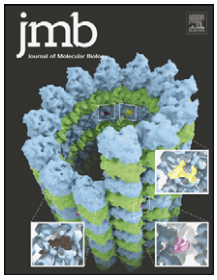
Review



Bacterial Signaling to the Nervous System through Toxins and Metabolites

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Abstract

Mammalian hosts interface intimately with commensal and pathogenic bacteria. It is increasingly clear that molecular interactions between the nervous system and microbes contribute to health and disease. Both commensal and pathogenic bacteria are capable of producing molecules that act on neurons and affect essential aspects of host physiology. Here we highlight several classes of physiologically important molecular interactions that occur between bacteria and the nervous system. First, clostridial neurotoxins block neurotransmission to or from neurons by targeting the SNARE complex, causing the characteristic paralyses of botulism and tetanus during bacterial infection. Second, peripheral sensory neurons—olfactory chemosensory neurons and nociceptor sensory neurons—detect bacterial toxins, formyl peptides, and lipopolysaccharides through distinct molecular mechanisms to elicit smell and pain. Bacteria also damage the central nervous system through toxins that target the brain during infection. Finally, the gut microbiota produces molecules that act on enteric neurons to influence gastrointestinal motility, and metabolites that stimulate the "gut–brain axis" to alter neural circuits, autonomic function, and higher-order brain function and behavior. Furthering the mechanistic and molecular understanding of how bacteria affect the nervous system may uncover potential strategies for modulating neural function and treating neurological diseases.

Introduction

Mammals host an incredibly complex community of commensal bacteria, with an estimated 10 trillion organism residents in an adult human gut [1]. Increasing evidence suggests that microbes residing in the gut, respiratory tract, genitourinary tract, and other barrier tissues actively participate in shaping and maintaining our physiology during development and homeostasis—almost as an extra "organ" [2]. In contrast, pathogenic bacteria have developed molecular strategies to survive within hosts, damaging physiological function and fitness through secreted toxins and metabolites. However, despite these differences, commensal and pathogenic bacteria share a common incentive to influence host physiology for their benefit. In this aspect, the nervous system is a desirable target as a master regulator of host function. By signaling to the nervous system, bacteria are granted a handle to influence a broad range of complex physiology, including motor coordination, sensation, metabolism, temperature control, mood, behavior, and cognition. In this review, we focus on two molecular classes of microbial signals that regulate the nervous system: bacterial toxins and metabolites.

From the perspective of the host, the nervous system provides a rapidly responsive and robust mechanism to detect bacterial cues and coordinate the appropriate defensive response. For example, peripheral sensory neurons densely innervate host barrier tissues, and are thus well positioned to detect unwanted microbes and microbial products when infection occurs [3,4]. Alternatively, in health, the gastrointestinal tract is densely inhabited by commensal bacteria, which easily outnumber local host cells by orders of magnitude [1]. As the gut microbiota is also an active producer of metabolites, its chemical signature allows the host nervous system to sample the status of gut bacterial communities and health.

An improved mechanistic understanding of how bacterial molecules act on the nervous system could yield improved therapeutics for treating neurological

diseases, as well as research tools for perturbing and analyzing the nervous system. Botulinum neurotoxin (BoNT) is a prime example where the toxin's ability to silence neurotransmission is currently utilized for the treatment of migraine and muscle spasticity [5]. The receptor-binding subunit of cholera toxin (CT) is widely used for retrograde tracing of neuronal connections as an experimental tool [6]. Identifying novel molecular interactions through which bacteria act on the nervous system, and characterizing known interactions in greater detail could highlight additional molecular pathways to be targeted or utilized. In the case of infectious disease, a better understanding of pathogenic mechanisms involving neuron–microbe interactions could also lead to novel antimicrobial approaches.

As such, here we highlight the molecular mechanisms through which commensal and pathogenic bacteria signal to the host central and peripheral nervous systems. Here, we focus mainly on recent work showing direct microbe–neuron molecular interactions, where bacterial molecules bind specifically or non-specifically to neurons to alter their biology and subsequent host physiology. We also introduce examples of indirect interactions where bacterial molecules act on an intermediary cell type such as endocrine or immune cells, which in turn produce neurochemicals and immune mediators that affect neurons. First, we discuss neurotoxins that specifically inhibit the vesicular release of neurotransmitters. Next, we introduce examples of bacterial molecules that affect smell and pain through their action on sensory neurons. In primitive organisms such as *Caenorhabditis elegans*, pathogenic bacteria are detected by chemosensation, which leads to avoidance behavior [7]. Recent work shows that similar mechanisms are also present in mammals, whose vomeronasal olfactory neurons and pain-sensing nociceptor neurons respond to bacterial effectors such as pore-forming toxins, N-formyl peptides, and various pathogen-associated molecular patterns (PAMPs). Bacteria also act on the intrinsic neurons of the enteric nervous system (ENS) to regulate gut motility through structural molecules such as polysaccharide A (PSA) or metabolites such as short-chain fatty acids (SCFAs). Bacteria-induced changes in gut motility could facilitate their colonization or maintenance of gut microflora composition. Lastly, we explore the increasingly complex area of research of the gut–brain axis, where neurotransmitters, toxins, and metabolites produced by the gut microbiota affect neural circuits, behavior, and central nervous system (CNS) function, with implications in neurological disorders such as autism.

Bacterial blockade of neurotransmission

Neurons transmit signals to each other at synapses through their release of neurotransmitters such as glutamate and γ-aminobutyric acid (GABA) stored within synaptic vesicles. Certain bacterial pathogens have evolved toxins that block this vesicular release. Depending on the type of neuron affected (e.g., motor or sensory neuron), inhibiting neurotransmitter release can cause diverging physiological consequences, such as flaccid paralysis observed with BoNT or spastic paralysis observed with tetanus neurotoxin (TeNT). Here we discuss the specific molecular mechanisms through which these bacterial toxins block neurotransmission (Fig. 1).

Botulinum neurotoxins

BoNTs and TeNT together compose the clostridial family of neurotoxins. BoNTs are the causative toxin for botulism in humans, which initially presents with blurred vision, difficulty swallowing and speaking, and muscle weakening, progressing to descending flaccid paralysis [8]. Among the known clostridial toxins, BoNTs are the most potent [9], and in the wild, amounts below the lethal dose are sufficient to paralyze a host and effectively terminate its survival [10]. Consequently, BoNTs play a central role in facilitating bacterial spread as cadavers provide an organic-rich, anaerobic environment that allows toxigenic clostridial species to proliferate [10].

BoNTs are produced by multiple strains of clostridia, including *Clostridium botulinum*, *Clostridium butyricum*, and *Clostridium baratii*. BoNTs are a genetically diverse group that can be classified into seven serotypes (BoNT/A–BoNT/G) based on immunoreactivity. An additional serotype (BoNT/H) was proposed, but recent studies suggest that it is a hybrid of BoNT serotypes A and F [11].

BoNTs are synthesized as a single-chain precursor, which is cleaved by clostridial or host proteases to yield a heavy chain (HC) and light chain (LC) that compose the mature toxin. The two chains are linked by a disulfide bridge and held together by a "belt region," a loop that extends from the HC and wraps around the LC [12–14]. In instances of food-borne or inhalation botulism where the toxin is first present in the lumen of the gut or respiratory tract, the HC mediates the transcytosis of the toxin across epithelial cells [15–17]. Subsequently, the intact holotoxin can enter the general circulation and accumulate at nerve terminals [18].

