Am J Physiol Gastrointest Liver Physiol 324: G196–G206, 2023.
First published January 10, 2023; doi:10.1152/ajpgi.00166.2022

american physiological society

AMERICAN JOURNAL OF PHYSIOLOGY
GASTROINTESTINAL AND LIVER PHYSIOLOGY

REVIEW

Who's talking to whom: microbiome-enteric nervous system interactions in early life

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Abstract

The enteric nervous system (ENS) is the intrinsic nervous system of the gastrointestinal tract (GI) and regulates important GI functions, including motility, nutrient uptake, and immune response. The development of the ENS begins during early organogenesis and continues to develop once feeding begins, with ongoing plasticity into adulthood. There has been increasing recognition that the intestinal microbiota and ENS interact during critical periods, with implications for normal development and potential disease pathogenesis. In this review, we focus on insights from mouse and zebrafish model systems to compare and contrast how each model can serve in elucidating the bidirectional communication between the ENS and the microbiome. At the end of this review, we further outline implications for human disease and highlight research innovations that can lead the field forward.

enteric neuron; enteric glia; ENS neuropathies; microbiota; zebrafish

INTRODUCTION

Gastrointestinal (GI) homeostasis depends on the integrative actions of the enteric nervous system (ENS) in motility, sensation, secretion, absorption, and communication with the immune system. The development of the ENS begins during early organogenesis and continues to develop once feeding begins, with ongoing plasticity into adulthood. There has been increasing recognition that the intestinal microbiota and ENS interact during critical periods, with implications for normal development and potentially disease pathogenesis. In this review, we focus on insights from animal model systems in our evolving understanding of the bidirectional communication between the ENS and the microbiome, and further outline implications for human disease and research innovations that can lead the field forward.

ENS DEVELOPMENT IN MODEL SYSTEMS

Model systems provide a powerful means to investigate new hypotheses and potential mechanisms in host-microbiota interactions. Although mice/rodent models are commonly used in biomedical research, all findings are not necessarily translatable to humans, and thus advancements in both basic science and clinical implications are further enrichened by exploring a range of model systems. In this section, the perspectives from mouse and zebrafish development will be highlighted.

The development of the ENS in both mice and zebrafish can be conceptualized into three stages: migration of progenitor cells, proliferation, and differentiation (Fig. 1). In the mouse, the ENS derives from the neural crest (1). The enteric neural crest-derived cells (ENCCs) that migrate to the GI tract delaminate from the neural crest at the vagal, truncal, and sacral axial levels. The majority of the ENCCs come from the vagal crest and colonize the entire bowel (2). A smaller set migrates from the sacral crest and only colonizes the postumbilical intestine (2–5) and the truncal crest contributes to the colonization of the esophagus (6). In addition to ENCCs, a population of Schwann cell precursors has been identified, which enter the caudal mid-intestine with extrinsic nerves and give rise to about one fifth of neurons in the colonic ENS, with ongoing postnatal neurogenesis (7). In zebrafish, the ENS is derived from a portion of the vagal crest that is located posterior to the developing ear, with the specific part of the vagal crest that gives rise to the ENS remaining to be discovered (8–10). Vagal crest-derived cells migrate ventrally from the postotic hindbrain toward the developing GI tract. Upon reaching the anterior portion of the developing GI tract, the enteric progenitor cells (EPCs) then migrate in two bilateral streams to reach the end of the intestine (8–11). In addition, trunk crest-derived neural crest stem cells, which are likely Schwann cell precursors, have also been suggested to contribute to postembryonic neurogenesis in zebrafish (12). The mouse ENCCs that migrate to the bowel constitute a heterogeneous population that changes progressively as a function of developmental age, both while precursor cells are migrating and after they have reached the GI tract (13–17). Similarly in zebrafish, the EPCs display an anterior to posterior gradient of different developmental states during colonization, based on their characteristics of migration and proliferation, as well as on their profiles of differentiation (10).

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Submitted 5 July 2022 / Revised 22 December 2022 / Accepted 4 January 2023

