

Figure 4. Number of tyrosine hydroxylase (TH)-immunoreactive cell bodies per square millimeter of tissue in SPF and GF mice. ***A,*** At P1, there was no significant difference in the number of TH-positive cells per area between SPF and GF mice in any region of the intestine examined. ***B,*** At P7, the number of TH-positive cells per square millimeter was comparable in SPF and GF mice in the duodenum, jejunum and large intestine, but was significantly decreased in the ileum of GF mice (*$p < 0.05$). ***C,*** Similarly, at P28, GF mice had significantly fewer TH-positive cells in the ileum than did SPF animals (*$p < 0.05$), with no significant difference in the colon at this time-point. TH-cell numbers were not quantified in the duodenum or jejunum of P28 animals

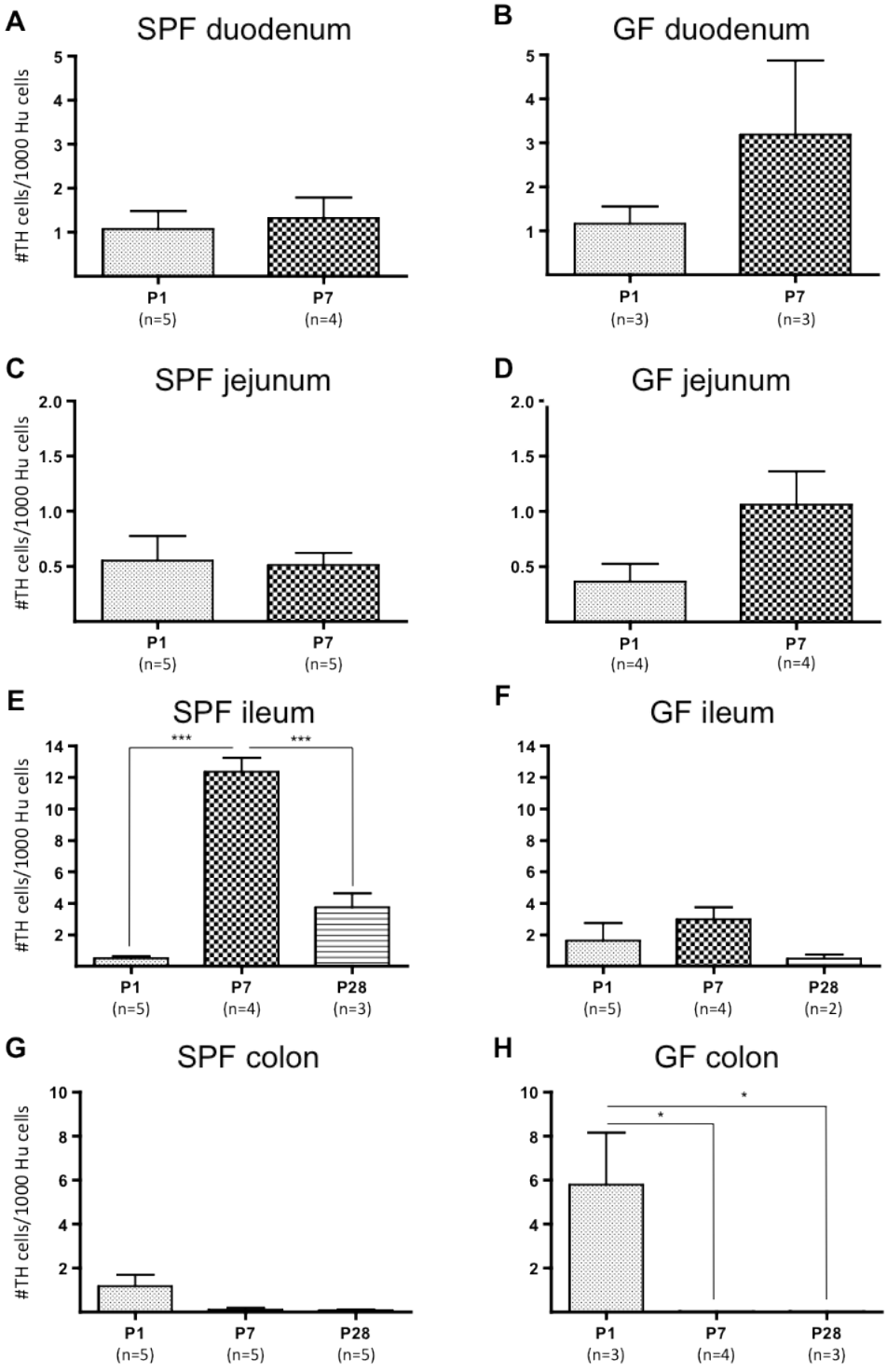
A
SPF duodenum
#TH cells/1000 Hu cells
P1 (n=5)
P7 (n=4)
B
GF duodenum
P1 (n=3)
P7 (n=3)
C
SPF jejunum
P1 (n=5)
P7 (n=5)
D
GF jejunum
P1 (n=4)
P7 (n=4)
E
SPF ileum
***
***
P1 (n=5)
P7 (n=4)
P28 (n=3)
F
GF ileum
P1 (n=5)
P7 (n=4)
P28 (n=2)
G
SPF colon
P1 (n=5)
P7 (n=5)
P28 (n=5)
H
GF colon
*
*
P1 (n=3)
P7 (n=4)
P28 (n=3)