The selective binding of BoNTs to nerve terminals is possible by their recognition of two independent host receptors, which synergistically increase apparent affinity [19]. Initial BoNT binding is to membrane polysialogangliosides (PSGs), mediated by the C terminal domain of the HC [20–22]. Subsequent binding occurs through proteinaceous receptors, mediated by a separate site within the C terminal domain of the HC. The synaptic vesicle protein SV2 serves as the proteinaceous receptor for BoNT/A [23,24], BoNT/D [25], BoNT/E [26], and BoNT/F [27]. BoNT/A binds

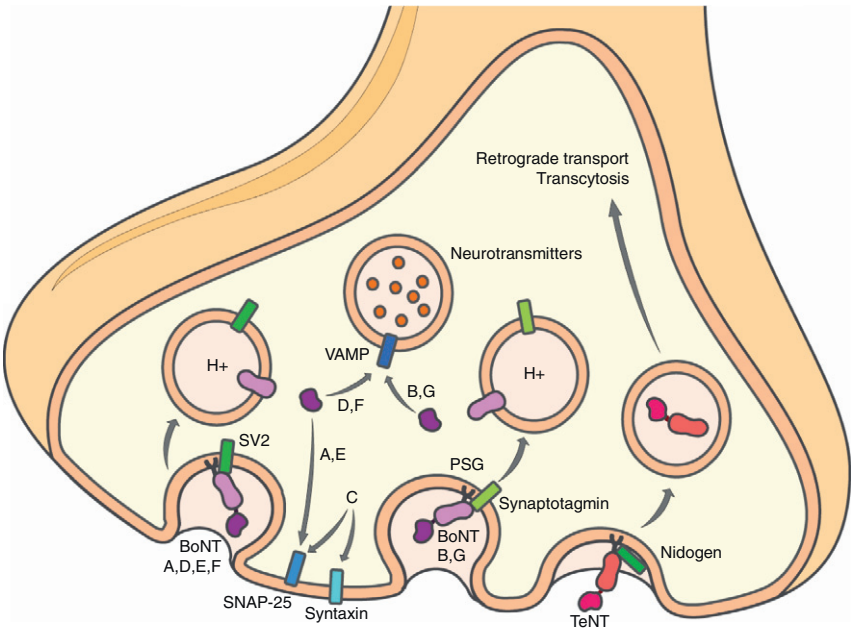

Fig. 1. Bacterial blockade of neurotransmission by clostridial neurotoxins. BoNTs from *C. botulinum* enter synaptic vesicles by binding to membrane PSGs and to a proteinaceous receptor such as the synaptic vesicle protein SV2 or synaptotagmin. Acidification of the vesicles triggers a conformational change allowing the HC of BoNT to translocate its LC into the cytoplasm. The BoNT LCs are metalloproteases that cleave components of the SNARE complex. TeNT from *C. tetani* binds to PSGs and nidogens in peripheral nerve terminals to enter an endocytic vesicle, which subsequently undergoes axonal retrograde transport. TeNT is then transcytosed to inhibitory interneurons in the spinal cord, where the TeNT LC enters the cytoplasm and cleaves VAMP to block neurotransmission.

selectively to the isoform SV2C, but not SV2A or SV2B [28]. Synaptotagmin is the host receptor for BoNT/B [29], BoNT/G [30], and the naturally occurring chimeric toxin BoNT/DC [31]. During synaptic vesicle recycling, SV2 and synaptotagmin become available in the extracellular space for binding. Recently, it was discovered that BoNT/A recognizes an evolutionarily conserved N-linked glycan on SV2C in addition to forming protein–protein interactions, allowing the toxin to bind to receptor variants across species despite diverging protein sequences [32].

Following endocytosis of the BoNT-receptor complex, acidification of the vesicle triggers a conformational change in the N-terminal domain of the HC (HCN; also known as the translocation domain). Acidification is mediated by the vesicular ATPase proton pump, whose function is to drive the re-uptake of neurotransmitters into the synaptic vesicle. HCN inserts into the lipid membrane, forming a channel that chaperones a partially unfolded LC into the cytosol, where it refolds [33]. It has also been proposed that LC and HCN undergo a concerted insertion into the membrane, where translocation of LC drives the formation of the HCN channel [34]. The LC is fully released into the cytoplasm as its interchain disulfide bond with the HC is reduced by the thioredoxin 1–thioredoxin reductase 1 system [35].

The key effector mechanism of BoNT-mediated neurotransmission blockade is the cleavage of SNARE proteins in neurons, which prevents the formation of the SNARE complex and thus blocking synaptic vesicle fusion and the release of neurotransmitters. BoNT LCs are all zinc-dependent metalloproteases which target specific molecular components of the SNARE complex. In particular, BoNT/A and BoNT/E cleave SNAP-25 [36,37]; BoNT/B, BoNT/D, BoNT/F, and BoNT/G cleave VAMP (also known as synaptobrevin) [38–40]; and BoNT/C cleaves both SNAP-25 and syntaxin [41,42].

BoNT/A has a prolonged duration of action, blocking neurotransmission in humans for up to several months, and is thus widely used for both cosmetic and therapeutic purposes. For example, its ability to block motor neuron neurotransmission at the neuromuscular junction and relax muscles is utilized to correct wrinkles or to treat muscle spasticity. Its action on peripheral sensory neurons is utilized to treat various pain conditions including chronic migraine [43].

Clostridium tetani TeNT

In contrast to the genetic diversity of BoNTs, TeNT is the only neurotoxin produced by *C. tetani*. TeNT is

the causative agent of tetanus, which presents with jaw cramping (lockjaw), muscle stiffness, and spastic paralysis. Infection occurs when *C. tetani* spores enter the body through puncture wounds. The lethality of TeNT is second only to BoNT [9]. Like BoNT, TeNT may facilitate bacterial spread by incapacitating the host and staking an anaerobic environment conducive to bacterial proliferation.

TeNT shares approximately 65% sequence homology with BoNTs, with most variance observed in the receptor-binding region of the HC [44]. As such, TeNT shares an analogous structure with BoNTs, composed of an LC and HC linked by a disulfide bond, but differ in aspects of receptor binding and subsequent trafficking within peripheral neurons. Unlike BoNTs that are internalized into recycling synaptic vesicles and translocate to the cytoplasm of peripheral neurons, TeNT is internalized into signaling endosomes that are retrogradely transported to the neuronal soma. TeNT is then transcytosed to inhibitory interneurons in the spinal cord, where it enters the cytoplasm to block neurotransmission.

As with BoNTs, TeNT has been proposed to bind two independent receptors [19], including PSGs and proteinaceous receptors. The HC of TeNT contains two separate binding sites for PSGs [45,46]. Nidogen-1 and -2 are the proteinaceous receptors for TeNT at the neuromuscular junction [47]. It is thought that the nidogens direct TeNT toward an endocytic pathway linked to retrograde transport and transcytosis, and away from recycling synaptic vesicles [47]. The retrograde pathway of TeNT is shared with that of neurotrophic factors such as NGF and BDNF, and transported to the cell body in vesicles marked by Rab7 [48]. In the spinal cord, SV2 has been proposed as a TeNT protein receptor on interneurons [49], although this finding contrasts with a later study [50].

