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Microbe-host interactions: Influence of the gut microbiota on the enteric nervous system

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ABSTRACT

The enteric nervous system (ENS), considered a separate branch of the autonomic nervous system, is located throughout the length of the gastrointestinal (GI) tract as a series of interconnected ganglionated plexi. Given the proximity of the intestinal microbiota to the ENS, it is perhaps not surprising that the gut microbiota can influence its development and function. However, these interactions are complex and may be either direct or indirect, often involving signalling initiated by microbe-derived components, metabolites or host-derived intermediaries which subsequently affect enteric nerve excitability and GI function. Individual microbes and strains can differentially influence ENS activity and neurochemistry. In this review we will briefly summarise the role of the microbiota on ENS development, and, in some more detail, explore the mechanisms by which the microbiota can influence ENS activity and function.

1. Introduction

The gastrointestinal (GI) tract is densely innervated by a network of 200–600 million neurones which comprise the enteric nervous system (ENS). This network of neurones innervates all regions of the GI tract and is located in distinct units between either the longitudinal and circular muscle layers of the intestine or in the submucosa as ganglionated plexi; termed the myenteric plexus or submucosal plexus respectively (Furness et al., 2014). The ENS can autonomously influence the physiology and function of the GI tract, however it also communicates in a bidirectional manner with the central nervous system (CNS) by both vagal parasympathetic and sympathetic pathways, whilst vagal afferent signalling from the ENS, circular muscle layers and mucosa is facilitated by intraganglionic laminar endings, intramuscular arrays and mucosal varicose nerve endings respectively. Within the distinct plexi are discreet populations of neurones which can be classified based on their function and morphology. These include intrinsic sensory neurones, motor neurones (muscle, secretomotor and secretomotor/vasodilator) and enteric interneurons which collectively regulate key functions of the GI tract including intestinal muscle activity, gastric peristalsis and secreto- and vasomotor control (Furness et al., 2014). By virtue of its location in the gut wall, the ENS may be considered "protected" from the contents of the lumen by the epithelial barrier, mucous layer, as well as by ion and fluid secretion (Saulnier et al., 2013). These barriers, to some degree, separate the ENS from the microbiota. The most heavily colonized area of the human body is the GI tract, with bacterial concentrations ranging from 10^1 to 10^3 cells per gram in the upper intestine to 10^{11}–10^{12} per gram in the colon (Derrien and van Hylckama Vlieg, 2015; O'Hara and Shanahan, 2006). The symbiosis between host and microbiota gives rise to a collective gene system referred to as the hologenome which represents the nuclear genome, organelles, and microbiome (Bordenstein and Theis, 2015). The genetic content of the microbial communities outnumber those of the host by approximately 150-fold (Qin et al., 2010). There are multiple ways by which gut microbes can influence the host including cellular components, biosynthesis of unique molecules and dietary modification (Koppel and Balskus, 2016). Several such mechanisms have been implicated in facilitating either direct or indirect communication between the microbiota and the ENS (Fig. 1).

