THE UNIVERSITY OF CHICAGO

MICROBIOTA-DEPENDENT EARLY LIFE PROGRAMMING OF GASTROINTESTINAL MOTILITY

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES

AND THE PRITZKER SCHOOL OF MEDICINE

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

INTERDISCIPLINARY SCIENTIST TRAINING PROGRAM:

MOLECULAR METABOLISM AND NUTRITION

BY

MARY ELEANOR FRITH

CHICAGO, ILLINOIS

DECEMBER 2023

To my parents.

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ACKNOWLEDGEMENTS

As PhDs tend to be, mine has been challenging. As I take a step back, though, I realize that I would have it no other way – I have always embraced challenges and have even been attracted to them. I think there is sometimes a common feeling among PhD students, while in the depths of the PhD, to feel like an island – that we, having adopted as our own the study of an unexpectedly complex research question, are on our own to figure it out.

However, while it is true that we have taken on significant intellectual responsibilities that we individually must address (i.e., the thesis) – we are not an island. We are "standing on the shoulders of giants" and truly there are so many giants who have elevated us to wherever we are today: our families who raise and support us, teachers who educate and inspire us, friends who walk alongside us, mentors who lead the way, and community members – scientific and otherwise, past and present.

Within this framework, I do have specific people to thank, in no specific order; "importance" is impossible to quantify here.

I'd like to thank my PhD mentor, Eugene (Gene) Chang. When I had rotated through other labs early in grad school and hadn't quite found a good fit, he welcomed me into his. Then, and throughout grad school, he allowed me to pursue my intellectual interests while keeping me rooted to the pursuit of making a significant impact on human health. (As he often says, in the Chang lab, "we don't do incremental science.") Thankfully – oh so very thankfully – Gene is not a "helicopter PI", meaning that his advisory style is not to hover over and critique trainees on every move. Rather, he respects us as scientists capable of independent thought, and he guides and inspires us along the road to discovery, providing the collaborative and material resources to pave the way. I

know that as I leave the PhD phase, I haven't left Gene's lab. We have more work ahead as there are several papers' worth of material still to publish – and I look forward to continuing working with Gene to share more of our work with the world.

I'd like to thank our collaborator Purna Kashyap, who hosted me in his lab at Mayo Clinic twice so I could learn essential experimental methods including transit testing and manometry. As a member of the Mayo Clinic Enteric Neuroscience Program, he has connected me with other brilliant scientists in the field. I am thankful to have had the opportunity to co-author a review paper about a clinical roadmap for microbiome research for functional bowel disorders.

The many past and present members of the Chang lab have also been essential to the success of this project, especially Katya Frazier, Vanessa Leone, Candace Cham, Jason Koval, Ashley Sidebottom, Orlando DeLeon, Marissa St. George, and Fran Vukovich.

I'd like to thank the Medical Scientists Training Program administrators and admissions committee for believing in my potential and supporting me along this path, particularly Marcus Clark, Alison Anastasio, Kristin McCann, Marisa Davis, and Shanetha Thomas.

I'd like to thank my thesis committee members, Matthew Brady, Xiaoxi Zhuang, and Erika Claud for their generosity of time, thoughtfulness, patience, and insights they have each shared with me.

There are so many others to thank at the University of Chicago and beyond, including Narayanan "Bobby" Kasthuri, Vandana Sampathkumar, Sebastian Pott, Oni Basu, Dinanath Sulakhe, Bingqing Xie, Mark D'Souza, Peggy Mason, Yogesh Bhattarai, and David Linden.

I would like to thank the Molecular Metabolism and Nutrition program for adopting me as one of their own. Matthew Brady especially deserves my thanks for his support as chair of the Metabolism program and his investment in my success. The past and present students of the

Metabolism Program have supported me along this journey as well, especially Katya Frazier, Avelino DeLeon, Jin Li, Sandra McClure, and Althea Bock-Hughes.

Many other friends and peers in the MSTP and BSD more broadly also merit thanks for their ongoing friendship and support, including Megan Kennedy, Joash Lake, Anthony Hu, Sarah Sun, Andrew Wang, Alexis Monical, Hendrik Glauninger, and many others.

I'd like to thank the staff of several UChicago core facilities that have enabled the present work, particularly the staff of the Light Microscopy Core Facility, Functional Genomics Core Facility, Gnotobiotic facility, and Animal Resources Center.

Of course, none of this would have been possible without my parents, each of whom have been and continue to be some of my top role models. I cannot thank them enough for their patience, love, care, respect, work ethic, generosity, and support that is beyond quantification. I am thankful to my siblings for their support as well; collectively, they have a way of making me laugh that is truly unmatched. My extended family has also provided invaluable support in raising me and to this day.

I want to thank my partner Donald Rodriguez, whom I am so grateful to have met here at UChicago 6 years ago. His companionship, love, and support have been a light throughout graduate school.

Finally, I want to thank my undergraduate research mentor, Dr. Julio Ramirez, for going out of his way to support me and cultivate my interest in scientific research when I was still on the fence. Without his guidance I would most likely not have chosen to pursue an MD/PhD.

There are many others not mentioned here that have helped me reach this point and to all I am grateful.

ABSTRACT

Much remains unknown about the role of gut microbes in the development of a functional enteric nervous system (ENS), a network of neurons and glia within the gastrointestinal tract (GI) that is fundamental to motility. We hypothesized that that many cases of chronic dysmotility in adults result in part from disturbances to gut microbes during a critical window of development—more generally, that microbial stimuli influence ENS development and thus long-term GI motility. In this study, microbe-lacking ("germ-free", GF) mice, which are known to have extremely slow GI transit, were given fecal microbiota transplantation (FMT) at weaning or as adults. We found that only the mice given FMT at weaning appeared to achieve normal overall transit, while those given FMT as adults failed to regain normal transit, showing only limited improvements. Providing clues into the mechanistic underpinnings of these functional differences, RNAseq of colonic muscularis propria revealed enrichments in neuron developmental pathways in mice exposed to gut microbes earlier in life, while mice exposed later – or not at all – showed exaggerated expression of inflammatory immune pathways. These findings highlight a microbiota-dependent sensitive period in ENS development, pointing to potential roles of the early life microbiome in later life dysmotility.

INTRODUCTION

Microbiota and human health

In humans and other animals, the gastrointestinal (GI) tract contains microbes that coexist with the host and collectively exert profound influence on development growth (Ronan, Yeasin, and Claud 2021). Known as the microbiota, this biological community carries out many key functions in the GI tract; these include digestion of fibers that cannot be broken down by the host, protection from pathogens and pathobionts through immune training and interspecies competition, and production of metabolites such as short chain fatty acids that nourish the epithelial cells lining the intestinal wall (Jandhyala et al. 2015).

Both the host and microbiota are shaped by the characteristics of the other. As such, functionally distinct regions of the GI tract have distinct microbial populations. This mutual influence is based in ecology; membership within a biological community requires that the conditions and resources within that community can sustain life for its community members. The conditions and resources within the GI tract that are relevant to microbes include: the nutrients available in an area, for example, dietary fibers that are digested specifically within the colon; the presence of oxygen, as some regions, such as the colon, are anaerobic; and potential threats to inhabiting organisms such as a highly acidic environment in the stomach. Therefore, microbes carry out their functions (such as fermentation of dietary fibers) because they benefit from being in that environment (nutrient availability) and because they can survive in that environment. Microbes can survive in that environment when the host (the organism providing the habitat for microbes) either benefits from or is unaffected by the presence of the inhabiting microbes.

Otherwise, the optimal habitat may be short-lived. As such, the composition of the gut microbiome depends both on microbial and host characteristics.

The bidirectional relationship between microbes and host has two notable implications. First, it suggests that studying the gut microbiota can yield insights about human health; second, that changes to the gut microbiota by external factors (diet, antibiotics, environment) may affect, or provide a means to improve, human health.

Given that the GI tract is the body region most intimately associated with gut microbes, it makes sense to study the GI tract when considering the effect of the microbiome on host health. After all, the GI tract is required for digestion, nutrient absorption, and removal of waste from the body, all critical functions for the host. As with any critical biological functions, problems can occur.

Motility disorders and the microbiome

Among the most common GI problems are motility disorders (also called functional bowel disorders, FBD). Gastrointestinal motility refers to the movements of the intestines. These movements facilitate churning of food for digestion and propulsion of intestinal contents along the GI tract. In FBD, these movements may be associated with pain, constipation, and/or diarrhea.

Irritable bowel syndrome (IBS), the most common FBD, affects up to 1 in 5 people in the United States (U.S.) and cost the U.S. an estimated $30 billion in 2003 (Leong et al. 2003). IBS has significant quality of life implications. One can imagine the impact that unpredictable bowel responses, particularly in the setting of food consumption, might have on a person's comfort with engaging in typical social activities. Chronic abdominal pain would also be expected to take a

psychological toll on the patient with IBS. This may contribute to the high levels of comorbidity between IBS and mental health conditions including depression and anxiety.

Given the absence of a singular diagnostic test for IBS and the chronic nature of the disorder (there is no cure), IBS is associated with recurrent healthcare visits. It is the most common reason for referral to a gastroenterologist (Thompson et al. 2000). As such, IBS poses a major economic burden to the U.S. health care system.

Enteric nervous system

Fundamental to the control of GI motility is the enteric nervous system (ENS), a network of neurons and glia within the gastrointestinal tract. In a well-functioning GI tract, enteric neurons monitor levels of nutrients, wastes, and bacterial products, directing a coordinated program of contraction and secretion. The ENS is semi-autonomous, meaning that it can continue to function without external neuronal input from the brain, spinal cord, and gut-extrinsic regions of the peripheral nervous system – yet ENS function can be modulated by input from these regions. The ENS also send signals from within the gut to these regions, for example, to communicate intestinal stretch for satiety and in the context of abdominal pain. A congenital lack of colonic enteric neurons, as seen in Hirschsprung disease, can be fatal without prompt surgical intervention.

Given its critical role in motility, the ENS has been implicated in other motility disorders, including IBS. While it is though that there is neuronal dysfunction in these disorders, the nature of this dysfunction is unclear.

The ENS has been called the “second brain” on account of its complexity and interactions with multiple other systems of the body as well as the environment, as well as its semiautonomous

nature. As mentioned previously, the ENS is able to continue to function and stimulate contractions of smooth muscle when separated from the brain. Like the brain, much remains to be discovered about the ENS.

Interactions between the ENS and microbiome

While knowledge of interactions between the ENS and microbiome is limited, there are a few ways in which such interactions are known to occur. First, microbes produce metabolites that can activate enteric neuron terminals directly if those metabolites are taken up in the bloodstream or diffuse across the epithelial barrier. It has been reported that certain bacteria produce metabolites that have the same structure as certain neurotransmitters, including GABA, acetylcholine, norepinephrine, dopamine, and serotonin (Strandwitz 2018). Another way in microbial signals can act on enteric neurons is indirectly through enterochromaffin (EC) cells, specialized epithelial cells that release large amounts of serotonin in response to stimulation by microbial products, such as by the short chain fatty acid (SCFA) butyrate. The serotonin released from EC cells can then act on serotonin receptors on enteric neuron terminals (Rezzani et al. 2022). A third mechanism of ENS-microbiome interaction is indirectly through the immune system. Microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) are recognized by innate immune cells such as macrophages, which respond by releasing cytokines and other products locally and into the bloodstream. These products can activate enteric neurons through cytokine receptors, such as CSF1R (Muller et al. 2014; H. Wang et al. 2022). Additionally, microbial metabolites, serotonin from EC cells, and immune products may activate vagal or other gut-extrinsic neuronal afferents

– or even cross the blood brain barrier – all of which may ultimately modulate enteric neuron function.

Highlighting the importance of the microbiome for gastrointestinal function, mice lacking gut microbes have extremely slow gastrointestinal motility. These so-called “germ-free” (GF) mice are raised in conditions that have no microbes. Used in microbiome research, GF mice live in isolators with a HEPA air filtration system. A special animal care staff handles the animals through build-in glove sleeves in the sides of the isolators.

Another well known problem with GF mice is their greatly enlarged ceca. Cecum weight can be ~3g for a GF mouse, while in SPF mice, the cecum weight averages around 0.6g (Hoces et al. 2022). Proposed explanations for the enlarged GF cecum include an excessive load of mucus, undigested material, and water; a decrease in cecal muscle tone; and a lower metabolism by myenteric neurons in part of the cecum. Related to the ENS, studies have reported a reduction in enteric neurons excitability in GF mice (McVey Neufeld et al. 2013; 2015).

Germ-free mice are useful for studying the microbiome because they provide a means for comparison between the presence and absence of microbes in a mammal and the effect of each of those on the health of the animal. GF mice also allow for mono- or co-colonization experiments to study the effect of one microbial strain or a defined consortium of microbes. While antibiotic treatment of mice greatly reduces the microbial load, the antibiotic treatment may have side effects that confound the experimental results. Additionally, antibiotic treatment does not eliminate all microbes, so it is not as “clean” of an experimental design. Nonetheless, antibiotic treatment, in contrast to complete absence of microbes, may be more representative of the conditions affecting humans.

Another method of manipulating the microbiome is through dietary changes, as microbial composition is greatly influenced by diet. As with antibiotic treatment, though, these experiments are limited in their ability to isolate the effect of microbes versus the effects of other potential confounders such as nutritional effects.

Nonetheless, even when using GF mice, there are caveats when interpreting results as a direct effect of the microbiome or specific microbial groups. Given the wide ranging effects of the microbiome on multiple organ systems as well as developmental processes, the differences observed in GF versus conventional mice in one specific tissue or organ system must be considered in the broader context of the animal's complete lack of microbes. Because GF mice must be housed under different conditions than conventional research animals, these environmental differences should be considered as well. Furthermore, although it is possible to study mice of the same strain under both GF and specific pathogen free (SPF) conditions, the inbreeding of the same mouse colonies over time can lead to genetic drift within the colony. This could be through random mutations or through inherited epigenetic changes affecting gene expression.

Critical periods in nervous system development

It is well documented that there are critical periods in nervous system development, best studied in the brain. Classic examples include the development of the visual system, development of which depends on visual stimuli during the first few weeks of life in mice and cats – an analogous developmental timeline has been observed in humans as well (Berardi, Pizzorusso, and Maffei 2000). Another example is the development of higher cognitive function which depends on social interactions during adolescence (Larsen and Luna 2018). The recurring theme with such

critical periods is that certain stimuli must be received during a specific stage of development; otherwise, the developmental window closes as subsequent developmental milestones are reached as the metaphorical house is built on a compromised foundation. The mechanism of critical period closure is thought to relate to formation of extracellular matrix structures called perineuronal nets around specific neurons and their projections, thereby acting as a physical constraint to the formation of new connections (Sigal et al. 2019). Another process that may contribute to critical period closure is the pruning of synapses deemed unnecessary due to lack of use (physiologically, as weaker synapses containing less recruitment of AMPA receptors to synapses which occurs when NMDA receptors are activated repeatedly upon stimulation) (Z. Zhang, Peterson, and Liu 2013; Piochon, Kano, and Hansel 2016). Once the nervous system has reached the new 'normal' levels of synapses as occurs in adulthood, the synapses that would have been stimulated by the earlier stimulus (e.g., visual patterns of a certain orientation) are no longer available. Mechanisms of critical period closure in the brain are postulated in some cases to relate to the differentiation potential of neuron progenitors through m6A modifications affecting pro-neural gene expression (Donega et al. 2018).

One study found that the expression of cAMP-induced transcription factors differed between mice with different early life stress and altered maternal handling associated with epigenetic changes to NGFI-A & AP-2 (Meaney et al. 2000). However, these measures were taken just 7 days after the conclusion of the handling period, so longer term effects were not reported.

Nonetheless, another study that included adult data showed similar results (O'Donnell et al. 1994). Additionally, they say that serotonin (5-HT) has been implicated due to immediate effects of handling on pups, but that 5-HT and 5-HT receptor (5-HTR) expression in adults is not

increased after handling. Additionally, 5-HT lesion in adult mice does not seem to affect GR density, so the mechanism remains unclear. One possibility is altered input to hippocampus. Authors speculate that handling affects the development of another neuronal system which then contributes to increased GR density (Berens, Jensen, and Nelson 2017).

Relating to manipulations during the adolescent period, another study discusses methylation changes in the GR gene (*Nr3c1*) promoter after childhood trauma, having a dose dependent effect (Perroud et al. 2011). Conceptually, this makes sense, as childhood trauma would have been highly stressful, increasing cortisol and affecting the expression of its receptor.

One study showed methylation changes for NT genes in adolescents who experienced early life adversity at a younger age. Another study showed reduced dendritic arborization throughout the brain in rodent early deprivation models. Further, a study showing hyperreactivity along the hypothalamic-pituitary-adrenal (HPA) axis still in adulthood after early life adversity suggested there was potential resistance to glucocorticoid GC feedback, leading to hyporeactivity. Other changes were altered GC receptors (GR) and other receptor types, hypermethylation of GR promoter in the hippocampus and in lymphocytes, GR sensitization & distribution suggestive of an inflammatory state. Maternal separation of rodents leads to later inflammatory reactivity and dysbiosis in the gut. In primates, dysbiosis and maternal separation also predicted later life immune dysfunction (Berens, Jensen, and Nelson 2017).

There is some data on molecular mechanisms of critical changes in the brain during adolescence. However, it is important to keep in mind that neuronal changes in the gut would not necessarily match those in the brain (by neurotransmitter (NT) type, etc.), as the neurons responding to emotional stimuli may differ in properties to those responding to gut microbes. In a

rat model of adolescent binge drinking, adults had lower levels of serotonin transporters (Crews, He, and Hodge 2007; Monti et al. 2005). Rats that were reared in darkness from birth until adulthood had impaired GABAergic signaling to the visual cortex (Morales, Choi, and Kirkwood 2002). Mouse "adolescence" is defined by some authors as the time between P25-P42+ (Crews, He, and Hodge 2007), which corresponds to shortly after weaning. Weaning generally occurs in mice between 3 and 4 weeks (w) of age.

ENS development

Most current knowledge about ENS development relates to the embryonic and prenatal period. During the prenatal period, different embryonic regions give rise to the beginnings of the nervous system divisions in the body. The bordering regions of the neural plate connect to become the neural crest, which gives rise to most of the neurons and glia of the peripheral nervous system (including the ENS), while the remaining portion of the neural plate becomes the neural tube, giving rise to the brain and spinal cord (Butler and Bronner 2015). Other cell types arise from these structures as well, such as melanocytes from the neural crest (Marmigère and Ernfors 2007).

The ENS is primarily derived from the embryonic neural crest. During embryonic development, neural crest stem cells migrate from the vagal and a portion of the sacral region of the neural tube to colonize the intestine as neural precursor cells that then terminally differentiate into mature neurons as they reach their target (Nagy and Goldstein 2017).

The Hedgehog (Hh) pathway is critical for this migratory process as one of its effects is to maintain the stemness of the migrating neural crest cells and prevent them from terminally differentiating before reaching far enough along the intestine (Nagy and Goldstein 2017). As such,

the Hh pathway also prevents hyperganglionosis in proximal regions of the intestine. Indeed, inhibition of sonic hedgehog (Shh) protein with cyclopamine in culture chick intestine results in the formation of ectopic, enlarged enteric ganglia, while Shh overexpression results in aganglionosis (Nagy et al. 2016). This failure to develop enteric neurons in the colon also occurs in Hirschsprung disease (HD). While HD is thought to involve many genes, some of the genes associated with HD risk are part of or interact with the Hh pathway (Nagy and Goldstein 2017).

What then is the role of the Hh pathway once the intestine is fully colonized with enteric neurons? The Hh pathway continues to regulate neuronal differentiation from neural precursor cells in the adult intestine, although the extent to which neuronal differentiation occurs in the adult intestine is currently subject to much debate (Kulkarni et al. 2017). At a minimum, however, there is a limited amount of neurogenesis in the adult intestine (Liu et al. 2009; Joseph et al. 2011; Laranjeira et al. 2011). The Hh pathway also regulates synapse formation and pathfinding by axons (Fuccillo, Joyner, and Fishell 2006; Hill et al. 2019).

What about the role of the Hh pathway after the embryonic period, but before full maturation – for example during adolescence? Less is known about the Hh pathway in ENS development in its later stages, that is, during postnatal ENS development including the period of sexual maturation or "adolescence" (this period is not typically referred to as adolescence in mice as it is in humans). However, given the known involvement of the Hh pathway in synapse formation and organization, it seems likely that the Hh pathway would be important in the process of synaptic refinement, which occurs during postnatal development, peaking around or after weaning in mice (Zeiss 2021). Indeed, evidence from brain research in this area does point to a role of the Hh pathway in synaptic refinement (Hill et al. 2019; Fuccillo, Joyner, and Fishell 2006).

