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► To cite this version:

François Leulier, Amélie Joly, Filipe de Vadder. Microbial Modulation of the Development and Physiology of the Enteric Nervous System. Trends in Microbiology, 2021, 29 (8), pp.686-699. 10.1016/j.tim.2020.11.007 . hal-03376787

HAL Id: hal-03376787

<https://hal.science/hal-03376787>

Submitted on 13 Oct 2021

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Microbial modulation of enteric nervous system development and physiology

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Abstract

The gastrointestinal tract harbors an intrinsic neuronal network, the enteric nervous system (ENS). The ENS controls motility, fluid homeostasis and blood flow, but also interacts with other components of the intestine such as epithelial and immune cells. Recent studies indicate that gut microbiota diversification occurring concomitantly to postnatal ENS maturation could be critical for ENS development and function. Here we discuss the possibility that this functional relationship starts *in utero*, whereby the maternal microbiota would prime the developing ENS and shape its physiology. We review the ENS/microbiota interactions and their modulation in physiological and pathophysiological contexts. While the microbial modulation of ENS physiology is now well established, further studies are required to understand the contribution of the gut microbiota to the ENS development and pathologies and to reveal the precise mechanisms underlying microbiota-to-ENS communications.

Keywords: enteric nervous system/microbiota/enteroendocrine cells/intestinal immune system/intestinal physiology

Anatomy and composition of the ENS

The gut is a densely innervated organ, with both an extrinsic and intrinsic innervation (the enteric nervous system, ENS). The ENS is a network of different nervous cell types located along the gastrointestinal (GI) tract, from the esophagus extending down to the anus. Because most of its component neurons are not directly connected to the central nervous system (CNS), the ENS represents an independent subdivision of the autonomous nervous system, receiving both sympathetic and parasympathetic outputs [1].

The anatomy of the ENS has been extensively described elsewhere [2]. Briefly, it comprises two ganglionated plexuses (**Figure 1**): the submucosal plexus, located at the interface of the submucosa and the circular muscle layer, and the myenteric plexus, located between the two muscular layers of the gut. Within each plexus, the neuronal cell bodies form groups called ganglia (**Figure 1**). Neurons in the ENS can be distinguished by their anatomy, their projections, as well as the different neurotransmitters they secrete. Classic neurochemical coding has shown that the ENS mostly comprises cholinergic excitatory and nitrergic inhibitory motor neurons [2]. However, new molecular biology techniques, particularly single-cell sequencing, have allowed to cluster and redefine new subtypes of enteric neurons, revealing several groups of myenteric neurons in mice and humans [3,4]. Besides neurons, the ENS contains glial cells, which form a unique lineage, distinct from other peripheral and central glia (**Figure 1**). Glial cells provide nutritional support to neurons but also play an active role in neurotransmission and exert neuroprotective roles on the ENS [2].

Unlike the central nervous system, which is separated from the surrounding environment by a blood-brain barrier, there is no physical separation between the ENS and the surrounding tissues. Thus, the ENS resides in close proximity with muscle cells, stromal cells, interstitial cells of Cajal, epithelial or immune cells [2]. In order to control gut physiology and homeostasis, the ENS requires coordinated regulation among these cell types.

Physiology of the ENS and impact of nutrients

The first evidence of the autonomous nature of the ENS can be traced back to the 18th century, with the work of Albrecht von Haller. However, it was not until the 1990s that first evidence showed that the ENS contained its own population of sensory neurons and that these neurons are involved in reflex circuits independent of the CNS (reviewed in reference [5]).

The ENS provides local control of motility, secretion, absorption, vasodilation and constriction (**Figure 1**) [6]. The coupling of intestinal secretion and vasodilation allows the movement of water and electrolytes across the epithelium. These functions are mainly regulated by vasomotor and secretomotor neurons from the submucosal plexus, often distinguished by their expression of vasoactive intestinal peptide (VIP) or ChAT [7].

Absorption and secretion are tightly linked to intestinal motility. Digestion of food and absorption of nutrients is conditioned by the presence of chyme content, which travels down the GI through the impulsion of tightly regulated motor movements. After food intake, mechanical and chemical stimuli activate mechanosensitive and intrinsic primary afferent neurons (IPANs) to stimulate motility [8]. In the myenteric plexus, motility reflex arcs induce propulsion through peristalsis and mixing of the chyme through segmentation.