As with BoNTs, the drop in pH in synaptic vesicles is thought to promote the entry of the TeNT LC into the cytosol *via* the translocation domain HCN [51]. The interchain disulfide between the TeNT LC and HC is then reduced by the thioredoxin 1–thioredoxin reductase 1 system [35]. In the cytoplasm, LC cleaves synaptobrevin at the same site as BoNT/B [38], thereby inhibiting vesicle release and neurotransmission. In contrast to BoNTs, TeNT is currently not pursued in therapeutic applications, likely because of the prevalence of neutralizing antibodies in the population due to childhood tetanus vaccination.

Bacterial Modulation of the Sensory Nervous System

Peripheral sensory neurons detect various external stimuli to be processed by the brain as smell, taste, touch, heat, cold, and pain, serving as an interface between the nervous system and the environment. Chemosensory olfactory neurons reside in the olfactory epithelium and the vomeronasal organ in rodents, and detect chemicals that are processed as smell. Nociceptor sensory neurons mediate pain, and their peripheral nerve terminals innervate various peripheral barrier tissues such as the skin, joints, and gut. Noxious and harmful stimuli such as heat or mechanical injury activate nociceptors, which then transmit these signals to their cell bodies within the dorsal root ganglia (DRG) and trigeminal ganglia, onward to the spinal cord and brain to be processed as pain. Recent work has shown that both olfactory and nociceptor neurons have the ability to directly detect bacteria to produce smell and pain, respectively. Interestingly, these mechanisms have been found in both the nematode *C. elegans* and in mammals. Conversely, certain bacteria can produce toxins that block pain sensation, potentially masking the infection and facilitating their spread between hosts. Here we highlight how olfactory and nociceptor neurons sense bacterial metabolites, toxins, and PAMPs through distinct mechanisms (Fig. 2).

Activation of olfactory chemosensory neurons

Odor-mediated pathogen avoidance by C. elegans

Lacking the means to detect visual or auditory cues, the nematode *C. elegans* relies heavily on chemosensation to identify favorable and unfavorable environments for survival [7]. Olfaction in *C. elegans* is mediated by 12 pairs of individually identified sensory neurons including AWB and AWC [7]. Bacterial metabolites are chemoattractive for *C. elegans*, as they feed on bacteria. However, *C. elegans* is also susceptible to infection by pathogenic bacterial species and can use associative learning to avoid pathogens following exposure [52]. A specific subset of sensory neurons expressing NPR-1, a receptor homolog to the neuropeptide Y receptor in mammals, critically mediates *C. elegans* avoidance behavior of the bacterial pathogen *Pseudomonas aeruginosa* [53]. This bacterial sensing is dependent on oxygen levels, and interestingly also leads to neuronal modulation of host defenses [53,54]. Certain bacterial metabolites act as innate chemorepellents, such as serrawettin W2—a lipidated cyclic peptide produced by the pathogen *Serratia marcescens*—which is detected by AWB neurons through a G-protein signaling pathway. (*S*)-3-hydroxytridecan-4-one (CAI-1), an autoinducer for *Vibrio cholerae* which allows the bacteria to detect population density, is detected by the AWC^{ON} sensory neuron [55].

Mammalian olfaction of N-formyl peptides

Mammalian olfactory neurons are also able to directly detect bacteria and mediate smell. N-formyl peptides in nature are found in bacteria or mitochondria

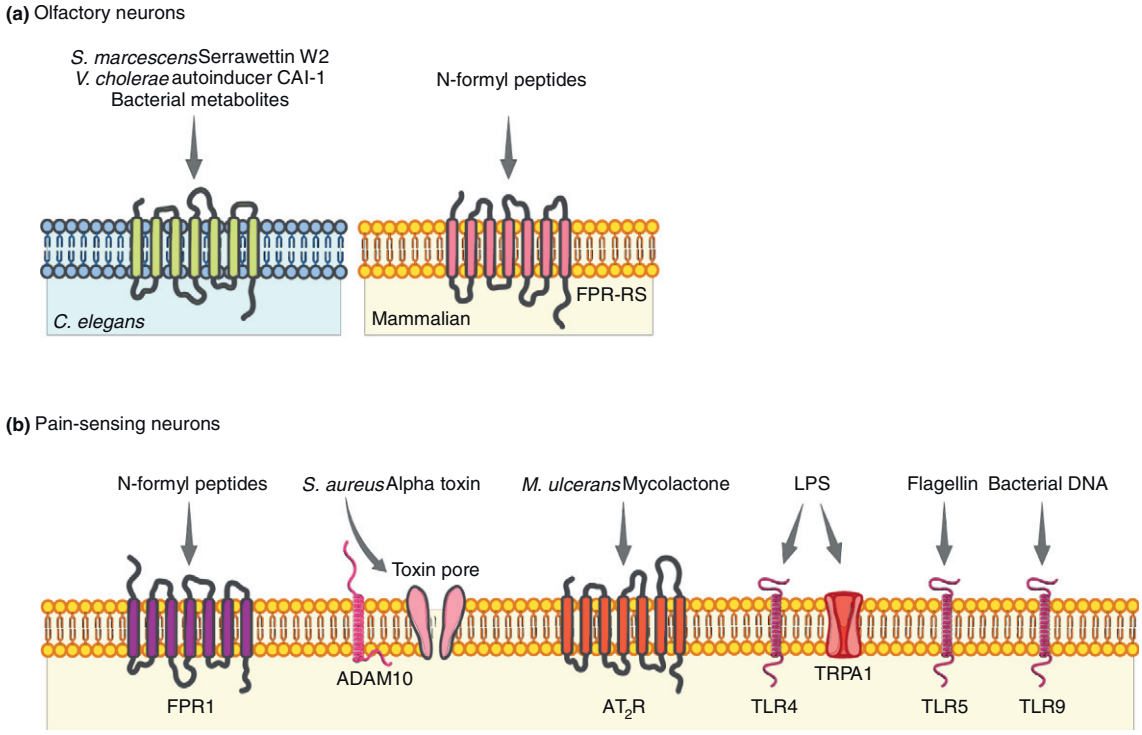

Fig. 2. Bacterial modulation of the sensory nervous system. (a) *C. elegans* olfactory neurons detect bacterial metabolites, autoinducers, and surfactants through G-protein coupled receptors to mediate bacterial foraging behavior and avoid bacterial pathogens. Mammalian vomeronasal olfactory sensory neurons express formyl peptide receptors (Fpr-rs1, Fpr-rs3, Fpr-rs4, Fpr-rs6, and Fpr-rs7), which allow them to detect bacterial N-formyl peptides. (b) Mammalian nociceptor sensory neurons detect distinct bacterial ligands to elicit or silence pain. N-formyl peptides activate nociceptor-expressed FPR1 to produce pain. *S. aureus* α-hemolysin perforates the neuronal cell membrane to cause ionic influx and pain. *M. ulcerans* mycolactone activates the AT_2R to silence pain through inducing hyperpolarization. Somatosensory neurons also detect LPS through TLR4 and TRPA1, bacterial flagellins through TLR5, and CpG motifs in bacterial DNA through TLR9.

due to their specific expression of N-formylases. The release of N-formyl peptides thus signals the presence of infection, inflammation and cell death. These peptides are recognized by mammalian formyl peptide receptors (FPRs), which are seven-transmembrane G-protein coupled receptors first found to mediate chemoattraction in phagocytic leukocytes [56]. However, FPR expression has since been discovered in certain neurons, allowing bacterial N-formyl peptides to serve as ligands of neuronal activation.