Synaptic refinement, also called synaptic pruning, is the process of removing synapses that the nervous system deems as unnecessary, largely based on the extent to which they are used. This is typically thought to be mediated by microglia and astrocytes in the brain [27,28](Hill et al. 2019; Geloso and D'Ambrosi 2021; Lim and Ruthazer 2021), so this may be a role of enteric glia and potentially tissue resident macrophages, since microglia are both glia and the tissue resident macrophages of the brain. Removal of unneeded synapses is beneficial because it reduces "noisy" or off-target signaling and allows for the important signals to reach their targets with less interference (Mimura, Kimoto, and Okada 2003). However, this also implies that neurons that are not stimulated according to the typical developmental timeline before synaptic refinement may lose the opportunity to establish certain connections – such as in the example of the visual system and the ability to perceive visual stimuli such as lines of a certain orientation (Uesaka, Nagashimada, and Enomoto 2015).

The probable glia-mediated role of the Hh pathway in ENS synaptic refinement during maturation (Hill et al. 2019) (that is, if the ENS is analogous to the CNS in this aspect of Hh signaling) also may relate to the nature of signals received from the gut microbiota. Recent evidence shows that the composition of the gut microbiota can activate developmental pathways through the Hh pathway (Hu et al. 2023). Considering the evidence for a potential microbiome-dependent critical period during ENS development as discussed above, it points to the Hh pathway as a potential mechanistic component, were such a critical period in ENS development to exist.

To study potential critical periods in postnatal ENS development, it may be instrumental to understand the trajectory of ENS development up the time of birth. In PNS neural crest specification, NCCs delaminate from the dorsal neural tube and follow the ventral path to produce

cells of the dorsal root ganglion (DRG) ("sensory" fate) or the sympathetic and enteric NSs ("autonomic" fate) or follow dorsolateral pathway to become melanocytes (skin) (Marmigère and Ernfors 2007). The cells that make the DRG then differentiate into sensory subtypes including mechanoreceptor, nociceptor, and proprioceptive.

The neural crest (NC) arises from the edges of the neural folds between the ectoderm and neural plate. Induction to NC from ectoderm and neural plate involves BMP and Wnt signaling. BMP is necessary for maintaining Wnt expression. Wnt factors promote the differentiation of cells at the edges of the neural folds to become neural crest cells (NCCs); here, the cells undergo an epithelial to mesenchymal transition into NCCs which involves cytoskeletal reorganization, downregulation of cadherin 6 and N cadherin, and changing of the cytoskeletal properties to become more motile. NCCs leave the neural tube as the basal lamina of the neural tube dissolves- the cells migrate to form the DRG in a ventral to dorsal pattern (Nagy and Goldstein 2017).

Neural crest cells differentiate to become precursor cells of several cell types, including melanocytes, neurons, and glia. Among these glia precursor cells are Schwann cell precursors (SCPs) (Adameyko et al. 2009), which can also differentiate into melanocytes, enteric neurons, and glia (Zirlinger et al. 2002). In vertebrates, migration occurs in 3 waves (Marmigère and Ernfors 2007). In zebrafish ENS, there are two phases of HH pathway function- first to migrate to anterior intestine ("pre-enteric phase") and then to migrate along intestine (Reichenbach et al. 2008).

ENS organization must be polarized to allow for propulsion of contents through peristalsis (Nagy and Goldstein 2017). That is, areas of the intestine that precede the bolus must contract, and areas after the bolus must relax to propel contents anally. The areas preceding the bolus receive signals from the ascending pathways originating from the neurons sensing the intestinal stretch,

while the areas after the bolus receive signals via the descending pathways originating near the bolus. This phenomenon is sometimes referred to as the "law of the intestine" (Bayliss and Starling 1899; Nagy and Goldstein 2017).

ENS is largest branch of ANS. Studies largely from chick have established the primary source for ENS to be the vagal neural crest (NC) with some contribution from the sacral neural crest. Neural crest cells (NCCs) migrate in chains to their final destination in the gut. As the NCCs migrate, exposure to retinoic acid (RA) is important for the commitment to EN differentiation. RA acts on retinoic acid receptors alpha, alpha2 and gamma and activates the expression of Ret, a tyrosine kinase that is critical for enteric neuron generation. A smaller contribution of NCCs to ENS is (most is from vagal NC) is sacral NC. Another group is Schwann cell precursors that have been reported to migrate along extrinsic nerve fibers to midgut via the mesentery, to avoid the cecum and cross over to the hindgut - 'transmesenteric' migration - and these may form the majority of the ENS population in the distal ⅔ of the hindgut.

According to one study, about 20 percent of colonic neurons are derived from Schwann cell precursors that migrate along the pelvic nerve to the ENS (Uesaka, Nagashimada, and Enomoto 2015). This study notes the importance of the processes of proliferation and migration and apoptosis in the proper organization of the ENS, involving MAPK, JNK, Ras and PI3K pathways. It is important that a sufficient pool of migrating cells is available to make it to the distal regions of the gut and to not differentiate too soon. Migration occurs best in chain like structures requiring cell cell contact so adhesion between cells is important for efficient migration and migration in sufficient numbers.

There is crosstalk between Wnt and Hh pathways (Ding and Wang 2017). *Sfrp1* is a key intermediary in that *Gli1/2* of the Hh pathway stimulates *Sfrp1* which negatively regulates Wnt pathway progression.

Hh signaling prevents migration of ENs outside of its designated area within the gut wall (Jin et al. 2015). Shh has been reported to enhance axon outgrowth & glutamate release from presynaptic terminals, and also to contribute to neuronal stem cell proliferation and differentiation (Yao, Petralia, and Mattson 2016).

Genes related to neural crest that are expressed in adults may reflect the "adult neural crest", that is, stem cell niches that are distributed across tissues (Mehrotra et al. 2020).

Hedgehog signaling takes place within the primary cilium, a projection from the cell body that is found on all cells. There is a pinwheel architecture of neural stem cell niches in adult mice; these niches are comprised of two types of ependymal cells (multiciliated and containing 2 cilia) along with neural stem cells (containing 1 short cilium) (Mirzadeh et al. 2008). The morphology of the primary cilium changes in neural precursor cells across different mitotic states (Matsumoto et al. 2019).

Another critical part of nervous system development is synaptic pruning. In the postweaning brain, phagocytic cells including microglia (tissue resident macrophages) and astrocytes eliminate synapses that are determined to be unnecessary (Neniskyte and Gross 2017).

Normal physiology of the adult ENS

Most cells of the ENS are in the myenteric plexus, which is between the circular and longitudinal muscle layers in the intestine. The myenteric plexus is the primary entity that senses

intestinal stretch and stimulates smooth muscle contractions, critical to motility. The submucosal plexus, located beneath the epithelial layer and above the muscularis propria, has fewer enteric neurons than the myenteric plexus but is important for secretomotor and sensory function and for communicating between the epithelium and muscle layers. While submucosal neurons have more control over chloride flux from crypt cells than myenteric neurons do, myenteric secretomotor neurons may be involved in secretion in some cases, such as during cholera infection (Cooke 1998). Each of these two major plexuses contain projections to the other as well as directly to the epithelium (Cooke 1998) or even out of the intestine to peripheral structures (for example, to communicate with other parts of the nervous system).

Very broadly, the two major classes of enteric neurons are excitatory and inhibitory. Most excitatory neurons use the neurotransmitter acetylcholine (ACh), while most inhibitory neurons use nitric oxide. The typical markers for cells that use these transmitters are choline acetyltransferase (ChAT or *Chat*) for cholinergic neurons and neuronal nitric oxide synthase (nNOS or *Nos1*) for nitrergic neurons.

According to one study in human colon, the neurochemical identity distribution of enteric neurons is as follows: ~48% nNOS+/ChAT-, ~43% nNOS-/ChAT+, ~4% nNOS+/ChAT+, ~5% nNOS-/ChAT- (Murphy et al. 2007). Another study in humans but specifically regarding colonic circular muscle motor neurons identified with retrograde tracing, the neurochemical and morphological identities were reported as follows (Porter et al. 1997): ~45-48% nNOS+; ~29% nNOS+/VIP-; ~19% nNOS+/VIP+; ~51% ChAT+. These cells had almost exclusively Dogiel Type I morphology (i.e. had a single long process). The cell bodies of nNOS+ neurons were generally larger than those of ChAT+, and the nNOS+ neurons had longer projections overall than

ChAT+ neurons. nNOS+ neurons mostly projected anally, while ChAT+ neurons mostly orally. This makes sense for facilitating colon emptying given the inhibitory and excitatory roles of Nos1+ and ChAT+ neurons, respectively. For each of these human studies, the subjects (n=10 in the 2007 study and n=13 in the 1997 study) whose tissue was used for this analysis had undergone a surgical resection for colon cancer and had a median age of 76 years and 68 years, respectively. The tissue used for measurement of these percentages was designated by pathologists as "non-diseased", as is generally the case at the borders of tissue resected for cancer elimination.

Despite the major roles of nNOS and ChAT in ENS neurotransmission, it is now well accepted that a single enteric neuron may use multiple neurotransmitters. For example, while nNOS has a major role in inhibitory EN neurotransmission, the neuropeptide vasoactive intestinal polypeptide (VIP) also contributes to inhibitory signaling in canine proximal colon (Keef et al. 1994). Additionally, In guinea pig small bowel, glutamatergic neurons in myenteric plexus generally coexpressed ChAT (100 percent) and Substance P (78 percent) (Liu et al. 1997).

Beyond neurochemical markers, another broad classification system of enteric neurons is based on morphology and electrophysiology (Mawe, Strong, and Sharkey 2009). At the simplest level, there are two groups of neurons using these criteria:

1. AH or Dogiel Type II neurons. These are characterized electrophysiologically by their long after-hyperpolarization (AH) period and morphologically by their several long projections, or processes (Dogiel Type II). These neurons are generally mechanosensitive motor or interneurons (rather than intrinsic primary afferent neurons, IPANs) and can stimulate a motor reflex (Sharkey and Mawe 2023). Acetylcholine (ACh) is the primary transmitter in

many cases, but sometimes nNOS and co-transmitters include tachykinins, GABA, calretinin, VIP, and others.

2. S or Dogiel Type I neurons: Electrophysiologically, these have fast excitatory post-synaptic potentials (EPSPs) and lack the long afterhyperpolarization period of AH neurons. Morphologically, Dogiel Type I neurons have a single long process or axon that projects to other ganglia (Brehmer 2021) and they have many short spiny processes on the cell body. These neurons include excitatory muscle motor neurons (which have ACh or tachykinins as primary transmitters, inhibitory muscle motor neurons (NO as the primary transmitter), ascending interneurons (primary: ACh), descending interneurons (filamentous)(ACh or ATP as primary), descending interneurons (NO as primary), and intestinofugal neurons (ACh as primary transmitter) (Brehmer 2021).

It is important to note that these broad classifications do not hold true across all organisms and bowel regions. For example, ChAT+ glutamateric neurons in the guinea pig small bowel almost all had Dogiel Type II morphology, and only ten percent coexpressed calbindin, which in previous studies had typically been associated with Dogiel Type II cells (Liu et al. 1997). Additionally, because not only glutamatergic neurons express AMPAR and NMDAR, other neuron types can also be responsive to glutamate. Therefore, the same neuron may both release and respond to multiple neurotransmitters (Liu et al. 1997). Nonetheless, these systems of classifications are useful frameworks for understanding the cellular population within the ENS, especially for characteristics of the ENS that are not well captured by brain-centered classification systems.

When making inferences about ENS physiology from studies in the CNS, an important difference to keep in mind is the higher prevalence of volume transmission in the gut, relative to the brain's predominant synapse-to-synapse neurotransmission. Volume transmission refers to the release of a neurotransmitter into a local area that can then act on multiple cells at once, including, for example, muscle cells. Volume transmission is slower than direct synaptic transmission. While synaptic connections also exist in the ENS, the slower method of neuronal signaling via volume transmission is suitable in many cases to promote the slower contractions and relaxations of smooth muscles, relative to the need for fast reflexes in the connections between the brain, spinal cord, and skeletal muscles.

The ENS also receives inputs from neurons with cell bodies outside of the intestine. These neurons are sometimes called "enteric-associated" neurons because they are not part of the ENS, but still may influence ENS signaling. The colon and rectum receive extrinsic innervation from separate sources – the colon receives splanchnic innervation, while the rectum receives sacral and pelvic innervation. The upper GI tract receives extrinsic innervation from vagal pathways. The extrinsic innervation includes both sensory and motor pathways (Brookes et al. 2016).

Enteric glia

Enteric glial cells (ECGs) are critical to the survival and function of enteric neurons. While they share similarities with glia in the brain and other parts of the peripheral nervous system, ECGs are distinct from other glial types in their gene expression profiles (Rao et al. 2015). They are most similar to oligodendrocytes (central nervous system, CNS) and Schwann cells (peripheral nervous system, PNS), two similar cell types that are best known for their role in myelination of axons.

Michael Gershon notes several distinctions between SCs and enteric glia (Gershon and Rothman 1991). These differences include enteric glia being more irregularly shaped, not synthesizing basal lamina proteins, not ensheathing individual axons but rather as a bundle, and having dense filaments (Gershon and Rothman 1991). Despite not being involved in myelination, enteric glia express some genes typically known for being expressed in myelinating Schwann cells (Boesmans et al. 2022).

Given that myenteric neurons are unmyelinated, and Schwann cells are primarily known for their critical role in myelination, what could this mean? It turns out that some SCs are non-myelinating (Baba et al. 2020) and some of these non-myelinating SCs are enteric glia (Harty and Monk 2017). These non-myelinating SCs form microvilli that extend to the axon at the nodes of Ranvier. Additionally, the non-myelinating SCs envelop small diameter axons (into structures called Remak bundles), whereas classic myelinating SCs generally wrap around a bundle of several axons rather than around individual ones. Remak bundles (and also immature SCs) express GFAP (Harty and Monk 2017).

One study reports that about 20 percent of colonic neurons are derived from Schwann cell precursors that migrate along the pelvic nerve to the ENS (Uesaka, Nagashimada, and Enomoto 2015). Looking further into the question of Schwann cell involvement in enteric neurogenesis, a paper notes that Schwann cell precursors (SCP) were in both submucosal and myenteric ganglia in large intestine but mainly just the submucosal ganglia in small intestine (which could explain some of the RNAseq differences I have seen between intestinal regions; see Results), showing that in the colon, approximately 20 percent of myenteric and submucosal neurons are SCP derived (Uesaka, Nagashimada, and Enomoto 2015).

Intramuscular and/or extramyenteric glia express PLP1 but not GFAP (i.e., enteric glia express PLP1 and GFAP), including colonic myenteric glia (Rao et al. 2015). The same study presented RNAseq array analysis showing that enteric glia have gene expression programs that are unique from other types of glia (Rao et al. 2015). Enteric glia share a subset of markers with myelinating glia but not all such markers. They also include some astrocytic related genes but again not all, as well as microglia and neuronal related markers. The paper notes that expression of myelin-related genes in non-myelinating cells occurs in some cells of the inner ear and perisynaptic Schwann cells; the function in non-myelinating contexts is unclear (Rao et al. 2015). The paper also notes that maybe the myelin related genes in ENS have scaffolding related functions and can influence differentiation pathways, such as to prevent differentiation into myelinating cells.

Ultimately the results here suggest that enteric glia are most similar to Schwann cells and oligodendrocytes of the CNS/PNS cells types. They have some similarities to astrocytes, but so do SCs and oligodendrocytes (Rao et al. 2015). However, another potential interpretation of these results about glia since multiple glial types were included is that there are many types of transcriptionally diverse glial types. One single-nucleus ENS profiling study reported the presence of three distinct glial populations in the ENS (Drokhlyansky et al. 2020).

Enteric glia and neurons closely interact. For example, Gabella noted the presence of neuroglial junctions in the ENS in several mammalian species using EM data (Gabella 1981). Interfering with enteric neuron to glia Ca2+ signaling reduces the magnitude of colonic contractions and slows motility (McClain et al. 2014). Glia-specific depletion of the hemichannel Cx43 increased fluid content in mouse stool, implicating a role of glia in intestinal secretion

(McClain et al. 2014). To measure contraction vs relaxation responses in mice with knockout (KO) of a glial protein, the authors used electric field stimulation (EFS) and showed a reduced magnitude of both contraction and relaxation. As to how the authors differentiated between stimulation to evoke relaxation vs contraction, they cited a previous paper, whose supplemental methods show they pulsed EFS for a certain duration and then measured subsequent contractions and relaxations (Gulbransen et al. 2012). In summary, the role of ECGs in motility is related to their interactions with ENs. Dampening ECG signaling reduces the magnitude of contractions and relaxations, at least *ex vivo*.

Despite the critical nature of enteric neurons and glia for motility, it is important to note that there are also myogenic (muscle-originating) and ICC-originating components of motility, including slow waves and rhythmicity (Huizinga 2016). ICCs, or interstitial cells of Cajal, are the pacemaker cells of the gut. However, more research is needed to fully understand the ionic mediators and mechanisms in the balance between ICCs, muscle cells, and enteric neuron types (Huizinga 2016).

Measuring GI motility

In experimental and clinical settings, GI motility is often measured with whole gut transit time (WGTT). In WGTT, transit time is typically defined as the time between ingestion of an inert dye to the appearance of the dye in the stool. Transit time is largely determined by propulsive movements within the intestine and therefore serves as a useful proxy for motility. A more direct, but more invasive, measure of motility is with manometry, in which electrodes are placed at defined points within the GI tract such that contractions are sensed from pressure changes. High

resolution optical manometry with many sensors in the region of interest within the intestine can track the propagation of individual pressure waves or contractions over a distance. Single electrode methods are feasible to do in rodents, but a downside is that propagation cannot be measured when a single electrode is used. Other clinical measures of motility include scintigraphy, which records the movement of a radioactive tracer within the intestine over time. In animal studies, the bead expulsion test is sometimes used to measure colonic motility; for this, a glass bead is placed into the colon at a defined distance from the anus and the time to bead expulsion is recorded. Another in vivo motility test is fecal output, or the number of stool pellets excreted within a defined period of time (Camilleri and Linden 2016).

Another experimental measure of motility is *ex vivo* spatiotemporal mapping, in which a segment of the intestine is removed from an animal and placed into an organ bath attached to force transducers on either end. The semiautonomous nature of the enteric nervous system allows the intestinal segment to continue with contractions for several hours even though it has been separated from the body. Other ex vivo techniques developed to study ENS include electrophysiology, in which the electrical activity of either individual neurons or a population of neurons is recorded (Fung and Vanden Berghe 2020).

One disadvantage of the *in vivo* motility tests relates to the sensitivity of the GI tract to external input, particularly in the context of stress (Fukudo, Nomura, and Hongo 1998). Changes to secretion and absorption of fluids may also affect the transit of intestinal contents without significant changes to colonic motility (Kumral and Zfass 2018). Of course, in contrast to *ex vivo* recording, an advantage of *in vivo* motility testing is that it better represents the physiologic state of the subject. Additionally, *in vivo* motility tests can be safely performed in both humans and

experimental animals, therefore having obvious ethical and practical advantages over *ex vivo* methods. For these reasons, despite its intrinsic variability, WGTT is often the most versatile and practical motility test. Fecal pellet output is less useful for microbiome related studies as germ-free mice at baseline have much higher water content in stool than mice with a complete set of microbes, such that slow motility could be masked when using this metric in GF mice due to their higher water content and thus higher stool volume. Notwithstanding, measures can be taken to account for contributions from other variables during in vivo testing, such as assessing fluid content of stool (to assess potential abnormalities in secretion or absorption), minimizing environmental disruptions during testing (to reduce additional stress) and testing the subject multiple times to compute an average.

Aims of this thesis

Returning to the topic of developmental critical periods, it is not known whether there are critical or sensitive periods in enteric nervous system development as observed in the brain. There are a few lines of reasoning that support the possibility that such sensitive periods exist in ENS. Retrospective patient accounts (P. Maxwell, Mendall, and Kumar 1997) and several other lines of evidence implicate early life microbiota-ENS interactions in adult dysmotility. First, disturbances of the gut microbiota during childhood, such as gastroenteritis and antibiotics, are risk factors for IBS (Chey, Kurlander, and Eswaran 2015; Chitkara et al. 2008; Janssen 2010; P. R. Maxwell et al. 2002; Spiller 2018; Krogsgaard, Engsbro, and Bytzer 2018). Second, animal models of IBS classically involve early life stressors such as maternal deprivation (Greenwood-Van Meerveld and Johnson 2018; Vannucchi and Evangelista 2018; Barreau et al. 2007; Ramalhosa et al. 2016),

and stress-related hormones alter gut microbial composition (Bailey 2016). Third, the early life microbiota directs the assemblage of the stable adult microbiota (Tanaka and Nakayama 2017; S. Subramanian et al. 2014), and persistent dysbiosis characterizes IBS (Jeffery et al. 2012; Chassard et al. 2012; Pimentel et al. 2002; Malinen et al. 2005; Lyra et al. 2009; Maukonen et al. 2006; Parkes et al. 2012). Fourth, microbial metabolites both directly and indirectly stimulate neurons, and activity-dependent synapse formation between neurons regulates long-term signaling patterns (Ramalhosa et al. 2016; Enthoven, de Kloet, and Oitzl 2008; Schwetz et al. 2005; Nishi, Horii-Hayashi, and Sasagawa 2014; De Vadder et al. 2018; Li et al. 2011; Hao et al. 2013; Bhattarai et al. 2017; Muller et al. 2014; Reigstad et al. 2015). Despite this evidence, no studies to date have mechanistically demonstrated how disruptions to the gut microbiota during early life influence the severity and reversibility of chronic dysmotility. The growing pervasiveness of microbial disruptions during childhood—such as antibiotics, non-traditional diets, and supplementation with putative probiotics (Vaz et al. 2014; Quin et al. 2018; Irwin, Davis, and Currie 2019)—underscores the urgency of such an investigation.