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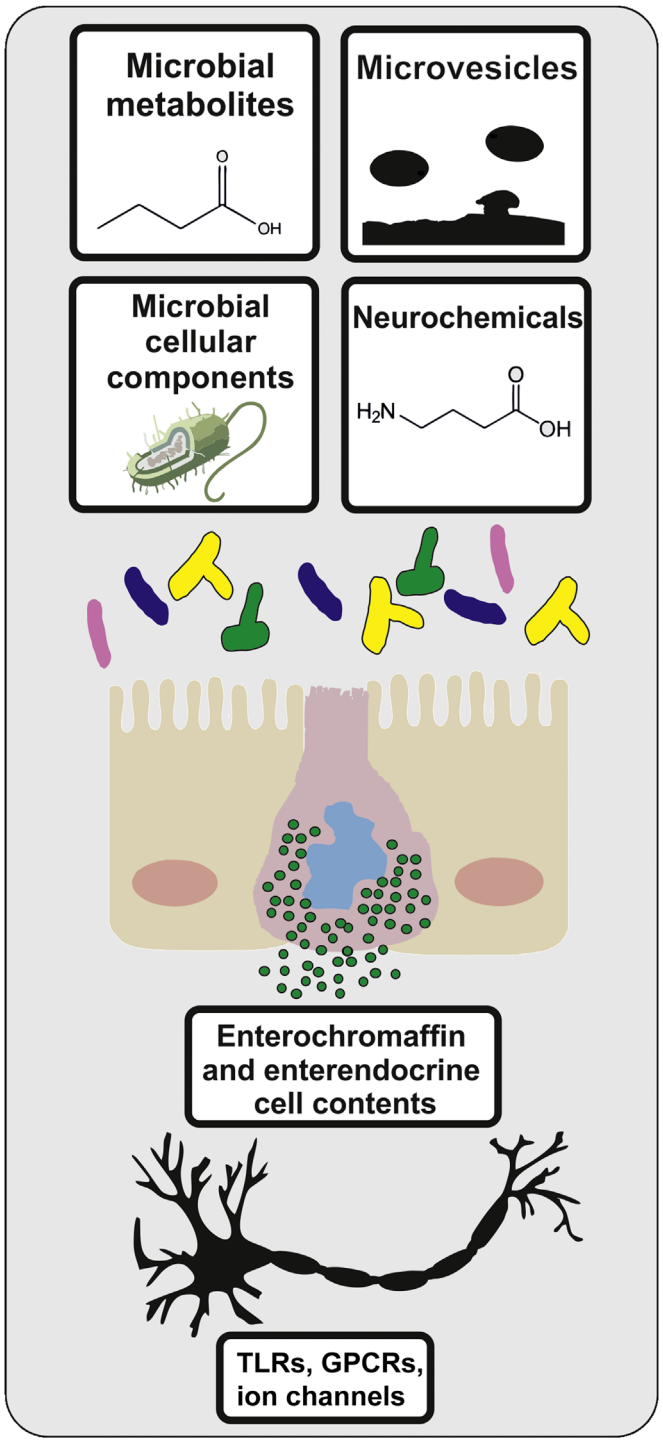

Fig. 1. Enteric neurones express Toll-like receptors, a vast array of neurochemical receptors as well as displaying sensitivity to microbial metabolites, and therefore the enteric nervous system (ENS) has the capacity to respond to microbes. Evidence suggests that the microbiota can either directly or indirectly influence the ENS through generation of microbial-derived components, neuroactive metabolites or by engaging with mucosal elements (e.g, enteroendocrine-cell derived mediators). TLR, Toll-like receptor; GPCR, G-protein-coupled receptor.