In humans, the FPR family has three members, including *Fpr1*, *Fpr2/alx* (also known as *Fprl1*), and *Fpr3* (also known as *Fprl2*) [57]. In mice, members include *Fpr1*, *Fpr-rs1*, *Fpr2* (also known as *Fpr-rs2*), *Fpr-rs3*, *Fpr-rs4*, *Fpr-rs5* (which is a pseudogene), *Fpr-rs6*, and *Fpr-rs7* [58]. Liberles *et al*. [59] and Rivière *et al*. [60] discovered that mouse vomeronasal olfactory neurons express subsets of FPRs (*Fpr-rs1*, *Fpr-rs3*, *Fpr-rs4*, *Fpr-rs6*, and *Fpr-rs7*). Accordingly, fMLF, a prototypical formyl peptide derived from *Escherichia coli* [61], induced calcium influx in vomeronasal sensory neurons *ex vivo* [60]. This finding suggests that mammals may be able to "smell" bacteria through N-formylated peptides, reminiscent of olfaction-mediated pathogen avoidance by *C. elegans*.

Bacterial modulation of nociceptor neurons and pain

Pain induction by N-formyl peptides

In addition to a role in olfaction (Mammalian Olfaction of N-formyl peptides section), bacterial formyl peptides also activate nociceptor neurons to produce pain during infection. Both fMLF (an *E. coli*-derived formyl peptide) and fMIFL (a *S. aureus*-derived formyl peptide [62]) induce calcium influx in nociceptor neurons to produce mechanical pain sensitivity when administered to mice [63]. Nociceptor sensory neurons express *Fpr1*, and the degree of neuronal activation and mechanical pain produced by bacteria is reduced in the absence of FPR1. The pain elicited by N-formyl peptides may discourage the organism from further contacting the infected area, potentially aiding wound healing and bacterial clearance.

Pain induction by Staphylococcus aureus alpha toxin

S. aureus is a major human bacterial pathogen, and infections cause painful abscesses, cellulitis, and in the most invasive cases, necrotizing fasciitis (flesh-eating disease). Although pain is a major component of the disease, the causative mechanisms of pain were not well defined. Recent work shows that *S. aureus* directly activates nociceptor neurons through alpha toxin and N-formyl peptides to produce pain independent of the innate and adaptive immune response [63].

Alpha toxin (also known as alpha-hemolysin or Hla) is one of many virulence factors produced by *S. aureus*. It is secreted as a monomer and binds to cell membranes *via* its receptor a disintegrin and metalloprotease 10 (ADAM10) [64]. Binding of the toxin to ADAM10 has been reported to up-regulate its metalloprotease activity, promoting cleavage of E-cadherin and compromising epithelial barrier function [65]. Alpha toxin also binds to phosphatidyl choline [66] and is capable of forming pores in artificial lipid membranes lacking protein receptors at higher concentrations [67]. Following binding, alpha toxin oligomerizes into a pre-pore complex composed of seven monomers. The pre-stem region of the monomer [68,69] then unfolds into a beta hairpin (the stem), which inserts into the membrane to line a beta barrel 2.6 nm in diameter [70]. The first 20 residues at the N terminus form a critical "amino latch" that binds the neighboring monomer and stabilizes the pore [70].

Subcutaneous infection of the community-associated methicillin-resistant *S. aureus* strain was shown to induce pain behavior in mice, in a manner correlated with bacterial load but not to immune cell influx [63]. Subsequently, the bacteria were found to directly activate pain-sensing nociceptor neurons *via* alpha toxin and N-formyl peptides. Nociceptor neurons—confirmed to express ADAM10—showed calcium influx, action potential generation, and neuropeptide release in response to alpha toxin. Neuronal activation by alpha toxin was dependent on its ability to form pores and did not require voltage-gated calcium channels or large-pore cation channels, suggesting that the toxin may directly depolarize neurons by allowing cations to enter the cytoplasm through the toxin pore. Furthermore, both the toxin and *S. aureus* supernatant induced nociceptor neurons to release the immunomodulatory neuropeptide CGRP [63], influencing the local inflammatory response and potentially bacterial clearance.

Pain induction by lipopolysaccharides

Nociceptor neurons have also developed molecular mechanisms to detect bacterial lipopolysaccharides (LPS), a PAMP and major outer-membrane component of all gram-negative bacteria. LPS contains three structural components: lipid A, a glucosamine-based phospholipid that anchors LPS; core oligosaccharides, which include sugars such as keto-deoxyoctulosonate (Kdo), heptose, and hexoses (glucose, galactose); and O polysaccharides (also known as O antigen), composed of both monosaccharides and non-carbohydrate components [71]. The O antigen can be incredibly diverse in its exact composition and structure, where *E. coli* notably produces approximately 170 O serotypes [71]. However, lipid A is the only component to be recognized by the innate immune system [72] through a receptor complex that includes LPS binding protein, CD14, toll-like receptor 4 (TLR4), and MD2 [73].

Components of this LPS-sensing receptor complex are expressed by peripheral sensory neurons, including nodose ganglion neurons [74], TRPV1-positive nociceptor trigeminal neurons [75,76], and DRG neurons [77–80]. Consistent with this observation, LPS has been reported to directly activate sensory neurons *via* TLR4, eliciting calcium influx in trigeminal [76,81] and DRG neurons [82]. LPS also sensitized the response of trigeminal neurons to capsaicin, the active ingredient in chili peppers that binds to the nociceptive ion channel TRPV1 and mediates pain [76,81].

In contrast, TLR4-independent nociceptor activation and pain production by LPS has also been reported. For example, Ochoa-Cortes *et al*. [79] observed that standard-grade LPS increases neuronal excitability in cultured DRG neurons, independent of TLR4. Meseguer *et al*. [83] further demonstrated that LPS can directly activate nodose and trigeminal nociceptor neurons *via* the nociceptive ion channel TRPA1, and that this activation was independent of TLR4. The lipid A moiety of LPS was found to mediate gating of TRPA1. Interestingly, LPS synergized with other TRPA1 agonists to increase calcium influx and CGRP release, supporting the role of TRPA1 as a molecular integrator of multiple noxious stimuli. LPS-induced mechanical pain sensitivity also required TRPA1 *in vivo* [83]. Outside of triggering depolarization, LPS has been reported to induce neuronal production of TNF-α [79], prostaglandin E_2 (PGE_2), and prostacyclin [80].

How neuronal sensing of LPS may contribute to host–pathogen defense is not fully determined. Following LPS activation, trigeminal and DRG neurons produce pain and nodose ganglia neurons modulate appetite, nausea, and fever. In the fruitfly *Drosophila melanogaster*, bitter-sensing gustatory neurons have been reported to detect LPS *via* TRPA1, allowing the organism to avoid food contaminated with bacteria [84]. We speculate that LPS-mediated defense mechanisms may also exist in mammals, such as inducing sickness behavior that promotes social isolation and limits the spread of pathogens.