On the topic of disruptions to the microbiota during postnatal development of the ENS, there was one study in 2014 that showed that neonatal mice lacking microbes had a reduced neuronal fiber density and altered neurochemical profile at postnatal day 3 (PND3). Because these mice were not followed to adulthood, the extent to which those abnormalities were recoverable was not assessed. A pair of studies did show that the electrical properties, namely excitability, of intrinsic primary afferent neurons (IPANs, one of the main neuronal cell types in the ENS) was significantly reduced in mice lacking microbes (germ-free [GF] mice) and that this excitability was partially recoverable upon colonization with a consortium of microbes (McVey Neufeld et al.

2013; 2015). This, combined with the ample data on the very slow transit times of GF mice, suggests that the lack of microbe-ENS interactions does affect GF mice into adulthood and that some of those effects may be reversed, but perhaps not completely.

From this background, we hypothesized that 1) depletion of gut microbes during a critical window of postnatal development impairs adult GI motility, and 2) this is mediated by changes in Hedgehog (Hh) pathway signaling that impede ENS maturation.

We proposed the following Aims:

Aim 1. Evaluate the extent to which GI motility during adulthood depends on the presence of microbial stimuli during a critical window of development. 1.1 Evaluate GI motility in adult gnotobiotic WT mice that were first exposed to gut microbes at 0, 4, 8, or 12 weeks of age (w). Motility assays include in vivo whole-gut transit time and colonic manometry. 1.2 Evaluate GI motility in adult SPF WT mice treated with antibiotics between 0-4, 4-8, 8-12 w, followed in some cases by treatment with a diet high in resistant starch (shown to enhance colonic butyrate). For Aim 1.1 and 1.2 each, assess longitudinal changes in microbial populations in stool collected from experimental animals using 16S rRNA amplicon sequencing and comparative phylogenetic analyses.

Aim 2. Based on leads provided by RNA-seq, explore the role of the microbiome in regulating Hh pathway-dependent differentiation and maturation pathways in cells of the ENS and the effects of this regulation on cell fate and spatial organization. 2.1 Evaluate the morphologic and organizational development of ENS of age-matched GF, SPF, and conventionalized mice using wholemount immunofluorescence and confocal microscopy. 2.2 Reconstruct microbiome-

sensitive regulatory networks of Hh signaling in cells of the ENS from RNAseq of ENS-enriched intestinal tissue from conventionalized, GF, and SPF mice.

Through the proposed studies, we hoped to gain insights into the fundamental significance of environmental disturbances during development, and the relevance of such disturbances for GI health. We anticipated that imprinting processes early in life may be reprogrammed by restoring key developmental factors, depending on the age at which intervention occurs. Such insights will be critical for the prevention of motility disorders and for developing effective therapies for the millions of patients whose quality of life is harmed by chronic dysmotility.

MATERIALS AND METHODS

Animals

All mice in the study were C57Bl/6. Germ-free female C57Bl/6 mice were given FMT at weaning between 3-4w of age (denoted as "4wC" for simplicity) or between 11-13w of age ("12wC"). Female and male mice were used; however, female mice are the focus of this study as noted in the text, because of the higher prevalence of functional bowel disordered in females (Kim and Kim 2018) and because male mice did not have the same motility impairments as in female mice. Germ-free and SPF adult mice between 12-25w of age were used as controls for similarly aged conventionalized mice. For antibiotic treatment studies, SPF mice between 0-16w were used.

All study procedures were approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC).

Transit testing

Transit testing was performed as previously described (Kashyap et al. 2013). Mice were placed individually into a cage with bedding removed and a steel wire rack placed on the floor of the cage so that fecal pellets would fall below. A white paper towel was placed underneath each cage to facilitate visualization of the pellets. At the start of testing, the mice were gavaged with 100-300µl 6% carmine dye in 0.5% methylcellulose and the time was recorded. Whole gut transit time was defined as the interval between the dye gavage and the appearance of a fully red stool pellet, verified by smearing the pellet onto a white paper towel to ensure it was red throughout the

pellet. This method has been well validated in conventional mice and there have been no reported adverse events.

Fecal microbiota transplantation

Fecal samples from female SPF mice between 8-16 weeks of age were collected over the course of several days and stored at -20 deg C until enough had been collected to last through the entire study. The stool samples were pooled, thawed on ice, and mixed thoroughly with a spatula. The pooled stool was divided into 100mg aliquots and sterile PBS was added to a total volume of 1mL. Each aliquot was vortexed for 1 minute with the PBS prior to freezing at -80deg C. On the day of the FMT, the aliquot was thawed, vortexed for 1 minute, then particulate matter was briefly spun down. The supernatant was transferred into a separate tube and this solution was used for oral gavage. For FMT, mice were transferred from the gnotobiotic facility to the barrier mouse facility on the day of the FMT.

Manometry

To measure colonic contractions, solid state manometry was performed as previously described (Gourcerol et al. 2009), using LabChart 8 rather than Spike 2 software.

To test the effect of colonization state on *in vivo* contractility, colonic manometry was used. Mice were briefly anesthetized with isoflurane inhalation (1-5% using vaporizer). Once a deep plane of anesthesia was confirmed, we placed the lubricated catheter (takes approximately 30 seconds). A miniaturized pressure transducer catheter (e.g., SPR-524 Mikro-Tip catheter, Millar Instruments, Houston, TX) lubricated with medical grade lubricant is introduced into the colon

such that the middle of the pressure sensor (3.5 F) lies 2-4 cm proximal to the anus. The catheter is secured to the tail with surgical tape and colonic contractions recorded in conscious mice for 60-90 minutes by placing them in an adequately vented size-specific restraint tube (Plas Labs Broome-Style Rodent Restrainer, 551BSRR). This procedure has been previously described and has no adverse effects on the mice (Am J Physiol Gastrointest Liver Physiol. 2009 May; 296(5):G992-1002). It is important to keep the mice conscious to get meaningful data on colonic contractility. Mice were promptly returned to their cages with access to food and water *ad libitum* after the procedure. Analysis of manometry data was performed as previously described (Gourcerol et al. 2009).

Treatment of SPF mice with antibiotics at different ages

We also examined whether changes in microbiota in early life affects motility later in life using antibiotic treatment. To invoke the change of pup's microbiota when they are born, we provided antibiotic in drinking water to pregnant females after confirmation of pregnancy. An antibiotic cocktail including vancomycin (0.5 mg/ml), neomycin (1.0 mg/ml), and cefoperazone (0.5 mg/ml) was added to drinking water for up to 42 days. Antibiotics were continued for the pregnant mother until birth and through weaning, therefore antibiotics for up to 6 weeks. To assess whether the effects of antibiotic treatment are age-dependent, we also treated 4, 8, or 12 week old mice with the same antibiotic cocktail as with the pregnant mice for up to 6 weeks. *In vivo* motility testing occurred 4 weeks after the conclusion of antibiotic treatment and/or at the age of 15-16 weeks, to minimize the potential confound of time after antibiotic treatment and age of motility testing when comparing between groups. Age-matched untreated SPF mice were used as controls.

Dietary carbohydrate manipulation

To assess the role of diet-induced alterations of the gut microbiota in gastrointestinal transit, some animals were fed special diets. The diets are based on AIN-93G and have either high (TD.200075) or low (TD.200074) amounts of a type of starch ("resistant starch") known to increase the production of butyrate (a bacterial metabolite) by gut flora (Vidrine et al. 2014). These diets were custom formulated by an animal nutritionist at Envigo. For diet studies, animals were fed either the high or low resistant starch diet for up to 12 weeks. To assess whether the benefits of dietary treatment depend on the age at which treatment begins, we began dietary treatment at 3 different ages (3-5, 6-9, or 11-16 weeks of age). To assess whether the benefits of dietary treatment depend on the presence of the gut microbiota, we tested both GF and SPF animals, as well as a subset of the antibiotic treated mice described above. *In vivo* motility testing and subsequent euthanasia and tissue harvest occurred at the ages of 7-8 weeks, 11-12 weeks or 15-16 weeks.

Tissue harvest

Mice were euthanized by CO_2 asphyxiation, the abdominal cavity was opened, and mice were transcardially perfused with PBS. Colon and cecum were dissected out on ice. Stool was removed from the colon and the colon was opened lengthwise. Approximately 1.5cm of the proximal and distal ends were collected for immunohistochemistry and pinned flat onto a silicon dish, washed with PBS, then 4 percent PFA in PBS was added while tissue harvest was completed. The remaining portion of the colon was placed into RNALater solution and stored at 4 deg C. The 4 percent PFA solution was removed at the end of the dissection and freshly made Zamboni's

fixative was added to the tissue, which was gently agitated overnight at 4 deg C, washed 3x in PBS, followed by washing in PBS + 10 percent sucrose solution at 4 deg for several hours, then agitated gently overnight in PBS + 20 percent sucrose + 10 percent glycerol solution, rinsed once in PBS and then stored in PBS + 0.1% sodium azide at 4 deg C. For both wholemount immunohistochemistry and for RNA extraction, the epithelial mucosa was removed from the colonic muscularis propria and only the muscularis propria was used for downstream applications.

Immunohistochemistry

For staining, intestinal segments were pinned into another silicon coated dish. Heat induced epitope retrieval (HIER) was performed in citrate buffer at 80-95 deg C for up to 50 min. Following protein block and permeabilization in Superblock buffer (PI37515, Thermo Scientific) with 0.3% TX100 for 30 min at 37 deg C, Mouse-on-mouse blocking was performed with MOM Blocking Reagent (Vector Labs; 5 drops per 9ml of PBS + 0.1% TX100) and incubated at 37 deg C for 1 hour. After washing with PBS + 0.1% TX100 ("PBST"), primary antibody incubation with 1:500 mouse monoclonal HuC/HuD (A21271, Invitrogen) and 1:2000 chicken polyclonal GFAP (ab4674, Abcam) or with mouse monoclonal 1:500 Tubb3/Tuj1 (Biolegend 801202) in Superblock w/ 0.3% TX100 at room temperature (RT) shaking overnight. After 5-7 washes with PBST over the course of 1-3 hours, secondary antibody incubation (Alexa Fluor dyes, Molecular Probes) was performed in Superblock with 0.3% TX100 shielded from light at RT shaking for 1 hour. Nuclear staining was performed with Hoechst 10ug/ml in PBST shielded from light at RT shaking for 10 minutes. 4-5 washes in PBST in 45 minutes then 3 brief PBS washes were performed prior to mounting in Prolong Gold in a glass bottom dish.

RNA extraction and sequencing

RNeasy Mini Kit from Qiagen (cat # 74104) was used to extract RNA. For the lysis and homogenation step, Qiagen PowerBead Tubes with Garnet 0.70mm (cat #13123-50) were used with a bead beater for 1 minute at 3450 oscillations/min (GlenMills, Beadbeater-16).

Next generation RNA sequencing was sequenced with Illumina NovaSeq 6000 (Oligo-dT mRNA directional paired end with 50-60M paired end reads/sample) at the University of Chicago Functional Genomics Core Facility.

RNA sequencing analysis

Raw files were downloaded from the UChicago Genomics Core server (using FileZilla, then uploaded to Globus, then transferred to UChicago Midway2 server endpoint) as fastq.qz files, with four files per sample: R1 and R2 each for two flow cells. UChicago Research Computing Center server was used for initial analysis using Python. Reads 1 and 2 for each sample were concatenated by flow cell. Reads were trimmed using Trimmomatic-0.39. Alignment was performed using STAR 2.7.9a and BAM files were sorted by coordinate. Samtools was used for indexing. featureCounts was used to generate counts using mm39 (.gtf file obtained from Ensembl.org).

Differential expression analyses were performed for the female colon samples using DESeq2_1.40.1. The SVA (3.48) package identified 3 surrogate variables which were included as technical covariates in DESeq design formula along with Condition (SPF, GF, 4wC, 12wC). For heatmap plotting, counts were normalized with the variance stabilizing transformation (vst) and

log fold changes were shrunk with apelgm (Zhu, Ibrahim, and Love 2019). The factoextra (Kassambara and Mundt 2020) package was used to create the PCA with ellipses and ComplexHeatmaps (Gu, Eils, and Schlesner 2016) was used to create the heatmap. Pathway enrichment was performed with fgsea (Korotkevich et al. 2021) using the Gene Ontology Biological Process gene set (m5.go.bp.v2023.1.Mm.symbols.gmt) from MSigDB.(A. Subramanian et al. 2005; Ashburner et al. 2000; The Gene Ontology Consortium et al. 2023)

To identify the early life microbiome dependent gene lists (i.e. those that returned to SPF levels in 4wC only), several pairwise DE (pvalue < 0.05) gene lists were intersected and differentiated. Specifically, for the genes that were recovered "down" to normal expression levels in 4wC relative to SPF (and not in 12wC relative to either group), the following intersections and set differences were made: downregulation in SPF versus GF, SPF versus 12wC, and 4wC versus 12wC; upregulation in GF versus 4wC; and no difference between GF versus 12wC. For “recovered - up" genes, the intersections and set differences were as follows: upregulated in SPF versus GF, SPF versus 12wC, and 4wC versus 12wC; downregulated in GF versus 4wC; and no difference between GF versus 12wC.

Microbial DNA extraction and 16S rRNA sequencing

DNA was extracted from freshly collected fecal pellets using the Qiagen PowerSoil DNeasy Kit (cat. #47016).

DNA was sequenced by Argonne National Laboratories Environmental Sample Preparation and Sequencing Facility for 16S amplicon library Illumina HiSeq2500 sequencing with 150bp length reads using primers for the bacterial 16S gene V3 and V4 regions.

Microbiome analysis

For microbiome analysis, FastQ files were unzipped with gzip for use with qiime2-2022.2 (Bolyen et al. 2019). These were in the format of emp-paired-end-sequences. Demultiplexing and quality filtering was performed with q2-demux and denoising with q2-dada2 (Callahan et al. 2016). Features were aligned using mafft (Katoh et al. 2002) and a taxonomy was constructed with fasttree2 (Price, Dehal, and Arkin 2010) and filtered at the sampling depth 11007, which maximized the number of samples and features retained. The Shannon metric was used for alpha diversity. ANCOM was used for differential abundance testing at the level of the taxonomic order (level 4) and samples from the three groups (SPF, 12wC, and 4wC) at approximately 16w of age were included in the analysis.

Fecal water content

Stool pellets were freshly collected, weighed, and allowed to dry overnight in an oven set to 55 deg C, then reweighed. The water fraction was calculated as 1 minus (dry weight / wet weight).

Statistics

Statistical analysis was performed using GraphPad Prism and R Studio. Selection of statistical tests (t tests, ANOVA, or nonparametric alternatives) was based on whether statistical assumptions were upheld. Multiple comparisons correction was performed as appropriate, based on the recommended methods in GraphPad Prism. For the statistics related to pathway and gene

enrichments, the default methods in DESeq2 (Love, Huber, and Anders 2014) and fgsea (Korotkevich et al. 2021) were used.

Microscopy

Whole mount immunofluorescence images were taken with a Leica SP8 microscope at the University of Chicago Light Microscopy Core Facility with a 20X oil immersion objective. Areas to image were selected to maximize the number of HuCD cell bodies in each field of view.

Imaging analysis

For glia:neuron ratio, Adobe Photoshop "Lasso" and "Count" tools were used to outline ganglia for ROIs and then count HuCD+ cell bodies (neurons) and GFAP+ cell bodies (glia). Specifically, glia were defined as (1) HuCD- nuclei (2) within the border of GFAP staining delineating ganglion (3) not having an elongated nucleus resembling that of a muscle cell.

For neuronal fiber density, Tubb3/Tuj1 staining was quantified per image as percent area of the field of view at plane at the level of the myenteric plexus. Tuj1 images were processed by smoothing with Gaussian blur with sigma = 0.7, subtracting background with a rolling ball radius of 50 pixels, and thresholded such that background staining was not included in the area calculation.

RESULTS

We first explored proof of concept for our hypothesis that a postnatal critical period in ENS development depends on the microbiota. To do so, we conducted a pilot study in which GF female mice were given FMT at either weaning ("4wC") or after 12w of age ("12wC"), then tested transit 4w later and compared to approximately age-matched controls. A schematic of our experimental approach is shown in Figure 1.

We found that indeed, animals conventionalized at 4, but not 12w of age recovered normal (SPF) transit times (Figure 1b).

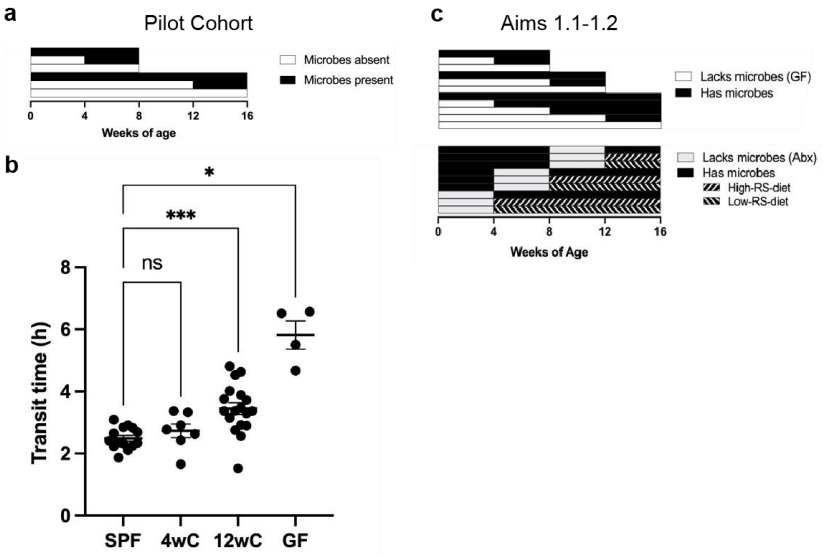

Figure 1. Motility remains impaired after FMT in mice lacking microbes post-weaning.
a Pilot study design: female mice given FMT at 4 or 12w of age. Transit testing was performed 4 weeks after FMT in each case. GF and SPF controls were female C57Bl/6 mice between 8-16w of age. **b** Mice given FMT after 12w of age (12wC) fail to recover normal SPF transit times, while the transit times of mice given FMT at weaning (4wC) are the same as those of SPF controls. Brown-Forsythe ANOVA with Dunnett T3 multiple comparisons correction. $F^*(3,10.56) = 24.85$. $p<0.0001$. **c** Schematic of experimental approach to follow up initial findings. In Aim 1.1, GF animals are conventionalized at various ages. GI motility is assessed either 4 weeks post-FMT or

Figure 1, continued
around 16 weeks of age. In Aim 1.2, SPF animals are treated with antibiotics from 0-4, 4-8, or 8-12 weeks of age, after which dietary manipulations begin (high or low resistant starch diet, or standard chow)

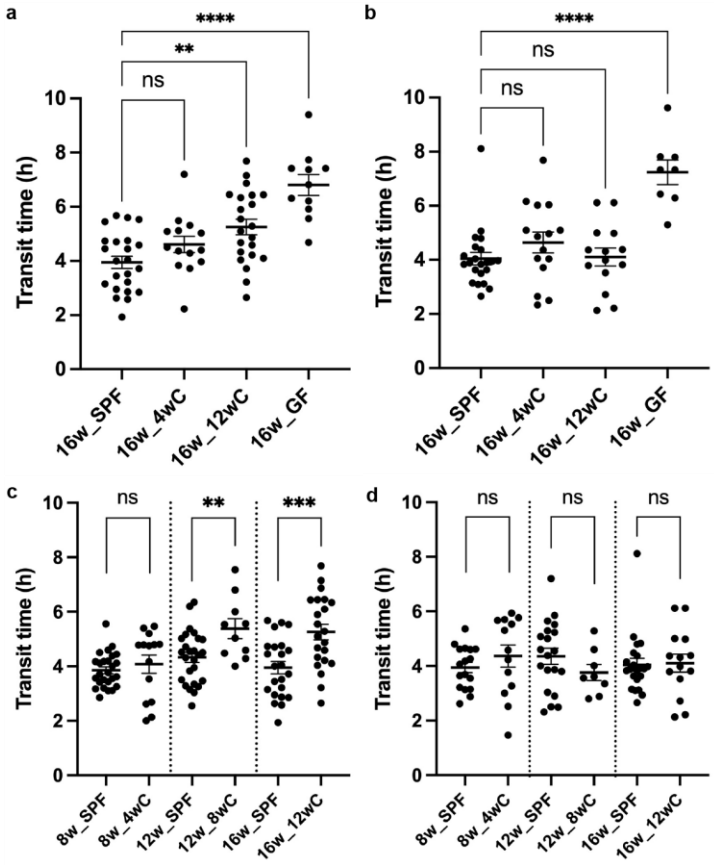

Figure 2. The critical window for encountering microbes for later life motility is female specific and not explained by differences in age of testing.

a,b Validation cohort transit data in female (**a**) and male (**b**) mice matched by age of testing. FMT was given at 4w or 12w of age and mice were tested at 16w of age. 16w-old GF and SPF mice were used as controls. For both: Ordinary one-way ANOVA with Dunnett's multiple comparisons test. **c,d** Validation cohort transit data in female (**c**) and male (**d**) mice for which conventionalized mice were matched by the interval between FMT and transit testing. FMT was given at 4, 8, or 12w of age and mice were tested 4w after FMT and compared with age-matched SPF controls. For both: Unpaired two-tailed t tests (or nonparametric equivalent) between each age-matched pair. (**a**) 16w_SPF n=23, 16w_4wC n=14, 16w_12wC n=22, 16w_GF n=11. (**b**) 16w_SPF n=22, 16w_4wC n=15, 16w_12wC n=14, 16w_GF n=8. (**c**) 8w_SPF n=26, 8w_4wC n=14, 12w_SPF n=26, 12w_8wC n=10, 16w_SPF n=23, 16w_12wC n=22. (**d**) 8w_SPF n=17, 8w_4wC n=13,

Figure 2, continued
12w_SPF n=20, 12w_8wC n=8, 16w_SPF n=22, 16w_12wC n=14. For all, mean +/- SEM is indicated. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ns=not significant.