2. Impact of the microbiota on enteric neural development and plasticity

Germ free (GF) animals have proven a useful tool for investigating the contribution of microbes to host function and the impact of the microbiota on the gut-brain axis, including the ENS (Luczynski et al., 2016). The first GF animals were developed as far back as the 1800s by aseptic caesarean section and the methodology used in the generation of GF mice remains largely unchanged today (Luczynski et al., 2016). Evidence of grossly abnormal myenteric plexus architecture and size in GF rats has been reported (Dupont et al., 1965). Moreover, GF rats have been used to demonstrate the impact of the microbiota on migrating myoelectric complex (MMC) activity, though no change in ENS neurochemistry was observed following colonisation of these animals (Husebye et al., 2001). More recently, the early postnatal developmental trajectory, neurochemical profile and function of the ENS has been investigated in GF mice (Collins et al., 2014; Lomasney et al., 2014b). In the context of myenteric nerve fibre density, a GF environment significantly decreased the development of enteric neural networks in a region-specific manner on postnatal day 3 relative to both offspring born in a specific pathogen free environment or to dams colonised with a simplified microbiota (Collins et al., 2014). Nerve density was similarly decreased in the jejunum and ileum of GF mice, though preserved in the duodenum. In terms of the number of neurones per myenteric ganglia, these were decreased in both the jejunum and ileum in which increased nitrergic neurones were also observed; though this may also represent a loss of non-nitrergic neurones (Collins et al., 2014). Whilst this study did not extend analysis further into the post natal developmental period or adulthood, others have reported that the number of nitrergic neurones in the myenteric plexus of the colon and distal ileum was decreased in 4-week old GF mice without any concomitant change in cholinergic neurone number (Anitha et al., 2012). Decreased calbindin positive neurones were also noted in the jejunum of GF mice relative to specific pathogen free animals (McVey Neufeld et al., 2015). Of note, however, when GF animals were colonised the number of calbindin positive neurones not only increased in GF ganglia but also significantly increased relative to specific pathogen free animals (McVey Neufeld et al., 2015). Therefore, there is converging evidence to suggest that the microbiota can influence the development of the ENS. However, the temporal nature of this influence is unclear and some interesting questions remain. For example, why normal ENS development occurs in some regions of the small intestine and not others of GF mice; and how a simplified eight strain flora, compared to a more complex microbiota, can have similar effects on ENS development? Nonetheless, there is evidence to suggest that putative probiotics can individually influence ENS neurochemistry (di Giancamillo et al., 2010; Kamm et al., 2004). *Saccharomyces boulardii* significantly decreased the number of calbindin positive neurones, and more particularly cholinergic/calbindin positive myenteric neurones in the pig (Kamm et al., 2004). On the other hand *Pediococcus acidilactici* significantly influenced ileal neurochemistry without affecting total neuronal numbers and did not affect caecal ENS neurochemistry (di Giancamillo et al., 2010). These studies support the plasticity of the adult ENS, and, furthermore, the selective influence of particular microbes on the ENS in distinct regions of the GI tract. More recently a role for the microbiota in modulating the flow of enteric glial cells from the ENS to the mucosa has been demonstrated in antibiotic treated *Sox10::Cre;R26RConfetti* mice (Kabouridis et al., 2015). Moreover, GF mice displayed a decrease in mucosal glial cell staining relative to conventional animals which could be restored upon colonisation. However, staining of S100β was not altered in the enteric plexi of GF animals (Kabouridis et al., 2015).

3. Toll-like receptors and the enteric nervous system

Despite the separation between the microbiota and ENS, enteric neurones express pattern recognition receptors, namely

Toll-like receptors (TLR) for which microbial components are agonists and represent a mechanism by which the microbiota communicates with the host (Koppel and Balskus, 2016). For example, viral and lipopolysaccharide (LPS)-recognising TLRs have been localised on enteric neurones (Barajon et al., 2009; Rumio et al., 2006). Protein for TLRs 3, 4 and 7 and TLRs 3 and 7 have been demonstrated in the murine and human ENS respectively and on neural elements innervating Peyer's patches which may provide a pathway for microbes to access the ENS (Barajon et al., 2009). Functionally, mice deficient in TLR4 display significantly decreased transit *in vivo* accompanied by significant changes in ENS neurochemistry characterised by a decrease in total ileal enteric neural numbers and more specifically in nitrergic ileal (nNOS) and colonic (NADPH diaphorase) myenteric neurones (Anitha et al., 2012). Moreover, GF and antibiotic-treated animals also display a similar neurochemical profile to mice lacking TLR4 thereby demonstrating the significance of microbe-ENS interactions in the context of host physiology as well as a role for LPS in promoting ENS survival (Anitha et al., 2012). Recent evidence, however, suggests that this effect of LPS on survival may be concentration-dependent and also dependent on the developmental time point at which the ENS is exposed to LPS (Voss and Ekblad, 2014). Other members of the TLR family, namely TLR2, have also been implicated in regulating host GI physiology and enteric neurochemistry (Brun et al., 2013). A decrease in distal ileal neuronal number, myenteric ganglion area and glial cells was observed in TLR2 knockout mice, and in a similar manner to mice deficient in TLR4, a decrease in myenteric nitrergic neurones was also observed (Brun et al., 2013). Consistent with alterations in the myenteric plexus, ileal contractility was dysregulated in tissues from mice lacking TLR2 (Brun et al., 2013). Structural abnormalities were also observed in the submucusal plexus of TLR2 knockout mice which manifested functionally as a decrease in neurally-driven secretory responses to cholinergic stimulation (Brun et al., 2013). One mechanism proposed to underlie the morphological and functional deficits in the ENS and intestinal physiology of TLR2 knockout mice may be explained by TLR2 regulation of neurotrophic factors which displayed sensitivity to TLR2/TLR1 and TLR2/TLR6 activation, but not to agonists of TLR4 or TLR9 (Brun et al., 2013). Therefore, collectively evidence suggests both that TLR4 (Anitha et al., 2012) and TLR2 (Brun et al., 2013) influence both the ENS and function of the small intestine with similar neurochemical changes observed in myenteric neurones in the proximal colon of TLR4 deficient mice (Anitha et al., 2012).