Other PAMPs

Pain-mediating nociceptor neurons have been reported to express the toll-like receptors TLR5 and TLR9, suggesting they may detect other bacterial PAMPs beyond LPS. TLR5 binds to bacterial flagellins, while TLR9 recognizes CpG motifs in bacterial DNA. Xu *et al*. [85] reported the expression of TLR5 in Aβ neurons in the DRG, which typically mediate light touch and proprioception but are sensitized in chronic neuropathic pain. Application of bacterial flagellin to Aβ fibers allowed a normally membrane-impermeable derivative of lidocaine (termed QX-314) to enter neurons and silence their activity. This effect was abrogated in $tlr5^{-/-}$ mice, suggesting that TLR5 is coupled to the opening of a separate ion channel or transporter that mediates the entry of QX314. DRG neurons have also been reported to respond *via* TLR9 to oligodeoxynucleotides containing CpG motifs, which are found in higher frequency in bacterial DNA compared to mammalian DNA. CpG induced DRG neurons to express inflammatory mediators including PGE_2 and IL-1, and also increased expression of TRPV1 [86].

TLR3 and TLR7 have also been reported to be expressed in sensory neurons. These are host receptors for viral associated double-stranded RNA and single-stranded RNA, respectively [85,86]. Interestingly, TLR3 and TLR7 are involved in mediating the sensation of itch [87,88]. TLR7 may additionally mediate pain when coupled to the ion channel TRPA1 [89]. Although TLR3 and TLR7 detect viral antigens, not bacterial, their expression in sensory neurons collectively underscores how the host somatosensory nervous system may be equipped to detect and respond to microbial molecules.

Pain blockade by Mycobacterium ulcerans mycolactone

M. ulcerans is the causative agent of Buruli ulcer, a skin disease characterized by large ulcerative lesions that are completely painless. It was mostly thought that *M. ulcerans* silences pain by damaging Schwann cells at later stages of infection and subsequently preventing neurotransmission [90,91].

Recently, Marion *et al*. [92] reported that the underlying mechanism of pain blockade and analgesia during the early stages of infection is mediated by the bacterial toxin mycolactone, which hyperpolarizes neurons *via* an angiotensin receptor-dependent pathway. Mycolactone is a 12-membered macrolide required for *M. ulcerans* virulence [93], which causes growth arrest and apoptosis in host cells [94]. Marion *et al*. demonstrated that mycolactone binds to the angiotensin II type 2 receptor (AT_2R) to activate phospholipase A2, which then releases arachidonic acid from membrane lipids. The cyclooxygenase COX-1 then converts arachidonic acid into prostaglandins including PGE_2, which activates TRAAK (KCNK4) potassium channels to cause neuronal hyperpolarization. The analgesic effect of mycolactone was abolished in AT_2R-deficient mice, as well as by chemical inhibitors of AT_2R and Cox-1. *M. ulcerans* mycolactone would thus be an interesting candidate as a potential novel analgesic for therapeutic applications to silence pain.

Bacterial Infection-Induced Brain Damage

Certain pathogenic bacteria infect the brain or produce toxins that reach the brain and produce significant neuronal damage. For example, *Streptococcus pneumoniae* secretes the pore-forming toxin pneumolysin, which is important for neuropathology and meningitis [95] and causes neuronal apoptosis in the brain [96]. *Clostridium perfringens* produces epsilon toxin, which reaches the brain through the circulation and induces neuronal injury. Here we discuss the reported action of epsilon toxin on cerebellar neurons and oligodendrocytes, triggering depolarization and neurotransmitter release.

C. perfringens epsilon toxin

C. perfringens is a spore-forming anaerobe that is found widely in the environment, including the gut microflora of healthy animals and humans [97]. However, it is also a pathogen that can cause gas gangrene (necrotic infection of the muscle), enteritis, and enterotoxaemia, where toxins produced in the intestine enter the circulation to damage other internal organs including the brain, kidney, and lung [97,98].

C. perfringens is classified into five toxinotypes (A–E) based on the expression pattern of four major toxins (alpha, beta, epsilon, and iota), and types B and D express epsilon toxin [99]. During *C. perfringens* infection, epsilon toxin preferentially accumulates in the brain and kidney [100]. The toxin is known to disrupt the blood-brain barrier (BBB) [101] and bind myelin [102]. Intravenously administered toxin has also been reported to cause excessive release of glutamate from the hippocampus and damage neurons [103,104].

Epsilon toxin is synthesized as an inactive protoxin and proteolytically activated by *C. perfringens* lamba protease, trypsin, or chymotrypsin [105]. Structurally, the toxin is composed of three domains similar to aerolysin, a pore-forming toxin produced by *Aeromonas hydrophilia* [106]. Epsilon toxin is thought to bind to receptor(s) residing in lipid microdomains. The hepatitis A virus cellular receptor 1 (HAVCR1) [107] and myelin and lymphocyte protein (MAL) [108] have been reported to play a role in epsilon toxin binding and cytotoxicity. Following binding, toxin monomers oligomerize into a heptameric complex

[109] with help from caveolin-1 and -2 [110] and neutral sphingomyelinase [111]. Conformation change allows two amphipathic β-strands in domain 2 to form a β-hairpin that inserts into the cell membrane to line the pore [112].

An analysis in cerebellar slices identified that the toxin binds to the cell body and dendrites of granule neurons (but not nerve terminals), as well as to oligodendrocytes. Accordingly, the toxin was shown to directly depolarize the soma of granule cells, leading to calcium influx, action potential firing, and glutamate release. It was suggested that the toxin may also reduce membrane integrity at high concentrations to cause leakage of glutamate [113].

Wioland *et al*. [114] reported that epsilon toxin, at low concentrations that do not form pores, stimulates glutamate release from oligodendrocytes and causes their intracellular calcium concentrations to oscillate, leading to demyelination. The metabotropic glutamate receptor type 1 (mGluR1) and NMDAR were implicated to play a role in this process. Similarly, Linden *et al*. [115] also reported that epsilon toxin targets oligodendrocytes to cause demyelination in cultured cerebellar slices. However, demyelination was observed to be a consequence of selective oligodendrocyte death, rather than a glutamate-mediated effect. In addition, the viability of neurons, astrocytes, and microglia was unaffected by the toxin [115].

Triggering neuronal depolarization through pore formation is a shared mode of action between *C. perfringens* epsilon toxin and *S. aureus* alpha toxin (Pain induction by *S. aureus* alpha toxin section). Further investigation could elucidate whether neuronal activation by bacterial pore-forming toxins is a general phenomenon, and if there are any structural or functional requirements for the pore to trigger depolarization other than sustaining the passage of cations through the plasma membrane.