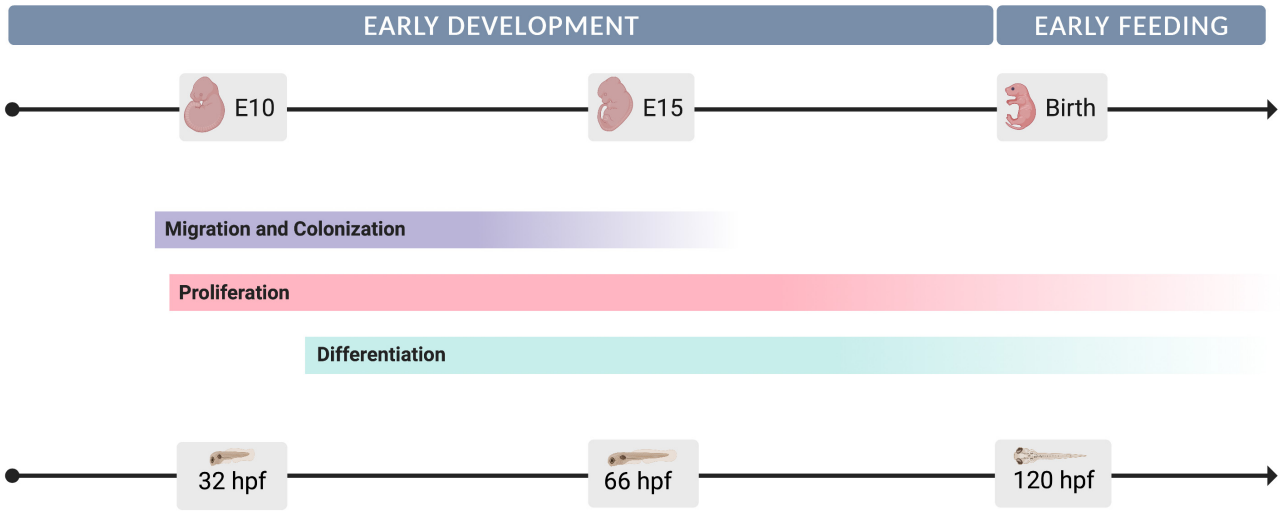

Figure 1. Schematic timeline of enteric nervous system (ENS) development in mice and zebrafish. Developmental stages in mice and zebrafish can be described by periods of early development and early feeding, at which point the gastrointestinal (GI) tract has greater opportunities to be colonized by microbiota. In both mice and zebrafish, the development of the ENS can be conceptualized into three stages: migration of progenitor cells and colonization within the gut wall, proliferation, and differentiation. Created with BioRender.com. hpf, hours postfertilization.

The colonization of the GI tract takes place over several days in mice, from E9 to E15 (18) and over multiple hours in zebrafish, from around 32 to 66 hours postfertilization (hpf) (10, 19) (Fig. 1). During this period, the GI tract is growing considerably in length with ongoing growth during the transition to feeding and digestion. To continue colonization in the caudal direction and to keep pace with the expanding length, mouse ENCCs must continue to proliferate while undergoing migration. Even with the ability to proliferate after reaching the GI tract, the starting pool of progenitor cells is still critical to ensuring complete colonization of the intestine (20–22). Additional insights can be gained from studies in zebrafish, in which EPC proliferation rates are heterogeneous depending on their location, with lower rates of proliferation in the anterior intestine coinciding with the onset of differentiation, and higher rates of proliferation in the more posterior regions of the intestine (10, 23). Once EPC migration is near completion, EPC proliferation rates then become more uniform along the anterior-posterior length of the intestine (10), consistent with the proposed model of EPC proliferation being a driving force of migration (24). Ultimately, similar to the mouse model, there is evidence of proliferation even in later stages of development, with the suggestion of the ongoing presence of EPCs into adulthood (10, 25).

Among the various signaling molecules and transcription factors that influence the survival and migration of EPCs are three regulators that are central to ENS development and highly conserved in both mice and zebrafish: transcription factor SOX10, the homeodomain transcription factor paired-like homeobox 2B (PHOX2B), and RET. In the mouse, all neural crest-derived progenitors express SOX10 as they delaminate from the neural tube and begin their migration into the GI tract. SOX10 is required for the survival of ENCC, and if missing, the result is aganglionosis in both humans and mouse models (26–28). The expression of SOX10 is also required to maintain ENCC in an undifferentiated and proliferative state (29, 30), with continued expression by enteric glial cells but turned off when ENCCs differentiate into neurons. In zebrafish, if SOX10 is missing, the mutant larvae demonstrate a marked absence in both enteric neurons and Gfap-positive enteric glia (31). In mice, PHOX2B is expressed by ENCC as they enter the intestinal mesenchyme (32) and promotes ENCC proliferation and survival (33). Similar to SOX10, deletion of PHOX2B leads to intestinal aganglionosis. (28, 33). In zebrafish, *phox2bb* is expressed is EPCs and later in enteric neurons (9, 34) with a knockdown of *phox2bb*, resulting in a range of decreased enteric neurons to complete aganglionosis in the distal intestine (9).

The expression of SOX10 is required for the expression of RET in both mouse (35) and zebrafish models (9). RET is a receptor tyrosine kinase that is activated by the GDNF family of ligands, a group of transforming growth factor proteins that activate RET in a complex with one of its family of corresponding coreceptors, GFRα1-4 (36, 37). These ligands bind initially to the GFRα1-4 coreceptors, but signal transduction is mediated by activated RET. In mice, to survive, develop, or both, vagal and sacral neural crest-derived precursors must express RET and its ligand-preferring GFR-α coreceptor. In zebrafish, *ret* and the two gene duplicates *gfra1a* and *gfra1b* are expressed in EPCs during migration toward the intestine (38). In transgenic mice that lack RET (39), GFR-α1 (40, 41), or GDNF (42, 43), there are no enteric neurons below the esophagus and the proximal stomach. Similarly, in zebrafish morphants and mutant larvae that lack *ret*, essentially no enteric neurons can be found in the intestine except for some neurons in the anterior-most intestine (38, 44). The RET pathway also plays a prominent role in ENCC and EPC migration. GDNF, expressed within the intestinal mesenchyme in mice and along the whole length of the developing intestine in zebrafish, is not only a factor for survival but also for chemoattraction of ENCC and EPCs (38, 45).