Figure 5. Developmental regulation of myenteric dopaminergic neurons in the small and large intestines of GF and SPF mice. ***A,B,C,D,*** The proportion of tyrosine hydroxylase (TH)-positive neurons in the duodenums and jejunums of SPF and GF mice did not change significantly between P1 and P7. ***E,*** A spike in the proportion of TH-positive neurons was seen in the ileums of SPF mice between P1 and P7, followed by a significant decrease between P7 and P28 (***$p < 0.001$). ***F***, Neither of these effects was seen in the ileums of GF mice. ***G,H,*** Whereas the proportion of TH-positive neurons in the colons of SPF mice remained stable across the first four weeks of life, a significant decrease was seen in the GF colons between P1 and P7. This effect persisted at P28 (*$p < 0.05$).

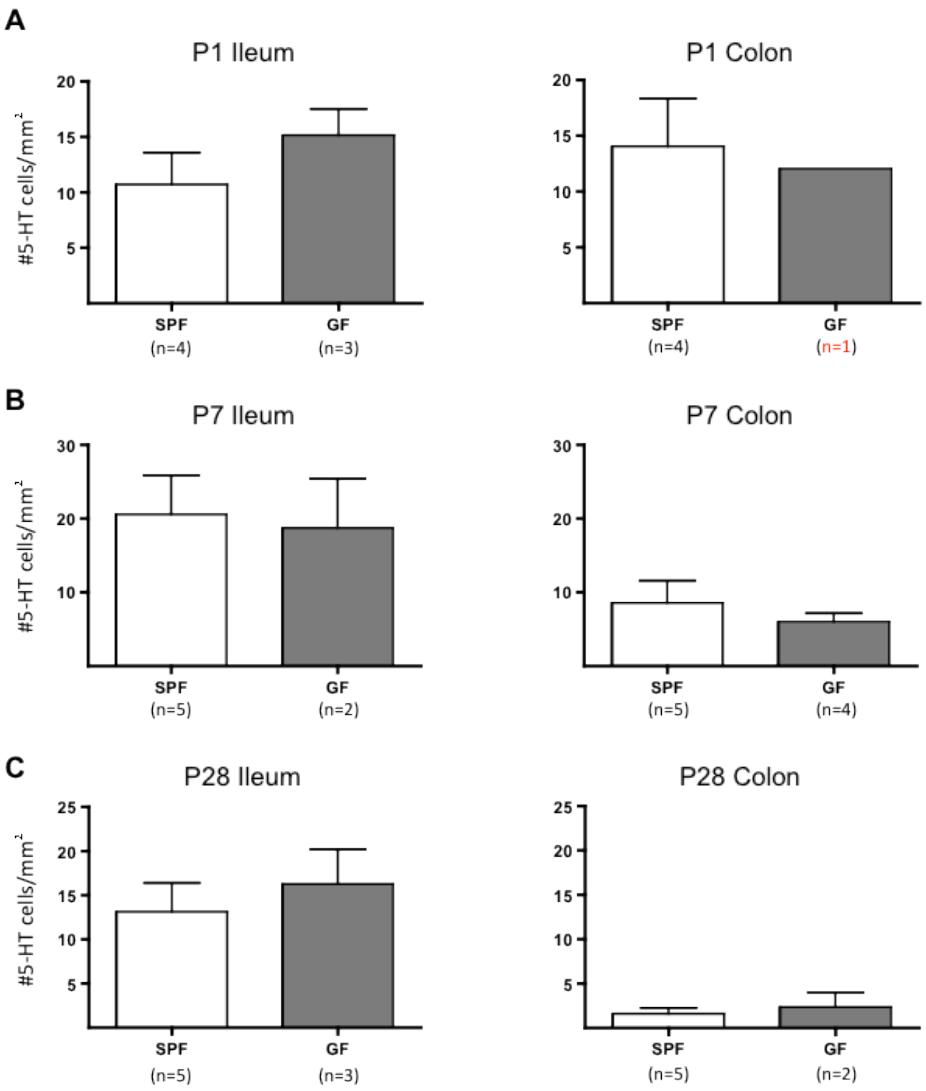

Figure 6. Number of serotonin-immunoreactive neurons per square millimeter of tissue in the ileum and colon of SPF and GF mice. ***A,B,C,*** There was no significant difference in the number of 5-HT-positive neurons per area between SPF and GF mice at any time-point examined.

