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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-03376787v1>

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

1 **Anatomy and composition of the ENS**

2

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

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

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

27

28 **Physiology of the ENS and impact of nutrients**

29

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

34 The ENS provides local control of motility, secretion, absorption, vasodilation and constriction
35 (**Figure 1**) [6]. The coupling of intestinal secretion and vasodilation allows the movement of
36 water and electrolytes across the epithelium. These functions are mainly regulated by
37 vasomotor and secretomotor neurons from the submucosal plexus, often distinguished by their
38 expression of vasoactive intestinal peptide (VIP) or ChAT [7].
39 Absorption and secretion are tightly linked to intestinal motility. Digestion of food and
40 absorption of nutrients is conditioned by the presence of chyme content, which travels down
41 the GI through the impulsion of tightly regulated motor movements. After food intake,
42 mechanical and chemical stimuli activate mechanosensitive and intrinsic primary afferent
43 neurons (IPANs) to stimulate motility [8]. In the myenteric plexus, motility reflex arcs induce
44 propulsion through peristalsis and mixing of the chyme through segmentation.
45 Several nutrients, such as glucose, fatty acids or amino acids indirectly modulate the physiology
46 of the ENS [9]. Indeed, enteric neurons can be activated by GI hormones secreted by
47 enteroendocrine cells (EECs) in response to nutrient detection, e.g. CCK secretion in response
48 to oleate infusion [10] or GLP-1 secretion induced by partial hyperglycemia [11]. The ENS also
49 mediates the effects of nutrients by modulating GI motility, and thus energy availability. For
50 example, application of the short-chain fatty acid (SCFA) butyrate into the colonic mucosa
51 induces peristaltic activity via activation of ENS pathways [12]. Besides modulating neuronal
52 excitability, nutrients also induce changes in the anatomy of the ENS. For example, long-term
53 exposure to high-fat diet induces a reduction in the number of enteric neurons, i.e. enteric
54 neuropathy [13].

55

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

57

58 Most ENS neurons are differentiating during embryonic development in humans [14] and mice
59 [15], *i.e.* in a context devoid of microbiota. However, evidence show that microbes can
60 influence the foetus development *in utero*. For example, vaginal dysbiosis linked to
61 *Ureaplasma parvum* infection induces a loss of enteric neurons and glial cells in ovine fetal
62 ENS [16]. Could maternal microbiota thus affect development of the ENS *in utero*?

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

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

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

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

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

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

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

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

114

115 **ENS, microbiota and juvenile life (Table 1)**

116

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

123 The early microbiome of the human infant is highly influenced by the maternal microbiota.
124 Notably, 24h after birth, 50% of the species present in the infant gut microbiota originate from
125 the mother's microbiota [36]. After birth, the infant's microbiota undergoes drastic changes
126 including an increasing diversity and a proliferation of specific phylum. For example,
127 *Bifidobacterium* are key components of the milk-fed newborn microbiota because they can
128 digest milk-derived oligosaccharides. They are predominant in the human gut microbiota
129 during the first year of life [37]. During the perinatal period, the colonization of the gut by the
130 microbiota is influenced by many factors such as the mode of delivery, gestational age at birth,
131 early-life stress, use of medication and mode of feeding (breast vs formula) [38].
132 Concomitantly, ENS neurogenesis, maturation and synaptogenesis continues. Notably, during
133 the first weeks of life, the production of late-born neurons is stimulated by the early born
134 serotonergic and cholinergic neurons [22] and the responsiveness of IPANs to 5-HT increases
135 [39].

136 Weaning, which occurs approximately 3 weeks after birth in mice and around 6 months of life
137 in humans, drives huge changes in the infant gut microbiota, simultaneously with changes in
138 dietary patterns associated with the interruption of breastfeeding and the passage to solid food.
139 Notably, *Bifidobacterium* are gradually replaced by *Clostridium* and *Bacteroides*, which are
140 more representative of an adult microbiome [37]. These bacteria are able to digest plant-derived
141 oligosaccharides and to produce SCFAs that are essential for the maintenance of a healthy gut
142 [26]. During the same period, the ENS undergoes significant chemical and synaptic remodeling
143 resulting in a maturation of the neuronal network. The number of enteric neurons and glial cells
144 increases in the myenteric plexus and changes in neuronal chemistry occur in the submucosal
145 plexus [40].

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

154 Even though several studies have focused on the ENS development in GF mice [41,43,44],
155 reports investigating the effects of antibiotics provide further evidence of the impact of gut
156 microbiota on ENS development. Vancomycin treatment in neonatal mice leads to ENS
157 modifications including neuronal loss, an increase of calbindin-positive neurons, a decrease of
158 nitrergic neurons and a higher speed of colonic contractions [45]. Similarly, antibiotics in
159 adolescent mice also affects the ENS with a decrease of cholinergic neurons, inducing an
160 expected reduction of intestinal motility [46]. It is noteworthy that a similar vancomycin
161 treatment has different functional consequences on neonates and adolescent mice. Indeed, it
162 does not affect the same neuronal subtypes and triggers opposite effects on colonic contractions
163 [45,46]. Moreover, P3 GF mice have an increased proportion of nitrergic neurons [34] whereas
164 P0-P9 vancomycin-treated mice have a decreased proportion of nitrergic neurons [45]. These
165 data suggest that the effects of microbiota on ENS maturation are dependent on the
166 developmental window considered as well as on the nature of the microbiota change.