4. Influence of the microbiota on enteric nerve activity

Intrinsic primary afferent neurones (IPANs) are characterised morphologically by their multiple axonal processes which extend into the gut mucosa and are therefore in a position to respond to changes in the gut lumen. Electrophysiologically they are characterised by their slow after hyperpolarisation (sAHP). The electrophysiological characteristics of IPANs isolated from GF mice differ from those observed in IPANs from conventional animals and reflect decreased excitability which can be restored to levels observed in neurones from control animals when mice were colonised with a conventional microbiota (McVey Neufeld et al., 2013). More recent data has also demonstrated a concomitant decrease in mesenteric nerve activity in GF mice, perhaps consequent to decreased AH activity, potentially representing connectivity across the microbiota-gut (ENS)-brain axis (McVey Neufeld et al., 2015). Moreover, fermented medium from *Bifidobacterium longum*, a bacterium demonstrated to affect behaviour by a vagal-dependent pathway, can influence ileal myenteric nerve activity (Bercik et al., 2011). Functionally, the secretomotor response to the sensory nerve stimulant, capsaicin was comparable in colonic preparations from GF and conventional animals (Lomasney et al., 2014b). This would perhaps suggest that in an integrated system a degree of functional reserve exists and supports the suggestion that the GF mouse is a valuable model system for *in vivo* studies of host–microbial interactions (Grover and Kashyap, 2014).

Whilst the collective replacement of the microbiome in GF mice restored enteric IPAN activity to that observed in control animals, individual microbes have also been demonstrated to influence the electrophysiological profile of enteric neurones. AH neurones from animals fed *Lactobacillus rhamnosus* displayed increased excitability whilst motor or S-type neurones were unaffected (Kunze et al., 2009). Functionally *Lactobacillus rhamnosus* decreased colonic motility *ex vivo* (Wang et al., 2010b). The effects of *Lactobacillus rhamnosus* on GI motility were dependent on the viability of bacterium and bacterial strain (Wang et al., 2010a). On the other hand a reduction in myenteric sensory nerve activity was observed in response to conditioned media free of live *Bifidobacterium longum*, but containing metabolic products released by the probiotic (Khoshdel et al., 2013).

Functionally *Lactobacillus salivarius* influences secretomotor function *ex vivo* via a largely tetrodotoxin (TTX)-sensitive response (Lomasney et al., 2014a). The magnitude of this acute response was similar in GF colon suggesting that the effect occurs independent of the presence of a more complex microbiota (Lomasney et al., 2014b). GF mouse colon does, however, display increased sensitivity to cAMP activation which may be driven by either neural or epithelial elements (Lomasney et al., 2014b). Heat-killed *Lactobacillus salivarius* had a similar effect on secretomotor function, suggesting that a component of the non-viable commensal either directly or indirectly affects this physiological response (Lomasney et al., 2014a).