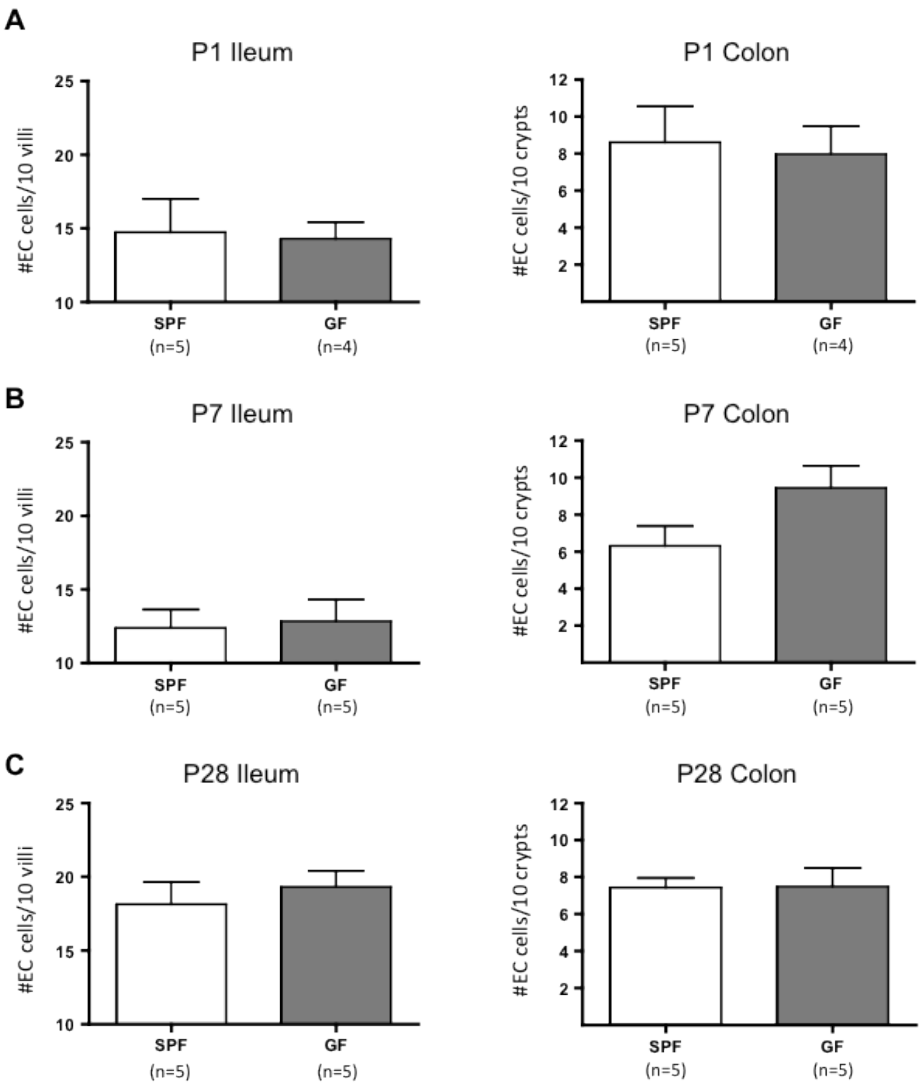

Figure 7. Enterochromaffin (EC) cell frequencies in the ileum and colon of SPF and GF mice at P1, P7, and P28. ***A,B,C,*** The numbers of 5-HT-positive cells per 10 villus-crypt units (ileum) and 10 crpyts (colon) did not differ significantly between SPF and GF mice at any time-point examined.

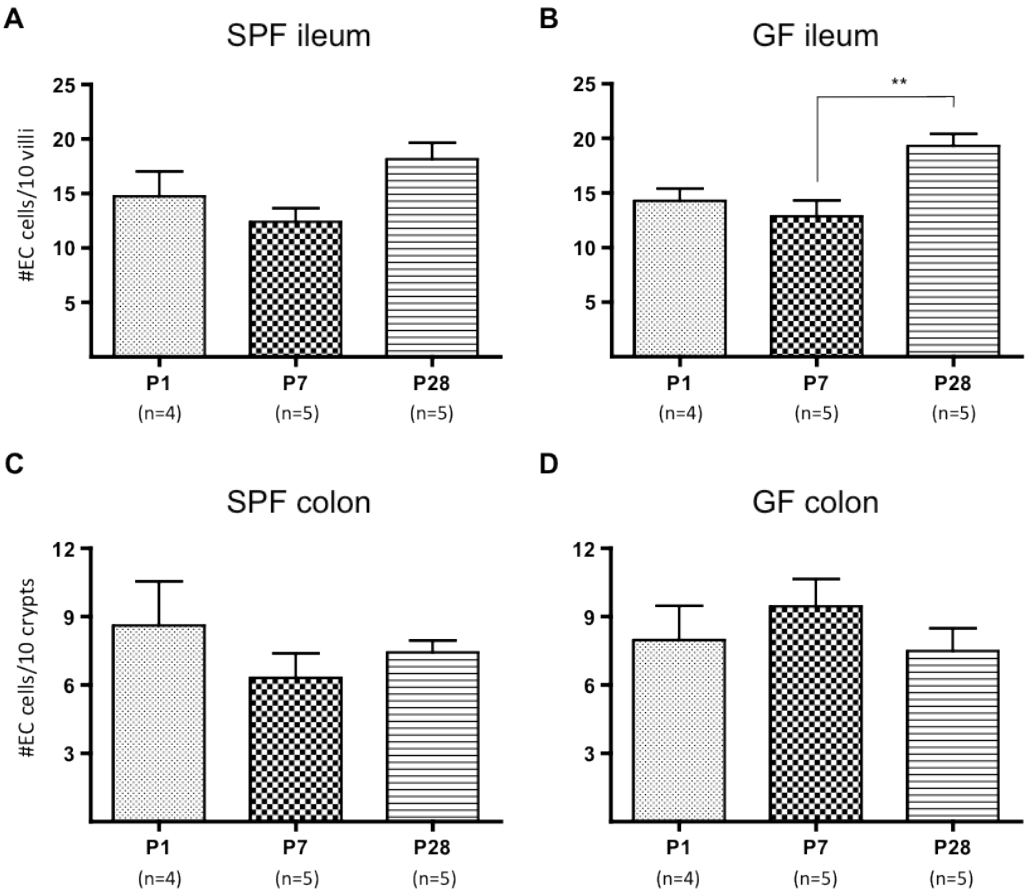

Figure 8. Developmental regulation of enterochromaffin (EC) cells in the ileum and colon of GF and SPF mice. ***A,C,*** The number of EC cells per 10 villus-crypt units and 10 crypts in the ileum and colon, respectively, of SPF mice remained stable across the first four weeks of postnatal life. ***B***, A significant increase in EC cell numbers was observed in the ileums of GF mice between P7 and P28 (**$p < 0.01$). ***D,*** EC cell numbers in the GF colon did not differ significantly across the three time-points examined.

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