Several nutrients, such as glucose, fatty acids or amino acids indirectly modulate the physiology of the ENS [9]. Indeed, enteric neurons can be activated by GI hormones secreted by enteroendocrine cells (EECs) in response to nutrient detection, e.g. CCK secretion in response to oleate infusion [10] or GLP-1 secretion induced by partial hyperglycemia [11]. The ENS also mediates the effects of nutrients by modulating GI motility, and thus energy availability. For example, application of the short-chain fatty acid (SCFA) butyrate into the colonic mucosa induces peristaltic activity via activation of ENS pathways [12]. Besides modulating neuronal excitability, nutrients also induce changes in the anatomy of the ENS. For example, long-term exposure to high-fat diet induces a reduction in the number of enteric neurons, i.e. enteric neuropathy [13].

***In utero* development of the ENS: an indirect influence of the microbiota? (Figure 2)**

Most ENS neurons are differentiating during embryonic development in humans [14] and mice [15], *i.e.* in a context devoid of microbiota. However, evidence show that microbes can influence the foetus development *in utero*. For example, vaginal dysbiosis linked to *Ureaplasma parvum* infection induces a loss of enteric neurons and glial cells in ovine fetal ENS [16]. Could maternal microbiota thus affect development of the ENS *in utero*?

The human GI tract houses a large population of microorganisms that form the gut microbiota. It consists of a diverse community of several hundreds of bacterial species playing a leading role for the host physiology, homeostasis and development [17].

Notably, recent studies showed that the gut microbiota can affect embryonic neuronal development. Indeed, germ-free (GF) mice have altered prenatal transcriptomic profile and

activation of microglial cells, i.e. resident macrophages of the brain, which are critical for neuronal development [18]. Functional equivalents to brain microglia also exist in the ENS, *muscularis* macrophages (MMs), which are involved in modulation of enteric neuronal networks (discussed below). Could MMs be influenced by the absence of maternal gut microbiota similarly to their brain counterpart? MMs colonize the fetal mouse bowel before enteric neurons [19], impacting the later ENS development. This possibility raises interesting questions regarding the potential effects of the maternal microbiota on the development of the fetal ENS and on their mediators. Unfortunately, to our knowledge, no study has focused on the developing ENS of GF mice to answer these questions. Interestingly, models of reversible gestational colonization based on the auxotrophic *Escherichia coli* HA107 strain have been recently elaborated [20]. This kind of experimental setup could be used to investigate the specific influence of the maternal gut microbiota and its modification on the ENS development *in utero*.

During fetal development, cells derived from the vagal and sacral neural crest colonize the bowel and give rise to enteric glial cells and numerous subtypes of enteric neurons in a sequential manner [21]. Early-born neurons such as serotonergic and cholinergic neurons can be impacted by products ingested by the mother [22]. These neurons influence the future fate of yet undifferentiated neurons. The maternal diet could therefore also impact late neuronal differentiation. Hirschsprung's disease, a life-threatening condition that affects 1 in 5000 infants, arises from a defect in ENS development, which fails to colonize part of or the entire colon [23]. Interestingly, studies in zebrafish have shown that a defect in retinoic acid supplying, which derives from diet-associated vitamin A, leads to a disorganized migration of neural crest cells along the gut and to the occurrence of subsequent aganglionic regions [24]. This observation supports the notion that *in utero* ENS development is impacted by the maternal diet. Consistently, maternal protein restriction induces a modification of the composition of the ENS and of its functioning in the offspring, with increased propulsive colonic activity [25]. Thus, an unbalanced maternal diet, with concomitant changes in microbiota composition, such as malnutrition or high-fat diet, could also have deleterious consequences *in utero*.

SCFAs are microbiota-derived metabolites [26] that could potentially be a molecular connection between the maternal microbiota and the fetal ENS development. Indeed, SCFAs produced by the microbiota circulate into the maternal blood and are thought to be able to affect the fetus after placenta crossing [27,28]. *In vitro*, SCFAs are critical for human neural cell progenitor proliferation [28], suggesting that SCFAs circulating into the fetal blood may impact ENS formation. To our knowledge, the effects of SCFAs on the developing ENS have not been

studied. A focus on the ENS of pups from mothers fed with a high-fiber diet (inducing production of SCFAs) could help verify this hypothesis.