To probe what could be happening molecularly between mice with microbes or without microbes, we performed bulk RNAseq with muscularis propria tissue from ileum and colon of female GF and SPF mice. We also sequenced homogenized tissue from the brainstem of the same mice, to provide a rough idea of whether the observed effect originated primarily from brain or gut differences.

We found that in both ileum and colon of those mice, synapse related pathways were strongly enriched in SPF over GF. There were fewer differences between brainstem gene expression in these groups, suggesting that the magnitude of the effect of the microbiota on gene expression in the enteric nervous system might be higher than in the cerebellum and brainstem as a unit. (There are probably individual regions that are more heavily affected than others in the brain; but those signals probably were diluted when measuring expression in homogenized tissue. Nonetheless, the more dramatic differences in the ENS as a whole supported focusing on this anatomical area rather than the brain.)

We then had a few next steps. First, we needed to repeat the FMT study with additional mice, as a validation cohort. We also decided to try an intermediate age point, 8w, for FMT in addition to the 4w and 12w time points. For the transit effect, we also wanted to test both the 4w-post-conv time point as before as well as all around 16w or more of age, to assess whether we would see the same effects in both study designs. Additionally, we needed to perform RNAseq of MP of colon and ileum from 4wC and 12wC mice to see which pathways were “recovered” in

these groups relative to SPF and GF. We were also curious to see whether the same effects we saw in female mice held true in male mice, so we set out to do the same for both females and males.

We again found that transit times were again restored in the female mice given FMT at 4w of age, while it was delayed for mice conventionalized at 12w of age (Figure 2). Additionally, the 8wC group also had delayed transit times relative to SPF, suggesting that the critical window for ENS-microbiome exposure for normal development closed prior to 8w of age – that is, the 8wC and 12wC mice seemed equivalent. Subsequently, then, we stuck with just the 4wC and 12wC groups for other analyses.

Strikingly, however, we found that male mice did not show the same effect as females (Figure 2). That is, although male GF mice had delayed transit relative to SPF, their recovery from delayed transit after FMT did not seem to depend on the age of FMT.

The RNAseq data showed strong separation in gene expression between ileum and colon (Figure 3). Visual separation of male and female expression was also visually apparent. In the ileum and colon MP of early versus late conventionalized male mice, SPF and GF male mice differed in gene expression as did the conventionalized mice, when compared to either SPF or GF. The differences between male SPF and GF colon MP were more dramatic in the colon than the ileum relative to the conventionalized groups; the differences between the male 4wC and 12wC colon MP were more dramatic in the ileum than in the colon relative to SPF and GF. These interactions between variables - microbiome status, age of conventionalization, sex, and intestinal region – highlighted the complexity of the microbiome's role in postnatal ENS development.

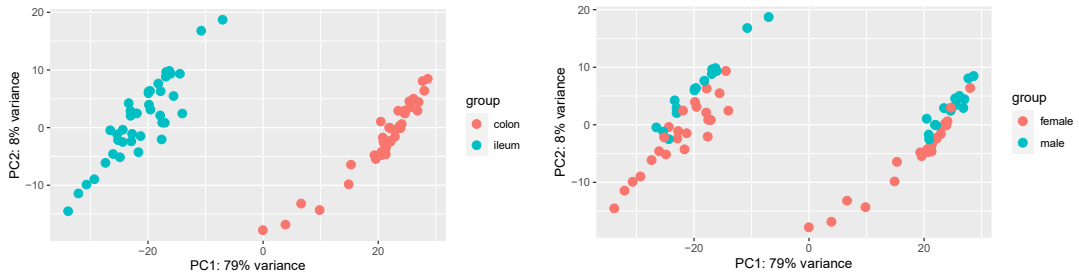

Figure 3. Muscularis propria gene expression differs by intestinal region and sex.
PCA plots of gene expression data when all samples (male and female, ileum and colon) were pooled. A strong separation of expression between colon and ileum is visually apparent. Female and male gene expression also differs within these regions. All mice were C57Bl/6 between the ages of 13-25w. The raw counts were transformed with the variance stabilized transformation (vst) before plotting.

Despite the differences in gene expression in colonic MP in male mice based on age of FMT, there were no differences in transit time. As such, functional impairments in conventionalized male mice relative to SPF were not captured here. As seen in the female conventionalized mice, the male conventionalized mice recovered normal fecal water fraction.

Although the region and sex differences were striking, we were finding that the story in even just female mice colon was sufficiently complicated for a thesis project; therefore we focus on just the female colon for the rest of the thesis. However, differences between sexes and intestinal regions are an area of future exploration. As such, unless otherwise specified, all subsequent results and discussion pertain to female mice, and specifically to colon data when related to tissue findings. We chose to continue with female colon as the motility effect was present in only females

and because it known that the majority of the time comprising whole gut transit concerns colonic transit rather than ileal.

Nonetheless, PCA plots for gene expression of ileum and colon in male and female are shown in Figure 4.

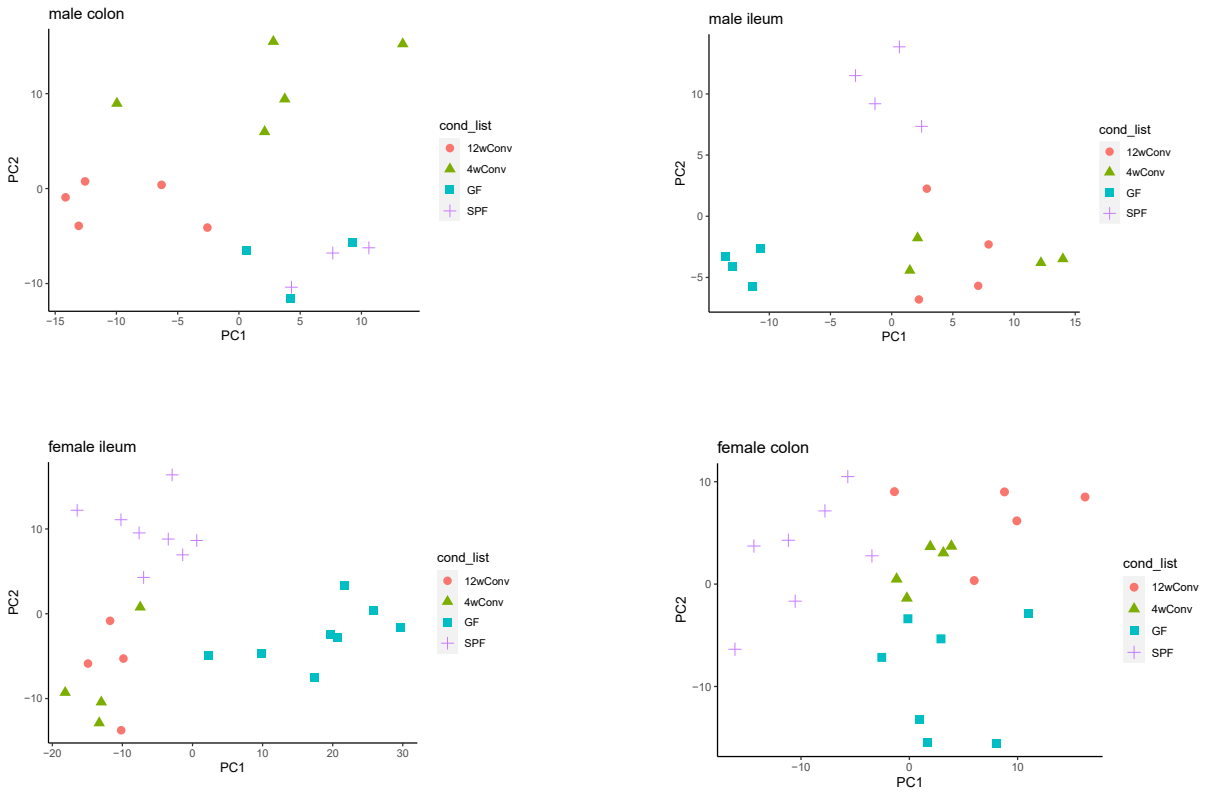

Figure 4. PCA plots of gene expression within each sex and region grouping.

PCA plots for axes 1 and 2 for analyses separated by sex and region. Color and shape of the points represent conditions (12wC, 4wC, SPF, GF).

From the female colon MP RNAseq data (SPF, GF, 4wC, and 12wC), we identified the genes whose normal expression required the presence of the microbiome before adulthood. We separated these genes based on those that were higher or lower in SPF than in GF. Conceptually, the "recovered-up" genes were those that 4wC restored to normal (SPF) levels from GF's lower expression, while 12wC's expression levels remained abnormally low. Similarly, the "recovered-down" genes were those that 4wC restored to normal (SPF) levels from GF's higher expression, while 12wC's expression levels remained abnormally high. The threshold for significance for these comparisons was pvalue < 0.05.

A heatmap of the expression data for the early life microbiome dependent genes, comprising the recovered-up and recovered-down genes (collectively referred to from here as the "recovered" genes) is shown in Figure 5.

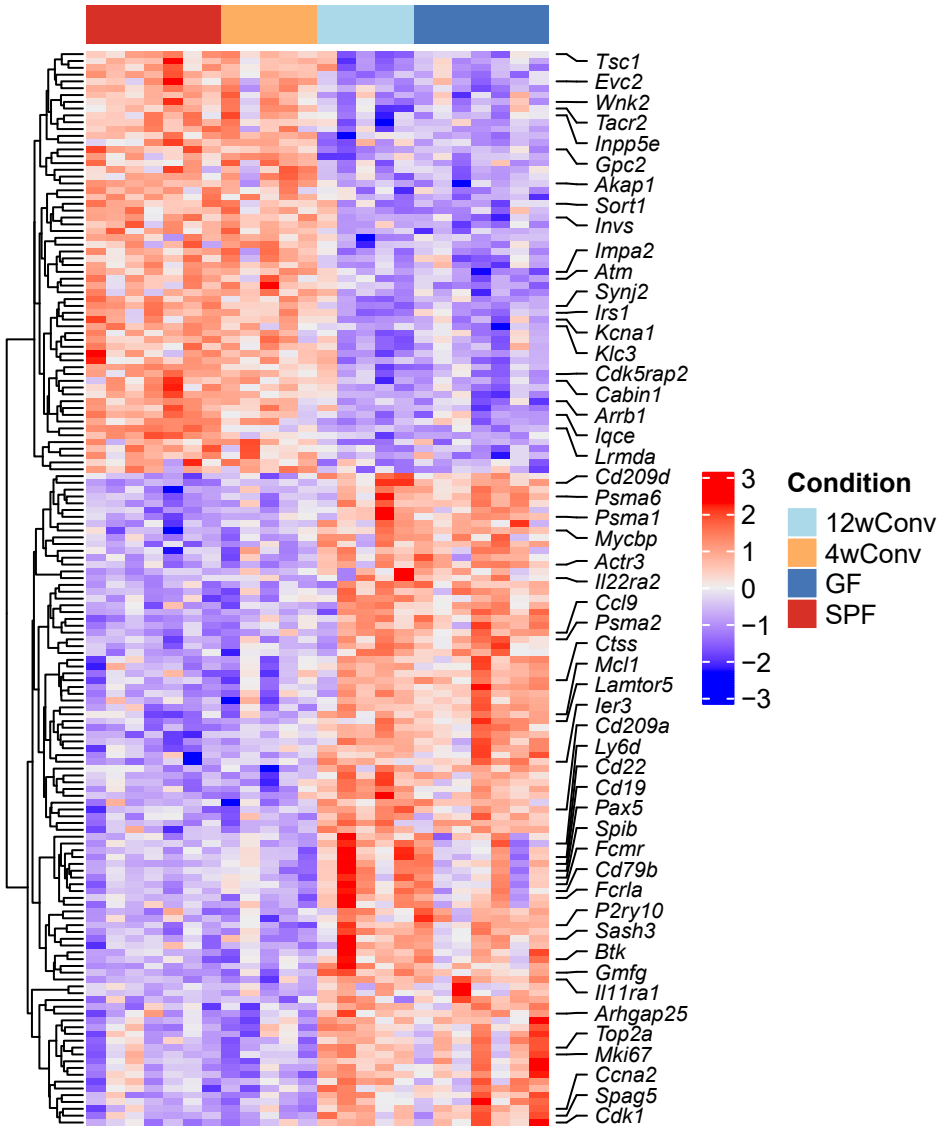

Figure 5. Early life microbiome-dependent gene expression.

Heatmap of genes whose expression returned to control (SPF) levels in mice first exposed to the microbiome at the time of weaning (4wC) but remained abnormal in mice first exposed to the microbiome during adulthood (12wC). SPF (n=7), GF (n=7), 4wC (n=5), 12wC (n=5).

A clear theme of the early life microbiome-dependent "recovered-up" genes was that they were related to the developmentally critical Hedgehog (Hh) pathway, also known as the Smoothened signaling pathway, and its interactions with the equally critical Wnt pathway. Conversely, a clear theme of the "recovered-down" genes was that they were involved in widespread immune activation, including both adaptive and innate components of the immune response. To represent some of the ways these themes came up in the pathway enrichments between SPF and GF and between 4wC and 12wC, Figure 6 is a bar plot showing a selection of Gene Ontology Biological Process gene sets containing one of either the Hedgehog effector *Smo* or Wnt regulator *Sfrp1*, or one of either immune signal transducer *Fcer1g* or immune response activator *Syk*.

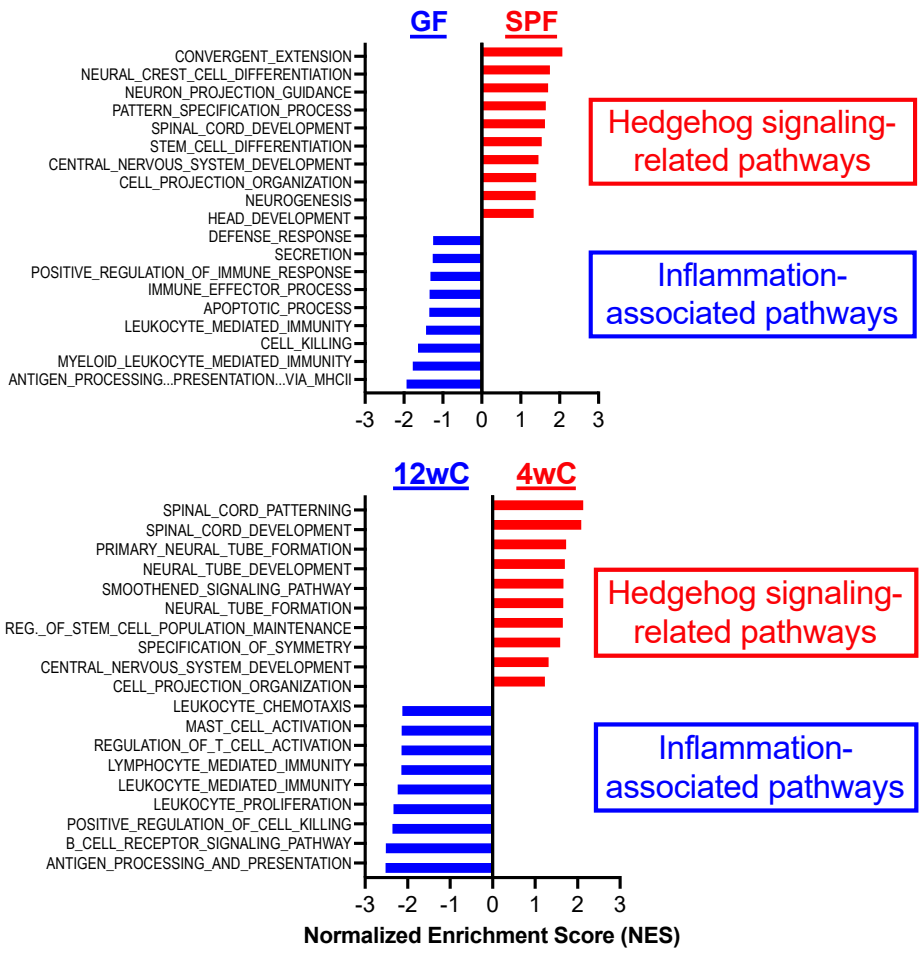

Figure 6. Key themes among early life microbiota-dependent pathway enrichments include Hedgehog, Wnt, and immune signaling.

Left: selected differentially expressed GOBP pathways in SPF versus GF. Positive scores indicate higher expression in SPF. Note that all the pathways shown for SPF and 4wC (i.e. red bars) include either Hedgehog effector *Smo* and Wnt regulator *Sfrp1*, while those for GF and 12wC (i.e. blue bars) include either immune signal transducer *Fcer1g* or immune response activator *Syk*. Enrichment analysis performed with fgsea. SPF (n=7), GF (n=7), 4wC (n=5), 12wC (n=5).

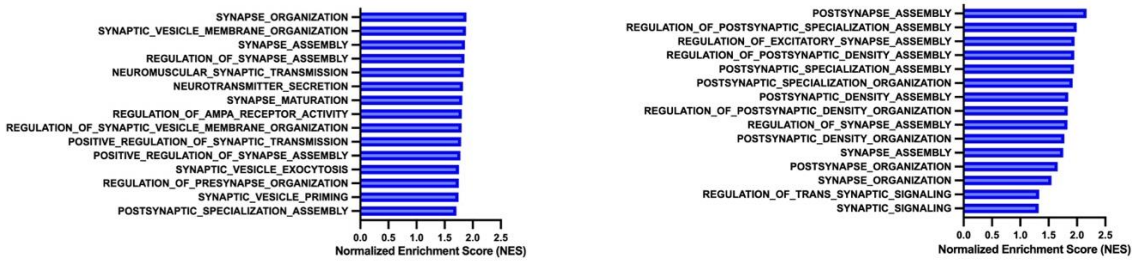

Figure 7. Synapse-related differentially expressed GOBP pathways in SPF versus GF ileum and colon.

In both ileum (left) and colon (right) MP, SPF mice had higher expression of synapse-related GOBP pathways compared to GF (non-exhaustive list). Positive scores indicate higher expression in SPF. SPF ileum (n=8), GF ileum (n=8), SPF colon (n=7), GF colon (n=7).

Given that the Hedgehog pathway functions to maintain the pool of glia and neural progenitor cells by inhibiting the terminal differentiation into postmitotic neurons, the next question was whether early life FMT restored proportions of glia and neurons from abnormal levels in GF, and the 12wC group remained impaired. As expected given the Hedgehog pathway expression differences, SPF had a higher glia to neuron ratio than 12wC and GF, and indeed, the ratio was restored in 4wC using whole mount immunofluorescent staining and quantification (Figure 8).