The mature ENS is composed of an extensive variety of neuronal cells type and glial cells, which have been increasingly distinguished based on morphology, immunohistochemical profiles, and electrophysiological properties in both mice (46–50) and zebrafish (19, 51, 52). Differentiation of mouse

ENCCs begins as early as during migration and is ongoing into the postnatal period (53) with evidence of ongoing plasticity in the adolescent (54) and potentially even in the adult periods (55), albeit the extent to which remains debated (56). In the zebrafish, neuronal differentiation coincides with ongoing proliferation and EPC migration. Neurons start to differentiate in the anterior developing intestine, whereas EPCs are still migrating toward the posterior end of the developing intestine (10). The proportion of neuronal subtypes and diversity change as a function of development, from larvae to adult stages (10, 51, 52). To generate the distinct classes of ENS neurons and glial cells, bipotential EPCs, capable of giving rise to both neurons and glial cells, progress during ENCC development to separate neural and glial progenitor cells, with further subdivisions into specific neuronal and glial types. Recent single-cell RNA-sequencing (scRNA-seq) analysis proposed a new model of neuronal diversification in the ENS, where postmitotic neurons continue to differentiate within a branch (57, 58). In zebrafish, the process of glial differentiation is less well characterized, but recent work has identified a population of enteric glial cells in adult zebrafish, which undergo self-renewing proliferation and neuronal differentiation during homeostasis (25).

The advancement of neuronal precursors through stages of progressive lineage restriction has classically been delineated through culture techniques and transgenic mice. The application of single-cell RNA-sequencing to the understanding of enteric neuronal diversification is identifying a new framework in which enteric neurons can be classified according to their expression patterns of transcription factors, neurochemical markers, adhesion markers, and other signaling molecules (57, 59, 60). Lineage sorting, furthermore, is mediated, in part, by the interactions of ENCCs and EPCs within the enteric microenvironment. The fates of enteric neuronal and glial cell precursors are thus determined by both intrinsic and extrinsic factors.

INFLUENCE OF THE MICROBIOTA ON THE DEVELOPMENT OF THE ENS

The time of birth, onset of feeding, and early postnatal period represent a significant period of microbial colonization of the GI tract. In humans, multiple factors have been found to influence the composition of the intestinal microbiota in early life, including gestational age, maternal diet and exposures, host genetics, mode of delivery, administration of antibiotics, and type of infant feeding (61–70). Mouse and zebrafish model systems to study ENS-microbiota interactions have largely focused on either germ-free (GF) models or the administration of broad-spectrum antibiotics.

The germ-free (GF) mouse, which exists in a sterile environment, has proven to be an important tool in elucidating host-microbiota relationships. GF mice are often compared with specific pathogen-free (SPF) mice, which comprise a complex commensal flora that is free of major pathogenic species and thus can serve as a control. Earlier work in neonatal GF mice has demonstrated the potential for intestinal microbiota to affect the development of the ENS (Fig. 2). The ENS of GF mice on postnatal *day 3* was found to be structurally abnormal compared with that of SPF mice, with a disruption in the lattice-like arrangement of the myenteric plexus, a reduction in nerve density and number of neurons per ganglia, and an increase in the proportion of nitrergic neurons. Interestingly, these structural changes were observed in the jejunum and ileum, but not in the duodenum of the GF compared with SPF animals (71). Colonization of GF dams with Altered Shaedler Flora (ASF), a simplified flora composed of only eight bacterial strains, was sufficient to restore the normal patterning of the ENS in their offspring (71). These structural observations were complemented by functional data showing impaired GI motility, with a reduction in the frequency and amplitude of intestinal contractions in the jejunum and ileum of GF mice (71). In contrast, GF zebrafish larvae do not show changes in ENS neuron numbers at 7 days (72). In GF zebrafish larvae, intestinal transit is not impaired, but GF larvae exhibit faster intestinal contractions compared with conventionally raised (CV) larvae along the length of the intestine (73, 74). Recent work in zebrafish has shown that specific bacterial strains can alter ENS-regulated intestinal motility via signaling through enteroendocrine cells in the intestinal epithelium demonstrating that communication between the microbiota and the ENS impacts ENS-regulated