In an effort to tease apart the combined effects of the microbiota and diet on the ENS, a recent study has investigated the consequence of a GF environment on the ENS in Ret heterozygote (+/−) mice (Dey et al., 2015). Specifically heterozygote GF mice were colonised with either high or low bile salt hydrolase-expressing microbial consortia and fed either a turmeric-supplemented or unsupplemented diet; the hypothesis being that turmeric's effect on GI physiology involves bile acid secretion/deconjugation and Ret signalling. Of note the impact of the interventions on motility were lost in GF-Ret +/− mice colonised with the microbial consortia. This would suggest that the impact of the microbiota and faecal bile acid profile on motility was directly related to a functioning ENS (Dey et al., 2015). This study highlights the complexity by which the microbiota can impact on the ENS and particularly the interplay which exists between diet, microbiota and host function.

Whilst microbes can impact neural activity, conversely, the activity of submucosal nerve fibres can also influence the way in which the host engages microbes (Chen et al., 2006). In particular, cocaine-induced accumulation of noradrenaline increased the caecal adherence of pathogenic *Escherichia coli* (Chen et al., 2006). And whilst more relevant in the context of susceptibility to infection, this study demonstrates that submucosal neural activity, albeit extrinsic, can influence host engagement with intestinal microbes. Moreover, in a mouse model of colorectal aganglionosis, significant differences in microbiota diversity over time were observed, and were characterised by increasing diversity in mutant aganglionic mice (Ward et al., 2012). This study further supports the concept that a dynamic bidirectional relationship exists between the microbiota and ENS.

5. Mechanisms implicated in facilitating microbe-enteric nervous system interactions

Bacterial toxins engage multiple mechanisms which can affect

neural function (Popoff and Poulain, 2010). Enterotoxins in particular can influence the ENS, for example both cholera toxin and *E. coli* heat labile toxin can either directly or indirectly influence ENS activity to stimulate secreto-motor reflexes and adversely affect host gut function (Popoff and Poulain, 2010). In the case of cholera toxin, neural and enterochromaffin cell-derived serotonin has been implicated in mediating the toxin's effects on gut function (Popoff and Poulain, 2010). Of note, serotonin has also recently been characterised as an important intermediary in mediating the effects of spore forming bacteria on the ENS (Yano et al., 2015). It has been proposed that particular toxins can target primary sensory neurons in the intestinal mucosa to stimulate intestinal secretion (Popoff and Poulain, 2010). In particular *Clostridium difficile* toxin A application increases the activity of both AH- and S-type submucosal neurones (Xia et al., 2000). Here again comparisons can be drawn between the effects of toxins on AH neural activity and that of commensal organisms. Ongoing efforts are now focussed on the elaboration of the mechanisms by which non-pathogenic organisms affect the activity of the ENS and have yielded thought provoking insights in this regard some of which will be discussed in more detail in this review. These include, for example, evidence that enteroendocrine cells (EC) can facilitate "synaptic" signalling from the gut lumen to the ENS (Bohórquez et al., 2015) and the description of cellular communication normally associated with eukaryotes being employed by bacteria to influence host function (Al-Nedawi et al., 2015).

5.1. Polysaccharide A

Oligosaccharides are one mechanism by which the microbiota can influence host function and represent bacterial-derived ligands for host receptors (Donia and Fischbach, 2015). Capsular cellular-associated polysaccharides, such as polysaccharide A (PSA) from *Bacteroides fragilis* signal via TLR2 to regulate host immune function (Donia and Fischbach, 2015). In a comparative study examining *Bacteroides fragilis,* mutant *Bacteroides fragilis* devoid of PSA and PSA itself on the activity of enteric IPANs, PSA mimicked the effects of *Bacteroides fragilis* (Mao et al., 2013). Further confirming the effect of PSA, mutant *Bacteroides fragilis* devoid of PSA failed to elicit such a response on ENS activity. In contrast LPS did not affect IPAN activity.