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

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

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

184

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

186

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

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

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

204 Hydrocarbon Receptor, a nuclear receptor activated by several tryptophan-derived metabolites.
205 The gut microbiota was able to induce expression of this receptor in these neurons, increasing
206 intestinal motility, among other patterns [50]. Chandrasekaran et al. studied the impact of the
207 widely used probiotic *Lactobacillus rhamnosus* GG and found that its presence in the GI tract
208 stimulated neuronal differentiation of cholinergic neurons and, *in fine*, intestinal motility [57].
209 How do gut microbes communicate with the host to modulate physiology of the ENS? As
210 mentioned before, there is no direct detection of luminal contents by components of the ENS.
211 The first line of detection of such contents are epithelial cells, which are in direct contact with
212 the gut lumen. Thus, interactions between neurons or glia and epithelial cells represent a key
213 mechanism of transmission of microbiota-mediated cues to the ENS [29]. Amongst gut
214 epithelial cells, enteroendocrine cells (EECs) of the gut barely represent 1% of the epithelium,
215 yet are essential to intestinal physiology [58]. Increasing evidence shows that connections
216 between enteric neurons and EECs regulate gut physiology. Recent work shows that some
217 EECs termed “neuropod cells” directly communicate with neurons through modified synapses
218 [59]. For example, a specific set of EECs, enterochromaffin cells, secretes 5-HT in response to
219 a wide variety of microbiota-derived chemicals in the colonic lumen [60,61]. Single-cell RNA
220 sequencing of enteric neurons shows that they express receptors of several gut hormones,
221 including *HTR3a*, *HTR4* (5-HT receptors) and could be therefore able to respond to
222 enterochromaffin cells activation [3]. Since the gut epithelium is renewed once every seven
223 days, this raises the question of the dynamics and plasticity of these synapses. Indeed, the ENS
224 is involved in the proliferation of the intestinal epithelium. Muscarinic acetylcholine (ACh)
225 receptors (named M1 to M5) are widely expressed in the epithelium and knockout of either
226 receptor M2, M3 or M5 increases intestinal proliferation [62], associated to the activation of 5-
227 HT2A receptors on cholinergic neurons [63]. Furthermore, *in vitro* co-culturing of intestinal
228 stem cells with enteric neurons and glia promotes epithelial proliferation [64] and
229 differentiation into EECs [65].
230 Enterochromaffin cells in the gut can act as mechanosensors, releasing large quantities of 5-HT
231 in response to mechanic distension [66–68]. 5-HT secreted from the EECs could directly impact
232 enteric neurons. Indeed, colonization of GF *Tph1*-deficient mice (*i.e.* mice with no production
233 of 5-HT in the epithelium) results in decreased number of myenteric neurons, showing that
234 EEC-derived 5-HT is crucial for the integrity of the ENS after colonization with gut microbiota
235 [41].
236 How can 5-HT secretion be directly linked to gut microbes and regulation of intestinal
237 homeostasis? 5-HT production in the gut is regulated by the presence of spore-forming bacteria

238 [69]. While 5-HT is mostly released from the basal side, several studies have found that EEC-
239 derived 5-HT can end up in the gut lumen and feces [69,70]. A recent study found that 5-HT
240 promotes intestinal colonization of the spore-forming gut bacterium *Turicibacter sanguinis*
241 [71]. Interestingly, both *T. sanguinis* and mucosal 5-HT have been found to be strongly pro-
242 inflammatory [72–74], a state that is classically linked to reduced motility of the intestine [75].
243 However, on the basal side, 5-HT has been described as a promoter of motility through
244 interactions with the ENS. 5-HT can activate the terminals of primary sensory neurons [76],
245 but recent data have challenged the concept that 5-HT is the initiator of motor activity in the
246 colon (widely discussed in reference [5]). Indeed, a transcriptomic analysis of enterochromaffin
247 cells of the small intestine has shown that these cells lack G protein-coupled receptors for lipid
248 and protein metabolites but highly express *Glp1r* in the basal side [60]. GLP-1 induces 5-HT
249 release *in vitro* [60]. Thus, L cells, a class of EECs which produce GLP-1 in response to
250 nutrients [60], could act as a mediator between nutrient sensing and 5-HT release to stimulate
251 intestinal motility in the small intestine.

252 Could it be that GLP-1 exerts opposite effects on intestinal motility in the colon and in the small
253 intestine? GF mice, which have reduced gastrointestinal motility, have elevated levels of GLP-
254 1 [77]. GF mice have reduced capacity of energy harvest [78] and thus the energy status of their
255 colon may be assimilated to chronic undernutrition. In such conditions, GLP-1 induces slower
256 transit time [77]. Thus, we can hypothesize that transit slowing is a physiological adaptation to
257 chronic undernutrition triggered by nutrient scarcity and an altered microbiota in order to
258 increase energy harvest and compensate for the reduced availability of nutrients in the lumen.
259 The aim now is to understand the intricate microbiota/EEC/ENS relationship and its
260 involvement in pathophysiology of diseases associated to impaired intestinal function.