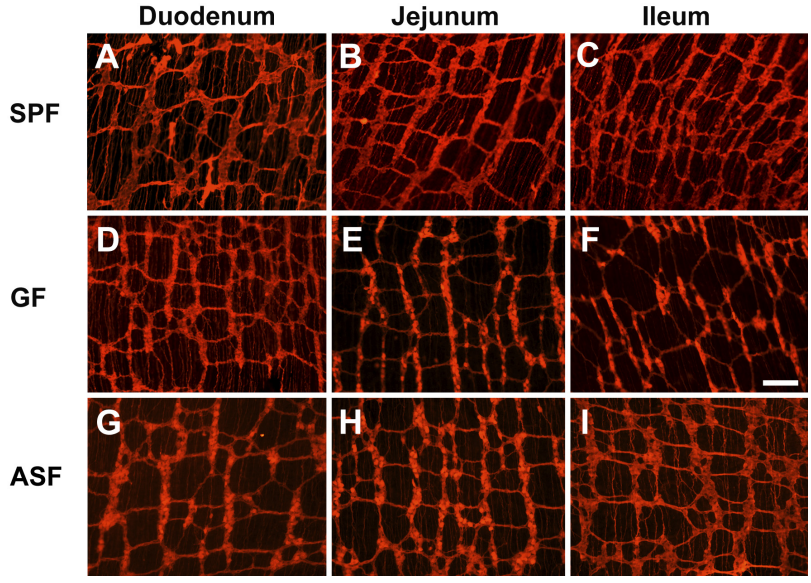

Figure 2. The myenteric plexus is hypoplastic in early postnatal GF mice. Myenteric nerves were visualized by immunolabeling with antibodies to PGP9.5 (red). *A–C*: myenteric plexus in the SPF duodenum, jejunum, and ileum is organized in a lattice-like network, with even spacing between ganglia and uniform thickness of connective nerve fibers. *D*: myenteric plexus in the GF duodenum resembles that of SPF duodenum. *E* and *F*: in GF mice, the myenteric plexus of the jejunum and ileum appears unorganized, with fewer ganglia and thinner connecting nerve fibers. *G–I*: In ASF colonized mice, the structure appears similar to that observed in SPF-colonized animals. Bar = 120 μm [from Collins et al. (71) with permission]. SPF: specific pathogen-free; GF: germ-free; ASF: Altered Shaedler Flora.

intestinal functions (75). The mechanistic basis of this microbiota-host interaction has been shown to work through the release of tryptophan catabolites by a specific gut bacteria strain, the pathogen *Edwardsiella tarda*. These catabolites activate a specific channel, the transient receptor potential ankyrin 1 (Trpa1), expressed by a subpopulation of enteroendocrine cells. Trpa1-mediated activation of enteroendocrine cells in turn activates enteric nerves, which results in changes in intestinal motility. This work is one of the few examples that have identified the mechanistic basis of microbiota-ENS interactions. Yet, whether the microbiota changes ENS neuronal numbers at other developmental stages, neuronal subtype composition or innervation patterns has not been tested in zebrafish.

Studies in GF mice have also shown a reduction in a population of enteric glial cells, which similar to enteric neurons, are also derived from the neural crest-derived precursors that colonize the GI tract. In this work, the average number and density of mucosal enteric glial cells were found to be significantly reduced in GF mice compared with CV mice at 8 wk of age, whereas the enteric glial networks within the myenteric and submucosal plexi were unaffected (76). If the GF mice were conventionalized at 4 wk of age, the network of mucosal enteric glial cells was found to be restored (76). The postnatal ability of mucosal enteric glia to invade the intestinal mucosa and form a normal network, therefore, seems to depend on the presence of intestinal microbiota.

These early-life abnormalities in the ENS of GF animals have been shown to persist to adulthood. Irregularities in the patterning of the myenteric plexus of the adult GF rat cecum were already described in the 1960s (77). GF mice in the postweaning period at 4 wk of age were found to have a significant reduction in neuronal numbers in the myenteric plexus of the colon (78). These persistent changes to the ENS in adult GF animals can manifest in functional deficits. The excitability of intrinsic primary afferent neurons, for example, has been found to be significantly reduced in adult GF mice compared with SPF controls, suggesting that commensal microbiota are necessary for the development of normal electrophysiological profiles in enteric neurons (79).

Animal models using antibiotics have ranged from single antibiotics to deplete the intestinal microbiota to broad-spectrum cocktails to more fully abolish the bacterial flora. In a neonatal model, mice were exposed to a single oral antibiotic, vancomycin, from birth to postnatal day 10, resulting in a significantly altered, but still present microbiota. In the vancomycin-exposed compared with control mice, there was reduced neuronal density in the myenteric plexus, decreased proportion of myenteric nitrergic neurons, increased proportion of calbindin-positive neurons, and increased colonic motility (80). When vancomycin was administered later in the postweaning period (6 wk of age), the pattern of ENS changes was different, with a decreased proportion of myenteric cholinergic neurons, an increased proportion of submucosal cholinergic neurons, and slower colonic propagating contractions (81). In a model system in which broad-spectrum antibiotics were given to juvenile mice at 3 wk of age, alterations in the ENS were found to include a decreased proportion of myenteric nitrergic neurons, altered cholinergic, tachykininergic, and nitrergic neurotransmission, and slower GI transit time (82). Changes in ENS structure and function can also be found in adult animals, at 8–12 wk of age, in which administration of broad-spectrum antibiotics led to observations of decreased neurons in both submucosal and myenteric plexus of the ileum and proximal colon, reduction of enteric glia in the myenteric plexus of the ileum and a reduction in GI transit time (83).