In the same study, though using *Lactobacillus rhamnosus*, the authors demonstrated that translocation across the epithelium by the microbe was not a prerequisite for influencing ENS function (Mao et al., 2013). The inference being that the effect on the ENS may be indirect, and for *Lactobacillus rhamnosus* this was demonstrated to be G-protein dependent. This elegant piece of work allows us to draw a number of conclusions; first that components of bacteria have the ability to influence enteric nerve activity independent of the intact microbe; secondly that microbes do not need to breach the epithelial barrier to exert their effects on the ENS; and thirdly that such effects on the ENS may be indirect and involve epithelial-derived mediators.

5.2. Microvesicles

Prokaryotic membrane vesicles facilitate movement of signals into the extracellular environment (Mashburn and Whiteley, 2005). Microvesicles, such as those generated by *Pseudomonas aeruginosa,* facilitate the packaging of hydrophobic content which can subsequently exert antimicrobial effects and facilitate cell-cell communication (Mashburn and Whiteley, 2005). Recent work has identified microvesicles formed by *Lactobacillus rhamnosus* as a mechanism by which it interacts with the host to ultimately influence ENS function (Al-Nedawi et al., 2015). Of note in this particular study, microvesicles were added to either the luminal domain of the epithelium or directly onto myenteric neurone preparations and AH nerve activity recorded. In preparations with an intact epithelium both *Lactobacillus rhamnosus* and microvesicles derived from the same bacterium increased the number of action potentials recorded (Al-Nedawi et al., 2015). However, when microvesicles were directly applied to enteric neurones, no effect was observed. Therefore, one can infer that a mucosal element is required in terms of transducing the microvesicle effect to the ENS. Whilst the same microvesicles influenced immune function via a TLR2-dependent mechanism, blockade of TLR2 signalling did not affect the mucosal-ENS response elicited by the microvesicles (Al-Nedawi et al., 2015).

5.3. Enterochromaffin and enteroendocrine cells

One intermediary implicated in facilitating communication between microbes and the ENS are enterochromaffin cells (EC; Ray, 2015; Yano et al., 2015). In particular spore-forming bacteria were demonstrated to increase transcription of genes in EC cells responsible for the biosynthesis of serotonin. To demonstrate an impact of spore-forming bacteria on the ENS, GF rodents were selectively colonised and activation of enteric neurones and motility assessed. To interrogate this pathway further, the effect of spore-forming bacteria on serotonin was confirmed by inhibiting serotonin biosynthesis and by immunohistochemical assessment of enteric nerve activation (Yano et al., 2015). Activation of both submucosal and myenteric 5-HT_4 receptor-expressing neurones was noted. Moreover, spore-forming bacteria increased the activation of calbindin-positive enteric neurones (Yano et al., 2015). However, the preceding events in microbial signalling to EC cells are less clear. Although several spore-forming bacteria-induced metabolites were identified in terms of their ability to influence serotonin biosynthesis, those responsible for impacting ENS activity could not be determined (Yano et al., 2015). Nonetheless, this interaction between microbes and EC cells represents a further indirect mechanism by which the microbiota can impact on enteric nerve activity and subsequently influence GI physiology. Recent evidence has also identified and characterised a pathway by which enteroendocrine cells (Peptide YY (PYY)-, cholecystokinin- and glucagon–like peptide 1-containing cells) engage sensory enteric neurones (Bohórquez et al., 2015). Neuropods have been demonstrated to connect or transduce signals from enteroendocrine cells to sensory (calcitonin gene related product and calbindin positive) neurones. Moreover, enteroendocrine cells were able to facilitate "synaptic" transmission of rabies virus administered into the gut lumen by enema to enteric nerves (Bohórquez et al., 2015), representing a putative mechanism by which the microbiota could interact via enteroendocrine cells with the ENS.