Bacterial metabolites and by-products activate the secretion of many gut-related hormones by EECs [29], which directly affect the ENS. For example, serotonin (5-HT) derived from maternal enterochromaffin cells, a subset of EECs, is an essential maternal hormone for *in utero* development [30]. Interestingly, antidepressant treatments that affect the maternal gut microbiota modify 5-HT levels in the mother, which is associated with increased risks of GI diseases development in the offspring [31]. Consistently, dysregulated levels of 5-HT were measured in the mucosa and the myenteric plexus of pups submitted to prenatal alcohol exposure, which also modifies maternal gut microbiota [32].

Through bacterial metabolites and microbiota-regulated signaling pathways, the maternal microbiota could then prenatally participate to the offspring's ENS development *in utero*.

ENS, microbiota and juvenile life (Table 1)

ENS development largely extends postnatally [33]. Studies in GF mice showed that very early post-natal alterations (as early as P3), such as a decrease in nerve density and increased proportion of nitrergic neurons, are detected in the ENS [34]. Moreover, alterations of the forebrain development of GF mice, including changes in neuronal death and microglial colonization, already occur a few hours after birth [35]. ENS development is thus likely affected by gut microbiota directly after birth.

The early microbiome of the human infant is highly influenced by the maternal microbiota. Notably, 24h after birth, 50% of the species present in the infant gut microbiota originate from the mother's microbiota [36]. After birth, the infant's microbiota undergoes drastic changes including an increasing diversity and a proliferation of specific phylum. For example, *Bifidobacterium* are key components of the milk-fed newborn microbiota because they can digest milk-derived oligosaccharides. They are predominant in the human gut microbiota during the first year of life [37]. During the perinatal period, the colonization of the gut by the microbiota is influenced by many factors such as the mode of delivery, gestational age at birth, early-life stress, use of medication and mode of feeding (breast vs formula) [38]. Concomitantly, ENS neurogenesis, maturation and synaptogenesis continues. Notably, during the first weeks of life, the production of late-born neurons is stimulated by the early born serotonergic and cholinergic neurons [22] and the responsiveness of IPANs to 5-HT increases [39].

Weaning, which occurs approximately 3 weeks after birth in mice and around 6 months of life in humans, drives huge changes in the infant gut microbiota, simultaneously with changes in dietary patterns associated with the interruption of breastfeeding and the passage to solid food. Notably, *Bifidobacterium* are gradually replaced by *Clostridium* and *Bacteroides*, which are more representative of an adult microbiome [37]. These bacteria are able to digest plant-derived oligosaccharides and to produce SCFAs that are essential for the maintenance of a healthy gut [26]. During the same period, the ENS undergoes significant chemical and synaptic remodeling resulting in a maturation of the neuronal network. The number of enteric neurons and glial cells increases in the myenteric plexus and changes in neuronal chemistry occur in the submucosal plexus [40].

There is therefore a significant temporal coupling between ENS and microbiota maturation during both perinatal and post-weaning periods in humans and mice. Interestingly, GF adult mice have a newborn-like ENS, with a capacity of plasticity that can be activated by microbiota colonization during adulthood [41]. ENS changes in adolescence could thus be related to changes in the gut microbiota. Moreover, breast milk, which shapes the infant's gut microbiota, also promotes enteric neurogenesis and neuronal growth during the perinatal period [42]. These data suggest the existence of direct and indirect mechanistical links between diet, gut microbiota and ENS development postnatally.

Even though several studies have focused on the ENS development in GF mice [41,43,44], reports investigating the effects of antibiotics provide further evidence of the impact of gut microbiota on ENS development. Vancomycin treatment in neonatal mice leads to ENS modifications including neuronal loss, an increase of calbindin-positive neurons, a decrease of nitrergic neurons and a higher speed of colonic contractions [45]. Similarly, antibiotics in adolescent mice also affects the ENS with a decrease of cholinergic neurons, inducing an expected reduction of intestinal motility [46]. It is noteworthy that a similar vancomycin treatment has different functional consequences on neonates and adolescent mice. Indeed, it does not affect the same neuronal subtypes and triggers opposite effects on colonic contractions [45,46]. Moreover, P3 GF mice have an increased proportion of nitrergic neurons [34] whereas P0-P9 vancomycin-treated mice have a decreased proportion of nitrergic neurons [45]. These data suggest that the effects of microbiota on ENS maturation are dependent on the developmental window considered as well as on the nature of the microbiota change.