INFLUENCE OF THE ENS ON MICROBIOTA COMPOSITION

The ENS regulates all important intestinal functions and interacts with the immune cells in the GI tract, thus significantly impacting intestinal homeostasis and function (Fig. 3) (84, 85). These functions include nutrient sensing and controlling GI motility and blood flow, thereby regulating digestion, nutrient uptake, and water absorption (Fig. 3) (85, 86). The ENS contributes to intestinal barrier function, which prevents bacterial products from crossing into the bloodstream (87). The ENS is also important for maintaining the chemical environment of the intestinal lumen by regulating luminal pH and mucus secretion (84, 85).

Because of the important role of the ENS in controlling intestinal functions, it is not surprising that changes in ENS development and function are connected to intestinal microbiota dysbiosis in various human disorders. For example, patients with Hirschsprung's disease, which is a congenital ENS disorder characterized by the absence of ENS neurons and glia in varying segments of the intestine, show microbiota dysbiosis and can develop Hirschsprung-associated enterocolitis (HAEC), a life-threatening intestinal inflammation (88–93). Additional examples of ENS dysfunction connected with altered intestinal microbiota range from neurodevelopmental disorders such as autism spectrum disorder to inflammatory bowel disease, diabetes, and neurodegenerative diseases such as Parkinson's disease (94–98). Animal models of ENS disorders have been instrumental in dissecting the impact of altered ENS function on microbiota colonization and composition (99, 100). In different mouse and zebrafish models of Hirschsprung's disease, a lack of ENS neurons results in dysbiosis and increased intestinal inflammation (89, 100–104). One fundamental question is which ENS-regulated intestinal functions are important for intestinal microbiota colonization and composition. Several studies using the zebrafish model system have identified mechanisms of how ENS functions impact the intestinal microbiota.

Zebrafish is an excellent model system to study the impact of ENS development and function on microbiota colonization and composition (19, 72, 99, 105). Because of their external, rapid development, large numbers of offspring, transparent larvae, and genetic and embryological tractability, various phenotypes can be visualized, in vivo, in a large number of related or unrelated individuals including *1*) ENS phenotypes, e.g., changes in ENS neuron number and ENS function; *2*) intestinal phenotypes, e.g., motility patterns, intestinal transit capabilities, luminal pH; *3*) inflammatory response; and *4*) microbiota composition. Using minimally disruptive microscopy approaches such as light-sheet fluorescence microscopy enables the identification of intestinal motility patterns and behavior of bacteria within the intestinal lumen at high

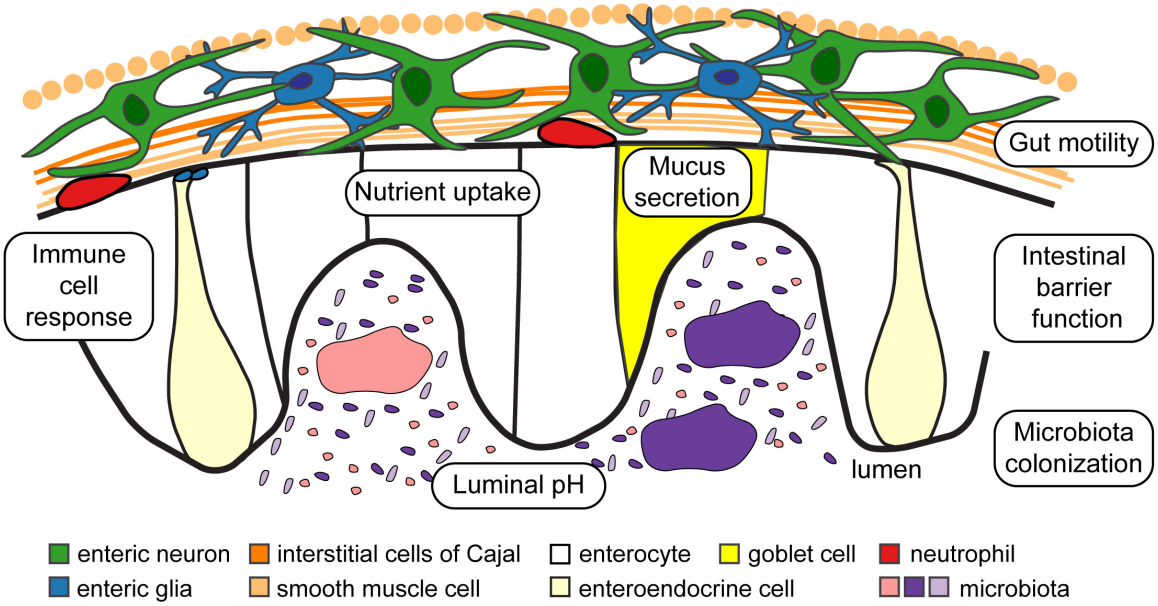

Figure 3. Summary of components of the intestine and enteric nervous system (ENS) functions. ENS neurons (green) and ENS glial cells (blue) interact with different intestinal cell types and control important intestinal functions (boxes). ICC: interstitial cells of Cajal. Modified from Ganz et al. (72) with permission.

resolution in a live animal (44, 106–109). Zebrafish embryos are highly amenable to gnotobiotic techniques and can be reared GF in large numbers (99, 110, 111). Zebrafish intestinal development is rapid, as their ENS and digestive tract are functional already at 5 days postfertilization (dpf). Zebrafish larvae can then be colonized by specific bacterial species by adding bacteria to the water column (99). Importantly, the genetic basis of ENS development is conserved between zebrafish and humans, allowing for the establishment of zebrafish models of human ENS disorders (19, 105).