Peptide YY is a significant peptide in the context of the gut-brain axis where it can regulate not only local enteric nerve reflexes but also centrally-mediated food intake and behaviour (Holzer et al., 2012). Moreover, PYY release can be influenced by the microbiota (Holzer and Farzi, 2014). Conversely, the PYY related neuropeptide, neuropeptide Y (NPY), present within the ENS, can exert an antimicrobial effect on *Escherichia coli*, *Enterococcus faecalis*, and *Lactobacillus acidophilus* (El Karim et al., 2008; Holzer et al., 2012).

5.4. Microbial endocrinology

Enteric neurones have the capacity to respond to a wide range of chemical messengers that signal through an even wider range of receptors (Furness, 2012). These receptors may provide actual or potential targets for microbial-derived neurochemicals - an area referred to as "Microbial endocrinology" (Lyte, 2011). The concept derives from the fact that microbial species have the ability to

produce an array of neurochemicals, for example, gamma-aminobutyric acid (Lactobacillus, Bifidobacterium), norepinephrine (Escherichia, Bacillus, Saccharomyces), serotonin (Candida, Streptococcus, Escherichia, Enterococcus), dopamine (Bacillus, Serratia) and acetylcholine (Lactobacillus; Lyte, 2011). Of course, it may be a very simplistic view to assume that luminally derived neuroactive metabolites reach the ENS (or indeed the CNS), but it is one worthy of consideration. However, further work is now required in this area not only to determine whether concentrations of neuroactive metabolites reach sufficient levels in the lumen of the GI tract, but also to determine whether they can successfully reach the ENS to affect activity and function or influence pathological states. In this regard a rubric for identification and characterisation of neurochemical-producing microbes has been proposed (Lyte, 2011).

6. Concluding remarks

Inferences have been drawn for some time from motility studies in GF rodents that the microbiota can influence the ENS (Caenepeel et al., 1989; Husebye et al., 1994, 2001). As summarised in this review, the GF rodent has further demonstrated its worth as a model for interrogating the role of the microbiota on ENS development, on the excitability of enteric neurones as well as on enteric neural plasticity. Studies in GF rodents combined with those examining the impact of commensal organisms and putative probiotics on enteric neural function have collectively demonstrated that microbial consortia, or individual microorganisms, can impact on enteric nerve activity with subsequent effects on GI physiology and potentially gut to brain signalling. Recent studies from our own group using GF animals also suggest a role for the microbiota in regulating CNS plasticity, with GF animals displaying increased hippocampal neurogenesis (Ogbonnaya et al., 2015) and increased prefrontal cortex myelination (Hoban et al., 2016). Of note, however, colonisation of GF rodents in adolescence did not restore hippocampal neurogenesis to levels observed in conventional animals (Ogbonnaya et al., 2015). In contrast, a number of studies discussed here suggest that the ENS (McVey Neufeld et al., 2015) and associated enteric glial cells (Kabouridis et al., 2015) display a degree of plasticity. Moreover, the neurochemical profile of the ENS displays sensitivity to microbial intervention (di Giancamillo et al., 2010; Kamm et al., 2004). This degree of plasticity perhaps suggests that the ENS remains more responsive to changes in the microbiota relative to the CNS thereby providing opportunities for intervention in disorders in which dysfunction of the ENS may underlie or contribute to disease pathogenesis. Nonetheless, targeting the microbiota during particular neurodevelopmental windows may have a long term impact on both the development and neurochemical profile of the CNS and behaviour (Borre et al., 2014). However, the functional and physiological outcome of the microbiota or microbiota-associated interventions on the ENS will quite likely be dependent on a number of host factors including diet and aging.

Conflicts of interest

None.

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