Interestingly, a conditional deletion of choline acetyltransferase (ChAT) in the ENS is related to altered microbial composition in the cecal and colonic microbiota [47]. Because antibiotic treatment induces a decrease of cholinergic neurons in adolescent mice [46], this finding

indicates a reciprocal effect between the ENS and microbiota composition. Changes in the gut microbiota composition may alter the ENS, thus inducing dysmotility, itself altering the microbial composition.

It is noteworthy that antibiotic treatment during juvenile life alters the expression pattern of brain-derived neurotrophic factor (BDNF), BDNF being a key regulator of ENS development and maturation, this data provides potential insights into the molecular mechanisms between the gut microbiota and the developing postnatal ENS. Similar modifications of BDNF signaling are also observed in mouse models of irritable bowel syndrome (IBS) [48]. IBS is a functional gastrointestinal disorder associated with disturbed defecation and abdominal pain notably due to ENS dysfunction. IBS pathogenesis is thought to be driven by genetic and environmental factors, and several studies have associated the development of pediatric IBS with an altered gut microbiota [49]. The relationship between microbiota, diet and early postnatal ENS development could thus have systemic and/or long-lasting effects impacting energy homeostasis and disease pathogenesis later in life.

Microbial modulation of ENS physiology: the role of enteroendocrine cells (Figure 3)

As commented before, the gut microbiota plays a crucial role in the development and maturation of the ENS. The relevance of microbiota-ENS interactions is highlighted by abnormalities in morphology and activity of the ENS of GF mice [34,41,43,50]. Detection of the gut microbiota, or their metabolites, either directly or indirectly via epithelial cells, is a core sensory system where alterations in microbial composition activate extrinsic sympathetic gut-projecting neurons [51]. GF mice exhibit delayed transit time, while re-colonization restores transit time and innervation of the mucosa [41].

Microbial factors may act through Toll-Like Receptors (TLRs), which are expressed in epithelial cells [52], glia [53] and neurons [54,55] and specifically recognize pathogen-associated molecular patterns (PAMPs). For example, TLR2 (a receptor that recognizes lipoproteins of the bacteria cell wall) signaling regulates inflammation by controlling ENS structure along with intestinal neuromuscular function [55]. Furthermore, in the adult mouse colon, TLR2 promotes neurogenesis after antibiotic treatment [56]. However, the fact that TLRs are expressed by several types of cells in the intestine makes difficult to identify the specific cells connecting the signaling between microbiota and the ENS.

Two recent studies gave further hints of the molecular coupling between gut microbiota and the ENS. Obata et al. showed that most neurons of the distal gastrointestinal tract express Aryl

Hydrocarbon Receptor, a nuclear receptor activated by several tryptophan-derived metabolites. The gut microbiota was able to induce expression of this receptor in these neurons, increasing intestinal motility, among other patterns [50]. Chandrasekaran et al. studied the impact of the widely used probiotic *Lactobacillus rhamnosus* GG and found that its presence in the GI tract stimulated neuronal differentiation of cholinergic neurons and, *in fine*, intestinal motility [57].

How do gut microbes communicate with the host to modulate physiology of the ENS? As mentioned before, there is no direct detection of luminal contents by components of the ENS. The first line of detection of such contents are epithelial cells, which are in direct contact with the gut lumen. Thus, interactions between neurons or glia and epithelial cells represent a key mechanism of transmission of microbiota-mediated cues to the ENS [29]. Amongst gut epithelial cells, enteroendocrine cells (EECs) of the gut barely represent 1% of the epithelium, yet are essential to intestinal physiology [58]. Increasing evidence shows that connections between enteric neurons and EECs regulate gut physiology. Recent work shows that some EECs termed “neuropod cells” directly communicate with neurons through modified synapses [59]. For example, a specific set of EECs, enterochromaffin cells, secretes 5-HT in response to a wide variety of microbiota-derived chemicals in the colonic lumen [60,61]. Single-cell RNA sequencing of enteric neurons shows that they express receptors of several gut hormones, including *HTR3a*, *HTR4* (5-HT receptors) and could be therefore able to respond to enterochromaffin cells activation [3]. Since the gut epithelium is renewed once every seven days, this raises the question of the dynamics and plasticity of these synapses. Indeed, the ENS is involved in the proliferation of the intestinal epithelium. Muscarinic acetylcholine (ACh) receptors (named M1 to M5) are widely expressed in the epithelium and knockout of either receptor M2, M3 or M5 increases intestinal proliferation [62], associated to the activation of 5-HT_{2A} receptors on cholinergic neurons [63]. Furthermore, *in vitro* co-culturing of intestinal stem cells with enteric neurons and glia promotes epithelial proliferation [64] and differentiation into EECs [65].