Bacterial Modulation of the ENS

The gastrointestinal tract is one of the most densely innervated organs in the periphery, by neurons that are either intrinsic or extrinsic to the gut. The intrinsic neurons of the gut form the ENS, which forms a complete sensorimotor reflex circuit consisting of intrinsic primary afferent neurons (IPANs), interneurons, and motor neurons contained entirely within the gut wall. These enteric neurons critically regulate gut motility and peristalsis. The two major populations of extrinsic neurons that relay sensory information from the gut to the CNS are cells located in the nodose/jugular ganglia that mediate nutrient sensation, nausea, appetite, and satiety; and cells located in the DRG that detect noxious stimuli and mediate pain. Here we discuss how bacterial molecules interact with the intrinsic enteric neurons of the ENS to influence GI motility and function. Bacterial signaling to the CNS through extrinsic afferent neurons of the gastrointestinal tract is discussed in The gut–brain axis section.

The gut exposes the host to the external environment with a surface area approximately 50 times larger than the skin [116]. Consequently, it interfaces intimately with commensal and pathogenic bacteria and the molecules they produce, including metabolites, neurotransmitters, hormones, bacterial antigens, PAMPs, and toxins. Bacterial molecules originating in the gut lumen may influence the intrinsic enteric neurons of the host ENS through direct or indirect mechanisms (Fig. 3). Direct interactions require the molecule to pass the epithelial barrier to access the nerve endings of enteric sensory neurons, which reside within layers of connective and muscular tissue immediately beneath the intestinal epithelium. In a healthy gut where the epithelial barrier is intact, bacterial products may cross epithelial cells through passive diffusion, active transport, or transcytosis [117]. In instances where the epithelial barrier is compromised by inflammation or other mechanisms (termed “leaky gut”), bacteria and bacterial products may also translocate paracellularly between cells whose tight junctions have become compromised [117,118].

Bacteria in the gut lumen may also interact with the ENS indirectly through non-neuronal intermediary cell types such as endocrine or immune cells of the gut. Sensory neurons innervating the gut typically detect paracrine signals that are released by enteroendocrine cells in the epithelium in response to chemicals—including nutrients—in the lumen [116,119,120]. In particular, a subset of enteroendocrine cells known as enterochromaffin cells (ECs) act as “signal transducers” [119] that convert bacterial stimuli into downstream neuronal responses. For example, *V. cholerae* CT or metabolites produced from the gut microbiota act on ECs to induce the release of 5-hydroxytryptamine (also known as serotonin), which act on enteric neurons to regulate peristalsis and secretions [121–123].

Bacteria also activate resident immune cells of the gut, which may signal to enteric neurons through immune mediators [124,125]. It is well appreciated that the commensal gut microbiota plays an integral role in the development and homeostasis of intestinal immune cell subsets, and in enhancing innate and adaptive immune responses against pathogens during luminal infections [126]. In turn, molecular mediators secreted by gut-resident immune cells can be detected by corresponding receptors in the ENS and affect enteric function. For example, muscularis macrophages of the gut secrete bone morphogenetic protein 2 (BMP2), which activates BMP receptors on enteric neurons to regulate gut motility [127]. Antibiotic treatment reduced the expression of BMP2 from muscularis macrophages,

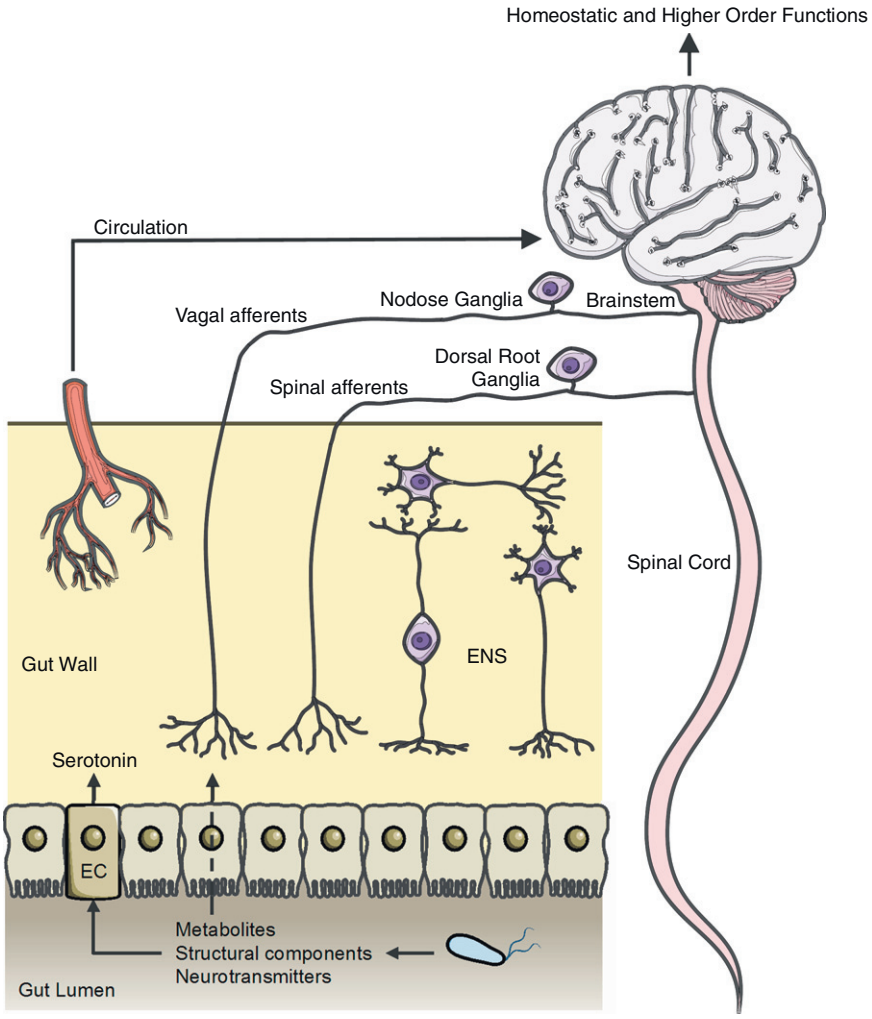

Fig. 3. Gut bacterial modulation of the nervous system. Bacterial metabolites and structural components produced in the gut lumen may act on neurons directly after crossing the epithelial barrier, or stimulate ECs to secrete neuroactive compounds such as serotonin. Within the gut lumen, bacteria can signal to the ENS to regulate gut motility. The ENS forms a complete sensori-motor reflex composed of IPANs, interneurons, and motor neurons. Beyond the gut, bacteria also signal to the brain through extrinsic afferent nerve fibers, or through secreted molecules that enter the circulation and reach the CNS. This gut microbe–neuron signaling is part of the "gut–brain axis." Extrinsic sensory nerves include vagal afferents that project from the gut to the brainstem and spinal afferents that project from the gut to the spinal cord. Bacterial inputs affect how the CNS regulates homeostatic functions such as metabolism and appetite, or higher-order brain functions such as anxiety and social behavior.

suggesting that the gut microbiota may signal to the ENS indirectly *via* this immunological intermediary.

Here we focus on examples of direct interactions between molecular products of the gut microbiota and enteric neurons, which subsequently influence gastrointestinal motility.

Bacteroides fragilis PSA

B. fragilis is a gram-negative bacteria found in the normal gut microbiota. It also causes gastrointestinal infections, leading to the formation of intra-abdominal abscesses due to host responses to its capsular polysaccharides [128]. PSA is an immunodominant component of the *B. fragilis* capsule, consisting of several hundred repeats of a zwitterionic tetrasaccharide [129]. Application of live *B. fragilis* or pure PSA evoked action potentials in IPAN enteric sensory neurons in *ex vivo* intestinal preparations. This occurred approximately 5 s after application of PSA, leading to increased excitability of the IPAN neuronal network within minutes [130]. The probiotic *Lactobacillus rhamnosus* (the JB-1 strain) also excited IPANs, although its molecular mediator(s) was not identified.