Using the experimental advantages of zebrafish has led to significant insights into the mechanisms by which ENS-mediated intestinal functions impact microbiota colonization and composition. Zebrafish mutants for the Hirschsprung's disease-associated genes *ret* or *sox10* show that changes in intestinal motility patterns and altered luminal pH affect intestinal microbiota colonization and composition, resulting in increased intestinal inflammation (Fig. 4). Zebrafish *sox10* mutant larvae completely lack ENS neurons and glia and consequently have defective intestinal motility patterns and intestinal transit (112, 113). Zebrafish *sox10* mutant larvae also show bacterial overgrowth connected with increased inflammation (112). The proinflammatory effect of the microbiota is transmissible, as transplanting the intestinal microbiota of *sox10* mutants into GF wild-type larvae induces inflammation in the wild-type larvae that have an intact ENS. The inflammatory response is connected to the presence of specific bacterial strains: high neutrophil accumulation correlates with a high relative abundance of the *Vibrio* genus (112). The hyperinflammatory response can be rescued by restoring ENS neurons in the intestine indicating that ENS function is sufficient to prevent the increase in inflammation (102). Restoration of ENS neurons in different mouse models of Hirschsprung's disease also decreased neutrophil numbers and restored essentially a wild-type microbiota composition (100). Alternatively, the addition of anti-inflammatory bacterial species such as *Escherichia* species or a *Shewanella* strain to CV zebrafish *sox10* mutants reduced intestinal inflammation (112), suggesting that interactions between proinflammatory and anti-inflammatory bacterial strains can balance the immune response of the host. In *sox10* mutants, intestinal permeability and intestinal transit are altered independent of the microbiota and precede the increased inflammation observed in *sox10* mutants. This suggests that microbial dysbiosis and inflammation depend on changed ENS function (74). As restoration of the ENS rescued the hyperinflammatory phenotype, the next question was to identify which ENS-regulated intestinal function results in intestinal dysbiosis and inflammation.

Recent work has shown that a decreased luminal pH is necessary and sufficient for the increase in inflammation and the abundance of proinflammatory bacteria in zebrafish *sox10* mutants (74). The ENS regulates luminal pH, which is decreased in both CV and GF *sox10* mutants indicating that the changed pH is not dependent on microbiota but due to the lack of ENS neurons and/or glia in zebrafish *sox10* mutants. Increasing the luminal pH in *sox10* mutants results in a rescue of the hyperinflammatory phenotype, which shows that an acidic pH is necessary for the increased inflammation. The reverse experiment, lowering the luminal pH in wild-type larvae, led to an increase in inflammation, indicating that luminal pH changes are sufficient to elicit an inflammatory response (74). This work provides a direct connection between ENS-controlled changes in the luminal environment, microbiota dysbiosis, and inflammatory response (Fig. 4, *A* and *B*).

In addition to changes in luminal pH, intestinal motility also has been suggested to impact microbiota colonization and composition (Fig. 4, *C* and *D*). As bacterial species colonize the intestine, they reside in specific spatial configurations within the intestine depending on their intrinsic behavior and