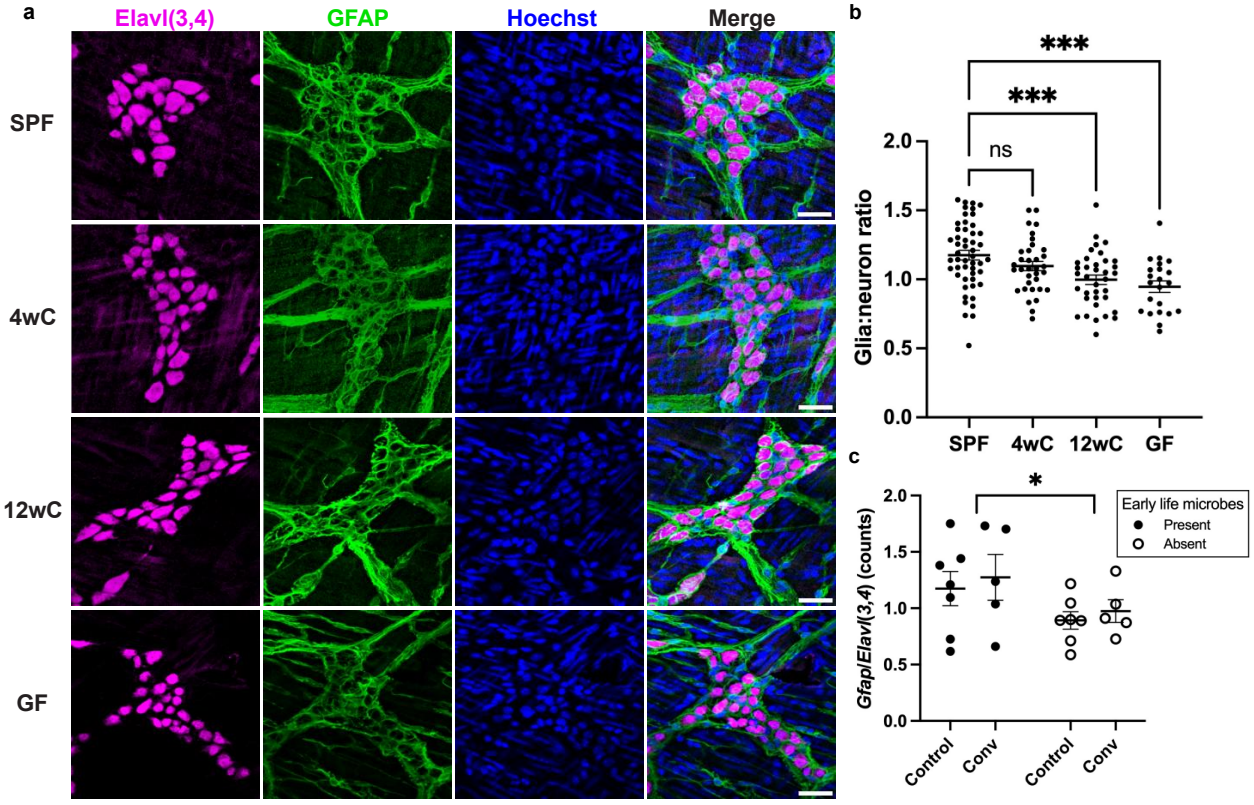

Figure 8. Glia:neuron ratio depends on the early life microbiome.

4wC regains a normal glia:neuron ratio, while 12wC and GF have a lower glia:neuron ratio compared to SPF. **a** Whole-mount immunofluorescent (IF) staining of myenteric plexus in colonic muscularis propria. Representative ganglia from SPF, 4wC, 12wC, and GF, stained for Elavl3/4 (HuCD; neuronal soma), GFAP (glia), and Hoechst (nuclei). Scale bar: 36µm. **b** Ratio of glia to neurons from IF quantification. Data are plotted by ganglion analyzed and include images from 3-6 animals per condition collected over 4 staining batches that each included samples from all conditions. Ordinary one-way ANOVA with Dunnett's multiple comparisons test. **c** Validation of IF quantification; RNA expression ratio of *Gfap* to the averaged counts of *Elavl3* and *Elavl4* (to correspond to the Elavl(3,4) antibody, which labels both proteins). Comparison is between mice for which microbes were either present or absent during the critical window (i.e., SPF and 4wC for "present", GF and 12wC for "absent"). Two-way ANOVA. *p<0.05, ***p<0.001, ns=not significant.

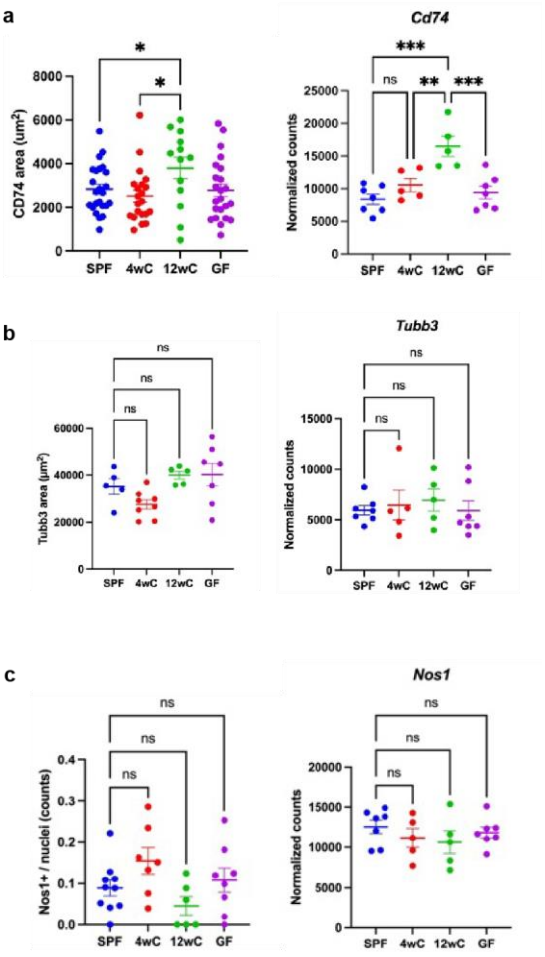

Figure 9. Expression of cell type markers and corresponding immunofluorescent quantification.

Expression of cell type markers and corresponding immunofluorescent quantification. **a** CD74 is a marker for antigen presenting cells (APCs) including B cells, dendritic cells, and macrophages. CD74 area: Ordinary one-way ANOVA with Holmes-Sidak multiple comparisons correction F(3,77)=2.626, overall p=0.0563. CD74 counts: Ordinary one-way ANOVA with Dunnett multiple comparisons correction. F(3,77)=2.626, overall p=0.0563.**b** Tubb3 (Tuj1) is a general neuronal marker. **c** Nos1 is a marker of nitrergic neurons.
*p<0.05, ns=not significant.

There were no differences in the overall expression of a general neuronal marker Tubb3 nor a nitrergic neuronal marker with both RNAseq and immunofluorescence quantification (Figure 9). Expression of Cd74, an antigen presenting cell (APC) marker, was higher in 12wC than SPF in both the RNAseq and IF quantification (Figure 9). These results support the validity of each metric and demonstrate that the groups did not significantly differ in overall neuronal protein or gene expression, nor in the proportion of nNOS+ to total neurons in colon. The higher expression of CD74 in the 12wC group is expected, given the many other enrichments in antigen presentation and other immune pathways relative to SPF and 4wC. Expression levels for several marker genes are shown in Figure 10.

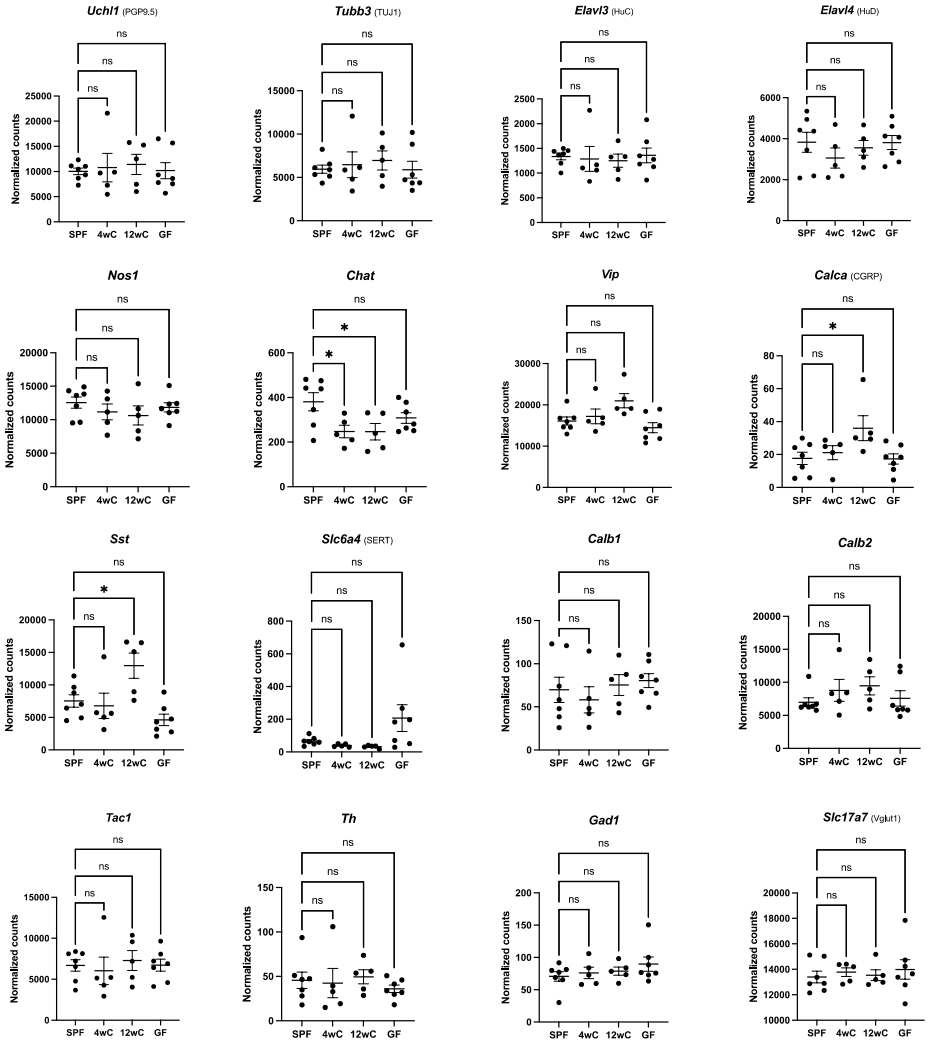

Figure 10. The recovery phenotype was not associated with significant shifts in general and type-specific neuronal markers.

Plots of expression of several marker genes for specific neuron subtypes show no clear relationship between specific neuronal subtype markers and the presence/absence of microbes during early life, suggesting the recovered signaling pathways are not specific to subtype or overall population of neurons. The markers represent the following cell types in the colon. Neurons, general: UChl1 (PGP9.5), Tubb3 (Tuj1), Elavl3 (HuC), Elavl4 (HuD); nitrergic: Nos1 (nNOS); cholinergic: Chat (ChAT); vasoactive intestinal polypeptide: Vip; CGRP: Calca; somatostatin: Sst; serotonergic: Slc6a4; calbindin: Calb1; calretinin: Calb2; tachykininergic (may include Substance P, Neurokinins A and B, and Neuromedin K) – Tac1; GABAergic: Gad1; dopaminergic: Th; glutamatergic: Slc17a7 (Vglut1). Ordinary one-way ANOVA.

In terms of functional measurements, late conventionalized mice exhibited hypercontractility for high amplitude contractions (>60mmHg) compared to SPF (Figure 11). This difference was also evident by manometry pressure area under the curve (pAUC) during the 40 minutes after the initial 20-min acclimation period.

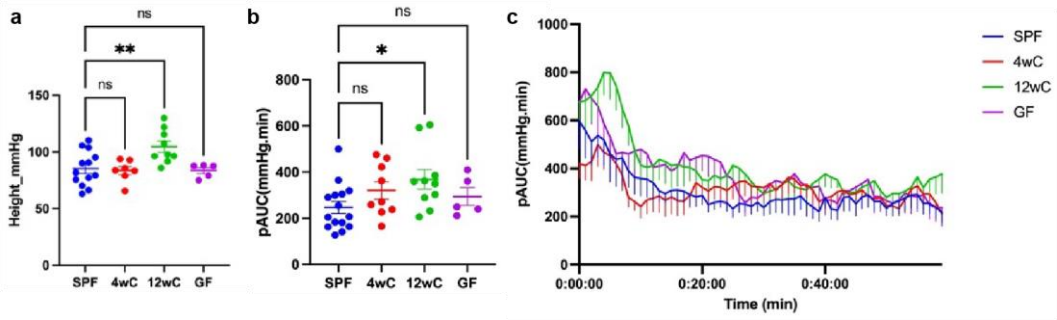

Figure 11. Manometry of conventionalized mice and controls.

a,b 16w-old conventionalized (12wC) mice had an elevated average peak height for high amplitude contractions (>60mmHg) (**a**) and an elevated presssure area under the curve during the testing period between 20-60 minutes (**b**). For both: Ordinary one-way ANOVA. **c** pAUC is shown as a rolling per-minute average, plotted as previously shown (Gourcerol et al. 2009). *p<0.05, **p<0.01, ns=not significant.

Summarizing the 16S data: there are differences in alpha diversity between conventionalization groups (Figure 12); however, since they were all given the same gavage material, the differences probably reflect host factors rather than microbiome originating factors.

Figure 12. Early life microbiota-dependent microbial diversity.

a Taxonomic composition of SPF, 12wC, and 4wC stool at 16w of age. Taxa identified as differentially abundant across groups (ANCOM) are marked with (*). **b** Weighted Unifrac distances to SPF showed that the composition of the 12wC fecal microbiota differed significantly from SPF, while 4wC resembled SPF. Brown-Forsythe ANOVA with Dunnett's T3 multiple comparisons test. **c** Shannon entropy scores; 12wC had lower fecal alpha diversity than SPF, while 4wC did not differ from SPF. Samples collected at 16w of age. One-way ANOVA with Dunnett multiple comparisons test. **p<0.01, ****p<0.0001, ns=not significant.

There were no differences in fecal water content in either of the conventionalized groups when compared to SPF. GF, however, had higher fecal water content than SPF (Figure 13).

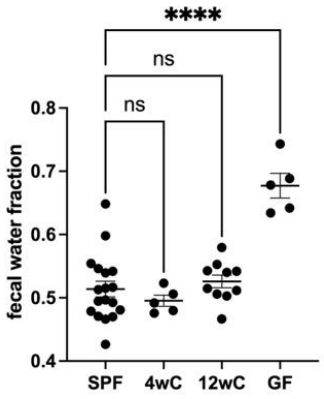

Figure 13. Age of FMT did not affect fecal water content.

Figure 13, continued
Fecal water content was not different between SPF and conventionalized mice, but it was elevated in GF mice. Mice approximately 16w of age. Ordinary one-way ANOVA with Dunnett multiple comparisons correction. $F(3,34)=20.65$, overall $p<0.0001$.
****$p<0.0001$, ns=not significant.

In another arm of the early life microbiome study on later life motility, we treated mice with antibiotics during ~4w blocks, either between birth and 4w of age, 4-8w, or 8-12w, then tested them on transit both 4w after the cessation of antibiotic treatment and as adults, ~16w of age in most cases (see Figure 1 for study design schematic).

This study had the added variable of dietary treatment groups after the cessation of antibiotic treatment, to allow us to assess whether dietary composition affected ability of the mice to recover either transit times or microbiome diversity after antibiotic treatment. The dietary groups included regular chow or one of two isocaloric simplified diets containing either high or low levels of resistant starch (RS). It was previously shown that diets high in resistant starch increased butyrate concentrations in the murine colon (Vidrine et al. 2014), and butyrate had been shown to increase motility when applied ex vivo to colonic tissue (Hurst et al. 2014). Because the transit times of the high resistant starch (hRS) treated mice did not differ from those of RC mice, and because regular chow contains moderate levels of resistant starch, the transit data for these two groups is pooled where indicated in the figures below. The lack of difference between the RC and hRS fed groups suggests that after a certain threshold, the amount of resistant starch in a diet may not further increase transit speed. If it did, the mice would be expected to have diarrhea.

From the antibiotic studies (with or without dietary treatment after antibiotics), we found a few things. First, regardless of the age of antibiotic treatment, the gut microbiome composition

from SPF antibiotic treated mice failed to recover to the same level of diversity as in SPF control mice, even when assessed 4 weeks or more from the cessation of antibiotics (Figure 14).

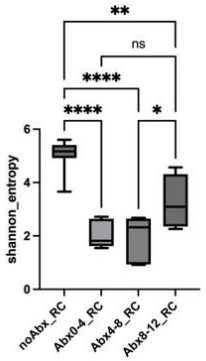

Figure 14. Previous antibiotic treatment causes longstanding impairments in microbial alpha diversity.

Fecal alpha diversity in mice previously treated with antibiotics remained impaired at 16w of age, despite treatment having ended at least 4 weeks prior in all cases. All mice were fed regular chow throughout the study ("RC"). Ordinary one-way ANOVA with Sidak multiple comparisons correction. $F(3,21)=34.02$, overall $p<0.0001$. *$p<0.05$, **$p<0.01$, ****$p<0.00001$, ns=not significant.

However, in these same groups, although transit times were significantly slower during antibiotic treatment, the transit times recovered in all groups by 4w after cessation of antibiotic treatment (Figure 15). This suggests that the microbial depletion using antibiotics was insufficient to promote the same amount of lasting transit impairment as seen in later conventionalized mice. An alternative explanation is that the period of development between weaning and early adulthood, when disturbed, has lasting effects on transit and motility, but that even just a small amount of microbial stimulation during that period – or during part of that period - is sufficient for later life restoration of normal transit.

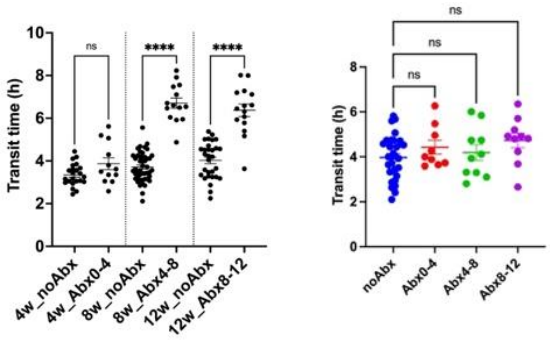

Figure 15. Antibiotic treatment impairs transit when treatment occurs after weaning, but the impairment is recoverable.

Antibiotic treatment slows transit when treatment occurs after weaning. However, when retested at 16w of age, all mice previously treated with antibiotics restored normal transit times relative control mice. RC and hRS fed mice are pooled in all groups in the 16w time point plot (right). Left: Brown-Forsythe ANOVA F*(5, 67.22) = 49.09, overall p<0.0001. Right: Ordinary one-way ANOVA with Dunnett: ns.

Other observations from this set of studies are that the mice treated with a low resistant starch (lRS) diet had slower transit than both RC-fed mice and high resistant starch (hRS) fed mice. However, this difference did not seem to depend on whether antibiotics had previously been consumed, and it also did not seem to depend on the age at which antibiotic treatment occurred. However, the difference did depend on the presence of microbes, as transit times of lRS-fed GF mice did not differ from those of hRS-fed GF mice (Figure 16).

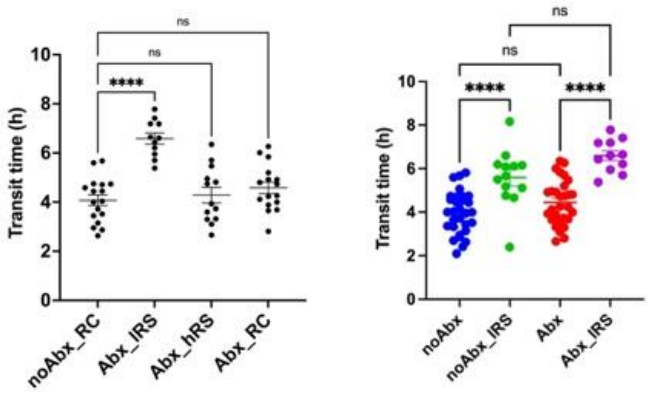

Figure 16. Dietary resistant starch is necessary for normal transit.

Low resistant starch (lRS)-fed mice previously had slower transit than mice fed with diets containing resistant starch, regardless of previous antibiotic treatment (hRS and RC diets are pooled in the "noAbx" and "Abx" groups in the right figure). The mice in each figure were around 16w of age at the time of testing.
Left: Ordinary one-way ANOVA with Sidak multiple comparisons correction. $F(3,53)=17.95$, overall $p<0.0001$. Right: Ordinary one-way ANOVA with Sidak multiple comparisons correction. $F(3,79)=21.27$, overall $p<0.0001$.
****$p<0.0001$, ns=not significant.

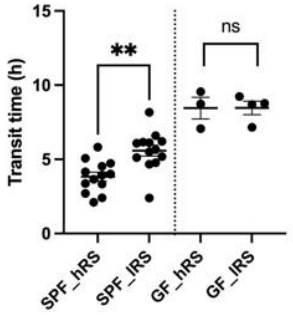

Figure 17. The effects of resistant starch on transit time are microbiome-dependent.