Based on the latency of the neuronal response, Mao *et al*. speculated that PSA may first act on enteroendocrine cells in the epithelium, which then activate IPANs through release of an intermediate mediator. As a potent immunomodulant, PSA may also exert effects on the nervous system *via* immune cells [131].

Short-chain fatty acids

SCFAs are bacterial fermentation products originating from starch and fiber that cannot be completely digested. In mammals, the gut microbiota is the primary producer of SCFAs, the majority of which are acetate, propionate, and butyrate. Following absorption into the circulation, SCFAs act on host G-protein coupled receptors (GPCRs) including GPR41 [also known as free fatty acid receptor 3 (FFAR3)] and GPR43 [also known as free fatty acid receptor 2 (FFAR2)] [132,133] to affect various aspects of host physiology including metabolism and energy homeostasis [134–136]. SCFAs are also known to inhibit the activity of histone deacetylases, with butyrate reported to be the most potent in activity [137,138].

Soret *et al*. [139] reported that butyrate, but not acetate or propionate, increased the expression of choline acetyltransferase in myenteric enteric neurons that innervate the circular muscle. Both bath application of butyrate and a diet rich in resistant starch that increases colonic concentrations of butyrate increased gut motility. It was proposed that butyrate acts directly in enteric neurons to increase histone acetylation and choline acetyltransferase expression, the effect of which was partially dependent on the expression of monocarboxylate transporter 2 in enteric neurons. Alternatively, SCFAs may also indirectly influence gut motility by inducing ECs to produce 5-hydroxytryptamine that act on enteric neurons [123,140,141].

Pathogen-associated molecular patterns

Similar to nociceptive sensory neurons (discussed in the Pain induction by LPS and Other PAMPs sections), enteric neurons also express toll-like receptors to detect bacterial PAMPs such as gram-negative bacterial LPS (*via* TLR4) and gram-positive bacterial peptidoglycans and lipoproteins (*via* TLR2) [78,142,143]. TLR4-mediated activation of enteric neurons regulates neuronal survival and gut motility [144], whereas TLR2 signaling affects ENS structure and intestinal contractility [143].

The gut–brain axis

A wealth of recent studies highlights the bidirectional communication that occurs between the gut microbiota and the brain, which is referred to as the "gut–brain axis." Germ-free (GF) mice have served as a valuable tool for investigating the physiological and neurobehavioral changes that occur in the absence of a gut microbiota. Controlled colonization of GF mice with defined species of bacteria has begun to identify mechanistic links [145]. These gnotobiotic studies have demonstrated that the microbiota plays a key role in modulating the levels and turnover of neurotransmitters, neurohormones, and neurotrophic factors in the brain and periphery [124]. Marked changes in neurophysiology have been reported, where GF mice display a leakier BBB [146] and hypermyelination in the prefrontal cortex compared with mice raised in a conventional environment [147]. At an organismic level, the presence and composition of the gut microbiota has been linked to affecting host mood, behavior, and cognition, including stress responses, anxiety-, and depression-like behavior; social behavior; learning; and memory [124,145,148–151].

Bacteria may signal to the brain through multiple routes, including neural, metabolic, endocrine, and immune pathways [151] (Fig. 3). Neural communication can occur through extrinsic innervations of the gut which connect to the CNS. Specifically, gut-innervating extrinsic sensory neurons include two types: (a) vagal sensory afferents, whose cell bodies reside in the nodose/jugular ganglia and project directly to the brainstem, and (b) spinal sensory afferents, whose cell bodies reside in the DRG and project to the spinal cord. Vagal sensory afferents typically detect gut nutrients, stretch, and hormones to mediate satiety and nausea, whereas spinal sensory afferents detect noxious and harmful stimuli to mediate pain. These sensory neuron afferents also detect bacterial molecules in the gut, triggering the CNS to modulate physiological functions through neural circuits that feedback to the gut through the sympathetic and parasympathetic efferent arms of the autonomic nervous system. In addition to direct sensing of bacteria by gut-innervating sensory neurons, metabolic communication in the gut–brain axis occurs when bacterial metabolites become absorbed into the portal vein and enter circulation. These bacterial metabolites may then cross the BBB to directly affect brain function [124]. The gut microbiota and their metabolites play a key role in regulating the phenotype of microglia, the resident innate immune cells in the CNS. Microglia and other immune cells play a major role in mediating neurodegeneration and pathology in neurological disease conditions.

Therefore, the gut microbiota can communicate with and shape brain function through multiple pathways. Despite overwhelming evidence suggesting that the gut microbiota actively influences host physiology and behavior during homeostasis and disease, the underlying molecular mechanisms often remain unknown. Here we discuss several examples and possibilities of

how molecules produced by the gut microbiota signal to the host brain to mediate its function.

Neurotransmitter production by the gut microbiota

Multiple bacterial species of the gut flora are known to produce neuroactive compounds, including neurotransmitters such as acetylcholine, dopamine, noradrenaline, serotonin, and GABA [152]. GABA is the main inhibitory neurotransmitter in the brain and is produced by glutamic acid decarboxylases that convert glutamate into GABA. Multiple strains of *Lactobacillus* have been found to express glutamic acid decarboxylase and produce GABA [153], including those found in fermented food products such as cheese and Korean kimchi [154,155]. In addition, *Lactobacillus brevis* and *Bifidobacterium dentium* were identified as one of the most efficient producers of GABA among groups of lactobacilli and bifidobacteria strains derived from the human intestine [156]. The levels of most neurotransmitters found in the gut are equal to or higher than those in the brain [124], suggesting that bacterial production of neurotransmitters may be a major molecular mechanism of bacteria–neuron communication.

Gut bacterial modulation of homeostatic function

In addition to influencing the ENS and gut motility (Bacterial Modulation of the ENS section), SCFAs have also been linked to the gut–brain axis through neural circuits that are activated by sensory afferents that feed into the hypothalamus and to efferent nerves within the sympathetic and parasympathetic branches of the autonomic nervous system. Indeed, Gpr41, one of the receptors for SCFAs, has been reported to be expressed in various parts of the peripheral nervous system that constitute the gut–brain axis, including nodose neurons, DRG neurons, and sympathetic ganglia neurons [157].

Kimura *et al*. [158] demonstrated that mice lacking Gpr41 display lower levels of heart rate, oxygen consumption and core body temperature. Intraperitoneal administration of propionate increased the heart rate and oxygen consumption in wild-type mice but not in Gpr41$^{-/-}$ mice, consistent with the observation that Gpr41 is expressed in the sympathetic ganglia and that propionate can directly activate primary cultures of sympathetic neurons. Propionate has also been implicated in promoting intestinal gluconeogenesis through periportal afferent nerves and corresponding regions in the brain—including the dorsal vagal complex, the parabrachial nucleus, and the hypothalamus—leading to increased activity of G6Pase (an essential enzyme for gluconeogenesis) in the intestine [159].