Enterochromaffin cells in the gut can act as mechanosensors, releasing large quantities of 5-HT in response to mechanic distension [66–68]. 5-HT secreted from the EECs could directly impact enteric neurons. Indeed, colonization of GF *Tph1*-deficient mice (*i.e.* mice with no production of 5-HT in the epithelium) results in decreased number of myenteric neurons, showing that EEC-derived 5-HT is crucial for the integrity of the ENS after colonization with gut microbiota [41].

How can 5-HT secretion be directly linked to gut microbes and regulation of intestinal homeostasis? 5-HT production in the gut is regulated by the presence of spore-forming bacteria

[69]. While 5-HT is mostly released from the basal side, several studies have found that EEC-derived 5-HT can end up in the gut lumen and feces [69,70]. A recent study found that 5-HT promotes intestinal colonization of the spore-forming gut bacterium *Turicibacter sanguinis* [71]. Interestingly, both *T. sanguinis* and mucosal 5-HT have been found to be strongly pro-inflammatory [72–74], a state that is classically linked to reduced motility of the intestine [75]. However, on the basal side, 5-HT has been described as a promoter of motility through interactions with the ENS. 5-HT can activate the terminals of primary sensory neurons [76], but recent data have challenged the concept that 5-HT is the initiator of motor activity in the colon (widely discussed in reference [5]). Indeed, a transcriptomic analysis of enterochromaffin cells of the small intestine has shown that these cells lack G protein-coupled receptors for lipid and protein metabolites but highly express *Glp1r* in the basal side [60]. GLP-1 induces 5-HT release *in vitro* [60]. Thus, L cells, a class of EECs which produce GLP-1 in response to nutrients [60], could act as a mediator between nutrient sensing and 5-HT release to stimulate intestinal motility in the small intestine.

Could it be that GLP-1 exerts opposite effects on intestinal motility in the colon and in the small intestine? GF mice, which have reduced gastrointestinal motility, have elevated levels of GLP-1 [77]. GF mice have reduced capacity of energy harvest [78] and thus the energy status of their colon may be assimilated to chronic undernutrition. In such conditions, GLP-1 induces slower transit time [77]. Thus, we can hypothesize that transit slowing is a physiological adaptation to chronic undernutrition triggered by nutrient scarcity and an altered microbiota in order to increase energy harvest and compensate for the reduced availability of nutrients in the lumen. The aim now is to understand the intricate microbiota/EEC/ENS relationship and its involvement in pathophysiology of diseases associated to impaired intestinal function.

Modulation of host physiology by the ENS and microbiota-neuroimmune interactions (Figure 4)

The intestinal immune system is crucial in maintaining homeostasis of the gut. Interactions between the ENS and immune cells have been thoroughly described as master regulators of gut physiology [79].

In the context of immune-mediated regulation of gut motility, *muscularis* macrophages (MMs) residing in close contact to the myenteric plexus have been shown to modulate peristalsis by secretion of bone-derived morphogenetic protein 2 (BMP2), stimulating enteric neurons, which in turn secrete colony stimulating factor 1 (CSF1) to regulate macrophage numbers [80]. In

addition, signals derived from the gut microbiota modulate the levels of both BMP2 and CSF1, tuning the interaction between enteric neurons and macrophages. However, other cell types are also involved in fine-tuning interactions between microbiota and the ENS. Type-3 innate lymphoid cells (ILC3) promote intestinal and metabolic homeostasis by integrating cytokine-derived signals [81]. ILC3 secrete interleukin 22 (IL-22), which promotes the production of antimicrobial peptides and mucin in the epithelium [82]. These cells express VIP receptor 2 (VPAC2) and lie in close proximity to VIPergic neurons in the lamina propria [83]. In response to feeding, particularly in the presence of fat in the luminal content, VIPergic neurons antagonize microbiota-induced secretion of IL-22 by ILC3, increasing lipid absorption and thus maintaining a trade-off between immune protection and the efficiency of nutrient absorption [83]. Modulation of microbiota-immune interactions may therefore be effective for the treatment of metabolic diseases, which are usually associated to inflammation in the gut.