The effects of the lRS diet were microbiome-dependent, as there was no difference in transit times between GF mice fed lRS or hRS diets. SPF mice fed the lRS diet had slower transit than SPF mice fed the hRS diet. Mice were around 16w of age at the time of testing. Unpaired two-tailed t tests. SPF: $t=3.723$, $df= 24$, $p=0.0011$. GF: ns.
**$p<0.01$, ns=not significant.

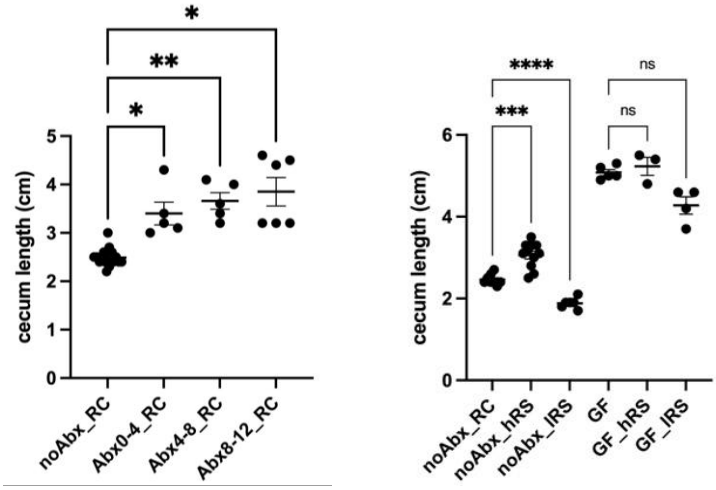

Figure 18. Ceca remained persistently enlarged after antibiotic treatment. Diets that varied in resistant starch affected cecal sizes in a microbiome-dependent manner.

Mice that were previously treated with antibiotics (with treatment ending at least 4w prior in all cases) had enlarged ceca relative to untreated mice, regardless of diet and age at which antibiotic treatment occurred. In the absence of antibiotic treatment in SPF mice, cecal sizes are larger when the mice are fed with hRS diets and smaller when fed with lRS diets. There is no difference between diets in cecal size in GF mice. This suggests that the effect of the diets on cecal size depends on whether a microbial disruption has occurred (including previous antibiotics and germ-free status). Left: Brown-Forsythe ANOVA with Dunnett's T3 multiple comparisons test. $F^*(3, 11.92)=15.04$, $p=0.0002$. Right: Brown-Forsythe ANOVA with Games-Howell's multiple comparisons test. $F^*(5, 9.476)=118.3$, $p<0.0001$.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.

As an overall view of how transit times varied with the antibiotic treatment and dietary manipulations, Figure 19 shows the transit times at 8, 12, and 16w of age in each of the groups.

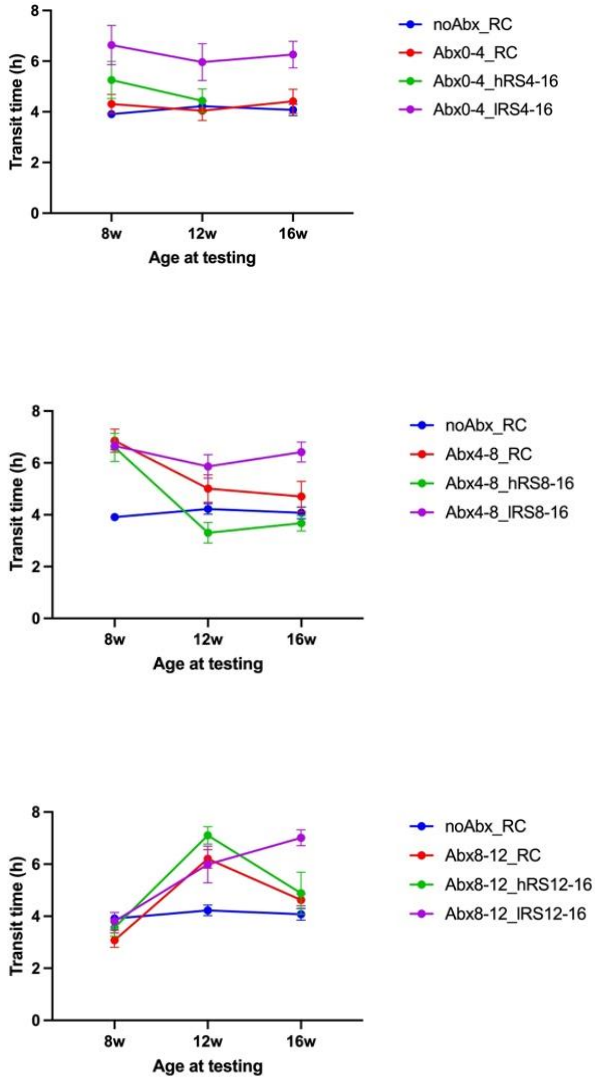

Figure 19. Longitudinal transit times for antibiotic and diet-treated mice.

Transit time at 8, 12, and 16w of age in untreated mice or mice treated with antibiotics between 0-4, 4-8, and 8-12 weeks of age, followed by feeding with regular chow (RC) or a diet that was low (lRS) or high (hRS) in resistant starch. Note that for the Abx0-4_hRS4-16 group, these mice had to be sacrificed at 12 weeks of age because the hRS diet ran out and additional diet could not be provided by the manufacturer in time due to production delays.

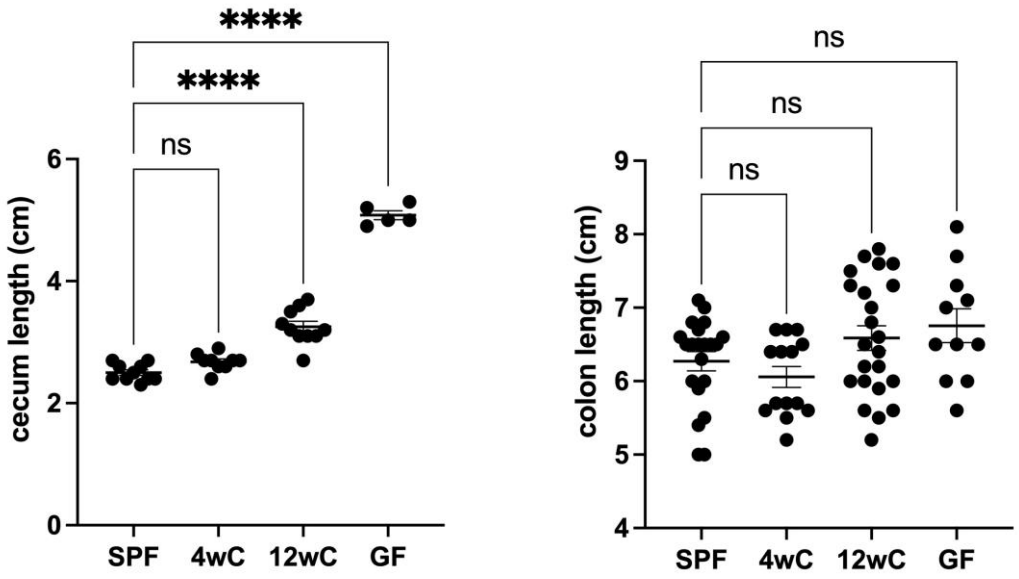

Figure 20. Ceca remained persistently enlarged in 12wC mice.

Cecal lengths are increased in both 12wC and GF mice relative to SPF. Ordinary one-way ANOVA with Dunnett's multiple comparisons test. $F(3,30)=206.5$, $p<0.0001$. Colon length is no different between groups. Ordinary one-way ANOVA with Dunnett's multiple comparisons test. $F(3,65)=2.921.04$, ns.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.

Finding the conventionalization studies most informative, we turned our focus back to the conventionalization studies in female mice.

In summary, normal post weaning development of ENS requires microbes for the following functions: maintenance of neural precursors, cell survival, immune tolerance, axon guidance, and a normal hedgehog pathway setpoint; reduce excessive mitosis and inflammation, hematopoietic stem cell proliferation, apoptosis, and TNF/NFKB signaling. Motility appears to be improved in both FMT groups but to a lesser extent in 12wC than in 4wC. The 12wC female also has increased inflammatory activity in the colon as shown by RNAseq pathway / gene enrichment and CD74

staining at the level of myenteric plexus. There was not much difference in manometry parameters - neither pAUC nor peak parameters for high amplitude contractions (>60mmHg) between 4wC, SPF, and GF. However, 12wC appeared to have hypercontractility based on the average peak heights and the average pressure area under the curve. This could relate to the excessive inflammation in the 12wC colon, as inflammation has been shown to increase colonic contractile activity in some studies (Bossone et al. 2001; Bassotti et al. 2004). Given that transit times of the 12wC mice were still slower than in SPF despite the higher contraction amplitude, this could suggest that while large contractions were higher in amplitude, they were not more effective at propelling contents.

DISCUSSION

Overview

Fundamental to our understanding of human development is that we need certain experiences during early life to develop normally as adults – this is particularly well known for vision, speech, cognition, and social intelligence.

The understanding that early life is a critical period for normal development shapes healthcare guidelines for children and fuels efforts to promote early childhood reading, speaking, and social interaction. While critical periods in brain development are well accepted, it has been unclear whether other parts of the nervous system - such as the enteric nervous system (ENS) - are similarly impacted by early life stimuli.

To explore the possibility of a microbiota-dependent critical period for postnatal ENS development and motility, we exposed germ-free (GF) mice to fecal microbes (FMT) at the time of weaning (4wC) or as adults (12wC) and compared them to age matched GF and conventionally raised (SPF) controls. We found that only the mice given FMT during early life developed normal motility, while those given FMT at 8w or later remained impaired. The transit differences were not explained by differences in colon lengths, cecal size, or fecal water content.

These findings revealed a microbiota-dependent critical period in ENS development affecting adult motility.

Probing potential mechanisms of the motility differences, we performed RNA-sequencing of colonic muscularis propria, where most enteric neurons are found. Specifically, we compared

adult female wildtype C57Bl/6 mice given FMT early (4wC) or late (12wC) in development with GF and SPF mice.

Transcriptomic analysis allowed us to identify the genes and pathways whose adult expression levels depended on earlier microbial exposure – that is, the genes and pathways that "recovered" in 4wC from GF's abnormal to SPF's normal expression levels, while remaining abnormal in 12wC. In other words, this allowed us to test which molecular programs depended on microbiome exposure before a critical age.

We found that expression of genes related to Hedgehog and Wnt signaling, which had low expression in GF mice, recovered up to normal levels in 4wC, but not 12wC, mice. Additionally, we found that expression of genes related to widespread immune activation, highly expressed in GF mice, were also highly expressed in 12wC, while 4wC's expression levels recovered down to those of SPF.

This revealed the presence of a microbiota-dependent critical period in ENS development between 4 and 12 weeks of age, and specifically for the development of a normal setpoint for the Hh and Wnt pathways and for reducing non-specific immune responses in the absence of pathogens.

One of the functions of the Hh pathway is to maintain a pool of neural progenitor cells by inhibiting premature differentiation into neurons. As such, deficient Hh pathway signaling can decrease the ratio of glia to neurons (Young, Stamp, and Hofstra 2015). Indeed, using wholemount immunofluorescence analysis, we found that 12wC and GF mice had lower glia:neuron ratios than SPF controls, while 4wC ratios did not differ from SPF. The reduced proportion of support cells relative to neurons may reflect a lack of molecular safeguards to maintain the glial population.

In a parallel study, when we treated SPF mice with antibiotics during 0-4, 4-8, or 8-12w of age, we found that while their transit times were slowed during the antibiotic treatment, they were still able to return to normal transit times when antibiotic treatment ended. This suggests that even low levels of microbial stimuli may be sufficient to retain the ability to bounce back to normal transit times once microbes are again allowed to freely colonize the gut. Interestingly, though the microbial alpha diversity of antibiotic treated mice remained impaired even several weeks after removal of antibiotics, indicating that microbial diversity alone could not predict transit abnormalities.

We also found that mice fed a diet either high or low in resistant starch differed in their transit times, and this effect depended on the presence of microbes, thereby demonstrating a potential role of the diet-microbiome interaction on ENS function. Specifically, a diet containing resistant starch promoted normal transit times, while a diet without resistant starch delayed transit. Importantly, this effect of diet was not seen in female GF mice fed either a high or low resistant starch diet, so the motility enhancing effect of dietary resistant starch appears to be microbiota-dependent.

These findings support a model in which disruption or depletion of gut microbes during a critical window of postnatal development impairs adult GI motility. Time-sensitive and microbe-dependent developmental signaling pathways may not be able to proceed in the absence or lack of certain microbial signals. Compensations may occur that ultimately 'wire' or configure the ENS in such a way as to not properly function or respond properly to other stimuli once the mouse has more fully matured. Among the signaling programs most impacted by lacking gut microbes during postweaning development is the Hedgehog pathway. While some of these impairments are

reversible, others are not, because subsequent developmental events may have built on the earlier ones and as such may have closed the critical window.

Is the change in Hh pathway related gene expression relevant though to understanding the "cause" of the differences between groups in this study after early versus late microbiome exposure? Beyond the broad difference in a Hh pathway setpoint, what more specifically may be occurring in the early versus late conventionalized mice? Might the differences in Hh related gene expression simply be downstream of other more microbiome-proximal pathways or events? The pathway components implicated by the recovered genes in this study are downstream of Smoothened, a Hh effector protein that is freed from Ptch1 when the hedgehog ligand (such as Sonic hedgehog, Shh) binds. In other words, Shh (or Dhh, Ihh, other hedgehog ligands) must be available for the subsequent steps of the Hh pathway to proceed – and these subsequent steps are the ones that involve the recovered genes.

Thus, either some source of the hedgehog ligand must be present for SPF and 4wC or Smo is being activated by some other mechanism. Because none of the hedgehog ligands Shh, Dhh, or Ihh were differentially expressed between SPF versus GF or between 4wC versus 12wC, it seems that the steps of the Hh pathway subsequent to Hh binding are most relevant to the differences between the groups by adulthood. Notably, though, the ligand levels may have differed earlier in life – such as during postweaning development – and through this ultimately the Hh pathway setpoint was determined.

There are many regulators of Smo activation. In the classic Hh pathway, the presence of the hedgehog ligand releases Smo from the suppressive action of Ptch1, allowing Smo to translocate to the nucleus. When Ptch1 is counteracted, Smo is able to be phosphorylated in many

sites, and the more phosphorylation occurs, the greater Smo's activity. Changes to trafficking within the cilium is also a way of regulating Smo's activity. In some cases, Smo can be activated independently of the Hh ligand. This occurs in the presence of oxysterols, a class of molecules derived from cholesterol (J. Zhang, Liu, and Jia 2021). Oxysterols may be stimulated in the context of inflammation (Foo, Bartlett, and Ronacher 2022). Therefore, one possibility is that the activation of Smo by oxysterols in the postweaning period conditioned Smo to be more active later, changing the changed the Hh pathway expression "setpoint". However, this argument does not explain why 12wC, which has higher expression of inflammatory molecules during adulthood, does not have greater expression of Smo and downstream proteins. Therefore, the most compelling explanation seems to be that a difference in the extracellular levels of Hh ligand would be the factor that promotes the higher levels of Hh pathway genes downstream of Smo, as in 4wC and SPF. Since none of the Hh ligands (Shh, Dhh, and Ihh) area differentially expressed between 4wC and 12wC or between SPF and GF, the cellular source of the Hh ligand could be outside of the colonic muscularis propria. However, this is just speculation; future research is needed to clarify the upstream factors to explain the higher expression of Hh pathway genes in the mice that encountered microbial signals during postweaning development.

Regardless of whether the changes to the Hh pathway originated from the ligand or the subsequent steps of the pathway, it is likely that changes to the Hh pathway did occur during the critical developmental period. Given that GF mice shared the downregulation of pathway components with 12wC suggests that the changes to the Hh pathway are unlikely to be a result far downstream of the late life conventionalization (as the GF controls were not conventionalized, unlike 12wC, yet they shared the Hh pathway differences). Rather, the high expression of antigen

presentation pathways and associated genes such as Cd74 (Figure 9) and the hypercontractility of 12wC – both of which are absent in GF – more likely reflects the downstream effects of receiving microbes against the backdrop of impaired immune tolerance in GF.

As such, along with the facts that Hh signaling is not only fundamental to development but also continues to have functions in adulthood, it seems likely that the Hh pathway regulation was a proximal target of the presence or absence of microbial stimuli during the postweaning period. Further support for this comes from the sex dependence we observed in the motility and gene expression differences, as Hh pathway activity is affected by sex hormones (Franco and Yao 2012). Moreover, several of the developmental phenomena normally occurring around or after the time of weaning have been shown to relate to Hh signaling and suggest potential mechanisms relevant to this study. These include (1) neuron differentiation, as certain neurochemical subtypes of neurons first arise around weaning – particularly peptidergic neurons; (2) pathfinding by neurites as the bowel continues to grow in size postweaning; and (3) synaptic pruning as part of refining synaptic connections for greater signaling efficiency.

Having identified the Hh pathway as a probable early target of microbial presence versus absence, how might these effects of Hh pathway expression play out among cells, their interactions, and in relation to other signaling pathways? To answer this, we will consider our findings in the context of the developmental phenomena normally occurring around the time of weaning.

What developmental processes occur around or after the time of weaning? At the cellular level, a few developmental phenomena of ENS during the postnatal period are synaptic pruning, neuronal differentiation, and pathfinding by neurites. At the organ system level, other pathways

whose developmental activities extend past weaning are immune tolerance development and sexual maturation. How might each of these relate to the ENS?

Neuron differentiation

As described in the Introduction, the Hh pathway is critical for inhibiting neuronal differentiation during migration of the neural crest during embryonic development so that the neural precursor cells can reach their targets – but what about in the postweaning period, once the neural crest derivatives have already migrated? What is the role of the Hh pathway in the postweaning period, and how might it relate the microbiome-dependent critical period for ENS development?

To address this question, it is important to assess what we already know about mechanisms of critical period closure in other parts of the nervous system, namely the brain. Mechanisms of critical period closure in the brain are postulated in some cases to relate to the differentiation potential of neuron progenitors through m6A modifications affecting pro-neural gene expression (Donega et al. 2018). In this study, we found that the proportion of glia:neurons was lower in 12wC and GF, suggesting an impairment in those groups in the ability to maintain the differentiation potential of neuron progenitors. What are the implications of this? First, as this shows a deficit in a function that is controlled by the Hh pathway, and we also found by gene expression that Hh pathway components were deficient in 12wC and GF, this finding strongly implicates the Hh pathway as a mediator of this difference between groups. Second, the involvement of the Hh pathway provides mechanistic insights into critical period closure between weaning and 12w of age.

Postnatal neurogenesis in ENS continues at least to the age of weaning, and peptidergic neuronal subtypes are thought to differentiate last (Pham, Gershon, and Rothman 1991). One possibility is that the differentiation of these late-born neuron subtypes require microbial signals, so lacking microbes during the period immediately postweaning (as in 12wC and GF) leads to a deficit in the numbers or function of these late born neurons. While we did not find evidence of a deficit in peptidergic neurons counts, we did find early life microbiome-dependent (recovered-up) expression of Tacr2, a receptor for a class of neuropeptides called tachykinins (also called neurokinins). Surprisingly, though, expression of the gene encoding for the precursor of the ligands that activate Tacr2 (Tac1) is significantly higher in 12wC than in 4wC (Tac1 expression also trended toward being higher in GF over SPF by log2FC, but the difference was not statistically significant). This could mean that responsiveness of cells to tachykinin levels via Tacr2 depends on microbial induction of tachykinin production before adulthood, as if the microbiome was not required for these peptidergic neurons to differentiate but was required for functional production of tachykinins. Then, upon microbial induction of neurokinin production via Tac1 expression by 12wC during adulthood, the ENS remained unresponsive to these higher levels of tachykinins, and similarly lacked negative feedback mechanisms to regulate tachykinin production. If this is true, this may highlight a neuron differentiation role of the Hh pathway that is specific to the fate decision between glia and neurons and has less of a role in differentiation between neuron subtypes. Simultaneously, other microbiome-dependent developmental processes may be occurring with regard to the function and neurotransmitter release by peptidergic neurons, once differentiated. Then, the responsiveness of the ENS to stimuli later in life may be impaired due to lack of adequate receptor stimulation during earlier life.

Tacr2 is also of interest given its role in enteric neuron-glia signaling during inflammation (Delvalle et al. 2018) (Steinhoff et al. 2014). Another study shows that neurokinin A (to which Tacr2 responds most strongly) has antiproliferative immune effects (Vishalakumar et al. 2006), which could potentially counteract the immune expansion that occurs highly in 12wC.

It proved difficult to quantify Tacr2 using immunofluorescence, as within a given group there could be wide variability in the staining pattern. Although we tried two independent Tacr2 antibodies from different vendors, we found the variable staining pattern in both cases. Notably, in the RNAseq colon data, Tacr2 expression was very mildly elevated in SPF compared to GF (by log2FC); so, a large effect in IF is not expected.