261

262 **Modulation of host physiology by the ENS and microbiota-neuroimmune interactions** 263 **(Figure 4)**

264

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

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

272 addition, signals derived from the gut microbiota modulate the levels of both BMP2 and CSF1,
273 tuning the interaction between enteric neurons and macrophages. However, other cell types are
274 also involved in fine-tuning interactions between microbiota and the ENS. Type-3 innate
275 lymphoid cells (ILC3) promote intestinal and metabolic homeostasis by integrating cytokine-
276 derived signals [81]. ILC3 secrete interleukin 22 (IL-22), which promotes the production of
277 antimicrobial peptides and mucin in the epithelium [82]. These cells express VIP receptor 2
278 (VPAC2) and lie in close proximity to VIPergic neurons in the lamina propria [83]. In response
279 to feeding, particularly in the presence of fat in the luminal content, VIPergic neurons
280 antagonize microbiota-induced secretion of IL-22 by ILC3, increasing lipid absorption and thus
281 maintaining a trade-off between immune protection and the efficiency of nutrient absorption
282 [83]. Modulation of microbiota-immune interactions may therefore be effective for the
283 treatment of metabolic diseases, which are usually associated to inflammation in the gut.
284 Indeed, during metabolic diseases such as type 2 diabetes, intestinal inflammation is associated
285 with dysfunction of enteric neurons [84]. Analysis of *lamina propria* and MMs showed that the
286 latter mainly expressed M2-type wound-healing and anti-inflammatory genes [85]. Moreover,
287 MMs express β 2 adrenergic receptors and reside in close proximity to enteric neurons. Infection
288 with *Salmonella typhimurium* spiB activates extrinsic noradrenergic neurons, increasing transit
289 time [85] and resulting in neuronal loss in the ENS [86]. By upregulating signaling via β 2
290 adrenergic receptors, MMs respond to infection and induce a neuroprotective effect in the ENS,
291 limiting infection-induced neuronal damage [86].
292 Extrinsic nociceptor neurons innervating Peyer patches have been shown to play a critical role
293 in protecting against infection with *Salmonella typhimurium* [87]. However, intrinsic
294 innervation also plays a role in host defense against pathogens. Particularly, enteric neuron-
295 derived interleukin-18 regulates the production of antimicrobial peptides in the epithelium,
296 targeting pathogens such as *S. typhimurium* [88].
297 Thus, immune cells also play a major role in coupling microbial signals from the gut lumen to
298 enteric neurons.
299 In addition to its role in sensing nutrients (as discussed before), a recent study showed that the
300 ENS also plays a crucial role in regulating glucose homeostasis. Muller, Matheis, Schneeberger
301 et al. studied the particular role of cocaine- and amphetamine-regulated transcript (CART)-
302 expressing neurons of the ENS. The number of CART-expressing neurons was increased by 2-
303 fold in mice with a gut microbiota, when compared to GF mice. Furthermore, CART neurons
304 were characterized as viscerofugal (i.e. with axons projecting to the viscera) and were
305 polysynaptically connected to the liver and pancreas via the celiac and superior mesenteric

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

311

312 **Conclusions and perspectives**

313

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

330

331 **Competing interests**

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

333

334 **Acknowledgements**

335 The authors would like to thank Anne Lambert (Institut de Génomique Fonctionnelle de Lyon)
336 for her help in obtaining images in Figure 1. F.D.V. is supported by a grant from La Fondation
337 des Treilles. A.J. is supported by a doctoral grant from the French Ministry of Research.
338 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-
340 01).

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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.

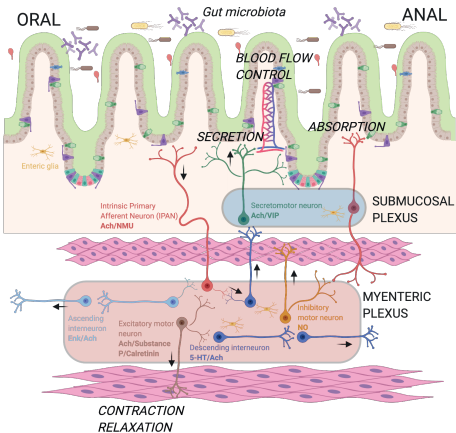
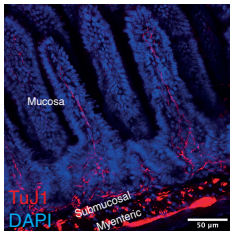
Muscularis macrophages (MMs), which reside in the *muscularis* layer, in close contact to myenteric neurons, secrete Bone Morphogenetic Protein 2 (BMP2), activating BMP2 receptor (BMP2R) in enteric neurons. In response, enteric neurons secrete Colony-Stimulating Factor 1 (CSF1), which binds CSF1 receptor (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.

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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