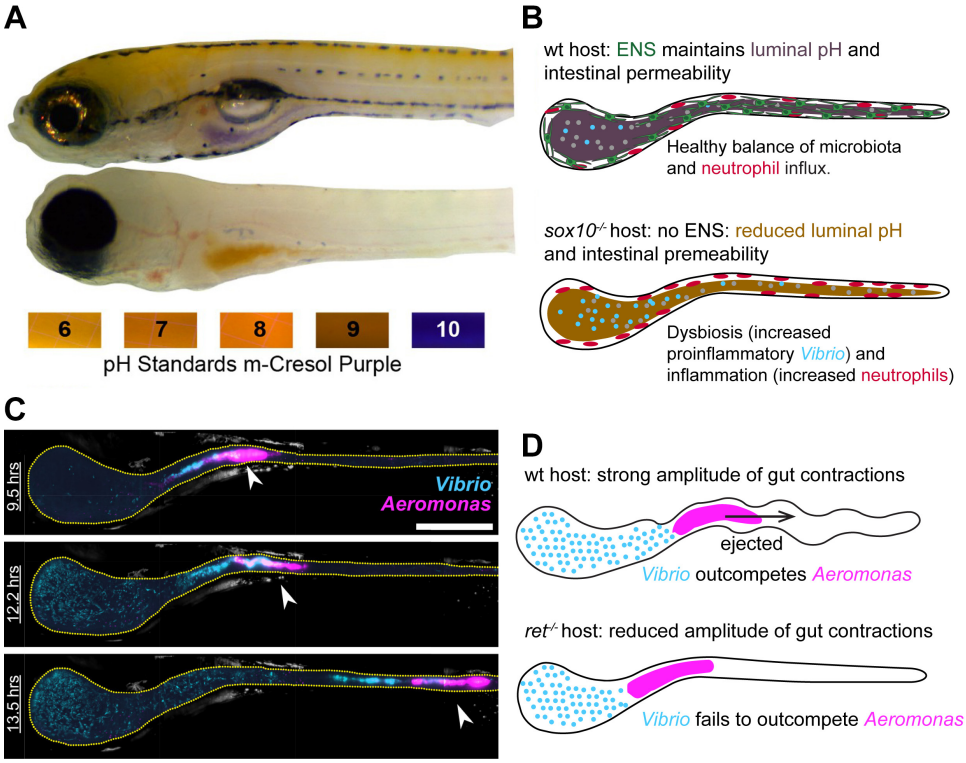

Figure 4. Examples of enteric nervous system (ENS)-regulated intestinal functions—luminal pH and intestinal motility—that impact the colonization and composition of the intestinal microbiota. *A*: luminal pH is lower in zebrafish *sox10* mutants (*bottom*) compared with wild types (*top*). *B*: in zebrafish wild type (wt), the ENS (*top*) regulates the chemical environment of the intestinal lumen and thereby maintains a healthy microbiota and neutrophil population. In zebrafish *sox10* mutants (*bottom*), the absence of ENS leads to a reduced luminal pH thereby increasing the abundance of proinflammatory *Vibrio* and neutrophils which results in an inflammatory response. *C*: example of competition between *Vibrio* (blue) and *Aeromonas* (magenta) in the zebrafish intestine over time using live imaging on a light-sheet microscope. *D*: intestinal contractions in the wild-type zebrafish host (*top*) promote the collapse of *Aeromonas* population when challenged by *Vibrio*. Lack of intestinal contractions in zebrafish *ret* mutants (*bottom*) prevents *Aeromonas* from being outcompeted by *Vibrio*. *A*: based on Fig. 4 from Hamilton et al. (74), which is licensed under CC BY 4.0; *C*: based on Fig. 3*A* from Wiles et al. (106), which is licensed under CC BY 4.0.

community architecture (108). In addition, the host environment impacts the colonization behavior of bacterial strains depending on their behavior and biogeographical distribution. Colonization and competition studies of specific bacterial strains in zebrafish have shed light on the impact of intestinal motility on intestinal microbiota composition. Two bacterial species native to the zebrafish intestine, *Aeromonas veronii* and *Vibrio cholerae*, have a competitive interaction that is strongly impacted by the host's intestinal motility patterns (106). Each species has characteristic, different behaviors, and biogeographical preferences when mono-associated: *Vibrio* cells are highly motile and are most abundant in the anterior part of the intestine. In contrast, *Aeromonas* forms dense aggregates with only a small population of motile cells and is primarily found in the midintestine (106). When an established culture of *Aeromonas* is challenged with *Vibrio*, the population of *Aeromonas* drops with significant population collapses (106). In vivo imaging of *Aeromonas* behavior with and without a *Vibrio* challenge within the zebrafish intestine illustrates that population collapses occur more frequently, are more drastic, and do not recover as readily in the presence of *Vibrio* (106). What are the impacts of the host on this competition? In vivo imaging of each bacterial population within the zebrafish intestine showed that both species are impacted differently by intestinal motility. The distribution of *Vibrio* was essentially not impacted by peristaltic motions along the intestine, presumably due to its position in the anterior part of the intestine and its highly motile characteristics (106). In contrast, *Aeromonas* populations were strongly impacted by intestinal contractions. As *Aeromonas* preferentially is located in the midintestine in dense aggregates, intestinal contractions could push them out, causing population collapses (106). Zebrafish *ret* mutants that lack ENS neurons along the intestine except for a few neurons in the anterior part of the intestine show changes in intestinal motility patterns particularly reduced intestinal motility amplitudes compared with wild-type siblings (44). In zebrafish *ret* mutants, *Vibrio* is not able to outcompete *Aeromonas*, indicating that their competition is neutralized by changes in ENS-regulated intestinal motility (Fig. 4*D*) (106)].