Indeed, during metabolic diseases such as type 2 diabetes, intestinal inflammation is associated with dysfunction of enteric neurons [84]. Analysis of *lamina propria* and MMs showed that the latter mainly expressed M2-type wound-healing and anti-inflammatory genes [85]. Moreover, MMs express β 2 adrenergic receptors and reside in close proximity to enteric neurons. Infection with *Salmonella typhimurium* spiB activates extrinsic noradrenergic neurons, increasing transit time [85] and resulting in neuronal loss in the ENS [86]. By upregulating signaling via β 2 adrenergic receptors, MMs respond to infection and induce a neuroprotective effect in the ENS, limiting infection-induced neuronal damage [86].

Extrinsic nociceptor neurons innervating Peyer patches have been shown to play a critical role in protecting against infection with *Salmonella typhimurium* [87]. However, intrinsic innervation also plays a role in host defense against pathogens. Particularly, enteric neuron-derived interleukin-18 regulates the production of antimicrobial peptides in the epithelium, targeting pathogens such as *S. typhimurium* [88].

Thus, immune cells also play a major role in coupling microbial signals from the gut lumen to enteric neurons.

In addition to its role in sensing nutrients (as discussed before), a recent study showed that the ENS also plays a crucial role in regulating glucose homeostasis. Muller, Matheis, Schneeberger et al. studied the particular role of cocaine- and amphetamine-regulated transcript (CART)-expressing neurons of the ENS. The number of CART-expressing neurons was increased by 2-fold in mice with a gut microbiota, when compared to GF mice. Furthermore, CART neurons were characterized as viscerofugal (i.e. with axons projecting to the viscera) and were polysynaptically connected to the liver and pancreas via the celiac and superior mesenteric

ganglia. Specific activation of CART in the ENS led to increased blood glucose and decreased insulin, while specific ablation of the neurons resulted in decreased blood glucose, increased insulin and decreased gluconeogenesis in the liver [89]. Overall, this study showed that specific neurons from the ENS can directly regulate blood glucose without the need of any input from the CNS.

Conclusions and perspectives

It is now well established that environmental signals that modulate the gut microbiota lead to persistent changes in the development and physiology of the ENS. Enteric neurons and glial cells' response to microbial cues are key drivers of these physiological adaptations. However, the specific molecular and cellular paths that drive the changes in the ENS development and function remain to be elucidated. Particularly, the scientific literature is lacking data on how the maternal microbial environment shapes the development of the ENS *in utero* and if these changes have long-lasting effect on the physiology and physiopathology of the GI tract. Furthermore, research needs to focus on the specific role of the ENS in maintaining energy homeostasis. The capacity of enteric neurons to respond to food-derived signals suggests that the ENS could be an important actor, along gut-brain communications, in the preservation of the energy balance. Finally, recent advances in imaging and single-cell sequencing technologies have greatly benefited the study of enteric neuron circuits and helped identify neurons subtypes owning a specific machinery to detect nutrients and microbiota-derived signals. Combining these advances with genetic manipulations and physiological analysis will allow to functionally dissect the contribution of these specific enteric neuronal subtypes to the modulation of intestinal development and physiology by microbes.

Competing interests

The authors declare no conflict of interest in relation to this publication.

Acknowledgements

The authors would like to thank Anne Lambert (Institut de Génomique Fonctionnelle de Lyon) for her help in obtaining images in Figure 1. F.D.V. is supported by a grant from La Fondation des Treilles. A.J. is supported by a doctoral grant from the French Ministry of Research. Research in F.L lab is supported by Université de Lyon, ENS de Lyon and CNRS and funded

339 by an FRM grant (Équipe FRM DEQ20180339196) and an ANR grant (ANR-18-CE15-0011-
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Figure legends

Figure 1. Anatomy of the Enteric Nervous System. (A) Cartoon depiction of the intestinal epithelium with the different layers and cell types of the ENS. The ENS contains several types of neurons, which ensure the regulation of various functions of the intestine (in italic capital letters). Intrinsic primary afferent neurons (IPANs) are activated by cues coming from the mucosa and activate ascending and descending interneurons, which stimulate inhibitory and excitatory motor neurons, as well as secretomotor neurons. Arrows indicate the sense of propagation of the signal. Ach: acetylcholine; Enk: enkephalin; NO: nitric oxide; NMU: neuromedin U; VIP: vasoactive intestinal peptide; 5-HT: serotonin. (B) Confocal image of a 100µm-thick intestinal roll showing structure of the intestine (in blue DAPI stains nuclei) and its very dense innervation that goes from the crypt to the tip of the villus (red, neuron-specific tubulin TuJ1). (C) Magnification of a myenteric ganglion showing the network of (top) glial cells (green, stained with S100β) and (bottom) neurons (green: pan-neuronal marker HuC/D; blue: neuron-specific tubulin TuJ1; green: inhibitory motor neuron marker nitric oxide synthase 1, NOS1).