One study found Tacr2 immunoreactivity in neuronal varicosities near enteric glia (Delvalle et al. 2018), and another source reports expression by neuronal varicosities, myocytes, and epithelial cells in mice (Steinhoff et al. 2014). In our staining of colonic muscularis propria, we see scattered and variable expression of Tacr2 in both neuronal soma and varicosities (data not shown). We see less staining in myocytes. Epithelial cells are not part of the muscularis propria, so we have not assessed Tacr2 expression in epithelial cells. However, there has been some conflicting information though about where Tacr2 is expressed.

While a single-nucleus RNA-sequencing (snRNAseq) study of ENS did not find Tacr2 expression in neurons, it is important to keep in mind that they sequenced nuclei rather than whole cells. Indeed, in a tsne plot for mouse from 10X sequencing (Mouse colon all cells (10X) tSNE) (Drokhlyansky et al. 2020), Tacr2 appears to be expressed in many different cell types including myocytes, neurons, and glia.

A study from 2008 describes Nk2r expression in myenteric neurons in colon in humans (Jaafari et al. 2008). That study shows that Tacr2 is co expressed with several types of neurons including those expressing TK, VAChT, NOS, VIP, GAD, in the soma as well as varicosities, and also intramuscular fibers closely opposed to circular muscle and longitudinal muscle. The authors did both protein and mRNA analyses. Another study, though, suggests that Tacr2 is only expressed in the gastric muscle layers in mice, and not neurons (Mulè et al. 2006); however, Tacr2 expression may differ in the stomach and lower intestinal regions.

Another study shows images with Tacr2 immunostaining in mice brains (Medja et al. 2006). While it is unclear based on these images which cell types within were expressing Tacr2, there is very little muscle in the brain, other than vascular smooth muscle. Therefore, it seems likely that the strong Tacr2 staining shown in the brain represents neurons and/or glia. Interestingly, this study shows that Tacr2 mRNA expression peaks before weaning (cortex) (NK2 = Tacr2 in this study).

It is possible that we and other authors have simply experienced cross reactivity of our Tacr2 antibodies with a variable set of cell types and components. However, the validity of our finding that Tacr2 is expressed in cell bodies and varicosities of myenteric neurons is supported by the fact that we tested with two Tacr2 antibodies from different manufacturers.

Further, a study presents findings that suggest that NK1 and NK2 are neuroprotective against excitotoxic neuron death (Medja et al. 2006), and another study suggests that murine NK2 participates in neuron signaling to muscles for contraction (Zhao and Shea-Donohue 2003). In one study, Tacr2 expression in full thickness colon tissue decreased in a rat after TNBS induced colitis (Martínez-Augustin et al. 2008).

What do I think Tacr2 would be doing? Tacr2 may be involved in neurogenic inflammation and immune tolerance, including induction of Tregs. Since it is more greatly expressed in 4wC and SPF, which appear to have less inflammatory signaling than in GF and 12wC, perhaps it relates to the development of immune tolerance for commensal microbes.

Regardless of the mechanisms by which Tacr2 expression is regulated, it is notable that Tacr2 has been associated with primary cilia, which are critical to neuron differentiation.

Enrichments in cilia-related pathways in 4wC and SPF relative to 12wC and GF may represent higher levels of neural precursor cell proliferation in 4wC and SPF; primary cilia undergo significant restructuring among neural precursor cells in different mitotic states (Matsumoto et al. 2019). Almost all cells in the body possess a primary cilium, which allows cells to respond to stimuli in the local environment and also aids in cellular migration. Neurons have a primary cilium with a distinct structure on the cell body, viewable with electron microscopy (Lee and Gleeson 2011). Neuronal primary cilia are important for neural development and this is also where Hh signaling occurs. Interestingly, zebrafish without cilia have impaired Shh signaling**,** but normal planar cell polarity (PCP) / Wnt signaling (Lee and Gleeson 2011), highlighting the unique importance of cilia for Hh pathway function.

The differential expression of cilium structural genes between mice that did or did not have gut microbes during postweaning development strengthens the case for the probable mechanistic relevance of the Hh pathway in the differences between groups; one of the ways Hh signaling (and thus neuron differentiation potential) is regulated is by alterations to cilium structure and its transport machinery. Interestingly, one of the enriched pathways in 4wC over 12wC was related to the regulation of proliferation of cerebellar granule cell neuron precursors, and cilia are required

for Shh-induced proliferation of cerebellar granule neuron precursors during neural development (Lee and Gleeson 2011). Since there is no cerebellum in the gut, this shows an overlap in genes that are involved in the regulation of neuron differentiation in the brain and colon.

Past postweaning development and into adulthood, Hh signaling may act to prevent migration of ENs outside of its designated area within the gut wall (Jin et al. 2015). Additionally, a study described the importance of Hh signaling postnatally, specifically to produce adult neural stem cells (Han et al. 2008). While the ablation of Hh signaling performed in the referenced study was embryonic, it nonetheless represents a phenomenon in which disruptions to Hh signaling during development led to long term impairments of Hh functions (namely, neural stem cell maintenance) that were still evident in adulthood. As Hh signaling also promotes the maintenance of a population of neural precursor cells in the adult brain, it is likely that it could do the same in the postweaning gut (Lee and Gleeson 2011; Palma et al. 2005). As such, this phenomenon could make sense for the present work, as 4wC and SPF may continue to renew their neural progenitor cell populations with the non-neuronal cells within ganglia, while 12wC and GF may have depleted their neural progenitor cell population. This could result in limited plasticity and adaptability of the ENS to new challenges for 12wC and GF.

It may be worth mentioning a few other “recovered-up” genes that may be involved in neuronal differentiation. Another one of the recovered (up) genes related to cellular differentiation, Lrmda, is known as a melanocyte differentiation gene. Why would such a gene be upregulated in the gut? The melanocyte differentiation pathway and Lrmda relate to neural crest, which is also the origin of enteric neurons. Because of the critical nature of the Hh pathway for the neural crest lineage, this gene can be conceptually grouped with the Hh pathway, as discussed above.

Another gene with relevance to neuronal differentiation (or inhibition thereof) is Wnk2. Wnk2 is a neuron enriched regulator of essential chloride - cation channels, and it is involved in regulation of cell volume and potentially regulation of GABAergic signaling. Furthermore, Wnk2 is a G-protein coupled receptor for purine and pyramidine nucleotides, preferentially adenosine and uridine. Wnk2 has been shown to be targeted by miR-18a (miRNA), leading to activation of p53 inhibitors and PI3K/AKT, MAPK, and Wnt pathways (Dong et al. 2018; H. Zhang et al. 2021). Wnk2 affects the balance of Rac1 and RhoA. More specifically, Wnk2 acts to activate RhoA to suppress GTP loading of Rac1, decreasing Pak1 stimulation (Moniz, Matos, and Jordan 2008) as Pak1 is an effector protein of Rac1 - thereby decreasing the affinity of Mek1 for Erk1/2 in response to stimulation by epidermal growth factor (EGF). Because Synj2, like Pak1, is a Rac1 effector protein, it seems like Synj2 would be suppressed if Rac1 is suppressed. The same paper shows that Wnk2 depletion leads to less RhoA activation, which leads to more GTP loading of Rac1, which leads to stimulation of Pak1 and phosphorylation of MEK1 at serine 298; together, this causes a higher affinity of MEK1 toward Erk1/2, whose interaction promotes cell proliferation. Ultimately this suggests a mechanism for Wnk2 suppression of cell proliferation.

Higher regulation of the proliferative response to growth factors in 4wC/SPF makes sense in the context of our results. With higher expression of Wnk2 in 4wC and SPF, one would anticipate fewer ‘growth’ type processes occurring, supported by the higher proportion of non-neuronal cells to neurons (suggesting less terminal differentiation) in 4wC and SPF in contrast to 12wC and GF. Alternatively, greater proliferative gene expression in 12wC and GF could simply reflect an increase in inflammatory cell presence, as many immune cells may turnover more quickly than other cell types.

Either way, it should be noted that the extent of adult neurogenesis in the intestine is somewhat controversial. While in the brain, adult neurogenesis is known to occur from radial glia-like cells even under normal conditions, such as in response to new experiences (Berg et al. 2018), the extent to which it occurs under physiological conditions in the intestine is under debate. It is also accepted that that enteric glia can generate neurons in response to injury (Subhash Kulkarni; Pieter Vanden Berghe; Jaime Belkind-Gerson 2023).

Pathfinding by neuron projections

The postweaning period is a time of continued growth for the murine intestine, and as it grows in size, it is necessary for the neuronal projections to keep pace so that the gut remains innervated throughout. Migrating neurites rely on extracellular guidance cues and the primary cilium is a key piece of machinery for sensing these cues. As such, the Hh pathway regulates synapse formation and pathfinding by neurites (Fuccillo, Joyner, and Fishell 2006; Hill et al. 2019). Therefore, factors that influence ciliary structure and/or Hh pathway activity can alter neuronal connectivity.

For example, of the defects of the ciliopathy Joubert syndrome are lack of proper contralateral connectivity in the brain. Additionally, if cell polarity proteins are disrupted, cilia loss or malformation may result. Certain posttranslational modifications (PTMs) including acetylation, palmitoylation, and others of neuronal tubulin may occur within cilia; these modifications may contribute to neurite outgrowth, maturation, and maintenance of neuronal morphology, allowing the cell to respond to the environment based on nutrient availability and extracellular cues (Lee and Gleeson 2011).

Additionally, Shh enhances axon outgrowth & glutamate release from presynaptic terminals in the hippocampus, enhances neural stem cell proliferation, and regulates neurogenesis (Yao, Petralia, and Mattson 2016). These observations illustrate the interdependence of cilia, Hh signaling, and pathfinding by neurites.

Changes in pathfinding may underly the critical period phenomenon we observe. How? As discussed previously, much of what we know about critical period closure is based on studies in the brain, which have implicated the formation of "perineuronal nets", ECM structures surrounding parvalbumin (PV) positive neurons. However, PV positive neurons have not been found in the ENS. This then raises the question of whether there are "analogous" neuronal subtypes in the ENS that could be involved in critical period closure. Additionally, there could be analogous mechanisms that similarly involve ECM structures preventing new connections by mature neurons. In the brain, these inhibitory neurons are sensitive to guidance cues and at specific times and places to allow them to reach their targets during migration. Relative to other cell types, PV interneurons have an extended migration period. The final destination may be cortex or hippocampus, and the farthest destination is the hippocampus pyramidal layer. Parvalbumin neurons synapse onto pyramidal neurons. High energy requirements also contribute to high sensitivity to inflammation and oxidative stress for PV neurons (Ruden, Dugan, and Konradi 2021).

Although we do not see differences in density of innervation between groups by immunofluorescent staining and analysis, it is possible that the connections that have formed in 12wC and GF are slightly off from how they would be under conventionally raised conditions. This may explain some of the motility defects observed by transit testing. However, to find the

answer to this beyond gene enrichments, electrophysiological studies or calcium imaging techniques are required and out of the scope of this thesis.

Refinement of connections

Synapse development and maturation of ENS occurs to a significant extent postnatally, potentially into adulthood (Foong 2016). Understanding the factors and processes that influence this maturational process may aid in prevention of several nervous system diseases having a gene-by-environment etiology.

Synaptic pruning, described in the Introduction, is the process of removing unneeded synapses during maturation of the nervous system. This makes signaling more efficient by reducing interference and noise. Pruning peaks around the time of weaning in mice, highlighting its potential relevance to the differences between 4wC/SPF and 12wC/GF. Pruning is mediated by several mechanisms, including phagocytosis from other cells such as glia and immune cells and autophagy in which a neuron endocytoses and degrades synapses that are deemed unnecessary. (Synapses that are deemed unnecessary are generally the ones that have been minimally used.) If synapses are pruned during development due to lack of use that will be needed later – such as for properly responding to microbial stimuli – this may preclude a normal response to microbes if they are administered later in life, after pruning has occurred.

As mentioned, a process that is required for pruning is autophagy. If synapses are being pruned, then they must be disposed of by some mechanisms. This is most likely through engulfment and digestion within the neurons themselves or from external cells, such as macrophages, that phagocytose the synapses.

Shh promotes autophagy in mature neurons which may be part of the pruning mechanism; this effect is mediated by Class 3 Pi3k complexes (Petralia et al. 2013). Both the Hh and Wnt pathways involve phosphatidylinositol signaling. Interestingly, several genes related to autophagy were among the "recovered – up" genes in for 4wC (e.g., Tsc1, Vps13c, Herc1).

Key to pruning and refinement of connections is the proper functioning of endo- and exocytosis (Lim and Ruthazer 2021). One of the recovered-up genes was Synj2, and its functions may help to pull together a few themes from the gene expression differences observed between conventionalization groups. Synj1, a paralog of Synj2, had previously been described as a brain specific protein critical for synaptic vesicle release (Malecz et al. 2000); Synj1 is a key enzyme for converting PIP2 to PI4P, which participates in the uncoating of clathrin coated vesicles. Later, a study identified Synj2 as an effector of Rac1 and then validated this with subsequent experiments. Two functions of Rac1 signaling were known to be actin reorganization, lamellipodia formation, and inhibition of endocytosis. These two functions were investigated for their dependence on Synj2. Through a variety of methods, the authors found that just the inhibitory effect of Rac1 on endocytosis depended on Synj2 (not lamellipodia formation), and furthermore, that Synj2 needed to localize to the plasma membrane to have this inhibitory effect. Synj2 was able to inhibit endocytosis of the transferrin receptor in Hela cells and of the EGFR in A431 cells (epithelial like). A version of Synj2 without the Rac1 binding domain did not have this effect (Malecz et al. 2000). The recovery of Synj2 expression only after early life exposure to microbes may reflect a microbiome-dependent process of establishing homeostasis in pruning pathways during postnatal ENS development.

While the postnatal function of Synj2 has not been explored, a study showed that Synj1 was particularly important for postnatal development of the nervous system, related to its role in allowing the uncoating of synaptic vesicles (Cremona et al. 1999). Indeed, mice deficient in Synj1 have an accumulation of synaptic vesicles at the presynaptic terminal; as a result, synaptic depression occurred more rapidly and took longer to recover in these mice. Interestingly, though, although Synj1 and Synj2 are paralogs, Synj2 inhibits clathrin mediated endocytosis (Svitkina 2013), potentially reflecting a homeostatic system that may be perturbed in 12wC and GF, relative to 4wC and SPF. Other studies showed that specific regulation of Rac1 around the time of weaning was required for normal synaptic pruning in mice (Riccomagno et al. 2012). Given that another recovered-up gene Wnk2 also regulates the activity of Rac1, it is plausible that the combined deficiencies in Wnk2 and Synj2 in 12wC and GF mice could reflect an impairment in synaptic pruning.

Immune training

Immune function has been connected with ENS function through interactions between muscularis macrophages and enteric neurons (Muller et al. 2014). One of the "recovered – down" genes across comparisons was P2ry10, a purinergic receptor typically associated with immune system processes. P2ry10 is officially an orphan receptor but has been shown to bind LysoPS (lysophosphatidylserine) (Chiu, von Hehn, and Woolf 2012), which was shown to inhibit T cell proliferation in vitro (Omi, Kano, and Aoki 2021). P2ry10 couples with Galpha12 and Galpha13 (Hwang et al. 2018).

One study demonstrated that P2ry10 contributes to cutaneous hypersensitivity and autoimmunity in certain animal models. Additionally, they show that P2ry10, in response to LysoPS and ATP, mediates RhoA activation in T cells to facilitate their chemokine induced migration (Gurusamy et al. 2021). LysoPS is a bioactive lipid (Omi, Kano, and Aoki 2021). The paper cited here mentions another study that found that LPS increased LysoPS in stimulated macrophages, and subsequently those macrophages released most of the LysoPS they contained while T cells accumulated LysoPS upon LPS stimulation and did not. LysoPS forms by deacetylation of phosphatidylserine; deacetylation is performed by PLA1 and PLA2. A table in the study cited above shows that in vitro studies found P2ry10 suppressed cytokine production in microglia and dendritic cells; in eosinophiles, P2ry10 promoted degranulation, survival, and formation of eosinophil extracellular traps, which serve as scaffolds for both immune defenses or pathogen attacks (Omi, Kano, and Aoki 2021). Additionally, B cells, T cells, and DCs strongly express P2ry10..

Transcription of P2y10 regulated by Ets transcription factors PU.1 and Spi-B. These also transactivate B cell related genes during B cell development. P2y10 is strongly expressed in immature and mature B cells, but not B cell precursors (Gurusamy et al. 2021). Suppression of LPS induced TNFalpha production in murine DCs and microglia (in vitro). LysoPS signaling through P2ry10 may act through ERK to induce eosinophil degranulation (Omi, Kano, and Aoki 2021). Under normal conditions, LysoPS levels are low; LysoPS production increases when platelets are activated. Therefore, LysoPS signaling through P2ry10 may indicate an inflammatory state (Omi, Kano, and Aoki 2021).

One paper suggests that P2ry10 could potentially promote autoimmunity by activating T cells through RhoA activation, promoting the migration of inflammatory cells to peripheral tissues (Gurusamy et al. 2021). It would therefore make sense for the animals with higher P2ry10 expression to have greater expression of pathways related to migration. Indeed, this is the case for 12wC and GF.

One of the P2ry10 promoter binding sites is recognized by NFKB (Safran et al. 2021; Stelzer et al. 2016). P2ry10 is part of purinergic signaling and the rhodopsin like receptors pathway. Looking more into the P2ry10 gene in relation to neurons, it is associated with transcriptional regulation of genes involved in immune activity, such as after brain injury (Hazy et al. 2019). Additionally, P2ry10 is upregulated in certain inflammatory conditions in humans (X. Wang et al. 2016). However, there is no evidence that P2ry10 is expressed in neurons.

In a study on single cell sequencing of lymphocytes infiltrating the spinal cord in a mouse model of multiple sclerosis, a disease characterized by neuroinflammation, it was found that a subtype of Th17 CD4+ T cells upregulated P2ry10 (Tischner et al. 2017). It seems likely that Th17 cells would be upregulated in 12wC in the context of an excessive inflammatory response to microbes.

P2ry10 associated GOBP pathways upregulated in 12wC over 4wC include regulation of signal transduction and regulation of intracellular signal transduction. However, there are no significantly enriched GOBP pathways that include P2ry10 in GF compared to SPF; this is likely related to a paucity of annotation for orphan receptors such as P2ry10. Nonetheless, because P2ry10 reflects inflammatory conditions, it may be secondary to developmental changes affecting immune tolerance in the 12wC and GF mice.

Sexual maturation

Related to sexual maturation, it is clear based on the contrasting ENS gene expression profiles of male and female mice that the microbiome has sex-dependent effects on the ENS (Results). It therefore makes sense that one of the strongest patterns among the early life microbiota-dependent genes would relate to the Hedgehog (Hh) pathway, which is known to function differently in males and females (Franco and Yao 2012). However, as previously mentioned, a deep dive into the role of sex hormones, although fascinating, is out of the scope of the present work.

Caveats and limitations

One possibility is that the ENS critical period effects are secondary to immune effects. While this cannot be ruled out by the present study, the reality is that the ENS effects exist regardless of whether the effects were mediated by other cells. Hopefully, future studies will explore these mediators. Additionally, much literature already exists describing a critical window for immune development during early life. In contrast, very little is known about microbiota-dependent critical periods in ENS development, and this work begins to address that gap.

Another criticism that could be posed of the present work is that it seems unlikely that there would not be any differences in manometry if transit times were so different between groups. Indeed, it is true that the manometry differences were few here. However, first, I should point out that our conclusions do not depend on the manometry findings. The manometry method we used in this study is the same that has been used by many others, and there are similar limitations to

those studies. Given the clear differences in transit time, this raises the question of the sensitivity of this measure. In humans, manometry is generally performed with many probes at defined locations within the intestine; however, this method in mice uses just a single probe and therefore does not assess propagation – just pressure changes. The major manometry peak difference (for high amplitude >60mmHg peaks) between the groups is 12wC having a higher amplitude of contraction, suggesting the capacity for a more powerful contraction in the 12wC than in others; this supports the idea that hypercontractility occurs in 12wC.

Hypercontractility could result from inflammation in the 12wC group. Signals from ascending or descending interneurons communicating instructions for contraction based on up/downstream activity or sensation, to facilitate coordinated contractions and appropriate responses to stimuli such as stretch; they might also receive inputs from the mucosa communicating the presence of immune activating patterns which they could use to increase inflammatory signals (neurogenic inflammation) in the myenteric plexus or to muscles to respond with stronger contractions if needed.

Relating this to manometry, what would then I expect to see? If myenteric neurons received fewer inputs from neighboring ganglia and projections from mucosa or extrinsic inputs e.g. from the vagus nerve, then they could have less of a response to external factors outside of their own specialized function (e.g., sensing stretch). This could result in a lack of difference between groups in the absence of external factors/stimuli. As a pellet passes through the colon - before it reaches the intestinal segment adjacent to the probe, myenteric neurons should have received descending reflex signals from more proximal segments of the intestine to relax and allow the pellet to pass.