The competitive interaction between *Vibrio* and *Aeromonas* is driven by specific features of each bacterial species. *Vibrio* mutants that are motility-deficient or chemotaxis-deficient lose the ability to outcompete *Aeromonas* (107). This change is mediated by an altered spatial distribution in the intestine. *Vibrio* motility and chemotaxis mutants form aggregates and reside mostly more posteriorly in the intestine in contrast to wild-type *Vibrio* forms that primarily reside in the anterior intestine (107). This change in cohesion and biogeography leads to significant impacts on population structure by intestinal motility movements; as for wild-type *Aeromonas*, aggregated *Vibrio* motility and chemotaxis mutants now are subjected to expulsion by intestinal contractions (107). The relevance of intestinal motility is confirmed by the rescue of the abundance and localization of both *Vibrio* mutant strains in zebrafish *ret* mutants. The study then tested if motility is necessary for colonizing or for persisting in the intestine after colonization using an elegant inducible CRISPR interference approach where the motility mutation is only induced in the *Vibrio* bacteria after colonization. *Vibrio* bacteria that acquire the motility mutation only after colonization still display the motility mutant phenotype indicating that *Vibrio* needs their swimming ability to occupy their wild-type intestinal niche (107). Performing a gain-of-function where Vibrio mutants reacquire their motility and chemotaxis abilities after colonization showed a full rescue of wild-type behavior, indicating that

these features are essential for occupying the wild-type luminal niche. Together, these studies identify different mechanisms of how ENS-regulated intestinal functions can impact microbiota abundance and bacterial biogeography in the intestine and provide insights into how these can be affected in ENS disorders with altered ENS functionality.

FUTURE DIRECTIONS

Taken together, studies in both mouse and zebrafish have highlighted the strengths of each model system in elucidating components of microbiota-ENS interactions. A common theme across studies, however, is the use of more drastic models of microbiota manipulation such as GF models, broad-spectrum antibiotics, or use of select bacterial strains, as well as models of severe ENS abnormalities such as aganglionosis, to generate proof of concept data of microbiota-ENS interactions. Looking forward, it would be important to determine whether more subtle changes in the intestinal microbiome can result in meaningful changes in GI function. For example, studies in pediatric patients with constipation have described changes in the composition of fecal microbiota compared with healthy control children (114), suggesting the potential for microbiota-ENS interactions under less extreme conditions to still manifest in altered GI function, the mechanisms of which remain to be understood. Similarly, determining how more subtle ENS defects impact the intestinal microbiota and inflammation will be important to show how ENS disorders with less severe changes in ENS composition or function may influence intestinal homeostasis and health.

Recent studies have highlighted the potential for newer methodologies in hypothesis generation and also in uncovering the mechanisms of microbiota-ENS interactions. For example, nuclear RNA-sequencing (nRNA-seq) was used to compare the nuclear transcriptome between the colons of GF and SPF mice identified a number of differentially expressed genes, with subsequent detailed investigations identifying the role of the aryl hydrocarbon receptor (AHR) signaling in enteric neurons in integrating cues from the luminal environment (115). In zebrafish, scRNA-seq studies of entire zebrafish larvae raised CV or GF (116) or of the intestine of CV versus GF larvae (117) provide great additional resources to determine microbiota-related transcriptional changes both within the intestine and beyond. It will be interesting to further explore the rich data set of differentially expressed genes between GF and SPF mice or zebrafish to generate further mechanistic hypotheses. This is particularly important as only a few studies have started to identify the cellular-genetic, mechanistic basis of microbiota-ENS interactions. Mutations in epigenetic modifier genes show changes in ENS and intestinal epithelium development connected to an intestinal inflammatory response (103, 104). As a next step, the combination of high-resolution scRNA-seq with assays that identify gene regulatory regions, such as chromatin accessibility assays and detection of specific chromatin features, for example, histone modifications, will open the door to characterizing the gene regulatory basis of microbiota-induced transcriptional changes that underlie the mechanisms of microbiota-ENS interactions.

Although this review has focused on the ENS, it is just one puzzle piece in the complex microbiota-gut-brain axis (118, 119). The gut-brain axis, composed of the ENS and bidirectional communication via the vagus nerve, is critical for normal GI homeostasis in both mice (120) and zebrafish (75). Alterations in the gut-brain axis have been shown to form the basis for the pathophysiology of disorders of gut-brain interaction (121, 122), which can present as early as infancy and occur at any point in the life span. Lack of microbiota alters neuronal development in the brain and subsequently changes behavior (121, 123–126), drawing attention to the significance of the microbiota milieu on the developmental programming of both the GI tract and the brain. We anticipate that future studies will not only better recognize the importance of the maternal and/or early feeding environment on the multiple levels of interaction in microbiota-gut-brain axis signaling, but also to further identify underlying mechanisms and potential avenues for therapeutic intervention.

ACKNOWLEDGMENTS

The authors acknowledge Ashwini Pugazhendhi for contributions to figure design (Fig. 1). Figure 1 was created using BioRender.

GRANTS

This work was supported by funds from the National Science Foundation CAREER Grant 2143267 (to J.G), the National Institute of Neurological Disorders and Stroke Grant R21NS123629 (to J.G.), Farncombe Family Digestive Health Research Institute (to E.M.R.), and the Natural Sciences and Engineering Research Council of Canada (to E.M.R.).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.G. and E.M.R. prepared figures; J.G. and E.M.R. drafted manuscript; J.G. and E.M.R. edited and revised manuscript; J.G. and E.M.R. approved final version of manuscript.

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