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Figure 2. The enteric nervous system (ENS) and the gut microbiota develop concomitantly and in close interaction during juvenile life and are affected by maternal and extrinsic factors. The development of the ENS (blue square) starts during prenatal life when the embryonic gut is colonized by cells derived from the vagal and sacral neural crest cells mainly. The first enteric neurons (early born neurons including serotonergic and cholinergic neurons) subsequently differentiate. ENS prenatal development is likely influenced by the maternal gut microbiota (orange square), itself shaped by the mother's diet and external cues including the use of antibiotics or xenobiotics. ENS development continues after birth with notably an intense neurogenesis, synaptogenesis and maturation during the first weeks of life. It also extends after weaning with synaptic and neuronal chemistry maturation. During the same time, the infant microbiota matures (Yellow square). For example, breast-fed neonates display a high prevalence of bacteria from the *Bifidobacterium* phylum. Bacterial diversity increases drastically, notably after weaning and the concurrent shift to solid food. The infant's microbiota composition depends on the mode of feeding and of delivery, the gestational age at birth, the use of antibiotics... (Green square). These factors also influence juvenile ENS development which suggests a strong influence of microbiota on ENS development. The mechanisms underlying microbiota effects on ENS development remain to be investigated.

Figure 3. Crosstalk between microbiota and epithelial cells regulates ENS physiology.

Enteric neurons can communicate with epithelial cells through paracrine ways or through enteroendocrine cells (EECs) with modified synapses, termed “neuropod cells”. Microbiota-derived cues (such as lipoproteins from the bacterial cell wall and tryptophan-derived metabolites) activate Toll-like Receptor 2 (TLR2) and aryl hydrocarbon receptor (AHR). Gut microbiota also modulates the production of several gut hormones, such as glucagon-like peptide 1 (GLP-1) and serotonin (5-HT). In enterochromaffin cells, spore-forming bacteria upregulate the expression of tryptophan hydroxylase 1 (TPH1), the rate-limiting enzyme of 5-HT synthesis. In turn, 5-HT stimulates growth of spore-forming bacteria and gut motility through activation of HT4 receptor (HT4R). Furthermore, secretion of acetylcholine (ACh) by cholinergic neurons activates epithelial proliferation through several muscarinic receptors.

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Figure 4. Microbiota-immune interactions and the ENS regulate host physiology.

Muscularis macrophages (MMs), which reside in the *muscularis* layer, in close contact to myenteric neurons, secrete Bone Morphogenetic Protein 2 (BMP2), activating BMP2 receptor (BMP2R) in enteric neurons. In response, enteric neurons secrete Colony-Stimulating Factor 1 (CSF1), which binds CSF1 receptor (CSF1R) in macrophages. These interactions are activated by gut microbiota and help maintain gut motility. *Salmonella* infection leads to neuron loss in the ENS. In order to respond to the infection, extrinsic neurons secrete noradrenaline, activating β_2 adrenergic receptor (β_2 AR), preventing neuron loss. Furthermore, enteric neurons respond to *Salmonella* infection through secretion of interleukin IL-18, activating goblet cells, which secrete antimicrobial peptides (AMPs).

Food intake (and particularly fat) activates VIPergic neurons, which secrete vasoactive intestinal peptide (VIP) in response. VIP activates VIP receptor (VPAC2) in type-3 innate lymphoid cells (ILC3). Production of interleukin 22 (IL-22), which is regulated by segmented filamentous bacteria, is inhibited by activation of VPAC2, increasing expression of lipid transporters, promoting fat absorption.

The gut microbiota stimulates the expression of cocaine- and amphetamine-related transcript (CART) in viscerofugal enteric neurons. These neurons project to the celiac and superior mesenteric ganglia (CG-SMG), which in turn innervate the liver and pancreas. Activation of CART neurons results in increased blood glucose and decreased insulin levels, showing that these enteric neurons are glucoregulatory.

Table 1. Studies focusing on the consequences of microbiota modification on the enteric nervous system (ENS) development during juvenile life.

A **B** **C**

Myenteric plexus