Once the pellet passed, the neurons in the area by the probe would need to signal a contraction to the muscles to continue to propel the pellet along the colon.

If communication between ganglia is impaired, then perhaps the signals to relax (return to baseline on manometry trace) would be absent or dampened while the signals to contract upon sensing stretch (i.e. while the pellet is passing by) are still present because those signals go directly to the cells in that area. So, perhaps there would be more activity for this reason, such as less relaxation when appropriate. However, the pAUC values were similar between groups. Maybe there would be more peaks expected per unit time, although I did not see that either. Previous studies have mentioned that certain manometry runs were not included in the analysis due to probable obstruction/occlusion by a pellet on the sensor (Gourcerol et al. 2009). So, I wonder if my pAUC data were diluted by cases such as this. To identify these, I could look back at my pAUC data and see which ones have very little activity, lower than most others. I could then remove those traces and see if it cleans up the data at all. The same might go for peaks characteristics, but this is less of a concern when the peaks are pooled across individuals. However, in the absence of clearly defined standards for keeping or removing manometry data from a study, it is unclear what criteria would distinguish between valid and invalid traces.

Data suggest that glia are involved somehow in the phenomenon I observe or at least dominate the "recovered" pathways after early conventionalization. Given this, it seems like one might predict that age matched 4wC versus 12wC female mice manometry data at 16w would show that the 4wC has a higher magnitude of large contractions relative to 12wC; also it seems like 4wC would have higher pAUC than 12wC. However, I did not see that. Additionally, lack of coordinated/efficient firing of ENs in 12wC and GF may have led to a 'noisier' signal in

manometry, but methods for quantifying this are uncertain. This lack of coordination could lead to slower transit, with a combination of immune training and neural development mediating the effect.

It was surprising that the Abx-treated mice did not show the same transit deficit as the 12wC: it turns out that the mice previously treated with antibiotics (prevAbx) still have an elevated fecal water fraction at 16w of age (i.e., at least 4w after Abx ended) relative to controls. The higher fecal water content could speed up transit time that would otherwise be delayed, therefore masking impaired motility in the prevAbx mice.

However, this raises the question of why the prevAbx mice continued to have higher fecal water content at 16w of age, while that of the 12wC mice normalized by 16w.

One potential explanation is that fecal water content is more strongly linked to present microbial diversity than to the early life microbiota. While 12wC mice had lower alpha diversity than SPF, the mice previously treated with Abx had even lower alpha diversity than 12wC. In other words, the magnitude of reduction in diversity could explain the residual high fecal water in prevAbx (but not 12wC) mice.

One final possibility is that the effect sizes of prevAbx on transit could be smaller than for 12wC mice due to the greater extent of microbial absence during early life in 12wC mice than in the prevAbx mice (Abx duration of 4w in all groups). Possibly, with more replicates, the prevAbx transit difference would reach statistical significance.

Another potential criticism of the present work is that it is necessary to characterize the proportions of neuron subtypes in each group because these would be expected to differ between groups. However, this is not necessarily the case – and in fact is probably not the case to explain

"recovery" of ENS development by 4wC in the present study – as there were few differences in neuron subtype gene expression among groups, particularly ones that were equivalent in expression in 4wC and SPF, with GF and 12wC both having expression levels of the marker that differed from that of SPF (Results).

Furthermore, there are limitations to wholemount immunofluorescence staining and analysis. Importantly, it is often unreliable to quantify biological differences by differences in fluorescence intensity of markers, as wholemount intestinal tissue is inherently variable in tissue thickness between and within samples. Thicker tissue is more likely to yield lower fluorescence intensity due to scattering and obstruction of light from tissue components. Even if the myenteric plexus is completely exposed and oriented such that the light will reach it before other tissue components, differences in extracellular matrix density and components surrounding the neurons and glia could still obscure light; and all cells of the myenteric plexus are not fully visible in a single image plane. Therefore, to capture cells that are in a slightly deeper imaging plane – which may systematically differ from those in the more proximal imaging plane – the light inherently must pass through other tissue components. Similarly, this variable thickness influences the plane (along the Z-axis) of the myenteric plexus, and there also maybe natural curvature in the plane of the myenteric plexus even if the tissue thickness remains uniform. The challenge in consistently getting one full field of view (in focus) of the ENS including multiple ganglia means that throughput is greatly limited as ROIs must be manually drawn when performing percent area calculations. Furthermore, the cell distribution between even neighboring ganglia in the ENS can vary dramatically – and even more so across intestinal regions – so obtaining reproducible results

for cell type distribution may require a large amount of tissue from a given animal; this then limits the tissue that is available for other experimental assays such as sequencing.

Another methodological limitation to keep in mind for wholemount immunofluorescence staining and analysis is that sample processing methods will greatly impact staining; even small differences in fixation time may affect antibody penetration. Tissue segment thickness and density of extracellular matrix proteins may also influence antibody penetration. It should also be noted that very few antibodies have been validated for use in multiple applications relevant to ENS research. One workaround is to use genetically encoded fluorescent tags that mark cells of interest – however, this may only work for a limited selection of cell types and with limited specificity given that ChAT expression, for example, occurs in multiple cell types with very different functions.

Another limitation of wholemount immunofluorescence is that intestinal tissue is inherently stretchy and the degree of tissue stretch can vary significantly across segments even when attempts are made to carefully control stretch during fixation. Tissue that is more stretched may appear to have larger cell bodies, proximity of cell types, and density of neurons within a given visual field. These differences can make or break an outcome when looking at subtle physiological differences such as those that may be present between individuals with and without motility issues.

These limitations may explain the conflicting results in the literature regarding cellular composition in mice under different treatment compositions, as noted in the Introduction.

A potential solution might be greater info sharing among ENS researchers to compile knowledge about staining methods and tissue processing. The appropriate methods inevitably will

differ between research questions as certain processing steps – such as tissue perfusion to remove blood – may improve visualization when studying blood vessel associated cell types, but may be out of the question for immune and/or endocrine research questions. Compiling this methodological knowledge is not an easy task but could greatly improve the productivity of current and future ENS researchers. Another solution when possible is using proportions of cell types rather than basing these values on area measurements, such as the present study for the glia:neuron ratios.

Of course, there are also limitations to transit testing as a method of assessing motility. Some of these limitations were discussed in the Introduction, but I have a few additional notes that may help those planning a motility study. First, one must be very clear and consistent with how the first positive pellet is defined. For example, when using carmine dye, does it count if just the tip of the pellet is red? Half of the pellet? Or throughout the pellet, such that the entire pellet including the inside is saturated with red dye (my criterion)? Note that special diets may affect the color of the pellet at baseline, which will influence how readily small amounts of dye can be detected by the observer.

Additionally, while the metal grating is placed at the bottom of the cage for the purposes of stool pellets falling out of reach of the test subject (mice are coprophagic), this will not work for all pellets because – as I have seen many times – mice often eat fecal pellets directly from the rectum in just a matter of seconds, which can easily be missed by the experimenter or a camera as the mouse may appear to just be grooming themselves, as they often do while assuming a similar position. (I have spent many hours observing mice undergoing transit testing.)

One more limitation to note about transit testing is that time of day of testing is another source of variability. Inconveniently, the sleep/wake cycle of mice does not match that of humans, and mice are most active at night. Mice will often fall asleep during transit testing, resulting in potentially several hours during which no pellet is observed – upon waking, the mouse may release multiple pellets in short succession, indicating that while they slept, stool pellets accumulated in the distal colon.

Despite its many limitations, transit testing is one of the best methods for experimentally testing motility, given its clear physiological relevance, minimal technical requirements, low cost, and reproducibility if enough replicates are included and conditions are reasonably controlled. It also may help to test the same mouse multiple times to obtain an average value – one must just make sure that the dye has completely cleared from the stool before re-testing. In my experience, SPF mice would clear the dye within 24 hours, while mice with transit delays could take 2-3 days (in these cases, it is probable that some of the dye accumulated in the cecum and continued to mix with intestinal contents as they passed from ileum to colon).

Conceptual framework for early life microbiome impact on ENS development

The central question to my study is how the early life microbiome affects the development of the ENS. What is different about the ENS in mice that did not have the necessary stimulation by microbes during postnatal development?

There are two clear effects of the early life microbiome that emerge from this study. First, microbial presence between 4-12w of age affects the "set point" of Hedgehog pathway expression in the ENS. Second, microbiome presence between 4-12w of age is necessary for immune

tolerance later in life. The focus of this discussion will be the former, as the latter has previously been characterized.

How would a pathway involved in embryonic neural crest cell migration and gut colonization (i.e., the Hh pathway) still be functionally relevant in the adult ENS? Literature from the brain (discussed in the Introduction) and data from the present study suggest that Hh signaling is responsible for maintaining the balance of neurons and glia in ENS; controlling the localization of neurons to the appropriate support cells; supplying new neurons through neurogenesis from progenitor cells as needed; and axon extension and neurite outgrowth in response to changing physiological demands requiring remodeling of connections.

One idea is that the late conventionalized mice missed out on a critical time of synaptic pruning within the ENS that depended on microbial stimuli. That is, proper pruning within the colonic ENS depends on microbial stimuli between 4w and 12w of age. The question remains though, would this deficit in pruning be specific to a certain set of processes and functions, give that microbial stimulation is likely not the only factor driving pruning, but may be critical just for certain pathways?

Another idea is a failure of the late conventionalized mice to develop proper inhibitory synapses during the “adolescent” or post-weaning, pre-adulthood period. It is known that in the brain, adolescence is a major time for the development of inhibitory synapses (Brennaman, Moss, and Maness 2014). Having fewer NPCs in the brain results in dysregulated inhibitory neuronal signaling, leading to excessive neuronal activity in the hippocampus (Hollands et al. 2017). While we did not directly measure inhibitory signaling, this is one hypothesis that future research could explore.

Additionally, since microbes likely influence the nature and quantity of axon guidance cues (Vuong et al. 2020), it seems plausible that in the absence of microbes, the proper connections fail to form before a critical point, and so when connectivity refinement & topographical organization occurred it caused the 12wC mice to have either improper connections or excessive loss of axons due to the nervous system "surveillance" system recognizing that the connections were not functional and therefore they were removed (Faust, Gunner, and Schafer 2021). Indeed, one of the pathways that returned to normal values after FMT in the 4wC mice only, the smoothened signaling pathway, is involved in axon guidance, formation of proper connections, and topographical organization (Faria et al. 2019). Synj2 could inhibit clathrin mediated endocytosis to prolong growth factor signaling for axon guidance (Barker et al. 2020). Therefore, the ENS of GF and 12wC may have aberrant connectivity.

Finally, the lower expression of Hh- and cilia-related genes in GF suggests they may have dysregulated cell growth, impaired sensing of the extracellular environment, and an impaired response to nearby cells and chemical stimuli.

One of the signaling hubs in 4wC and SPF was Trp53 (TP53), which is a critical regulator of the cell cycle. Interestingly, in presence of Bax and Bak, which were upregulated in 12wC and GF, TP53 signals apoptosis, while TP53 activation of Tsc1 and Tsc2 (upregulated in 4wC and SPF) can lead to autophagy instead (Fan and Zong 2013). Therefore, these gene expression differences related to the cell cycle may reflect steps in pro-autophagy (survival) signaling in SPF and 4wC yet pro-apoptotic (death) signaling in 12wC and GF. It is important to keep in mind though that the increased expression of apoptosis and cell cycle progression related genes in 12wC

and GF could just reflect a higher immune activation in these groups, since immune effector cells are shorter lived and more proliferative than neurons and glia.

Enteric glia could account for some of the enrichments in the RNAseq data, and it has been shown that glia affect motility (Rosenberg and Rao 2021). Reflecting the close interactions between enteric neurons and glia, Gabella described "neuroglial junctions" within the ENS of several species using electron microscopy (EM) (Gabella 1981). These neuroglial junctions involved an axon appearing to synapse onto an enteric glial cell. It is potentially relevant to note that one function of neuron-glia communication is autophagy related to misfolded proteins (Damulewicz, Szypulski, and Pyza 2022). Therefore, an upregulation of autophagy related genes in 4wC and SPF could reflect more neuron-glia communication and proper removal of cellular waste. If what happens in the brain holds true for the enteric nervous system, inadequate waste removal could lead to an excessive buildup of misfolded proteins and neuron degeneration. Therefore, impairments in neuron-glia communication could lead to more apoptosis in GF and 12wC.

Mediators of hypercontractility in 12wC could include greater inflammatory response to commensal or even self-antigen signals and that this leads to a potentiation of muscle contractile response or properties. In this case it seems like the immune system is what is dysregulated and that there is a lack of tolerance for commensal microbes (or even for self - such as in the case of GF). To test this, one would need to suppress the immune system in 12wC female mice to see if hypercontractility remained. Additionally, if one were to treat 4wC or even SPF female mice with inflammatory stimuli then I would expect to see the same hypercontractility response seen in 12wC. GF did not appear to have hypercontractility in the same way as 12wC compared to SPF.

One idea is that 12wC (female) “rebounds” or “overshoots” relative to GF when exposed to microbiota because immune tolerance for commensal microbes failed to develop. As a result, the excess inflammatory conditions in 12wC may impair contractility.

Tacr2 (one of the recovered-up genes) may be important for tolerance to microbes, including for cells of the ENS, given that it skews T cells toward a more tolerant phenotype and is expressed on several cell types including on neuronal varicosities. This combined with more effective neuron-glia communication could allow 4wC female to have more normal immune function relative to 12wC.

Lacking a microbiome between 4-12w of age in female mice may lead to an autoimmune phenotype because mice fail to develop tolerance commensal microbes. The downregulation of pathways related to primary cilia in 12wC and GF may also suggest a mechanism by which these groups are impaired in the ability to sense and appropriately respond to extracellular cues. Potentially this is not only a problem of the current inflammatory environment but also the neurons themselves, having lower expression of ciliary proteins, may be impaired in their ability to sense certain stimuli required for neurite outgrowth and axon guidance.

It should be noted that important processes are probably not showing up in the GO terms list given less research having been performed on many of the recovered genes; hence, relevant GO terms for these genes have not been created. For example, many of the strongly enriched genes (including Wnk2 and other recovered-up and recovered-down genes), did not show up in the pathways gene set enrichments. Because of this, it has been necessary to consider the available literature on individual top DE genes to try to understand and reason through their functions in the present study.

Signaling by Hh family would keep terminal differentiation in check for neural precursor and stem cells to maintain the stem cell niche, to avoid the low proportions of glia:neurons in 12wC and GF ganglia. There may be more noise in the manometry signal from 12wC and GF maybe since inefficient and unrefined synaptic firing, but this has proven difficult to quantify.

All in all, the differences between mice exposed to the microbiome early versus later in life form part of a complicated system involving multiple cell types – but pertaining to the ENS the function 4wC "gets back" that 12wC does not may regard balanced neuronal versus glial differentiation, axon guidance, and the ability to remodel connections as needed. The Hh pathway is critical here because it reflects a system that is sensitive to the extracellular environment and regulates cell fate decisions, such as precursor proliferation vs terminal differentiation into neurons, as well as the proper localization of neurons and their projections.

The ENS of 4wC and SPF seems to represent coordinated maintenance of a healthy adult nervous system, including prevention of excessive signaling while also allowing for neurogenesis and axon guidance as needed; maintenance of glial cells and neural progenitor cells; and the ability to sense and adapt to environmental conditions.

In summary, this project has shown that the microbiome affects maturation pathways in ENS. If microbial signaling is absent, this affects motility. This microbial signaling needs to occur within a certain time frame or it cannot be fully recovered later. The ENS is sensitive to early life disturbances similar to how the brain is sensitive to such disturbances.

Potential clinical implications

The results of this thesis raise the possibility that motility disorders are in some cases related to an early life microbiota-dependent critical period in ENS development that has closed. This may explain why motility disorders in some cases are so hard to treat, i.e., if some developmental processes slowly or never return – this would be a strong rationale for identifying early life drivers of ENS development, assessing the clinical trajectory of gut motility development, and intervening in a precision way to restore early life microbiome eubiosis. This would represent a shift in the traditional paradigm of reactive medicine to one of prevention. The gut microbiome, unlike other vital organ systems, is easily manipulated. It can of course be replaced (FMT), but is it far better and easier to fix early life dysbiosis than to correct it in later life when host systems are less responsive.

Conclusion

This thesis is built on known principles including critical periods of neural and immune development – and potentially 'neuroimmune training' - after weaning. What had not previously been shown is how these principles might apply to ENS.

Exposures to environmental stimuli during early life shape long term immune, brain, and metabolic health, and microbial exposures are increasingly recognized as key environmental stimuli during this period. However, the role of the early life microbiome in gastrointestinal motility and ENS development is unclear.

To address the question of whether microbial stimuli are needed for ENS development, gut transit (a readout of ENS function) was tested in mice exposed to microbes either at weaning or

once fully mature adults (~12w of age). Indeed, gut transit was impaired in the 12wC mice relative to SPF controls while it was not in the 4wC mice, suggesting the existence of a microbiota-dependent critical period in postnatal ENS maturation and motility. This effect was present only in female mice, so females were the focus of this study. Notably, functional bowel disorders are more prevalent in females than males. To explore potential mechanisms for this difference, we performed RNAseq of colonic muscularis propria as an ENS-enriched population of cells. We found that the ENS and associated cells of mice conventionalized at weaning (4wC) were more like the SPF ENS than that of mice conventionalized at 12w of age (12wC), while 12wC ENS was more similar to the GF ENS. More specifically, 4wC and SPF mice had greater expression of genes related to axon guidance and Hedgehog signaling compared to 12wC and GF, which had greater expression of genes related to mitosis and immune activation.

Our study highlights a microbiota-dependent sensitive period in ENS development, pointing to potential roles of the early life microbiome in later life dysmotility.

It is important to note that although the mice conventionalized at weaning recovered normal transit times and expression of several key pathways, there were some unrecovered pathways whose expression may depend on microbial exposure prior to weaning. Many others have shown the importance of pre-weaning microbiome exposure on long term health outcomes.

One of the canonical principles in psychology and neuroscience is that certain stimuli must be encountered during specific developmental periods for the brain to develop normally. This principle has prompted public health metrics such as the ACE score for long term health outcomes based on traumatic childhood experiences. Screening for early life microbiome disturbances might be just as important for clinicians to assess health risks. It may also be worth noting that this study

largely focused on a developmental period that could be considered murine "adolescence" – the transition period between early childhood and adulthood, encompassing sexual maturation, establishment of a metabolic setpoint, and development of higher order cognition. Early life studies often justifiably focus on the pre-weaning period in mice to maximize effect sizes when comparing early and later life; however, development continues after weaning and disruptions to this period may have health consequences that are distinct from those resulting from earlier disturbances.

Potential future areas for further investigation

1. What are the mechanisms by which the "setpoint" of Hh signaling is established in ENS? Beyond motility, what are the other functional consequences for ENS development?
    a. Further characterization of Hh signaling differences in 4wC and 12wC, including staining for Hh pathway proteins, qPCR for Hh pathway genes, single nucleus RNAseq, and quantification of patterning differences, such as fewer or more cells per ganglion, and uniformity between the positioning of the ganglia.
    b. Failure of neural population homeostasis: depleted ratios of neural progenitor cells to total glia and neurons; staining for markers of DNA damage and cell death to assess which if any cell types other than immune cells might be apoptosing.
    c. Are there specific microbial metabolites that contribute to the Hh setpoint establishment?
        i. In vitro cell stimulation experiments (with microbial metabolites or sources of immune-activating molecular patterns, such as LPS).

2. What other ENS morphological differences depend on the early life microbiota, such as clustering patterns of synapses and neuroglial junctions and innervation of smooth muscles?
    a. Axon guidance: in vitro studies with chemoattractants; however, the deficit is likely multifaceted and best replicated in vivo; alternatively, staining for neural targets to muscle, or EM analysis of differences in muscle innervation, potentially quantified by less surface area in contact between neuronal processes and muscles.
3. To what extent do the ENS and motility deficits depend on the immune response to the microbiota?
    a. Aberrant immune activation: staining for spatial relationships of a variety of immune cell types to neuronal ganglia in imaging; quantification of average size of these other immune cells, and their counts.
4. How does ENS development and its sensitivity to early life microbiota differ between sexes?
    a. Can the sex differences be recapitulated through hormone manipulation and/or gonadectomy?
5. What is the contribution of the extracellular matrix (ECM) to early life microbiota dependent motility impairments?
6. Do 4wC and 12wC differ in propagation patterns of contractions, using high resolution colonic manometry?
7. How and why does the microbiota differentially affect colon and ileum ENS and what implications might this have for motility disorders affecting small versus large bowel?

8. What other body systems might have early life microbiota-dependent critical periods in development?
    a. Metabolic impairments: staining for levels of lipids, body composition measurements using DEXA scan, monitoring of activity using metabolic cages.
    b. Other parts of the nervous system, including cardiovascular function.

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