Acetate derived from the gut microbiota has been implicated in regulating weight gain and appetite. Perry *et al*. [160] reported an increase in acetate production by the gut microbiota in response to a high-fat diet, which in turn promoted glucose-stimulated insulin secretion (GSIS) and ghrelin secretion by activating the parasympathetic nervous system. Local administration of acetate to the nucleus tractus solitarius in the brainstem replicated GSIS stimulation, whereas parasympathetic blockers abrogated it. It was proposed that this gut microbiota communication with the CNS forms a positive feedback loop that promotes obesity. In a physiologically contrasting manner, Frost *et al*. [161] reported a role for acetate in suppressing appetite *via* actions on the hypothalamus. Intraperitoneally administered acetate was detected in the arcuate nucleus, a region of the hypothalamus, and correlated with increased expression of the anorectic neuropeptide melanocortin precursor pro-opiomelanocortin, decreased expression of the orexigenic neuropeptide agouti-related peptide, and an overall reduction in food intake. Therefore, gut bacteria and their metabolites actively regulate homeostatic function through molecular and neural circuits involving the brain.

Gut commensal microbes also modulate visceral pain sensitivity. Rousseaux *et al*. [162] found that oral administration of *L. acidophilus* induced analgesia in a colonic distension tests for visceral pain that was comparable to the efficacy of morphine. They found that this may be due to induction of mu-opioid receptor MOR1 and cannabinoid receptor CB2 in gut epithelial cells, and the analgesic effects were abrogated with CB2 or MOR1 antagonists [162].

Gut bacterial modulation of neurons through the immune system

The gut microbiota can also influence brain function and behavior by signaling through the immune system, both peripherally and in the CNS. For instance, the gut microbiota affects circulating levels of pro- and anti-inflammatory cytokines [124,151], which can act on corresponding receptors in the brain endothelium, microglia, astrocytes, and neurons [163] to induce behavioral changes such as sickness behavior and depression [164].

The microbiota also strongly influences the phenotype of microglia, the resident myeloid immune cells of the CNS to affect neuronal health and degeneration. Microglia play critical roles in synapse pruning, clearance of apoptotic bodies, and signaling to neurons in the brain in health and neurological diseases. It was discovered that SCFAs produced by the gut microbiota modulated microglia maturation and activation, influencing the innate immune functionality of microglia in the brain [165]. The commensal microbiota promotes disease progression in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis, by driving the

development of myelin-reactive CD4+ T cells [166]. A recent study showed that the gut microbiota promotes motor dysfunction in α-synuclein transgenic mice, a mouse model of Parkinson's disease, by potentiating microglial activation and neuroinflammation [167]. Interestingly, microbiota transplants from Parkinson's disease patients enhanced disease progression in α-synuclein transgenic mice, but not microbiota transplants from healthy human donors [167].

Gut bacterial modulation of mood, anxiety and behavior

The gut microbiota also affects the functions of the brain mediating mood, anxiety, and behavior. Several studies have shed light on the molecular mechanisms that may enable this process. Bravo *et al*. [168] reported that chronic administration of the gut probiotic *L. rhamnosus* (JB-1) reduces stress-induced levels of corticosterone and anxiety- and depression-like behavior. The vagus nerve was implicated in relaying *L. rhamnosus* signals to the host brain, as vagatomy abrogated this effect, and application of *L. rhamnosus* to the intestine *ex vivo* increased the firing rates of vagal sensory afferent fibers within minutes [169]. In the brain, *L. rhamnosus* treatment altered neuronal expression levels of GABA receptor subunits [168] and increased the concentrations of neurotransmitters including glutamic acid/glutamate and GABA [170].

Hsiao *et al*. [171] reported that microbially driven metabolic changes can trigger anxiety-like behavior in a mouse model of autism spectrum disorder. A maternal immune activation (MIA) model was employed where pregnant females received poly(I:C) to produce offspring with impaired communicative and social behavior representative of autism spectrum disorder. MIA offspring displayed a leaker gut and compositional alterations in the gut microbiota and serum metabolome, as well as deficits in a series of behavioral traits related to autism. Strikingly, chronic administration of the bacterial metabolite 4-ethylphenylsulfate (4EPS), which was elevated in MIA offspring, was sufficient to induce anxiety-like behavior. Treatment of mice with *B. fragilis* restored gut permeability and serum levels of 4EPS, and reduced anxiety-like behavioral deficits in this autism model.

Recently, Buffington *et al*. [172] reported that compositional changes in the gut microbiota induced by a maternal high-fat diet (MHFD) impaired oxytocin production and synaptic plasticity that mediate social behaviors. MHFD offspring were found to have a compositionally altered gut microbiota, as well as lower levels of oxytocyin production in the paraventricular nuclei region of the hypothalamus. In addition, oxytocin-activated neurons in the ventral tegmental area were impaired in inducing long-term potentiation following interactions with a stranger. However, treatment of mice with *Lactobacillus reuteri,* the most significantly reduced species in the MFHD microbiota, rescued oxytocin production and sociability in MHFD offspring. Although the exact mechanism of how *L. reuteri* induces oxytocin production is unknown, its molecular and cellular modulation of the CNS is a striking example of the potentially extensive reach of the gut microbiota.

Conclusions

Bacteria can exert a varied and far-reaching impact on our physiology by signaling to the host nervous system. Bacteria produce toxins, metabolites, and structural components that are detected by peripheral and central neurons through corresponding receptors. Bacteria also alter neural function indirectly through relayed signaling through endocrine and immune cells. Smell and pain, two prototypic sensory neuron functions, are directly modulated by bacteria and their molecular products. Moreover, the gut microbiota has been found to mediate gastrointestinal motility by communication with the ENS, and signal to the brain through the gut-brain axis to affect higher-order brain function such as anxiety and social behaviors. Although not discussed in the present review, resident bacteria and microbes of other important barrier surfaces and anatomical niches such as the skin, esophagus, stomach, and vagina [173] may also produce neuroactive molecules, and their effects on the central and peripheral nervous system warrant further study. The implications of molecular bacteria–neuron interactions are profoundly changing our view of host–pathogen defense and host–microbe symbiosis. There is a great need for further investigations to understand the molecular mechanisms underlying these interactions, as they may be translated into therapeutic applications. For example, bacterial neurotoxins are already in use to treat neurological diseases including pain. Future identification of bacterial modulators that silence or modulate specific subtypes of neurons will enable targeted treatment of neurological disease.

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Abbreviations used:
BoNT, botulinum neurotoxin; CT, cholera toxin; PAMPs, pathogen-associated molecular patterns; ENS, enteric nervous system; PSA, polysaccharide A; SCFAs, short-chain fatty acids; CNS, central nervous system; GABA, γ-aminobutyric acid; TeNT, tetanus neurotoxin; HC, heavy chain; LC, light chain; PSGs, polysialogangliosides; HCN, N-terminal domain of the HC; DRG, dorsal root ganglia; FPRs, formyl peptide receptors; LPS, lipopolysaccharides; TLR4, toll-like receptor 4; PGE_2, prostaglandin E_2; AT_2R, angiotensin II type 2 receptor; IPANs, intrinsic primary afferent neurons; GF, germ-free; BBB, blood-brain barrier; MHFD, maternal high-fat diet; GSIS, glucose-stimulated insulin secretion.

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